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Continuous biphasic enzymatic reduction of aliphatic ketones

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ABSTRACT

Biphasic reactions offer an attractive alternative for the utilisation of enzymes for conversion of hardly water soluble substrates. Especially, the alcohol dehydrogenase from *Lactobacillus brevis* was successfully used for the reductive synthesis of enantiopure secondary aliphatic alcohols. With the enzymatic catalyst and the cofactor effectively retained in the reactive aqueous phase, the continuous operation was demonstrated by continuous addition and withdrawal of the non-reactive phase. The four tested substrates 2-heptanone, 2-octanone, 2-nonanone, and 2-decanone showed that the space time yield and turnover numbers (TON) of the enzyme decrease as the availability of the substrate decreases with increasing partition coefficients. Nevertheless, a TON_{LbADH} of up to 478×10^3 could be achieved. Remarkably, the cofactor utilisation turned out to be very high and a TON_{NADP^+} of more than 20×10^3 was easily achievable for both 2-heptanone and 2-octanone by substrate coupled cofactor regeneration with excess of 2-propanol.

1. Introduction

The synthesis of enantiomerically pure alcohols is either carried out by kinetic resolution or by direct asymmetric synthesis. Kinetic resolution was almost exclusively carried out by using hydrolases [1], for which prominent examples for robust and versatile enzymes can be found. The maximum yield for a kinetic resolution is 50%, which is unfavourable both economically and ecologically. The asymmetric reduction of prochiral ketones with alcohol dehydrogenases (ADH) offers an attractive alternative [1] which is more and more introduced in the fine-chemical and especially API-production [2–4]. Chiral aliphatic alcohols are of special interest as they are widely applicable as building blocks in the fine-chemical- and in the pharma-industry. For example, chiral aliphatic alcohols can serve as cosurfactant in capillary electrophoresis for resolution of the enantiomers of ephedrine-derivatives [5], as a building block for the synthesis of chiral liquid-crystals with interesting optical properties [6], or as a derivative for polymeric aniline to produce solid electrodes for the recognition of chiral compounds [7]. The direct enantioselective reduction of ketones to the corresponding alcohols represents a reaction with high added value in the range of 100-fold. Classical chemical routes like hydrogenation with modified noble metal-catalysts or Corey–Bakshi–Shibata reduction provide product alcohols with high enantiomeric excess (*ee*) only from functionalised ketones [8,9]. Enantiopure, aliphatic non-branched and non-functionalised chiral alcohols are hardly accessible *via* non-enzymatic synthesis-routes. For 2-heptanol an *ee* of 51% [10] and for 2-octanol an *ee* of 60% [11], 52% [10], 76% [12], and 28% [13] are reported. The main challenges for the enzymatic asymmetric reduction lies in the dependency of the ADH on high-priced nicotinamide cofactors (NAD(P)H) as redox mediator and the utilisation of both cofactor and enzyme.

To recycle biocatalysts and cofactors, several methods are available such as immobilisation [14], retention by ultra- or nanofiltration membranes [15–17], entrapment approaches [18], or the use of biphasic systems [19,20]. Immobilisation often implicates laborious steps to derive an active catalyst for the specific reaction system. When an immobilisation protocol is available, the flexibility of the system is restricted. Membrane techniques allow a straightforward use of the biocatalyst. If isolated/free enzymes are used, the retention of cofactors remains an issue. By means of nanofiltration, a partial retention of the cofactor is possible [15]. However, substrates, products or auxiliary agents can be in the same range of molecular weight as the cofactors and thus render nanofiltration impossible to selectively retain the cofactor. An alternative to immobilisation on solid supports and membrane based retention is the application of aqueous–organic biphasic systems. The enzyme and the cofactor are dissolved in the aqueous, reactive

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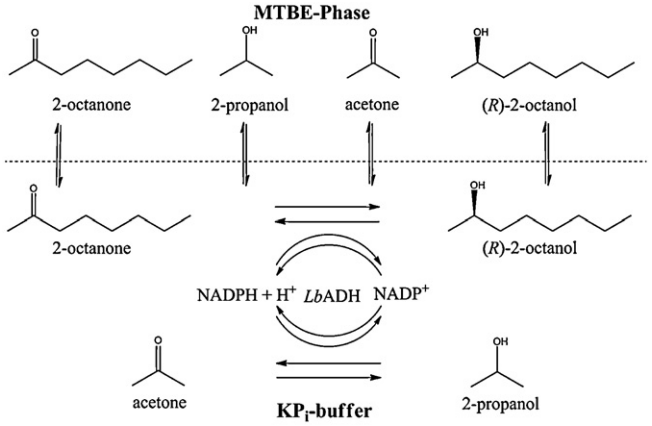

Fig. 1. Biphasic reaction system for the enantioselective reduction of aliphatic ketones.

phase, whereas the non-reactive phase acts as a substrate reservoir and extraction phase, respectively.

An enzyme showing outstanding (enantio-)selectivity, activity and robustness is the alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*ADH) [21]. The enzyme is depending on NADPH as cofactor and compulsory needs Mg^{2+}-ions to maintain its activity [22]. *Lb*ADH is (*R*)-selective. It has been shown previously that *Lb*ADH is stable in the presence of organic solvents, especially in the presence of methyl-*tert*-butyl-ether (MTBE) [19]. Although a slight increase in the reaction rate has been reported using an ionic liquid as second phase [23], MTBE was preferred as second phase due to its lower viscosity, which simplifies the dosage. Another reason is the low boiling point of 55 °C for MTBE which allows for an easy down-stream-processing as well as the lower price when compared to most ionic liquids. In this report, we investigated the continuous exchange of the MTBE non-reactive phase allowing high turnover numbers ($mol_{product}/mol_{catalyst}$, TON) for the cofactor and the enzyme as well as the integration of product separation into the reaction.

The aim of this project was to use this approach to continuously synthesise long-chain (*R*)-2-alcohols. A biphasic approach was chosen for the afore mentioned advantages such as easy reuse of cofactor and enzyme as well as the facile product separation. As the non-reactive organic phase, methyl-*tert*-butyl-ether (MTBE) was chosen as it has already been proven that *Lb*ADH remains stable in the presence of this solvent [19]. The system has already been tested with 2-butanone as model substrate and turned out to be highly suitable for this purpose [24,25]. However, short-chain ketones such as butanone are water miscible, which is not the case for long-chain aliphatic ketones such as 2-octanone. So, the continuous enantioselective reduction is a way to allow the conversion in water as the reactive phase. To be able to conduct continuous synthesis, substrate coupled cofactor-regeneration with 2-propanol is an appropriate choice as it can be continuously added with the non-reactive phase and the coupled product acetone can be continuously removed (Fig. 1). Most regeneration agents for an enzyme-coupled approach, such as glucose or formate, are not soluble in organic media and can therefore not be dosed conveniently. Also, coupled products would accumulate in the non-reactive phase in the case of gluconic acid, the coupled product of the glucose-based cofactor regeneration. Furthermore, both coupled products, gluconic acid as well as CO_2 will strongly influence the pH of the aqueous solution. In order to determine appropriate reaction conditions, preliminary investigations on stability, kinetics, partitioning, equilibrium, and interphase formation were conducted. Results from these experiments were later transferred to the continuous syntheses of (*R*)-2-heptanol, (*R*)-2-octanol, (*R*)-2-nonanol, and (*R*)-2-decanol.

2. Experimental

Reporting our experimental data, we followed the standards given by Gardossi et al. [26] as closely as possible. All ketones and alcohols were purchased from Sigma–Aldrich, Schnelldorf. All other chemicals came from Carl-Roth, Karlsruhe. *N*-methyl-*N*-trimethylsilyl-trifluoracetamide (MSTFA) came from CS-Chromatographie-Service, Langerwehe. 2-Propanol and acetone were of technical grade. Ultrapure water was obtained by reverse osmosis and ultrafiltration in an ELGA purelab ultra-system and used throughout the experiments.

All reactions were carried out in potassium phosphate buffer with a concentration of 50 mmol L^{-1}, containing 2.5 mmol L^{-1} $MgCl_2$ at pH = 7.0. First, a buffer solution with a phosphate-concentration of 500 mmol L^{-1} was prepared by dissolving the respective amounts of KH_2PO_4 and K_2HPO_4 in H_2O. The desired buffer-solution was then prepared by dissolving the respective amount of $MgCl_2 \cdot 6H_2O$ in H_2O and adding the respective volume of concentrated buffer-solution to give three quarters of the desired final volume. For stock solutions of 2-octanone, 2-octanol, 2-propanol or acetone, the respective amount was added. The pH was subsequently adjusted to the desired value by adding dropwise KOH or H_3PO_4 and the solution was filled to the final volume. For the kinetic measurements and determination of the protein content (see below), no pretreatment was carried out. For the determination of partition coefficients and use in the continuous synthesis, the buffer was saturated with MTBE by adding 10% of the buffer volume. The buffer was then shaken and equilibrated for at least 24 h at room temperature (23–26 °C).

The protein content of the *Lb*ADH-lyophilisate was determined according to the literature [27] with a commercial kit (Carl Roth, Germany). Calibration was carried out with bovine serum albumin. The protein-content of the enzyme preparation was 48%, all activities and enzyme concentrations refer to the protein content of the enzyme preparation (specific activity).

Samples of *Lb*ADH, NADPH and $NADP^+$ were stored at 25 °C, 300 rpm in a thermomixer MKR13, HLC. For the *Lb*ADH, one sample was taken immediately after dissolution and in distinct time intervals, the activity was measured at 25 °C, $c_{2\text{-octanone}}$ = 1.8 mmol L^{-1}, c_{NADPH} = 0.5 mmol L^{-1}. For the half-life of the cofactors, $NADP^+$- and NADPH-solutions were dissolved. Samples were withdrawn and mixed with substrate solution (final concentrations: $c_{2\text{-octanone}}$ = 3.6 mmol L^{-1} and $c_{2-\text{octanone}}$ = 1.8 mmol L^{-1}). A concentrated *Lb*ADH-solution was added and the extinction at λ = 340 nm was measured before and after adding the enzyme. From the difference in extinction, the remaining amount of cofactor was deduced. For both, *Lb*ADH and the cofactors, a first order degradation kinetic was assumed and the half life was determined by fitting a first order exponential function [28].

If not stated otherwise, the kinetic measurements were carried out with non-MTBE-saturated buffer at 25 °C in a UV/VIS-multiplate reader (Powerwave HT, BioTek). The reaction volume was 180 μL and prepared in 96-well plates (greiner bio-one). 10 μL of a buffer solution containing 10 mmol L^{-1} NADP(H) was added to give a final concentration of 0.5 mmol L^{-1}. The reaction was started with 10 μL of a solution containing 50 mg L^{-1} *Lb*ADH preparation (≅ 24 mg L^{-1} protein) to give a final concentration of 2.5 mg L^{-1} *Lb*ADH preparation (≅ 1.2 mg L^{-1} protein) in each well. Reaction progress was observed by following the formation or degradation of NADPH at 340 nm. For the oxidation of 2-octanol to 2-octanone and for inhibition experiments, (rac)-2-octanol was used. The concentration of (*R*)-2-octanol was obtained by halving the total concentration.

Kinetic measurements with 2-heptanone as substrate were carried out as described for 2-octanone, but at 30 °C and in KP_i-buffer with a concentration of 100 mmol L^{-1} and 1 mmol L^{-1} $MgCl_2$.

For the determination of partition coefficients in ternary systems, samples of 2-octanone and 2-octanol with concentrations between 20 and 100 mmol L^{-1} were prepared in water-saturated MTBE. These samples were analysed *via* GC to verify the 2-octanone- and 2-octanol-concentration and 5 mL of these samples were placed into screw capped test tubes. 5 mL of MTBE-saturated buffer was added. The test tubes were closed, vigorously shaken and stored at 25 °C in a water bath. The organic and the aqueous phase were then separately analysed *via* GC.

For the determination of partition coefficients in multi-component systems, the aqueous phase was prepared as described above. Concentrated solutions of 2-octanone, 2-octanol, 2-propanol and acetone were prepared in water-saturated MTBE and mixed together to represent conversions of $X_{2\text{-octanone}}$ = 0, 20, 40, 60, 80, and 100% if the reaction had started with a 2-octanone concentration of 100 mmol L^{-1} and a 2-propanol concentration of 1000 mmol L^{-1} in the organic phase. A sample of the mixtures in MTBE was withdrawn and analysed *via* GC.

Monophasic batch experiments were carried out in a standard non-MTBE saturated buffer solution. The different mixtures were prepared by weighing the respective amounts of 2-octanone, 2-octanol, 2-propanol and acetone into a test tube with screw cap. 1000 μL of a $MgCl_2{\cdot}(H_2O)_6$ solution with a concentration of 25 mmol L^{-1} was then added as well as 1000 μL of the concentrated buffer-solution. Water and $NADP^+$ stock-solution were added to obtain a final concentration of 0.1 mmol L^{-1} $NADP^+$. The reaction was started with *Lb*ADH-solution, the final protein content was 12 mg L^{-1} protein. The overall volume was 10 mL. The test tubes were shaken at 25 °C at 300 rpm. Samples were withdrawn and analysed for conversion.

Biphasic batch experiments were carried out by preparing 2-octanone and 2-propanol stock-solutions in water-saturated MTBE. Defined volumes of both solutions and water-saturated MTBE were put in a test tube with screw cap. The aqueous phase was prepared with pre-MTBE saturated buffer concentrate and water as described for the monophasic batches with a Mg^{2+}-concentration of 9.7 mmol L^{-1}. A $NADP^+$-solution was added to obtain a final concentration of 0.1 mmol L^{-1} and the reaction was started by adding *Lb*ADH-solution so that the protein content was 250 mg L^{-1}. The overall aqueous volume was 4 mL just as the volume of the organic phase. Samples were withdrawn in defined time intervals from the organic phase.

Investigation of the interphase was conducted by mixing KP_i-buffer with saturated MTBE in combination with 2-propanol and *Lb*ADH lyophilisate. The samples were stirred at ≈500 rpm and in all phases *Lb*ADH activity was determined.

For the continuous synthesis, a biphasic reactor was used as described previously [24]. The upper, MTBE phase was stirred by using a magnetic stirrer (Variomag Micro, Thermo Scientific). The lower phase was stirred using a teflonated magnetic stirring bar. The mobile MTBE-phase was prepared by dissolving the respective ketone and 2-propanol in water saturated MTBE to give final concentrations of $c_{2\text{-ketone}}$ = 100 mmol L^{-1} and $c_{2\text{-propanol}}$ = 1000 mmol L^{-1}. The substrate solution was then stored with a slight excess of water at 25 °C. The aqueous phase was prepared with MTBE-saturated KP_i-buffer. *Lb*ADH and $NADP^+$ were dissolved in buffer and a defined volume was added to obtain final concentrations of 480 mg L^{-1} and 0.1 mmol L^{-1} respectively. Buffer was added to a final volume of 5 mL aqueous phase. The capillaries and the pump were flushed with substrate solution, and 5 mL of organic phase was placed above the aqueous phase. Substrate solution was continuously pumped into and out of the reactor by two countercurrent working syringe pairs in a syringe pump (MDSP3f, MMT Micromechatronic, Siegen/Germany). The operating temperature of the reactor was 25 °C. A flow cell (V = 107 μL) was integrated in the product stream and samples were taken automatically by an autosampler and analysed by GC.

The analysis of the organic phase was carried out by taking a 200 μL sample, mixing it with 400 μL of a solution of 20 mmol L^{-1} 1-octanol in ethanol and analysing the mixture by GC. For analysis of the product stream in the continuous synthesis, 0.5 μL sample and 1 μL of a solution of 20 mmol L^{-1} 1-octanol in ethanol were taken from a flow cell with an autosampler and immediately injected into a GC for analysis. In the case of the 2-heptanone and 2-nonanone experiment, the internal standard was 1-octanol and 1-nonanol respectively in the same concentrations as for the 2-octanone experiments. For the analysis of the monophasic batches, 200 μL sample was mixed with 400 μL of a 20 mmol L^{-1} ethanolic solution of 1-octanol. The mixture was immediately extracted with *n*-heptane by vigorous agitating on a vortex-shaker. After the phase-separation, the *n*-heptane phase was separated from the aqueous phase to prevent further conversion. To get reliable data for the concentrations in the aqueous phase for the partitioning experiments, a concentration by extraction had to be carried out. 800 μL of the aqueous phase was mixed with 200 μL of a 1 mmol L^{-1} solution of 1-octanol in water. The whole mixture was extracted with 200 μL of *n*-heptane and subsequently analysed *via* GC. All samples were measured on Trace GC Ultra or Focus GC (both Thermo Scientific, Dreieich/Germany) on a CP-ChirasilDex CB column (Varian; l = 25 m, ID = 0.25 mm, film = 0.25 μm) as described previously [29]. *ee* was determined after derivatisation with *N*-MSTFA as described previously [29].

3. Results and discussion

A key requirement for a successful continuous process is the stability of the involved catalysts (respectively enzymes) and cofactors. Thus, the stability of *Lb*ADH, $NADP^+$ and NADPH were examined independently by storing samples of each compound in pure buffer as well as in MTBE-saturated buffer with and without addition of 2-propanol, and acetone. The half life of *Lb*ADH was between 9 and 14 days, for NADPH between 0.56 and 0.65 days, and for $NADP^+$ between 155 and 370 days (see ESI for detailed information). Therefore, the stability of the oxidized cofactor $NADP^+$ is not a limiting factor for the synthesis. With ≈10 days, the half-life of *Lb*ADH is lower, but this value is still sufficient for a continuous setup. Due to the short half-life for NADPH this is limiting for the continuous approach.

Kinetic measurements are an important tool to characterise an enzyme. In order to obtain a deeper insight, initial reaction rates were determined for all four reactions, M, M′, R and R′ (Table 1) with 2-octanone as substrate. For every reaction, the reactant concentrations as well as the effect of the MTBE content were varied (see Supporting information). Investigation of the effect of the respective co-substrate on a specific reaction (effect of acetone on M for example) was not reasonable. As only the concentration of the reduced cofactor NADPH can be determined spectrophotometrically, it is not possible to distinguish between the conversion of 2-octanone and acetone in this special case. An extended Michaelis–Menten based model.

$$v = v_{max}[S][S_{\mathrm{NADP(H)}}] \left[K_{\mathrm{M}}\left(1+\frac{[P]}{K_{I,P}}\right)\left(1+\frac{[P']}{K_{I,P}}\right)+[S]\left(1+\frac{[S]}{K_{I,S}}\right)\right]^{-1} \left[K_{\mathrm{NADP(H)}}\left(1+\frac{[P_{\mathrm{NADP(H)}}]}{K_{I,P_{\mathrm{NADP(H)}}}}\right)+[S_{\mathrm{NADP(H)}}]\right]^{-1} \quad (1)$$

Table 1
Reactions characterised by initial rate measurements.

	S	$S_{NADP(H)}$		P	$P_{NADP(H)}$	P'
M:	2-Octanone	+NADPH	→	(*R*)-2-octanol	+NADP$^+$	2-Propanol
M′:	(*R*)-2-octanol	+NADP$^+$	→	2-Octanone	+NADPH	Acetone
R:	2-Propanol	+NADP$^+$	→	Acetone	+NADPH	2-Octanol
R′:	Acetone	+NADPH	→	2-Propanol	+NADP$^+$	2-Octanone

Table 2
Parameter values of Eq. (1) for 2-octanone/2-octanol (dashes indicate that the corresponding inhibition was not found to be significant).

	M	M′	R	R′
v_{max} (U mg^{-1})	51.0 ± 1.7	15.4 ± 0.4	4.8 ± 0.1	21.6 ± 0.6
K_M (mmol L^{-1})	0.056 ± 0.005	0.511 ± 0.042	3.417 ± 0.511	0.064 ± 0.009
$K_{I,P}$ (mmol L^{-1})	0.031 ± 0.004	–	0.035 ± 0.006	0.010 ± *n.a.*
$K_{I,P'}$ (mmol L^{-1})	0.96 ± 0.11	5.5 ± 0.4	0.004 ± 0.0006	–
$K_{I,S}$ (mmol L^{-1})	–	–	–	374 ± 30
$K_{NADP(H)}$ (mmol L^{-1})	0.160 ± 0.018	0.036 ± 0.002	0.067 ± 0.007	0.032 ± 0.006
$K_{I,P_{NADP(H)}}$ (mmol L^{-1})	0.088 ± 0.011	0.012 ± 0.001	0.155 ± 0.042	–

with limiting rate v_{max}, substrates S, products P, inhibitor P', Michaelis–Menten constants $K_{NADP(H)}$ and K_M, as well as inhibition constants K_I were then used to describe the measured initial rates. The parameters were estimated by a non linear fitting to the experimental data (Origin 8G, OriginLab Corp.). The parameter values are given in Table 2 and plots of the respective models are shown in ESI. MTBE concentration showed no significant influence and was not considered in the model (see Supporting information).

Apparently, the reduction of 2-octanone M is fast compared to its reverse-reaction M′. The v_{max} is more than threefold higher for M than for M′ (Table 2). Furthermore, the reductive cofactor regeneration R is slower than the cofactor oxidation R′, with a fourfold lower v_{max} for the latter. The respective K_M-values reflect this trend in the same manner. Unfortunately, product- and co-product-inhibition, reflected by the parameters $K_{I,P}$ and $K_{I,P'}$ were highly pronounced especially for the two reactions M and R which are important for producing the desired substance, (*R*)-2-octanol. This means, that the main reaction M is inhibited by high concentrations of (*R*)-2-octanol as well as by high concentrations of 2-propanol. In view of the high excess of 2-propanol needed to obtain a reasonable conversion (see below), and the partition coefficient which leads to a 2-propanol-concentration of 420 mmol L^{-1} in the aqueous phase, these inhibitions are disadvantageous. The same is true for the inhibition by the corresponding product-cofactor, represented by $K_{I,P_{NADP(H)}}$. Nevertheless, these inhibitions are intrinsic properties of the enzyme and their adjustment is not within the scope of our research. We focussed on the elaboration of a process within the imposed boundaries.

For comparison, 2-heptanone was also tested as a substrate (Table 3). In turn, 2-heptanone is less well accepted than 2-octanone by *Lb*ADH which is mirrored by the lower value of v_{max} and from the higher Michaelis–Menten constant K_M. For both 2-heptanone and 2-octanone oxidation M′ no inhibition by the corresponding ketone was observed. Initial rate experiments for 2-nonanone/2-nonanol proved to be futile as the solubility limit [30] of less than 1 mmol L^{-1} led to inconclusive results.

Table 3
Parameter values of Eq. (1) for 2-heptanone/2-heptanol at 30°C (dashes indicate that the corresponding inhibition was not found to be significant).

	M	M′
v_{max} (U mg^{-1})	41.8 ± 1.4	14.6 ± 0.139
K_M (mmol L^{-1})	0.134 ± 0.02	0.315 ± 0.013
$K_{I,P}$ (mmol L^{-1})	0.223 ± 0.03	0.026 ± 0.001
$K_{I,P'}$ (mmol L^{-1})	38.36 ± 4.88	–
$K_{NADP(H)}$ (mmol L^{-1}	0.21 ± 0.012	0.053 ± 0.002

3.1. Partitioning

The partition properties of 2-octanone and 2-octanol were determined in ternary systems (C_8-component, MTBE and KP$_i$-buffer) and for multiple systems with concentrations chosen to represent extents of conversion ($X_{2\text{-octanone}}$) in the system consistent of 2-octanone, 2-octanol, 2-propanol, acetone, MTBE, and aqueous KP$_i$-buffer (see Supporting information).

In a two phase-system the partitioning of a substance i is described by the Nernst partition law, where P_i is the partitioning coefficient of i. $c_{org,i}$ represents the concentration of i in the upper organic phase, $c_{aq,i}$ is the concentration of i in the lower aqueous phase:

$$P_i = \frac{c_{org,i}}{c_{aq,i}} \quad (2)$$

In the ternary system, the average partitioning coefficient P for 2-octanone is 330 ± 24 and 350 ± 29 for 2-octanol. Both are concentration independent. In the multiple component system, the partitioning coefficient for 2-octanone is unchanged with 330 ± 49 compared to the ternary systems. The observed partitioning coefficient for 2-octanol in this system of 400 ± 40 is higher than in the ternary system (see Supporting information). A slight increase of P as a function of $X_{2\text{-octanone}}$ up to 40% conversion could be observed in the multiple component system with $P_{2\text{-octanol}} > P_{2\text{-octanone}}$.

Partitioning of 2-propanol and acetone was investigated in ternary systems with MTBE and KP$_i$-buffer (ESI). For both acetone and 2-propanol, P is concentration independent with $P_{acetone} = 1.47 \pm 0.06$ and $P_{2\text{-propanol}} = 1.38 \pm 0.04$. Hence, a selective extraction of acetone is not possible. With a starting concentration of 1 mol L^{-1} 2-propanol in the organic phase, the equilibrium concentration is 0.42 mol L^{-1} in the aqueous phase. At this concentration range, the reaction rate of *Lb*ADH is pseudo-zero order, thus the biphasic system will not influence the cofactor regeneration rate (Fig. 2 and ESI).

An aqueous 2-octanone concentration of 0.3 mmol L^{-1} results for a concentration of 100 mmol L^{-1} in the MTBE-phase at a volume ratio of one. The kinetic investigations revealed a sufficient *Lb*ADH activity for 2-octanone concentrations of 0.3 mmol L^{-1} in Fig. 2. Nonetheless, a twofold decrease in the reaction rate for 2-octanone reduction M is expected.

3.2. Batch experiments and prediction of equilibrium conversions

It has been shown previously that the equilibrium conversion X_{eq} in a monophasic system is depending on the initial ratio of the starting materials [31–33]. In a biphasic system, factors like

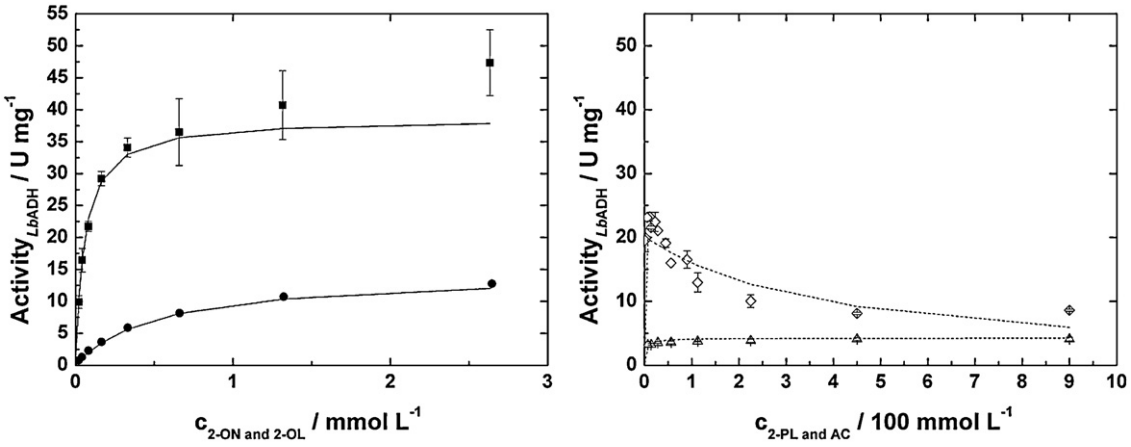

Fig. 2. Kinetic measurements with *Lb*ADH, variation of the respective substrate; full squares: reduction of 2-octanone (reaction M); full circles: oxidation of 2-octanol (reaction M′); open triangles: oxidation of 2-propanol (reaction R); open diamonds: reduction of acetone (reaction R′); solid lines: models for C_8-reaction; dashed lines: models for C_3-reactions; all reactions carried out at 25 °C with concentrations if not stated otherwise: $c_{KP_i} = 50\,mmol\,L^{-1}$, $c_{MgCl_2} = 2.5\,mmol\,L^{-1}$, pH = 7.0, $c_{NADP(H)} = 0.5\,mmol\,L^{-1}$, $c_{2\text{-octanone}} = 2.63\,mmol\,L^{-1}$ or $c_{2\text{-octanol}} = 1.32\,mmol\,L^{-1}$, $c_{LbADH} = 1.2\,mg\,L^{-1}$.

the phase volume ratio and the partitioning coefficient further determine X_{eq}. If the phase volume ratio is one and the partition coefficients of the two corresponding ketones and alcohols are similar, the equilibrium is determined by the initial ratio of $R_0 = c_0(2\text{-propanol})/c_0(2\text{-octanone})$. With the equilibrium constant K, the X_{eq} can be calculated according to

$$X_{eq} = K\frac{1 + R_0 - \sqrt{(R_0 - 1)^2 + (4R_0/K)}}{2(K - 1)} \quad (3)$$

for the reduction of 2-octanone. For the oxidation of (*R*)-2-octanol the equilibrium conversion X'_{eq} is calculated according to

$$X_{eq} = \frac{1 + R'_0 - \sqrt{(R'_0 - 1)^2 + 4R'_0 K}}{2(1 - K)} \quad (4)$$

with $R'_0 = c_0(\text{acetone})/c_0(2\text{-octanol})$.

To determine R_0, and K, batch syntheses were carried out in one- and two-phase systems with various R_0. The reduction of 2-octanone and the oxidation of (*R*)-2-octanol were investigated. In Fig. 3 the obtained X_{eq} vs. R_0 and X'_{eq} vs. R'_0, respectively, are shown. K could be determined to be 0.38 which is in the range of previously reported values ($K = 0.425$ for acetophenone/2-propanol and $K = 0.536$ for 2-heptanone/2-propanol) [34,35]. The fact that both the equilibrium conversion of monophasic and biphasic batches is described with the same model underlines the validity of the findings that the partitioning coefficients P of 2-propanol and acetone can be considered equal which also accounts for 2-octanone and 2-octanol.

The initial turnover frequencies (TOF/s^{-1}) for *Lb*ADH for monophasic and biphasic batches were determined as a function of R_0 (Fig. 4). As expected from the initial rate measurements, the rates for the biphasic experiments are lower than those for the monophasic experiments. The lower reaction rate in the biphasic system even at high R_0 is in line with the lower 2-octanone concentration in the aqueous phase due to the partitioning. Noteworthy, the initial TOF_{LbADH} in the monophasic system correlates linearly with R_0. A pseudo-zero order in R_0 is observed in the biphasic cases due to the partitioning. Although, high 2-propanol concentrations will increase the equilibrium conversion, they do not necessarily increase the reaction rate. Additionally, the concentration of the long-chain ketone in the aqueous reactive phase is unaffected by 2-propanol concentration.

In the course of the biphasic batch-wise syntheses, the formation of a third, emulsion-like phase or crud between the organic

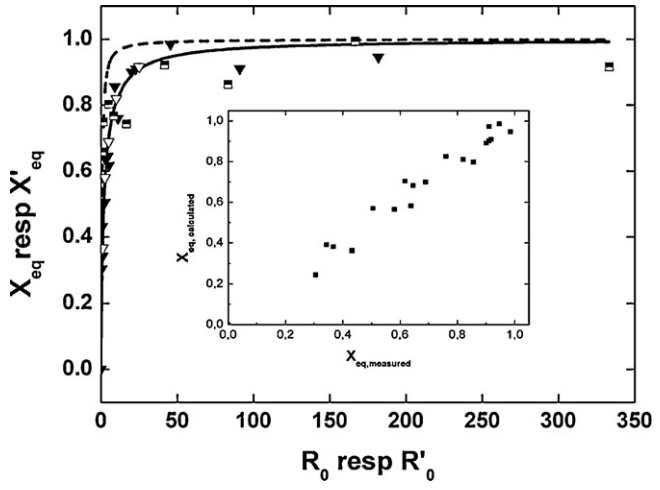

Fig. 3. Measured and calculated X_{eq} resp X'_{eq} as a function of R_0 respectively R'_0, with $K = 0.38$ full triangles: monophasic reduction; open triangles: biphasic reduction; squares: monophasic oxidation; full line: model for the reduction; dotted line: model for the oxidation; conditions: see Section 2.

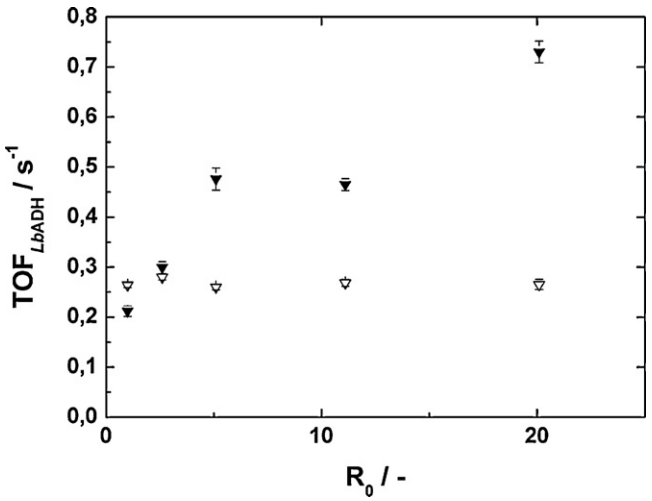

Fig. 4. Initial turnover frequencies (TOF) for the monophasic (full triangles) and biphasic (open triangles) reduction reactions; conditions: see Section 2.

and the aqueous phase has been observed. When these phases were separated, the emulsion-like phase spontaneously split up into two phases: The lower phase was clear, the upper phase was emulsion-like. Further investigations revealed that the emulsion-phase is only formed when *Lb*ADH is present in the aqueous phase. On first view, emulsion-forming seems to be advantageous due to the enhanced mass transfer. However, for the continuous synthesis a good phase separation is required. Otherwise, parts of the aqueous, reactive phase can be carried out of the reactor. This leads to leaching of the catalyst. For this reason, we determined the enzyme activity in all three phases. The highest volumetric activity was found in the aqueous phase, less in the interphase and no activity was detectable in the organic phase (see Supporting information). 2-Propanol did not influence the phase behavior, but reduced the volumetric activity as already indicated by the kinetic measurements. These results show that emulsion forming needs monitoring to prevent loss of the aqueous phase.

3.3. Continuous syntheses

The continuous synthesis offers advantages over batchwise synthesis such as better catalyst utilisation, constant product quality and easy automatisation [36]. As reactor, the biphasic mini-reactor as described previously [24] was chosen. This can be considered as a continuous stirred tank reactor (CSTR). For information gathering, a CSTR offers more detailed information on the catalyst system long term activity and stability than a plug flow reactor (PFR). A biphasic approach with pure substrate as second phase was considered [37] but turned out to be challenging if carried out continuously due to the aforementioned difficulties with the co-substrate dosage and removal. Only the substrate coupled approach with 2-propanol would be feasible. Then, a mixture of 2-propanol and 2-octanone could be dosed into the reactor. Due to the miscibility of 2-propanol with water, the initial phase ratio would change and removal of acetone and remaining 2-propanol is no longer guaranteed. Thus, an approach where the 2-ketone and 2-propanol are dissolved in MTBE was preferred. Both, the organic MTBE-phase and the aqueous buffer-phase, were pre-saturated prior to use in the continuous synthesis in order to prevent loss of the aqueous phase due to solubility in MTBE. Initial *Lb*ADH concentration was 480 mg L^{-1} (=2.4 mg lyophilised enzyme preparation) and $NADP^+$-concentration was 0.1 mmol L^{-1} if not stated otherwise. The outlet concentrations were monitored by using a flow cell *via* online gas chromatography.

2-Octanone was chosen as substrate for comparison with previous studies, in which either a solubiliser was used in both batch and continuous synthesis in a single phase [29,30,35], or biphasic approaches with pure substrate as second phase [37] and a biphasic approach with ionic liquid or MTBE as non-reactive phase in a batch [23,35]. The reaction conditions were chosen based on the results of the kinetic investigations and batch-experiments. All continuous syntheses were carried out at $R_0 = 10$ with a ketone concentration of 100 mmol L^{-1} and 2-propanol concentration of 1.00 mol L^{-1}.

The influence of stirring speed in the aqueous phase and cofactor-concentration were determined. Apparently, increasing the stirring speed to more than 400 rpm led to no further increase in the conversion. It also turned out that an initial stirring speed of 200 rpm was beneficial for the long time enzyme performance. So, the aqueous phase for all subsequent experiments was stirred at 200 rpm for 2 h (except for the 2-decanone-experiment, here the initial stirring time was 20 h) and increased to 500 rpm afterwards. As increasing the cofactor concentration from 0.1 mmol L^{-1} to 0.2 mmol L^{-1} augmented the conversion from 25% only to 30% (data not shown), the lower concentration was fixed in view of a better cofactor utilisation.

Conversion as a function of time of a typical experiment with 2-octanone as substrate is shown in Fig. 5. With the initial reaction conditions, a conversion of 31% could be observed with an apparent deactivation of 0.1% h^{-1}. After 170 h on stream, the initial enzyme concentration of 480 mg L^{-1} (=2.4 mg lyophilised enzyme preparation per 5 mL aqueous phase) was increased to 1480 mg L^{-1} (=7.4 mg lyophilised enzyme preparation per 5 mL aqueous phase) which led to a doubling of the conversion from 26% to 48%. A doubling of the residence time from 4 to 8 h showed less effect on the conversion (from 48% to 56%) but led to a decrease in the space-time-yield (STY) from 142 mmol L^{-1} d^{-1} to 82 mmol L^{-1} d^{-1}. After readjusting the residence time to 4 h, the conversion dropped to 42%, which, assuming a constant deactivation rate, is in accordance with the steady-state before increasing the flow rate of the non-reactive phase. An overall turn-over number (TON) of 186×10^3 for the *Lb*ADH has been achieved. For the cofactor TON_{NADP^+} was 26×10^3, which is exceptionally high for an *in vitro* application [38]. The conversion in the continuous synthesis is with 56% at its best not reaching the estimated equilibrium conversion of 81%. In this experiment emulsification was observed to a very low extent, so that continuous decantation was operable.

Although the kinetic model is in line with the measured initial reaction rates, it was not possible to predict the course of the biphasic batch-reaction or the continuous syntheses. Reasons for this may lie in the complex reaction system. Partitioning of the substrates and products is not included in the model and may be not ideal. The prediction of a continuous experiment starting from initial reaction rates is not straightforward, especially if a biphasic experiment is described with data from monophasic initial rate experiments. Conducting biphasic initial reaction rate experiments might be helpful, but not straightforward to carry out.

To show the potential and limitations of the approach, we extended the experiments to homologous alkanone substrates with higher and lower solubility in water. Subsequently, 2-heptanone, 2-nonanone, and 2-decanone were employed under the same reaction conditions as for 2-octanone (Fig. 5 and Table 4). Kinetic data for 2-heptanone hint towards lower reaction rates when compared to 2-octanone experiments (Table 2). However, in the continuous synthesis, with 2-heptanone as substrate, a conversion of 49% could be realised. Under the same reaction conditions with 2-octanone as substrate, a conversion of only 31% could be achieved. At a reaction time of 140 h, the residence time was decreased from 4 h to 3 h which led to a decrease in conversion to 29%. After increasing the residence time again to 4 h, the conversion regained 45%. In total, a TON_{LbADH} of 478×10^3 and a TON_{NADP^+} of 22×10^3 were achieved. It is noteworthy that the conversion with 2-heptanone as substrate is higher than for 2-octanone, even though the kinetics with a lower $v_{max,2\text{-heptanone}}$ hint to a lower rate for 2-heptanone. Here, the higher availability of 2-heptanone seems to play the key role for the higher reaction rate and thus a higher conversion. Still, the equilibrium conversion of 85% with $K = 0.536$ [34,35] was not reached either. In this experiment, emulsification was not significant.

In contrast to the high conversion reached with 2-heptanone as substrate, the maximum conversion achieved was 24% with 2-nonanone as a substrate – under comparable reaction conditions. Due to the formation of an interphase making decantation of the organic phase impossible, the experiment was stopped after 72 h. Still, an overall TON_{LbADH} of 6.4×10^4 and a TON_{NADP^+} of 3.0×10^3 were reached. Similar results were achieved with 2-decanone as a substrate. The maximum conversion was 22% although the residence time was 8 h instead of 4 h for 2-nonanone. Again, the formation of an extended interphase caused the end of this experiment. Slightly higher TON were achieved when compared to the 2-nonanone experiment ($TON_{LbADH} = 8.2 \times 10^4$; $TON_{NADP^+} = 3.8 \times 10^3$).

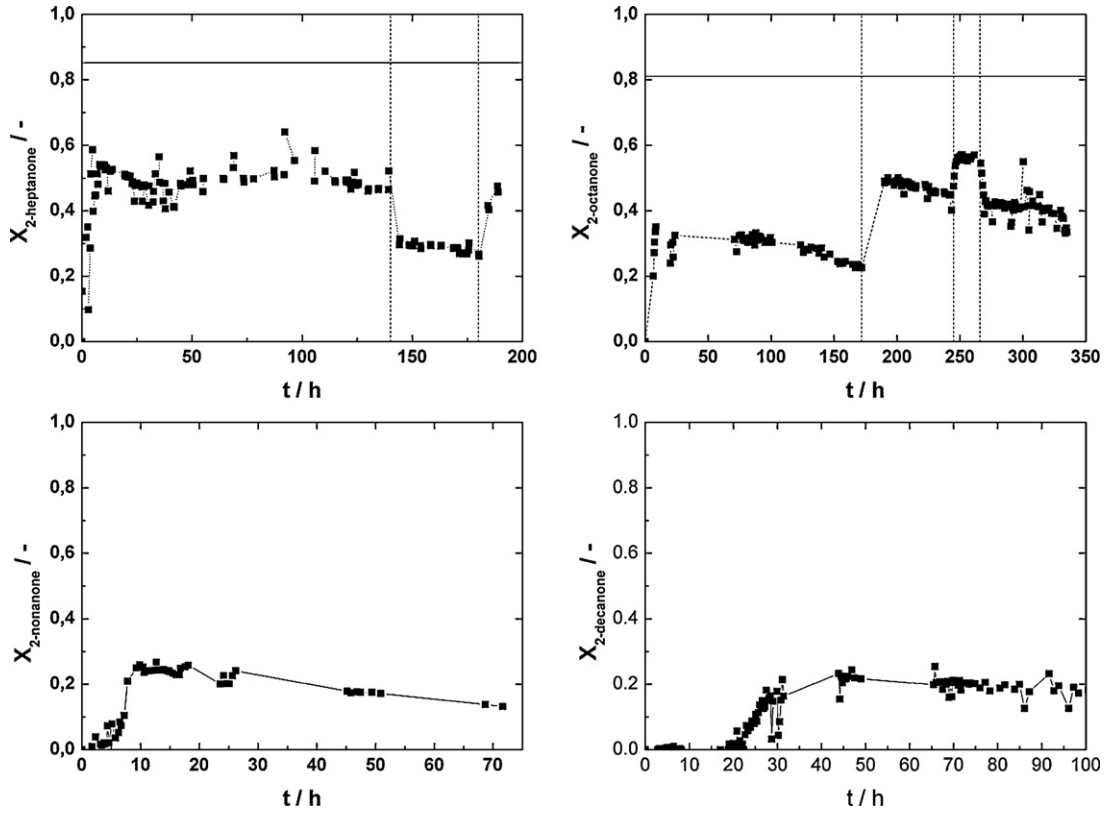

Fig. 5. Conversion as a function of time for the continuous syntheses of (*R*)-2-alcohols in the biphasic reactor, solid lines indicate the limiting conversion X_{eq} and dashed vertical lines indicate change of reaction parameters (all: $c_{2\text{-ketone}_{MTBE}} = 100\ mmol\ L^{-1}$; $c_{2\text{-propanol}_{MTBE}} = 1000\ mmol\ L^{-1}$; $\dot{V} = 1.25\ mL\ h^{-1}$; $\tau = 4\ h$; $c_{NADP^+} = 0.1\ mmol\ L^{-1}$; $c_{LbADH} = 480\ mg\ L^{-1}$; 2-heptanol: $t = 140\ h$, $\tau = 3\ h$; $t = 180\ h$, $\tau = 4\ h$; 2-octanol: $t = 172\ h$, *Lb*ADH concentration 1480 mg L^{-1}; $t = 245\ h$, $\tau = 8\ h$; $t = 266\ h$, $\tau = 4\ h$); 2-decanone: $\tau = 8\ h$).

Table 4
Overview of the key performance indicators of the biphasic continuous syntheses.

Substrate	t_{total} (h)	$X_{2\text{-ketone}}$ (%) [a]	STY[a] (mmol L^{-1} d^{-1}	TON_{NADP^+} [b] (10^3)	TON_{LbADH} [b] (10^3)
2-Heptanone	180	49	147	22	478
2-Octanone	320	31	94	26	186
2-Nonanone	72	24	73	3.0	64
2-Decanone	90	22	54	3.8	82

[a] Values in steady state with initial reaction conditions.
[b] Values based on the accumulated amount of product produced.

Comparing the biphasic experiments with 2-octanone and 2-heptanone, the effective reaction rate is much higher in the case of 2-heptanone. The lower rate for the longer-chain ketones in the continuous biphasic system is most probably due to their lower solubility in water. A similar trend for a biphasic batch reaction has also been observed [35]. This is in contrast to the monophasic acetonitrile-buffer approach [35] where almost the same initial reaction rates were observed for the reduction of 2-heptanone, 2-octanone and 2-nonanone. Furthermore, a similar trend for apparent reaction rates in monophasic batch reactions with an ionic liquid as solubiliser was observed for the homologous series 2-octanone, 2-nonanone and 2-decanone [30].

4. Conclusion

Our investigations demonstrate that the proposed reaction and reactor system are suitable for the continuous synthesis of enantiopure (*R*)-2-alcohols with at least a minimum solubility in aqueous solutions ((*R*)-2-heptanol and (*R*)-2-octanol). Longer chain alcohols are also accessible *via* the same approach, but, the STY, apparent rate, and the conversion drop drastically when the solubility decreases in the series 2-heptanone, 2-octanone, 2-nonanone, and 2-decanone as substrate. Thus, a major limitation of the system is the effective aqueous concentration of the respective substrate.

One major advantage of the biphasic continuous synthesis is the cofactor utilisation. In monophasic continuous systems with retention of the enzyme(s) *via* ultrafiltration, the TON_{NADP^+} is always directly coupled to the substrate solubility [29,30]. A TON_{NADP^+} of 26,000 is an extremely good value. An overview for *Lb*ADH catalysis is given in the literature [21]. In academia typically values between 3 [39] and 20,000 [40,41] are obtained, whereas in an industrial process conducted by Wacker, Germany, TON_{NADP^+} is given with 74,000 [42,43].

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2012.09.017.

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