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Development of *in*-situ product removal strategies in biocatalysis applying scaled-down unit operations[†]

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Abstract:

An experimental platform based on scaled-down unit operations combined in a plug-and-play manner enables easy and highly flexible testing of advanced biocatalytic process options such as *in-situ* product removal (ISPR) process strategies. In such a platform it is possible to compartmentalize different process steps while operating it as a combined system, giving the possibility to test and characterize the performance of novel process concepts and biocatalysts with minimal influence of inhibitory products. Here the capabilities of performing process development by applying scaled-down unit operations are highlighted through a case study investigating the asymmetric synthesis of 1-methyl-3-phenylpropylamine (MPPA) using ω-transaminase, an enzyme in the sub-family of amino transferases (ATAs). An on-line HPLC system was applied to avoid manual sample handling and to semi-automatically characterize ω-transaminases in a scaled-down packed-bed reactor (PBR) module, showing MPPA as a strong inhibitor. To overcome the inhibition, a two-step liquid-liquid extraction (LLE) ISPR concept was tested using scaled-down unit operations combined in a plug-and-play manner. Through the tested ISPR concept, it was possible to continuously feed the main substrate benzylacetone (BA) and extract the main product MPPA throughout the reaction, thereby overcoming the challenges of low substrate solubility and product inhibition. The tested ISPR concept achieved a product concentration of 26.5 $g_{MPPA} \cdot L^{-1}$, a purity up to 70 % $g_{MPPA} \cdot g_{tot.}^{-1}$ and a recovery in the range of 80% $mol \cdot mol^{-1}$ of MPPA in 20 hours, with the possibility to increase the concentration, purity and recovery further.

Introduction:

Biocatalysis is an established technology in the chemical and pharmaceutical industries, that potentially provides an environmentally friendly alternative to many chemical synthesis routes (Bornscheuer et al., 2012; Huisman and Collier, 2013; Nestl et al., 2011). It is recognized that the industrial application of biocatalysis provides many advantageous features, such as exquisite selectivity, mild operating conditions and few synthetic steps with high atom efficiency, to name a few (Choi et al., 2015; Kohls et al., 2014; Narancic et al., 2015). However, some common limitations often hamper the industrial application and economic feasibility of many biocatalytic processes. For example, it is often the case that inhibitory and toxic effects from substrates and products greatly reduce the activity and stability of biocatalysts (Woodley et al., 2008). Consequently, it is often necessary to consider both protein and process engineering strategies, as a means to overcome these limitations and challenges (Ringborg and Woodley, 2016; Woodley, 2013). The focus of this work is intensification of biocatalytic processes, through the development and testing of process engineering strategies in modular scaled-down unit operations, making it possible to compartmentalize multiple process steps operating in parallel. As a result it is possible to test and develop novel and/or complex biocatalytic process in a simple manner, which are not only dependent on operating new processes based on conventional batch based technologies. The benefit of applying scaled-down unit operations is the possibility of compartmentalizing different process steps, which is difficult at best to put in place with conventional batch based platforms. In this work, that benefit is illustrated by showing how modular scaled-down unit operations can be applied for the development of process engineering strategies, such as *in-situ* product removal (ISPR) and substrate supply strategies based on liquid-liquid extraction (LLE). Commonly, such strategies are applied to overcome inhibitory and solubility related process limitations. ISPR refers to the continuous removal and recovery of product(s) during the biocatalytic reaction course (Wohlgemuth et al., 2015), e.g. by linking reaction steps with the following downstream product recovery step(s).

Application of modular scaled-down unit operations for continuous manufacturing concepts is a research topic in chemical synthesis (flow chemistry) that has matured in recent years (Aota et al., 2009; Jensen et al., 2014). Therefore, the aim of this work is to transfer that knowledge base and scale-down concepts to biocatalysis, in order to establish a scaled-down experimental platform that can assist the development of novel and robust biocatalytic processes. For example, modular scaled-down unit operations enable compartmentalization of different process steps, which is not possible to the same extent with conventional batch based technologies (Heintz et al., 2016). Furthermore, combining scaled-down unit operations in a plug-and-play manner enables easy, robust and flexible experimentation with the possibility of automated operation. In addition, the application of scaled-down technologies will ensure that scarce and valuable resources are used efficiently, which is especially important in the early development phase where resources are limited (Fagaschewski et al., 2012). Likewise, the small characteristic length scale and the large surface-to-volume ratio in small scale (μL and mL) enables fast heat and mass transfer (Wohlgemuth et al., 2015). The fast heat and mass transfer will be beneficial in overcoming/minimizing inhibitory effects of substrates and/or products, by putting in place fast acting substrate supply and ISPR strategies. However, the fast heat and mass transfer properties of scaled-down unit operations are difficult to achieve across scales, which will be briefly discussed in this work.

The implementation of ISPR strategies will result in intensified biocatalytic processes. Despite this, such strategies often fail to reach industrial implementation, e.g. due to increased process complexity (Van Hecke et al., 2014; Stark and von Stockar, 2003). Furthermore, it is frequently difficult to test complex ISPR process options with conventional batch based technologies, since it is difficult to compartmentalize and link multiple process steps at the same time (Heintz et al., 2016). Therefore, we demonstrated herein how combining modular scaled-down unit operations in a plug-and-play manner provides an experimental platform for developing ISPR options, where upstream and downstream process steps can be linked, compartmentalized and tested in combination. This is shown through a case study, where a two-step LLE ISPR strategy is studied for an ω-transaminase reaction (see figure 1 and scheme 1, respectively). The given

system made it possible to reach high product purity and titers, with a biocatalyst with severe product inhibition. At the same time the proposed process concept overcame limitations of substrate solubility in the reaction mixture, by continuously feeding the main substrate.

Combining scaled-down/ microfluidic unit operations in a plug-and-play manner to test multi-step synthetic systems is to some extent already established (Andrade et al., 2014; Aota et al., 2009; Fagaschewski et al., 2012; Novak and Znidarsic-Plazl, 2013; Sahoo et al., 2007). This work is different in as much as streams are recycled between modular scaled-down unit operations, as well as by compartmentalizing upstream and downstream process steps, making it possible to test biocatalysts under conditions where limiting effects caused by substrates and/or products are minimized.

Case study:

As a case study, the asymmetric synthesis of optically pure chiral amines using ω-transaminases (ω-TAs) has been selected. ω-TAs are frequently cited as promising biocatalysts in the synthesis of optically pure chiral amines for active pharmaceutical ingredients (APIs) (Guan et al., 2015; Nobili et al., 2015; O'Reilly and Turner, 2014). The exquisite regio- and stereo-selectivity, along with potentially fewer process steps compared with conventional chemical synthesis routes, motivates the industrial application of ω-TAs for chiral amine synthesis (Savile et al., 2010). Furthermore, ω-TAs are versatile biocatalysts that can be applied for (dynamic) kinetic resolution and asymmetric synthesis (Koszelewski et al., 2010; Malik et al., 2012). In scope here is the asymmetric synthesis, where ω-TAs catalyze the transfer of an amine group from an amine donor to a prochiral acceptor ketone substrate (Koszelewski et al., 2010; Schätzle et al., 2011). The transfer is facilitated by the cofactor pyridoxal-5'-phosphate (PLP) (Cassimjee et al., 2015; Steffen-Munsberg et al., 2015). Despite the promising aspects of the application of ω-TAs their industrial application is often limited by some fundamental challenges. For example, the ω-TA specific activity is often low, which challenges the economic feasibility of such processes (Narancic et al., 2015). Furthermore, the activity is often significantly reduced by substrate inhibition and further reduced over the course of the

reaction due to inhibitory effects of the products (Malik et al., 2012; Martin et al., 2011; Shin and Kim, 2009; Truppo et al., 2010). In addition, some ω-TA synthesized reactions suffer from unfavorable reaction thermodynamics, making it challenging to achieve sufficiently high reaction yields (Gundersen et al., 2015). For all these reasons, it is often necessary to consider ISPR process strategies to overcome these challenges and intensify the performance of ω-TA based processes.

Tested and characterized in this work is a scaled-down two-step LLE ISPR process concept (figure 1), which enables simultaneous substrate supply and ISPR. The given ISPR concept is tested for the asymmetric synthesis of 1-methyl-3-phenylpropylamine (MPPA) from benzylacetone (BA). Isopropylamine (IPA) is applied as the amine donor (scheme 1).

This particular reaction system has several features that make it an interesting choice for testing the proposed ISPR process concept. First, the thermodynamic reaction equilibrium is reported to be close to unity, i.e. $K_{eq} = 0.74$ (Tufvesson et al., 2012), making it suitable to shift the reaction equilibrium simply by applying an excess of the amine donor (Fesko et al., 2013). Hence, the testing and characterization of the proposed ISPR concept will focus on overcoming inhibitory and solubility related challenges. Secondly, the pK$_a$ values of MPPA and IPA are very similar i.e. 10.64 and 10.68, respectively. Therefore, it is suitable to separate the reaction species based on differences in hydrophobicity, i.e. MPPA (log P of 2.18) and BA (log P of 1.67) are hydrophobic compared to IPA (log P of 0.21) and Ace (log P = -0.24) (Royal Society of Chemistry, 2015).Thirdly, the solubility of BA in aqueous solution is rather low, ~1.6 $g \cdot L^{-1}$, which makes it interesting to consider an efficient feeding strategy. Finally, it is likely that the reaction species will have a significant impact on the rate of reaction (e.g. due to inhibition), which also makes it interesting to consider simultaneous substrate supply and product removal strategies.

Materials and Methods:

Chemicals

Recombinant Amino transferase ATA-v was provided by c-LEcta GmbH (Leipzig, Germany). Immobilization of ATA-v in lentil shaped polyvinyl alcohol (PVA) particles was performed and provided by LentiKat's a.s. (Czech Republic, www.lentikats.eu). The PVA particles contained 50 mg ATA-V per g PVA. Benzylacetone (BA; synthesis grade) was purchased from Merck KGaA. Potassium carbonate (99+%) was purchased from Acros Organics. All other chemicals were purchased from Sigma-Aldrich and were of analytical grade (1-methyl-3-phenylpropylamine, pyridoxal 5'-phosphate monohydrate, acetone, isopropylamine, citric acid monohydrate, sodium citrate dehydrate, sodium bicarbonate, undecane, xylenes, toluene, and heptane).

Equipment

For temperature control an IKA® C-MAG HS7 magnetic stirrer (IKA® - Werke GmbH & Co. KG, Staufen, Germany) was used. The pumps used in the experimental set-ups were TECAN cavro® XLP6000 OEM pumps (Tecan Systems Inc., San Jose, CA, USA) equipped with TECAN cavro® XLP 250 μL syringes (Tecan Systems Inc., San Jose, CA, USA). The pumps were controlled with LabView (National Instruments, Austin, TX, USA). Standard HPLC PEEK fittings and connectors were used to connect pumps, tubing and modular scaled-down unit operations. Manual sampling was performed with a 10 μL 10F syringe (SGE Analytical Science, Trajan Scientific Australia Pty Ltd). For on-line HPLC measurements, an 8 port dual sample injector valve was used with 2 μL PEEK sample loops (Valco Instruments Co. Inc.).

Analytical methods

The concentrations of the substrates and products in aqueous solutions were determined by HPLC (Dionex Ultimate 3000) with UV detection (Dionex Ultimate 3000 PDA detector). One method was applied to determine BA and MPPA concentrations and another was applied to determine IPA and ACE concentrations. Both methods were operated isocratically at 30℃ with the same column, a Gemini-NX C18 column (100mmx2mm, 3μm, 110Å, Phenomenex, Torrance, CA, USA). The method applied to determine BA

and MPPA was operated at a flowrate of 0.450 $mL \cdot min^{-1}$ (35 % $vol \cdot vol^{-1}$ acetonitrile and 65% $vol \cdot vol^{-1}$ Milli-Q water adjusted to pH 11 with addition of NaOH). The retention times for MPPA and BA were 2.9 min and 3.6 min, respectively. Both components were detected at 215 nm for off-line measurements and at 265 nm for on-line measurements. It was necessary to apply a wavelength with lower absorbance of the two components for the on-line measurements in order not to saturate the absorbance, as a consequence of the outlet reaction mixture not being diluted before injection onto the HPLC. The second method was solely used for off-line measurements and was operated with a flow rate of 0.300 $mL \cdot min^{-1}$ (5 % $vol \cdot vol^{-1}$ acetonitrile and 95 % $vol \cdot vol^{-1}$ Milli-Q water adjusted to pH 11 with addition of NaOH). The retention times for Ace and IPA were 1.6 min and 2.7 min, respectively. ACE was detected at 265 nm, while IPA was detected at 200 nm.

Scaled-down PBR characterization

Packed bed reactor (PBR) modules for characterization of the performance of ATA-V entrapped in PVA particles consisted of PTFE tubes (Length 5 cm, OD 1/8", and ID 1/16", giving a working volume of app. 100 µL). The PVA particles for the characterization were lentil shaped, with diameters of 1-1.5 mm and a thickness of 0.1-0.2 mm. The catalyst loading in the reactor modules was determined by the weight of wet PVA particles. For characterization of the reactor module with the immobilized catalyst, some standard conditions were applied (fixed: 0.100 $mL \cdot min^{-1}$, temperature at 30℃, 100 mM pH 9.5 carbonate buffer, and 0.1 mM PLP). The concentrations of all other reaction species were varied sequentially, by changing the flow ratio to the reactor from different substrate reservoirs. Standard experiments with 10 mM BA and 500 mM IPA at various flowrates were performed at the start and end of each experiment for comparison of tested reactor modules and loss of biocatalyst activity. For each experimental set point, a minimum of 5 reactor volumes was flushed through before commencing the sampling and all samples were repeated 4-6 times. For these experiments, an on-line HPLC setup, with 2 µL sample loops, was put in place in order to accelerate the characterization, automate the setup and thereby minimize manual handling. The diagram of the on-line HPLC setup is shown in the supplementary material B.

Two-step liquid-liquid extraction *in-situ* product removal testing

A combined scaled-down system was put in place to test the promising ISPR concept. The combined system consisted of a PBR module and two LLE modules. The setup of the combined system is shown in figure 1. The flows in the system were operated by 4 Tecan syringe pumps with 250 µL syringes. Each pump had a large inner diameter (ID) PTFE tube attached to the suction side (ID 1 mm, L=300 mm, 236 µL), to prevent cavitation, and a smaller ID PTFE tube on the flow discharge side (ID 0.25 mm, L=200 mm, 10 µL). The tubes were connected to the pumps and the scaled-down unit operations by standard PEEK flangeless fittings. The applied PBR reactor module was a custom built reactor, i.e. a glass cylinder with PEEK connector in the ends (ID 10 mm, L=11 mm, ~864 µL). The reactor was connected to the first LLE module by a PTFE tube (ID 0.25 mm, L=300 mm, 15 µL). The LLE modules consisted of PTFE tubing (ID 0.25 mm, L=300 mm, 15 µL). The applied solvent undecane and the aqueous streams were combined and formed slugs before the LLE modules in a standard PEEK Y-connector for 1/16" tubing. The mixing in the LLE modules is dominated by diffusion at the phase interface at the end of each of the slugs and by internal mixing effects within the slugs, known as Hadamard-Ryczniski circulation (Bird et al., 2002). All the tubes from the suction side of the pumps and the outlets from the two LLE modules went into the two reservoirs, through a PTFE septum. 2 mL borosilicate glass test tubes (8x70 mm) were used as reservoirs/settlers. The reservoirs contained 1000 µL aqueous solution (same pH 9.5 mixture with reaction species as for the standard PBR characterization experiment or pH 3 buffer solution) and 750 µL solvent solution (undecane with 1 M BA). Undecane was chosen as the solvent since it enabled extraction of MPPA and simultaneous feeding of BA, see supplementary material A. During experiments, the reactor was submerged into a water bath (30℃) on a magnetic stirrer. The reservoirs and the two LLE modules were operated at ambient conditions (22±1℃). All pumps were operated at a flowrate of 0.300 $mL \cdot min^{-1}$, unless otherwise stated. For testing the system performance, an aqueous solution with pH 9.5 (100 mM carbonate buffer) containing 10 mM BA, 500 mM IPA and 0.1 mM PLP was prepared as the reaction phase. Another aqueous solution at pH 3 (100 mM citrate buffer) was prepared as the second extraction phase. Finally, a solvent solution of 1 M BA in

undecane was prepared as the extracting phase. Before starting the testing of the scaled-down system, tubes and pumps in the system were primed by flushing through with the respective solutions. Priming is done to remove air and fill dead volume with the prepared standard solutions. Furthermore, various flowrates of the reaction solution were flushed through the prepared PBR to evaluate its performance. The PBR was prepared by filling in a known amount of PVA particles containing ATA-V.

Results and discussion:

ATA-V characterization

For the analysis and characterization of immobilized ATA-V performance in the scaled-down PBR an on-line HPLC system was integrated with the PBR module (setup shown in supplementary material A). The combination of the scaled-down reactor and on-line HPLC setup enabled simple and fast characterization of the relative influence of the different reaction conditions (e.g the effect of changing composition of the reaction species, on the overall rate of reaction in the reactor). Additionally, the setup made it possible to perform characterizations without manual sample handling, which is recognized as an important feature to improve the accuracy, throughput, and reproducibility of data collection.

Figure 2 shows the variation and change of substrate and product concentrations influence the rate of reaction in the applied PBRs using immobilized ATA-V at fixed temperature and flows as described in the materials and methods section. The characterization profiles for varying BA and IPA concentrations in the reactor module indicate that the reaction rate increased with increasing substrate concentrations, in the entire studied range up to 500 mM IPA and 10 mM BA (figure 2.A and 2.B, respectively). The reaction rate profile of varying BA concentrations (figure 2.A) indicates that the overall reaction rate in the PBR can be increased significantly by increasing the BA concentration. However, the aqueous solubility limit of BA (~11 mM) prevents operation with higher BA concentrations. Therefore, the optimal operation of the proposed ISPR system with aqueous media will be achieved by saturating the reaction mixture with BA at all times. At

the same time it is desirable to maintain the IPA concentrations in the range of 500 mM (~50 times excess), to achieve as high a reaction rate as possible. The implication of these operating conditions on the ISPR process performance are discussed in the two-step LLE ISPR results section.

The reaction rate profiles for varying initial MPPA and ACE concentrations entering the scaled-down PBR module, show that the reaction rate decreases with increasing concentrations (figure 2.C and 2.D, respectively). In particular, the MPPA causes a drastic decrease in the relative reaction rate at relatively low concentrations, while it requires somewhat higher ACE concentrations for significant inhibition. Therefore, to ensure that the PBR operates with the highest possible reaction rate in aqueous solution it is necessary to remove the products as fast as possible during the reaction course to operate the PBR module as efficiently as possible. In this work, the focus has been kept on removal of MPPA due to the significantly higher inhibitory potential of MPPA.

Immobilization of ATA-V by entrapment in PVA particles was chosen since this formulation showed sufficient stability and activity at pH 9.5. The lentil shaped PVA particles, which are recognized to have low diffusional limitations (Neto et al., 2015) are cheap, long term stable, biologically inert and non-toxic immobilization material (Zajkoska et al., 2014).

Operating with a relatively high pH, close to the pK_a value of MPPA, is essential for the given ISPR concept to extract MPPA adequately. The immobilized enzyme preparation was stable under such conditions. (See supplementary material B for stability data at pH 9.5.)

Two-step liquid-liquid extraction *in-situ* product removal

To address the solubility and inhibitory issues of substrates and products, respectively, a two-step liquid-liquid extraction (LLE) ISPR process concept for the ATA process using modular scaled-down unit operations in combination was developed (figure 1). The two-step LLE ISPR process concept enables simultaneous substrate supply and product removal, making it a valuable process concept for intensifying ATA processes. The investigated two-step LLE ISPR concept exploits differences in both hydrophobicity and charge to

separate the hydrophobic amine product from hydrophilic amine donor and ketone substrate. The concept is inspired by the principles that have been exploited in the solvent bridge concept reported by Yun and Kim (Yun and Kim, 2008) and the supported liquid membrane (SLM) concept by Adlercreutz and co-workers (Börner et al., 2015; Rehn et al., 2014; Rehn et al., 2016). This work goes a step further by incorporating simultaneous substrate supply and product removal, along with enabling continuous extraction in two scaled-down LLE modules and settlers. The LLE modules were designed to ensure the two LLE steps operate as equilibrium steps (see supplementary material C).

The first LLE step exploits the pK_a values of the amine containing compounds. By operating the reaction close to the amine product pK_a value, a significant fraction of the (protonated) product can be extracted. However, a fraction of the available amine donor will also be extracted. The quantity of extracted amine donor relative to the amine product is dependent on differences in hydrophobicity and partitioning to the applied solvent. For example, the selectivity of this extraction step will improve if the amine product has a lower pK_a value than the amine donor, since it will ensure a higher ratio of the amine product to be protonated. In cases with similar pK_a values of the two amines, the separation selectivity is dictated by the differences in hydrophobicity.

The second LLE step also exploits the charge of the amine compounds and has increased aqueous solubility at pH values significantly lower than the pK_a values. Hence, mixing the amine-containing solvent with a low pH aqueous stream allows extraction of the amines from the solvent. Small quantities of the ketone substrate will be lost in this step. Low aqueous solubility of the ketone substrate is beneficial as it minimizes losses of the ketone substrate in the system, without the need for additional extraction steps to recover the ketone substrate.

Reaction profiles

Illustrated in figure 3, is the change in MPPA concentration in the two aqueous reservoirs over time, which clearly indicate that MPPA is efficiently extracted from the high pH reservoir (reaction reservoir) to the low

pH reservoir. This enables minimization of MPPA inhibition on the biocatalyst. The tested ISPR concept achieved MPPA concentrations of about 26.5 $g \cdot L^{-1}$ in the low pH reservoir in approximately 20 hours, resulting in a specific space-time yield (STY) of $\sim 0.27 \, g_{MPPA} \cdot L^{-1} \cdot h^{-1}$ based on the volume of the overall system. Under the used reaction and operating conditions the initial PBR activities for the experiments were estimated to be in the range of $\sim 1.6 - 2.5 \, U \cdot mg_{PVA}^{-1}$ ($\sim 32.1 - 50.1 \, U \cdot mg_{ATA-V}^{-1}$). As this STY also takes into account the volume occupied by the primary recovery step, a comparison of the STY with conventional batch based systems will be biased. If the STY is solely based on the volume of the high purity product solution, the low pH reservoir, then the STY is estimated to $\sim 1.3 \, g_{MPPA} \cdot L^{-1} \cdot h^{-1}$. The low STY is a significant limitation to the specific system, which has to be resolved in future work. In this context it is important to emphasize that the product solution reached MPPA purities in the range of 70 % $g_{MPPA} \cdot g^{-1}$ with a degree of recovery of approximately 80 % $mol \cdot mol^{-1}$ (see supplementary material D). I.e. the STY might be low, but it is possible to reach high product purity and recovery in the outgoing stream, which simplify the following downstream recovery. An additional, benefit of the given ISPR concept and the model reaction is that ACE is to some extent extracted as well, which delays the ACE buildup causing significant inhibitory effects (figure 4). Furthermore, the experimental results did not suggest that simultaneous extraction of IPA is a cause for concern (see supplementary material D).

Nevertheless, there are some challenges associated with the ISPR concept. BA is saturating both aqueous reservoirs during operation (see supplementary material D), which will cause a certain quantity of BA to be lost, dependent on the size of the low pH reservoir. Furthermore, it is necessary to operate the system until all the available BA in the system has been converted, as a means to approach full conversion. In this proof-of-concept study, the maximum achievable yield and degree of conversion was not tested since the focus was on achieving high product titers in the low pH reservoir. Alternatively, recycling the solvent can be used to exploit the available BA in the system. Additionally, IPA is applied in a large excess (more than 46-fold) relative to the maximum solubility of BA. It has previously been identified by Tufvesson and co-workers that applying more than 10 times IPA excess relative to available ketone greatly challenges the

economic feasibility of ATA processes (Tufvesson et al., 2015). However, this can be addressed by recycling IPA and BA to approach full conversion. Despite the fact that the experimental results presented in this work did not show IPA extraction in the setup, there is a possibility that small quantities of the available IPA will be extracted over time. For example, in the SLM system presented by Rehn and co-workers it was shown that their SLM system experienced simultaneous IPA extraction to a greater extent than seen in the scaled-down two-step LLE ISPR process (Rehn et al., 2014). It is possible, of course, that small quantities of IPA are extracted simultaneously in the two-step LLE ISPR process, but in that case the quantities are lower than the detection limit of the applied analytical method, see supplementary material D. Based on that it is not possible to assess if there is an actual difference between the scaled-down system and the SLM concept or if it is merely the result of altered response times due to the different volume ratios in the system.

Discussion

Optimization options

There are several ways to optimize the proposed two-step LLE ISPR process concept. One possibility for optimization is to minimize the volumes of the reservoirs. This will improve the response times in the system and thereby greatly accelerate experimental throughput. Furthermore, by improving response times in the system as well as the reducing the reservoir sizes then the time to reach high MPPA concentrations in the low pH reservoir will greatly decrease, improving the STY which is a major target for the further development of this system. This will enable the identification of the capacity of the system in a short time, in terms of achievable MPPA concentrations, purity and recovery. Furthermore, faster response times in the system will also reduce the build-up rate of the reaction species in the high pH reservoir over time. This is important since it will potentially allow to operate the biocatalyst while experiencing minimal product inhibitory effects. The latter is an essential prerequisite for evaluating the performance of the applied biocatalyst without the influence of inhibitory products.

Other multiphase biotransformations reported in the scientific literature, mainly focus on improving the space-time-yield (STY) and solubility of the applied reaction species (product removal and substrate feeding) (Bolivar and Nidetzky, 2013). The tested reaction in the two-step LLE ISPR process concept did not achieve high STY. The low STY is only in part related to the performance of the tested two-step LLE ISPR system, i.e. it is also related to the low activity of the applied biocatalyst. Despite not reaching high STY, the tested system also enabled to simultaneously remove, purify and recover the product and at the same time continuously feed the main substrate. High product titer and purity in the outgoing stream is an important feature for ISPR as it can greatly simplify the downstream recovery cost and complexity.

Implementation of on-line monitoring and control strategies in the two-step LLE ISPR concept can also improve the experimental throughput and data resolution, thereby making it possible to optimize the process in a fast manner, with minimal manual handling and interference. This will also benefit potential industrial applications of such ISPR concepts, since as it will provide a platform for monitoring and control of the process in an easy manner. For example, measuring the pH and titrating the high pH and low pH reservoirs with the amine donor and an acid, respectively, will make it possible to operate the system without application of expensive buffers. This concept is shown in supplementary material E. Titrating the reservoir will only cause slight dilution of the phases, provided concentrated solutions are applied. Keeping the reservoirs in control through pH and titration will make it possible to operate the system continuously by replacing the MPPA from the low pH reservoir over time with fresh acid solution. Application of spectroscopic methods (e.g. NIR has the potential for monitoring the composition of the extracting solvent in the system, which can be useful for following and refilling BA into the solvent).

Biocatalyst formulation

This work has mainly focused on the application of immobilized ATA by entrapment in PVA, since it was a straightforward way to apply and automatically recycle the biocatalyst in the scaled-down system. This choice of formulation makes it possible to avoid the biocatalyst contact with the liquid-liquid interface in

the LLE modules while achieving the large surface area present in the system (a high surface to volume ratio is a characteristic of such scaled-down systems). Immobilization in PVA was chosen since this formulation showed excellent performance in the conditions that were tested. Other immobilization strategies can be applied in the scaled-down system if required, which might solely require a change of reactor module. Bolivar and Nidetzky have previously reviewed the scientific literature on the subject of biotransformations operating with multiphase systems in different scaled-down reactor (Bolivar and Nidetzky, 2013). As an example, a promising alternative immobilization strategy is the quick and easy immobilization of whole cells in chitosan (Rehn et al., 2013). Tests with the combined scaled-down system were performed with this immobilization strategy also, but are not included here, but can be found in supplementary material section F.

Alternatively, if the applied biocatalyst is compatible with the surface interface then there is no problem in applying soluble enzyme in the system. This option will neglect the need for having dedicated reactor modules since the high pH reservoir could also act as the reactor. Membrane modules can be applied for product extraction in cases where the biocatalyst is not compatible with the surface interface of the extracting solvent. This has previously been shown by Fagaschewski and co-workers (Fagaschewski et al., 2012).

Overall, scaled-down reactor modules are quite versatile and can handle various biocatalyst formulations and thereby enable the possibility of testing a larger variety of reactor configurations compared to conventional batch based technologies.

Scale-up / scale-out

A key element for the application of scaled-down unit operations in a plug-and-play manner is the possibility of knowledge transfer across scales, to enable the transition to industrial scale production. However, it is difficult to achieve similar fast heat and mass transfer properties in large scale (Kockmann and Roberge, 2011). Processes that are developed in small scale, as presented here in scaled-down unit

operations, are either scaled-up or scaled-out (Jensen, 2001). Scale-out is based on operating scaled-down units in parallel, in order to reach the desired productivity. While scale-up is based on increasing the dimensions of the scaled-down unit operations to reach the desired production throughput. The advantages and disadvantages associated with both scaling strategies and the choice of the scaling strategy is highly dependent on the process and the applied biocatalyst. For example, scale-out has the benefit that it is possible to maintain heat and mass transfer properties, while scaling up will be a tradeoff in terms of heat and mass transfer properties. Increased heat and mass transfer limitations is also a common challenge when scaling-up from batch based small scale testing platforms, e.g. microtiter plates. Furthermore, scale-up gives the possibility of retrofitting new processes in pre-installed production equipment. Here, we foresee that computational fluid dynamics (CFD) will be increasingly used to support efficient scale-up, with a minimum number of required experiments.

Conclusions:

We have presented a robust scaled-down two-step liquid-liquid extraction (LLE) *in-situ* product removal (ISPR) process concept for ω-transaminase (ω-TA) processes, which enables simultaneous substrate supply and product removal. The given process concept is effective for addressing and overcoming several commonly experienced ω-transaminase process limitations (e.g. substrate solubility, substrate inhibition and product inhibition). This concept was validated through a case study focusing on the asymmetric synthesis of 1-methyl-3-phenylpropylamine (MPPA) using ω-TA. For the given case study, the tested two-step LLE ISPR concept made it possible to continuously feed the primary substrate benzylacetone (BA) and extract the primary product MPPA during the reaction course, thereby overcoming the problems of low substrate solubility and product inhibition. Remarkably, the tested ISPR concept made it possible to achieve a product concentration of 26.5 $g \cdot L^{-1}$ reaching a purity up to 70 % $g_{MPPA} \cdot g^{-1}$ and a recovery towards 80 % $mol \cdot mol^{-1}$ of the produced MPPA. In contrast, it would not have been possible to reach similar purity and product titers, with conventional batch or fed-batch operational modes, due to the required

excess of the amine donor and severe losses in catalytic activity with increasing product titer, respectively. Increasing STY remains a target for the future application of such systems for production, although for testing of new substrates and biocatalysts the system already shows great potential.

Overall, it was found that scaled-down unit operations combined in a modular plug-and-play manner enables easy and highly flexible testing of advanced biocatalytic process options, such as ISPR process strategies, which are difficult to test and operate in conventional batch mode. A key benefit of the plug-and-play experimental strategy is the possibility to compartmentalize the different scaled-down process steps while operating the combined system. Therefore, the application of scaled-down unit operations in a plug-and-play manner has great potential for the future development of new and advanced biocatalytic processes that are challenging to operate in conventional batch based technologies.

Acknowledgements:

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Schemes:

Scheme 1: ω-transaminase (ω-TA) reaction scheme for the synthesis of 1-methyl-3phenylpropylamine (MPPA) from benzylacetone (BA) using isopropylamine (IPA) as the amine donor. The co-product of this synthesis route is acetone (ACE).
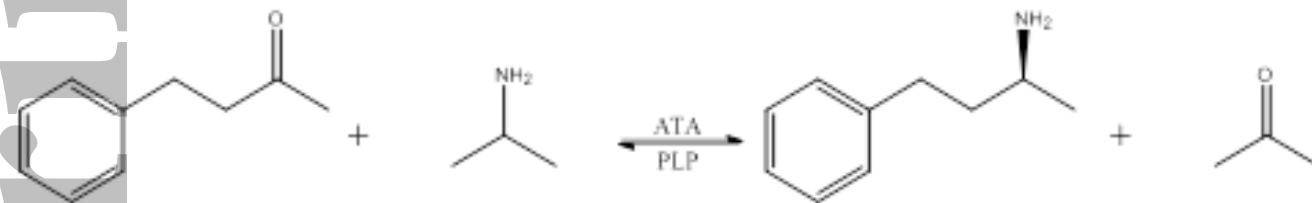
Figures:

Figure 1: *Top:* PI diagram of a scaled-down two-step liquid-liquid (LLE) *in-situ* product removal (ISPR) process concept for amino transferase (ATA) processes, which simultaneously can supply prochiral ketone substrate and remove hydrophobic chiral amine products. *Bottom:* Separation principle of the two-step LLE ISPR process concept. P1-P4: pumps operating the flow between the different unit operations in the system. R: scaled-down reactor module. LLE1: Liquid-liquid extraction step 1. LLE2: Liquid-liquid extraction step 2. V_1: High pH reservoir and settler 1. V_2: Low pH reservoir and settler 2. S_1: solvent reservoir and settler 1. S_2: solvent reservoir and settler 2.

Figure 2: Reaction rate profiles of packed-bed reactors containing PVA particles with entrapped ATA-V. A: fixed [BA] (10 mM) and varying [IPA] (50-500 mM). B: fixed [IPA] (500 mM) and varying [BA] (2-10 mM). C: fixed [BA] (10 mM) and [IPA] (500 mM) with varying [MPPA] (0-17 mM). D: fixed [BA] (10 mM) and [IPA] (500 mM) with varying [Ace] (0-200 mM). All experiments were performed at 30 ℃, in the presence of 0.1 mM PLP and applying a feed of 100 µL/min (~1 minute residence time).

Figure 3: Experimental results of the time dependent MPPA concentrations in the pH 9.5 (Left) and pH 3 (Right) aqueous reservoirs, with three different reactor loadings: 0.44 g_{PVA} (0.02 g_{ATA-v}) (□), 0.54 g_{PVA} (0.03 g_{ATA-v}) (✻), and 0.68 g_{PVA} (0.03 g_{ATA-v}) (Δ).

Figure 4: Experimental results of the time dependent ACE concentrations in the pH 9.5 (Left) and pH 3 (Right) aqueous reservoirs, with three different reactor loadings: 0.44 g_{PVA} (0.02 g_{ATA-v}) (□), 0.54 g_{PVA} (0.03 g_{ATA-v}) (✕), and 0.68 g_{PVA} (0.03 g_{ATA-v}) (Δ).
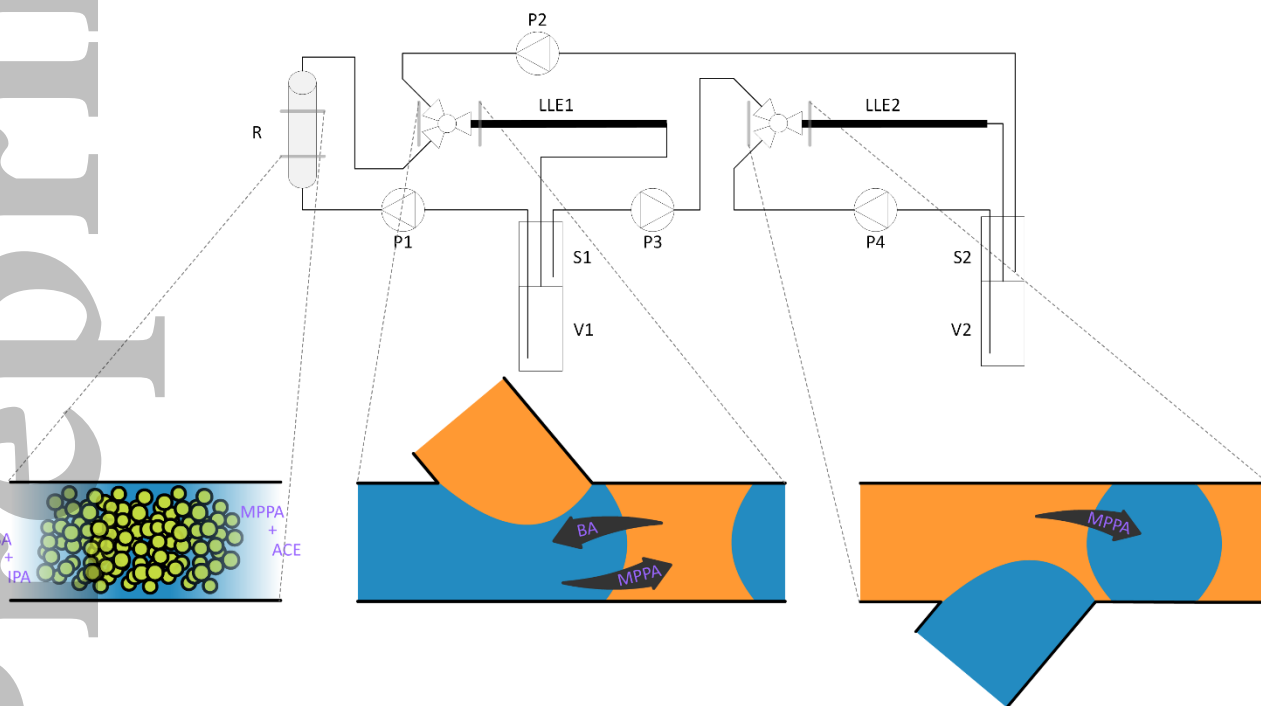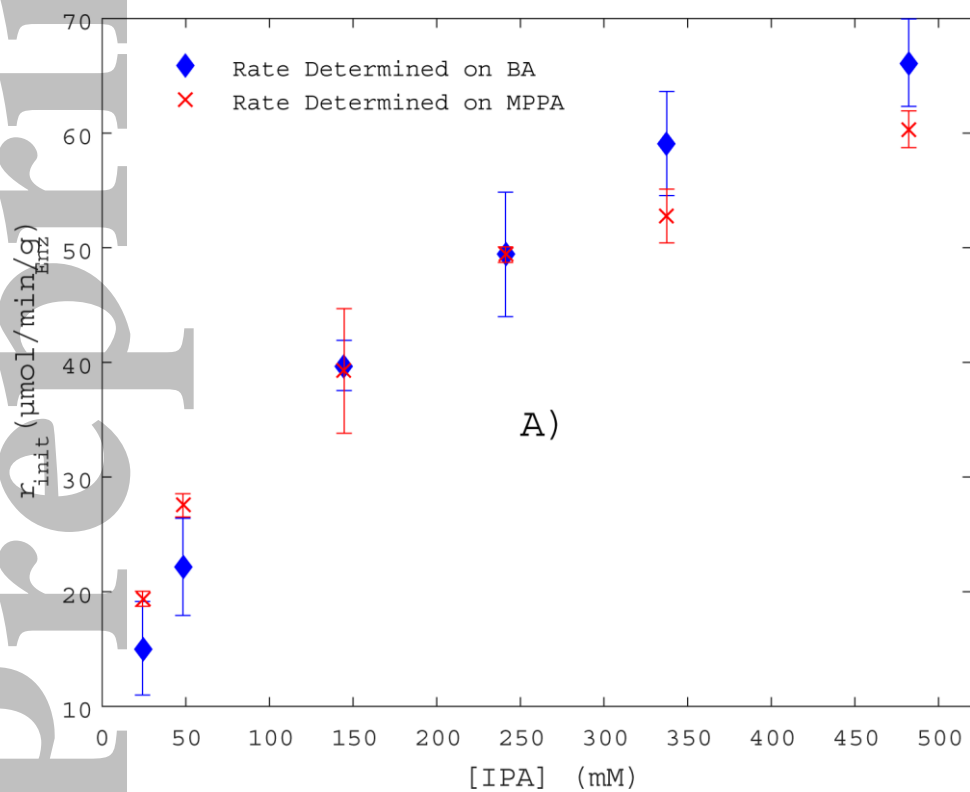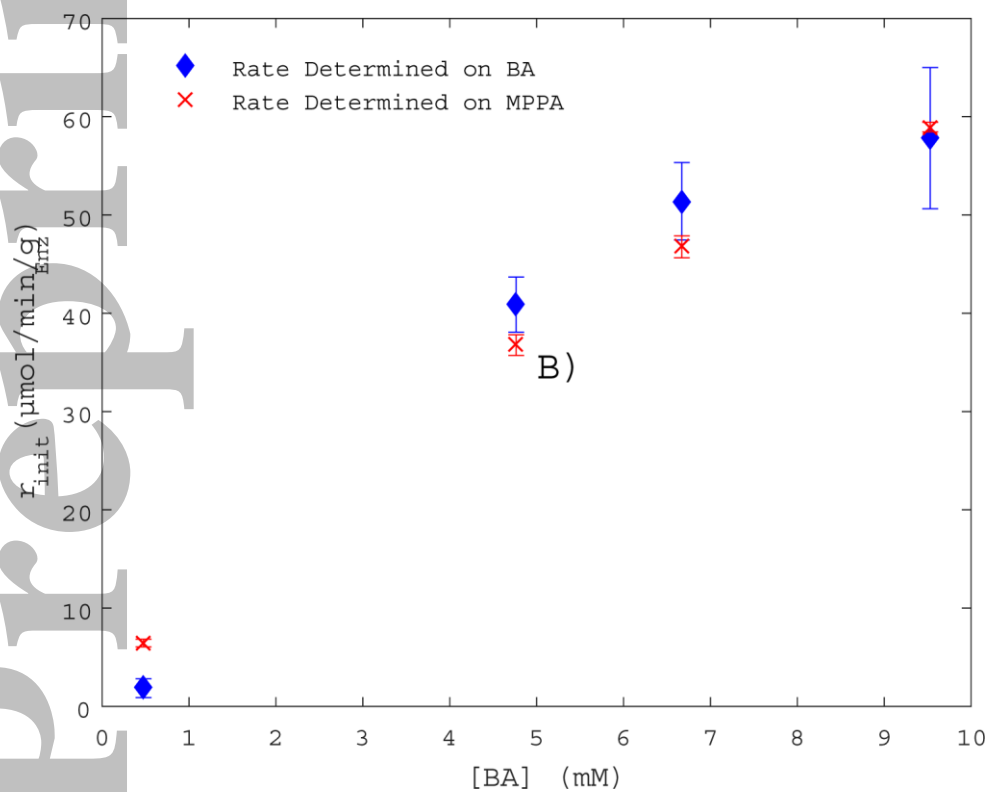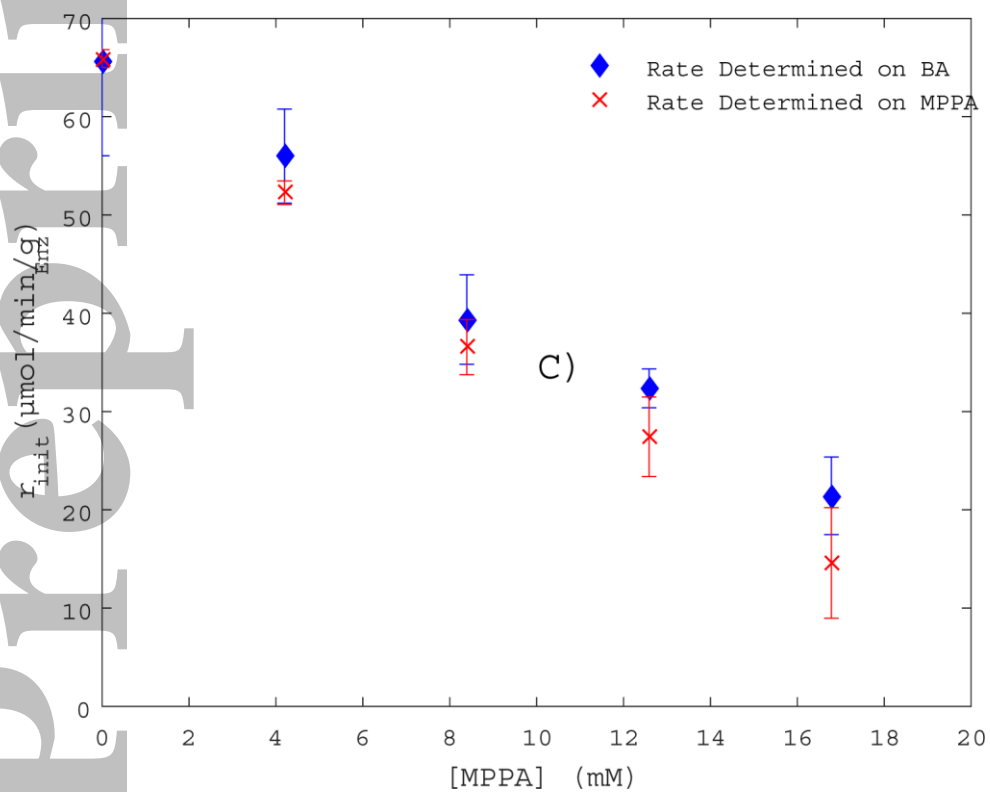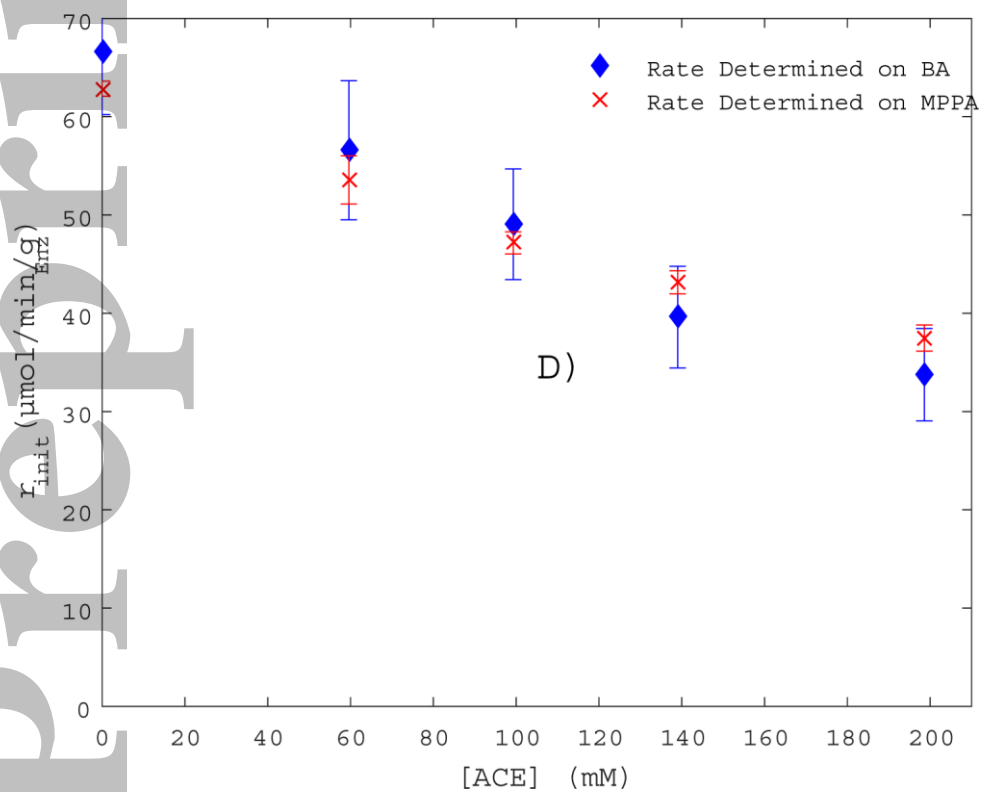
Scheme 1

Figure 1

Figure 2a

Figure 2b

Figure 2c

Figure 2d

Figure 3

Figure 4