Very Important Publication

Efficient Nicotinamide Adenine Dinucleotide Phosphate [NADP(H)] Recycling in Closed-Loop Continuous Flow Biocatalysis

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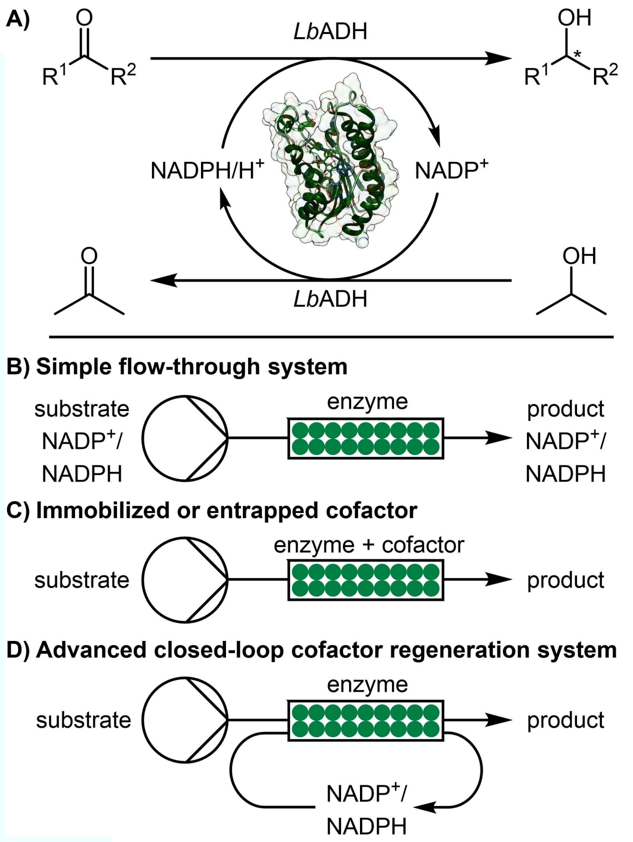
Abstract: Biocatalytic redox reactions regularly depend on expensive cofactors that require recycling. For continuous conversions in flow chemistry, this is often an obstacle since the cofactor is washed away. Here, we present a quasi-stationary recycling system for nicotinamide adenine dinucleotide phosphate utilizing an immobilized alcohol dehydrogenase. Four model substrates were reduced with high enantioselectivity as a proof of concept. The two-phase system enables continuous production as well as quick substrate changes. This setup may serve as a general cofactor regeneration module for continuous biocatalytic devices employing (co-)substrates being miscible in organic solvent. The system resulted in space-time yields up to 117 $\mathrm{g\,L^{-1}\,h^{-1}}$ and total turnover numbers for nicotinamide adenine dinucleotide phosphate higher than 12,000 mol/mol are possible.

Keywords: cofactor regeneration; enzyme catalysis; flow chemistry; oxidoreductases; phase separation

Introduction

Biocatalysis is playing an ever-increasing role in the synthesis of fine chemicals, pharmaceuticals, and natural products.[1] Enzymes are already widely used in industrial applications as they readily enable access of highly functionalised products due to their high regio-, stereo- and enantioselectivity.[2] At the same time, also flow chemistry approaches affected many fields of synthesis including biocatalysis:[3] the catalyst is immobilised within a reactor, the substrates are passed by and the final product should rinse out ideally without any need for purification.[4] However, while this looks conceptually simple, the implementation is often challenging, especially when it comes to cofactor regeneration.[5] In the present work a standalone module for chemoenzymatic reactions in continuous flow with regard to closed-loop cofactor regeneration was developed. The proof of principle was demonstrated using a nicotinamide adenine dinucleotide phosphate [(NADP(H)]-dependent alcohol dehydrogenase (ADH, Scheme 1.A). Three different conceptual approaches were developed in the past,[6] which will be briefly explained below (Scheme 1.B–D).

The first concept (Scheme 1.B) is a simple flow-through system. Usually, a mixture of substrate, cofactor, buffer etc. is pumped through a reactor filled with immobilised enzyme. The substrate is converted into product and the cofactor is oxidised/reduced equimolarly. The entire solution leaves the reactor again including the cofactor. Different cofactor regeneration methods are used, but since it is not recirculated but washed out, the cofactor consumption and costs, respectively, will scale proportional to the substrate amount.[7] The second concept towards continuous cofactor regeneration (Scheme 1.C) utilises (co-)immobilised or (co-)entrapped cofactor, allowing a stream of substrate, buffer etc. to pass by and



Scheme 1. A) Overview of the *Lb*ADH from *Lactobacillus brevis* catalysed asymmetric reduction of ketones including a substrate-based cofactor recycling.[7a,b,f,i,8c,11] B) Simple flow-through system. A substrate together with the cofactor either NADP$^+$ or NADPH are pumped through a reactor filled with immobilised enzyme. C) Flow-through systems with immobilised enzyme and immobilised or entrapped cofactor. D) Closed-loop cofactor regeneration system. The cofactor remains inside the aqueous phase, while the product and acetone (co-product) are extracted into the organic layer to leave the column. The protein structure was generated from pdb:1zk4.[12]

perform several reaction cycles.[3i,6b,8] In case of immobilisation, just recent work by *Scott et al.* demonstrated the use of PEG-immobilised NAD(H) as well as ATP and made use of the flexibility of a linker to reach the active catalytic centre. Excellent space-time yield (STY) and total turnover number (TTN) over 10,000 mol/mol could be achieved.[6a] Moreover, in recent years the (co-)entrapment of the cofactor in matrices has also moved into focus and led to impressive results, such as remarkable TTNs over 14,000 mol/mol.[7f,g] Unlike the first concept (Scheme 1.B), no continuous addition of cofactor is required. However, these methods depend on chemical modifications of the cofactor and enzymes tolerating these modification and entrapment, respectively. Thus, the method cannot be considered as a general solution.

Scheme 1.D shows a closed-loop cofactor regeneration system separating an aqueous layer including the ionic cofactor (*e.g.* NADH) from the organic layer containing the product. This discrimination in polarity allows recirculation of the cofactor-layer. *Šalić et al.* used two micromixers in series for this purpose, one for product formation and one for cofactor regeneration. The process remained stable for three days under continuous conditions without addition of fresh cofactor.[9] Another innovative system was recently published by *Paradisi* and *Contente* performing a functional group interconversion from amines into alcohols. After phase separation the aqueous layer was refed into the system. A high TTN of 2,000 mol/mol for NADP$^+$ was achieved.[6a,10] Nevertheless, it was reported that a dilution of the substrate feed could not be prevented.

The presented continuous cofactor regeneration systems demonstrated impressively the proof-of-principle. However, an ideal cofactor regeneration system serving as a blueprint for future applications would need to fulfil several requirements:

- *Catalytic use of the cofactor*: The cofactor should remain within the oxidoreduction-module or should be recirculated. There should be no further addition of cofactor to run the system continuously. In addition, the cofactor should not be diluted during the course of reaction.
- *Modularity*: The system should be a standalone module that can easily be integrated into flow cascades. Various substrates can be applied to the very same setup without cross-contaminations. The cofactor regeneration can be used for various enzymes rather than single cases.
- *Easy handling*: There should be no need for chemical modifications of the respective cofactor to ensure the natural function of the enzyme. The system should be independent of the kind of immobilisation of the biocatalyst.

To setup a new modular system, we based our work on the studies of *Döbber et al.* using an immobilised Halo-tagged alcohol dehydrogenase from *L. brevis* (*Lb*ADH). It catalyses the enantioselective asymmetric reduction of prochiral ketones to the corresponding secondary alcohols (Scheme 1.A).[7a,b] We propose a standalone closed-loop cofactor regeneration system using a membrane-based phase separation technique. It enables the usage of cofactor-dependent enzymes in continuous flow chemistry and previously impossible rapid substrate changes without changing the recirculated cofactor solution.

Results and Discussion

Simple Flow-Through System

Initially a simple flow-through system (*cf.* Scheme 1. B) with co-substrate (2-propanol)-coupled cofactor regeneration was established. This has been extended by a *Syrris Asia FLLEX* (Flow Liquid Liquid EXtraction) system (see Figure 1). It allows a simple phase separation of an aqueous cofactor-containing layer and an organic product-containing layer. As organic solvent, ethyl acetate was introduced via channel *2*. The setup was utilized to determine phase separation efficiency as well as a screening for preliminary process parameters. HaloTag-immobilised *Lb*ADH was used in a packed-bed reactor to reduce acetophenone (**1 a**) asymmetrically yielding (*R*)-phenylethan-1-ol (**2 a**) as the product (Scheme 2).

Various reactions were carried out. For this purpose, the flow rates (FR) were varied from 30 up to 500 µL/min and different concentrations of the co-substrate 2-propanol were used. For a concentration of 50 mM of **1 a**, 10% (v/v) 2-propanol and a flow rate of 30 µL/min, a turnover of 92% was achieved (lit. 95%[7a]). This turnover remained constant even at higher flow rates of up to 65 µL/min, which means a doubled productivity and STY with respect to previous results.[7a] An increase in the 2-propanol concentration to 15% (v/v) resulted in marginal increase of conversion of approx. 1%. For further details see supporting information (SI). Furthermore, the phase separation was possible and thus was applied in the advanced systems described below. Although many organic substrates are to a certain extend water soluble, they preferably solve in organic layers whilst the charged cofactor strictly remains within aqueous layers. So, it is possible to carry-out the reaction in aqueous phase or even in an aqueous/organic-mixture and extract the cofactor afterwards to feed it again to the enzyme reactor.[10a]

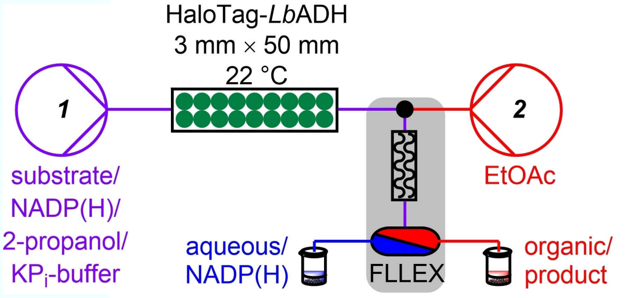
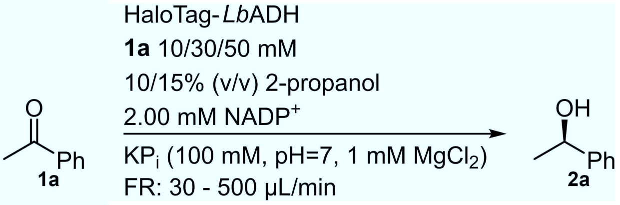


Figure 1. Schematic representation of the simple flow-through system. It consists of two pumps: One for the reaction mixture (channel *1*) consisting of the substrate, NADP$^+$, 2-propanol and KPi-buffer and one for the organic extraction solvent (channel *2*). The packed-bed-reactor is filled with Halo-tagged *Lb*ADH and a FLLEX is applied for phase separation. Colour code: Blue indicates the aqueous, red the organic and purple the mixed stream. Acetone and 2-propanol are found in both layers after extraction.



Scheme 2. Reduction of acetophenone (1a) to (*R*)-phenylethan-1-ol (2a) by substrate-coupled regeneration of NADPH using an immobilised *Lb*ADH variant.[7a]

Closed-Loop Cofactor Regeneration System

Structure of device: The new advanced closed-loop cofactor regeneration system is shown in Figure 2. In contrast to the simple flow-through system (Figure 1), a third channel (channel *2*) providing the substrate and the co-substrate 2-propanol was added to the biocatalyst cartridge and the aqueous layer containing the cofactor (channel *1*) is refed to the cartridge after passage and phase separation, respectively. This has

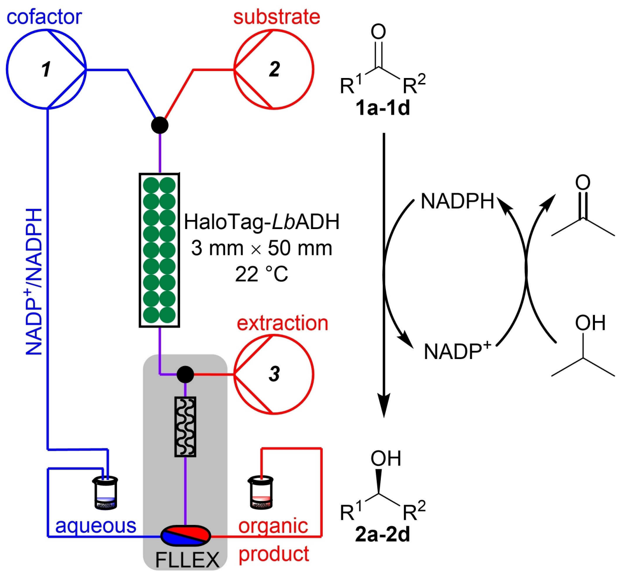


Figure 2. Schematic overview of our system for continuous cofactor regeneration. It consists of two pumps containing four separated channels in total. Channel *1* is used for the aqueous/cofactor stream and *2* for the substrate and co-substrate (2-propanol). Channel *3* delivers extraction solvent. *1* and *2* are connected via a y-piece. The column is filled with immobilised HaloTag-*Lb*ADH as catalyst. The last and important device is the Asia FLLEX for extraction and phase separation. Colour code: Blue indicates the aqueous, red the organic and purple the mixed stream.

two advantages: the cofactor can be used in catalytic amounts and multiple turnovers are performed during the retention time in the enzyme reactor allowing for full-conversion in dependence of the flow-rate and catalyst loading. Once the mixture leaves the enzyme reactor an organic solvent (channel *3*) is added for product extraction. Finally, the layers are separated by the *FLLEX*-system, which also prevents any layer from dilution over time, which is one of the issues described in literature.[10a] The organic layer contains the product, while the aqueous, NADP(H)-containing layer is again mixed with fresh substrate dissolved in the co-substrate 2-propanol. The substrate/co-substrate is fed and the product is extracted continuously, respectively, whilst the cofactor remains within the recirculated aqueous layer. Due to this closed-loop set up NADP(H) is no longer removed with the product, but efficiently regenerated, which enables a further decrease to catalytic amounts (0.1 mol% relative to the substrate).

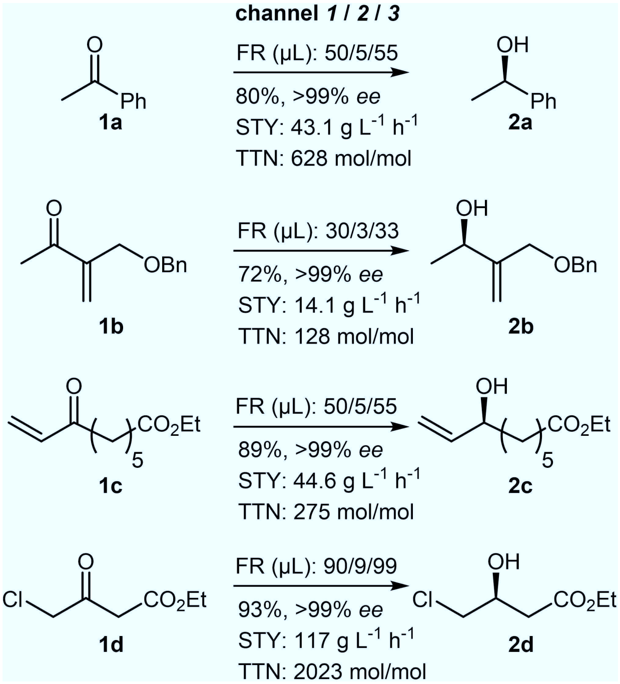
Comparing simple flow-through vs closed-loop system: A comparison of the simple flow-through system with the closed-loop has been performed. Therefore, the flow rates were optimised and compared with the previous results (see SI Table 2 and ref.[7a]) of the very same reaction (Scheme 2). Two mixtures were prepared: 1.00 mL of 0.6 mM NADP$^+$ in KP$_i$ for channel *1* (aqueous layer) and 500 mM acetophenone (**1 a**) in 2-propanol for channel *2*. Conversions were determined by gas chromatography (GC) and the flow rates and results are shown in Table 1. The screening results show that the closed-loop system produces comparable results to the simple flow-through system. For example, for a combined flow rate of approx. 30 μL/min, a conversion of 96% was achieved (simple flow-through system 92% and lit. 95%).[7a]

Application on different substrates and continuous run: The reliability of the system has been demonstrated for the reduction of acetophenone (**1 a**). But as *Lb*ADH shows a broad substrate range,[7a,c,f,11a,c,d,13] the closed-loop cofactor regeneration system was tested for three further substrates as well. Different ketones **1 b**–**1 d** were reduced to the corresponding secondary alcohols **2 b**–**2 d**. To obtain high STY, some flow rate optimisation was carried out and for the ketones **1 a** and **1 d**, the NADP$^+$-concentration was lowered from 0.6 mM to 0.3 mM. More details of the studies can be found in the supporting information and the respective conditions are shown in Scheme 3.

Moderate to high space-time yields from 14 g L^{-1} h^{-1} up to 117 g L^{-1} h^{-1} and high to very high total turnover numbers from 128 mol/mol up to 2023 mol/mol could be achieved with excellent enantioselectivities (>99%). Furthermore, the aqueous phase was extracted and analysed with respect to the absence of substrate/product. In fact, NMR-signals neither for substrate nor for product could be detected in the extracted aqueous layer (see SI). Moreover, three different extraction solvents such as dichloromethane, ethyl acetate, and diisopropyl ether were tested successfully, which offers a certain flexibility for follow-up reactions in future studies. Furthermore, the system including closed-loop substrate-coupled cofactor regeneration demonstrated its reliability by continuous runs over 32 h without loss in performance. In total, 1.36 g of alcohol **2 d** could be obtained with a STY of 121 g L^{-1} h^{-1} (96%). Even longer runs were possible without loss on performance (Figure 3). The

Table 1. Optimisation of flow rates with closed-loop cofactor regeneration system according to Figure 2 (transformation of **1a**). The given errors are technical standard deviations (SD) from individual runs (N = 3).

flow rate [μL/min]			conversion
channel *1*	channel *2*	channel *3*	[%] ± SD
27.0	2.7	29.7	96.6 ± 1.8
36.0	3.6	39.6	92.5 ± 0.4
45.0	4.5	49.5	92.4 ± 0.4
54.0	5.4	59.4	91.4 ± 0.1
59.0	5.9	64.9	93.0 ± 1.4



Scheme 3. Reduction of different ketones **1 a**–**1 d** to the corresponding alcohols **2 a**–**2 d** using HaloTag-*Lb*ADH in flow chemistry with continuous cofactor regeneration system (Figure 2). Isolated yields after purification. The enantiomeric excesses were determined by GC and HPLC, respectively. The configuration was validated by optical rotatory power. Space-time yield (STY) was calculated as shown in the supporting information. The total turnover number (TTN) refers to NADP$^+$ consumption.

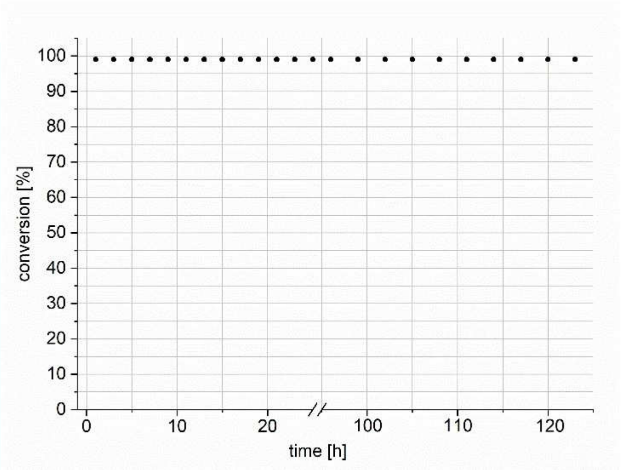


Figure 3. Performance of closed-loop continuous cofactor regeneration system (see Figure 2) over 123 h. A 500 mᴍ solution of ketone **1 d** was pumped through the system at a flow rate of 90/9/99 µL/min (channel *1*/*2*/*3*) and converted into alcohol **2 d** by using one stock solution of NADP$^+$. Conversion was determined by ^{1}H-NMR. The sampling was done in time mode and samples were collected for 2 h per test tube.

TTN could be drastically increased to 12,855 mol/mol, which is to the best of our knowledge the highest value in literature for closed-loop continuous cofactor regeneration systems.[6a,9–10]

Application on serial conversions: The sound phase-separation system offers the opportunity for using the very same setup including the very same batch of enzyme and cofactor for several transformations. To prevent cross-contaminations it was necessary to have an intermediate flushing giving the benefit of total product recovery from the device (Figure 4). Following the setup shown in Figure 2, only a rinsing step after each reduction was added. After the reduction of the first ketone (**1 d**, step 1), a solution of a water miscible organic solvent (here 300 mᴍ of acetone) in KP$_i$ was pumped through the system (step 2). Then, the second ketone (**1 c**) was reduced (step 3), followed by the same rinsing step (step 4). In fact, this procedure worked well as neither the products nor the aqueous phase were contaminated with substrate/product from a previous run. Alcohol **2 d** could be received in 92% and alcohol **2 c** in 87% yield. Furthermore, in another experiment it was found that the washing step has no influence on the activity of *Lb*ADH (see SI, Table 8).

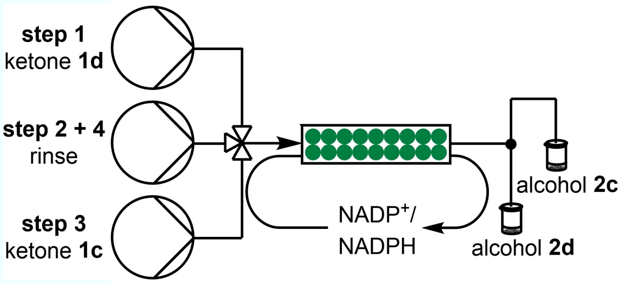


Figure 4. Schematic overview of a consecutive experiment using the setup shown in Figure 2 with one cofactor solution for the whole experiment. Substrate 1 [500 mᴍ in 2-propanol, flow rate: 90/9/99 µL/min (channel *1*/*2*/*3*)]. is used for the reaction (step 1), followed by a short rinsing step with 300 mᴍ acetone in KP$_i$ (step 2). Then substrate 2 (200 mᴍ in 2-propanol, FR: 50/5/55 µL/min) is reduced (step 3) and followed by another rinsing step (step 4).

Conclusion

In summary, a well-functioning system for ADHs and water-insoluble substrates was developed with the help of a phase-separation system (here *Asia FLLEX*). As a basis, the already established system of *Lb*ADH and 2-propanol as co-substrate was used. Since a permanent recycling and regeneration of the cofactor was possible, only catalytic quantities were required. It is a closed-loop setup allowing for continuous runs without subsequent supplementation of cofactor and the concentration of which should be constant within the biocatalytic cartridge (steady-state) since no dilution takes place. We demonstrated excellent performance with full conversion for the reduction of ketone (**1 d**) in a 123 h run. Four different ketones could be reduced to the corresponding alcohols in good to very good yields and excellent enantioselectivities. Different organic solvents were successfully tested for extraction of the products using the *Asia FLLEX* module. The system is a closed module, which can be used for serial biotransformations with the very same setup. As we have demonstrated, different ketones can be reduced with just one batch of cofactor solution, when implementing rinsing steps.

Finally, while the system has its limitations if exclusively water-soluble substrates including cofactor-regenerating substrates such as glucose are used, it can be regarded as a generic module that could also be used with other oxidoreductases, either in separate or parallel mode. If this approach is not possible, one can also implement enzyme-coupled cofactor regeneration by including a second oxidoreductase for that purpose. In addition, the system might be combined with other preceding or follow-up reaction modules in organic solvent.

Experimental Section

General Information

The starting materials used in this work were commercially purchased by *Merck KGaA*, *TCI International* and *Alpha Aesar* with the exception of ketone **1 c** and **1 d**. They were synthesised according to literature procedures.[14] The solvents used were in GC grade purity. For flash chromatography *Macherey-Nagel* 60 M (0.040–0.063 mm, 230–400 mesh) was used as silica gel. For analytical TLC finished films from *Macherey-Nagel,* type *POLYGRAM® SIL G/UV254* with fluorescence indicator were used. The plates were developed using a UV lamp at a wavelength of 254 nm and/or by staining with an aqueous potassium permanganate solution. NMR spectra were measured on a *Bruker 600 Ultra Shield™* with a frequency of 600 MHz for ^{1}H– and 151 MHz for ^{13}C-spectra. Only deuterated solvents were used. Chemical shifts were determined by using solvent or tetramethyl silane as internal standard and are given in ppm. Coupling constants *J* are given in Hz. Multiplets are described as s (singlet), d (doublet), t (triplet), q (quartet) and p (pentet). Furthermore, for evaluation DEPT-(135°-pulse)-, ^{1}H–^{1}H–COSY-, ^{1}H–^{13}C–HSQC- and ^{1}H–^{13}C–HMBC-spectra were taken. IR: Infrared spectra were recorded on a *PerkinElmer FT-IR SpectrumTwo* spectrometer. Measurements were taken in ATR mode. The absorption bands were given in $\tilde{\upsilon}$ [cm^{-1}]. The rotary power was measured on a *KRÜSS P8000-TF.* Probes were solved in $CHCl_3$ and measured in a 5.00 cm cuvette at 589 nm (sodium-D-line). The specific rotation was calculated with the *Biot* equation. HPLC: HPLC enantiomeric analysis was done on a *Thermo Scientific Dionex UltiMate 3000.* As stationary phase a *Phenomenex Lux Amylose* and as mobile phase *n*-heptane:2-propanol (90:10) was used. GC: Conversion and enantiomeric excess were measured with gas-liquid chromatography. GC-chromatograms were measured on a *Thermo Scientific TRACE® GC Ultra.* The probes were dissolved in MTBE. Carrier gas was H_2 at 0.6 bar. For the detection, a FID-detector was used. For exact HPLC and GC methods see SI.

Syrris Asia Flow Devices:

Syringe Pump: For the attempts with a flow rate up to 200 µL/min, the pumps were equipped with yellow syringes (50.0 µL/100 µL). For greater flow rates, they were equipped with blue ones (500 µL/1.00 mL).

FLLEX: The *FLLEX* module was equipped with original *FLLEX* PTFE or Merck FHLP02500 PTFE membranes. In the last case, the polyethylene support was removed. The total system pressure was set to 3.00 bar and the cross-membrane pressure (CMP) was dependent on the solvent (100 mbar for ethyl acetate, 50.0 mbar for CH_2Cl_2 and 50.0 mbar for diisopropyl ether).

Automated collector: The collector, type: *Gilson FC 203B*, was used for the collection of products.

Software: The controlling of the devices was done with the *Asia* Manager PC Software (1.69 beta) and herein presented flow rates were defined in the pump settings.

Enzyme Digestion and Immobilisation Procedure

2.00 g cell pellet of HaloTag-*Lb*ADH produced in *E. coli* BL21 (DE3) was resuspended in 8.00 mL KP_i (100 mM, pH 7, 1.00 mM $MgCl_2$). For cell lysis ultrasonication has been applied and the crude cell extract has been centrifuged (7084 rcf). Ultrasonication was carried out using a *Bandelin SONOPLUS* with a *SONOPLUS KE76* cone tip. The mode used was 2 × 5 min, 5 × 10% cycle at 35% power. The supernatant was used for immobilisation without further filtration steps. Immobilisation was done in flow mode. Therefore, an *Omnifit* column (3 mm × 50 mm) was filled with HaloLink resin (Promega, ~400 mg wet weight) and plugged to an *Asia* syringe pump. Afterwards, it was washed with KP_i-buffer (100 mM, pH 7, 1.00 mM $MgCl_2$) for 30 min at a flow rate of 250 µL/min. Then, 6.00 mL of supernatant was pumped through the column at 30 µL/min and collected afterwards. Finally, the washing step was repeated.[7a] The total activity before immobilisation was 768 U and afterwards 406 U. The difference of 362 U is the theoretical maximum activity of enzyme in the column.

(*R*)-Phenylethan-1-ol (2 a): Screening with Simple Flow-Through System

A stock solution of acetophenone (**1 a**) in different concentrations (10, 30, 50 mM), 10/15% (v/v) 2-propanol, KP_i (100 mM, pH 7, 1.00 mM $MgCl_2$) and $NADPNa_2$ (2.00 mM) was prepared and filled in channel *1* (see Figure 1). As extraction solvent ethyl acetate was used. An *Omnifit* packed-bed reactor (3.00 mm × 50.0 mm) filled with Halo-tagged *Lb*ADH was used as catalyst and reactions were performed twice with flow rates ranging from 30.0 to 500 µL/min. The results are presented in Table 2 (SI). The determination of conversion was done by GC.

(*R*)-Phenylethan-1-ol (2 a): Screening with Closed-Loop Continuous Regeneration System

Three different channels were needed to perform the screening with closed-loop continuous regeneration system (cf Figure 2). Channel *1* (aqueous cofactor containing layer) was filled with 1.00 mL 0.5 mM $NADPNa_2$-solution in KP_i. Channel *2* (substrate and co-substrate containing layer) was filled with 500 mM solution of acetophenone (**1 a**) in 2-propanol. Channel *3* (organic extraction solvent) was filled with ethyl acetate. As catalyst, an immobilised Halo-tagged *Lb*ADH was used and filled into an *Omnifit* (3.00 mm × 50.0 mm) column. Different flow rate combinations were used (see Table 1 and SI Table 3). Recirculation of the aqueous layer started right after the dead volume of the system was overcome. After finishing the reactions, the conversion was determined by GC. The results are shown in Table 1 and SI Table 3. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.44–7.32 (m, 4H, ArH), 7.31–7.25 (m, 1H, 5-H), 4.91 (q, $^3J_{1,1'} = 6.5$ Hz, 1-H), 1.81 (s, 1H, OH), 1.51 (d, $^3J_{1',1} = 6.5$ Hz, 1'-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 146.15 (C-2), 128.86 (C-3,7/4,6), 127.84 (C-5), 125.73 (C-3,7/4,6), 70.80 (C-1), 25.53 (C-1'). IR (ATR): 3339, 3064, 3029, 2973, 2928, 2877, 1493, 1450, 1368, 1284, 1261, 1203, 1096, 1076, 1029, 1010, 997, 897, 759, 697, 606, 539. $R_f = 0.40$ (*n*-pentane:EtOAc 8:2), $[\alpha]_D^{20} = +44{,}6$ (c = 1.16 in $CHCl_3$)

(*R*)-3-[(Benzyloxy)methyl]but-3-en-2-ol (2 b): Synthesis with Closed-Loop Continuous Regeneration System

Based on the setup shown in Figure 2, channel *1* was filled with 1.00 mL 0.5 mM $NADPNa_2$-solution in KP_i. Channel *2* was filled with a 200 mM solution of ketone **1 b**. Therefore, 23.2 mg (0.12 mmol) were solved in 583 µL 2-propanol. As extraction solvent in channel *3* diisopropyl ether was used. As catalyst an immobilised HaloTag-*Lb*ADH filled into an *Omnifit* column (3.00 mm × 50.0 mm) was used. The flow rate combination was set to 30/3/33 µL/min (aqueous/organic/extraction). Both the collection of the organic layer and the recirculation of the aqueous layer started right after the dead volume of the system was flown through. After the reservoir of channel *2* was empty, 2-propanol was added, and the run was continued. After completion, the organic layer was evaporated and a ^{1}H-NMR was taken. A conversion of 80% (20% of substrate) was achieved and 21.0 mg of product/substrate mixture was collected. Purification of the product was done via flash chromatography (*n*-pentane:EtOAc 8:2) and the product was obtained as yellow oil in 72% yield (16.7 mg, *ee* < 99%) with a STY of 14.1 $\mathrm{g\,L^{-1}h^{-1}}$ and a TTN of 128 mol/mol. ^{1}H NMR (600 MHz, DMSO-d_6): δ 7.38–7.31 (m, 4H; ArH), 7.31–7.26 (m, 1H; 9-H), 5.11 (s, 1H; 2'-H), 5.02 (s, 1H; 2'-H), 4.78 (s, 1H, OH), 4.46 (m, 2H, 5-H), 4.17 (q, $^3J_{3,4} = 6.8$ Hz, 1H; 3-H), 4.01 (q, $^3J_{1\mathrm{gem}} = 13.2$ Hz, $^3J_{1,2'} = 1.2$ Hz, 1H; 1-H), 1.17 (d, $^3J_{4,3} = 6.5$ Hz, 3H; 4-H). ^{13}C-NMR (151 MHz, DMSO-d_6): δ 150.39 (C-2), 138.47 (C-6), 128.24 (C-7,11/8,10), 127.41 (C-9), 127.36 (C-9), 109.24 (C-2'), 71.28 (C-5), 69.83 (C-1), 66.69 (C-3), 22.58 (C-4). IR (ATR) 3401, 3030, 2976, 2859, 1652, 1496, 1454, 1366, 1260, 1204, 1070, 1040, 1028, 908, 735, 696, 602, 467 $\mathrm{cm^{-1}}$. HRMS (ESI): m/z for $C_{12}H_{16}O_2 + Na^+$: calcd: 215.1043 $[M+Na]^+$; found: 215.1044 $[M+Na]^+$; calcd: 216.1076 $[M+Na]^+$; found: 216.1079 $[M+Na]^+$. $R_f = 0.33$ (PE:EtOAc 8:2). $[\alpha]_D^{20} = +16$ (c = 1.00 in $CHCl_3$).

Consecutive Experiment

Based on the setups shown in Figure 2 and Figure 4, a stock solution of $NADPNa_2$ was prepared. Therefore 0.5 mg (0.64 µmol) $NADPNa_2$ was dissolved in 1.00 mL KP_i (100 mM, pH 7, 1.00 mM $MgCl_2$) and filled into channel *1*. With this batch, two different ketones were converted to the corresponding alcohols. Diisopropyl ether in channel *3* was used as extraction solvent. An *Omnifit* column (3.00 mm × 50.0 mm) filled with immobilised HaloTag-*Lb*ADH was used as biocatalytic cartridge. For step 1, channel *2* was filled with a 500 mM solution of ketone **1 d**. Therefore, 22.9 mg (0.14 mmol) were solved in 260 µL 2-propanol. The flow rate combination was set to 90/9/99 µL/min (aqueous/organic/extraction). Both the collection of the organic layer and the recirculation of the aqueous layer started right after the dead volume of the system flown through. After the reservoir of channel *2* was empty, 2-propanol was added and the run was continued. After completion, an intermediate flushing with 300 mM acetone in KPi (100 mM, pH 7, 1.00 mM $MgCl_2$) was performed for 10 min (step 2). Then, step 1 was repeated for ketone **1 c** (step 3). This time, 21.0 mg (0.11 mmol) were solved in 512 µL 2-propanol resulting in a 200 mM solution. The remaining steps were carried out as described above, but the flow rate combination was set to 50/5/55 µL/min and the intermediate flushing was performed for 15 min. The collected organic layers were evaporated and a ^{1}H-NMR was taken. A full conversion was achieved and product **2 d** yielded in 92% as yellow oil (*ee* < 99%, 21.0 mg, STY 114 $\mathrm{g\,L^{-1}h^{-1}}$, after step 1 and 2). For product **2 c**, a product/substrate mixture of 97/3 was collected, which was further purified by flash chromatography (*n*-pentane:EtOAc 8:2). Afterwards, the product was collected as yellow oil in 87% yield (*ee* < 99%, 18.2 mg, STY 30.9 $\mathrm{g\,L^{-1}h^{-1}}$, after step 3 and 4). Product **2 c**: ^{1}H NMR (600 MHz, DMSO-d_6): δ 5.82–5.76 (m, 1H; 8-H), 5.12 (d, $^3J_{9\text{-trans},8} = 17.2$ Hz, 1H; 9-trans-H), 4.97 (d, $^3J_{9\mathrm{cis},8} = 10.5$ Hz, 1H; 9-cis-H), 4.65 (d, $^3J_{\mathrm{OH},7} = 4.80$ Hz, 1H; OH), 4.04 (q, $^3J_{1',2'} = 7.10$ Hz, 2H; 1'-H), 3.89 (m, 1H; 7-h), 2.26 (t, $^3J_{2,3} = 7.38$ Hz, 2H; 2-H), 1.50 (p, $^3J_{3,4} = 7.36$ Hz, $^3J_{3,2} = 7.38$ Hz, 2H; 3-H), 1.38–1.33 (m, 2H; 6-H), 1.33–1.23 (m, 4 h; 4-H, 5-H), 1.17 (t, $^3J_{2',1} = 7.10$ Hz, 3H; 2'-H). ^{13}C NMR (151 MHz, DMSO-d_6): δ 172.70 (C-1), 142.53 (C-8), 112.78 (C-9), 70.77 (C-7), 59.46 (C-1'), 36.60 (C-6), 33.32 (C-2), 28.30 (C-4), 24.46 (C-3), 24.33 (C-5), 13.98 (C-2'). IR (ATR): 3443, 2987, 2934, 2860, 1733, 1463, 1423, 1373, 1266, 1180, 1119, 1095, 1029, 991, 919, 737, 669, 591 $\mathrm{cm^{-1}}$. $R_f = 0.26$ (PE:EtOAc 8:2). $[\alpha]_D^{20} = 5.32$ (c = 1.08 in $CHCl_3$). Product **2 d**: ^{1}H NMR (600 MHz, $CDCl_3$): δ 4.28–4.23 (m, 1H; 3-H), 4.19 (q, $^3J_{1',2'} = 7.2$ Hz, 2H; 1'-H), 3.64–3.58 (m, 2H; 4-H), 3.12 (d, 1H; OH), 2.67–2.59 (m, 2H; 2-H), 1.28 (t, $^3J_{2',1'} = 7.2$ Hz, 3H; 2'-H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 172.15 (C-1), 68.32 (C-3), 61.39 (C-1'), 48.48 (C-4), 38.78 (C-2), 14.50 (C-2'). IR (ATR): 3452, 2983, 1722, 1406, 1374, 1304, 1259, 1188, 1151, 1086, 1058, 1030, 951, 894, 850, 803, 756, 709, 557, 473 $\mathrm{cm^{-1}}$. $R_f = 0.30$ (PE:EtOAc 8:2). $[\alpha]_D^{25} = -26.6$ (c = 1.25 in $CHCl_3$).

Calculation of Space-Time Yield (STY)

The STY was calculated according to the following equation:

$$\mathrm{STY} = \frac{\text{Yield [g]}}{\text{column volume [L]*time [h]}}$$

column volume = $3.53 \cdot 10^{-4}$ L

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