

# Stable Continuous Operation of a Biphasic Enantioselective Enzymatic Reduction

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**Abstract:** Continuous operation of alcohol dehydrogenase (ADH) catalysed enantioselective reduction in a biphasic system showed gains in productivity and stability of the overall reaction system. The total turnover numbers obtained for the cofactor NADP<sup>+</sup> are high with up to  $1.5 \times 10^4$ . Productivity for (*R*)-2-butanol with *Lactobacillus brevis* ADH was up to 26 kmol(mol enzyme)<sup>-1</sup>. Enantioselectivity was greater than 0.99 for (*R*)-phenylethanol and up to 0.99 for (*R*)-butanol.

**Keywords:** Biocatalysis, continuous biphasic synthesis, enantioselective reduction, alcohol dehydrogenase from *Lactobacillus brevis*.

Alcohol dehydrogenases (ADH) are versatile catalysts for the enantioselective reduction of prochiral ketones to chiral alcohols. However, their applicability is mainly limited to aqueous buffers as the reactive phase. This is implied not only by the enzymes themselves but also by the insolubility of the required redox cofactors NADH or NADPH. Water as the only available reactive solvent limits productivity by solubility for a broad range of substrates. Using biphasic reaction systems with a non-reactive phase as a reservoir for substrates and products opened up both the use of sparingly water soluble substrates as well as expanding the thermodynamic boundaries of conversion limited systems [1, 2].

Specifically, the ADH from *Lactobacillus brevis* (*Lb*ADH, NADP<sup>+</sup> dependent) has been applied under biphasic conditions [3]. Apart from the common model substrate acetophenone our interest is in the production of enantiomerically enriched short chain alcohols. In these reactions enzymes are unmatched in enantioselectivity compared to chemical homogeneous catalysts. The most challenging substrate is butanone where to the best of our knowledge the best results for hydrogenation yielded enantiomeric excess  $ee=0.72$  (enantiomeric ratio  $er=6$ ) utilising Ru based catalysts, so far [4-6].

As non-reactive phase we evaluated methyl *tert*-butyl ether (2-methoxy-2-methyl-propane, MTBE) and the ionic liquid (IL) [N-pentyl N'-methyl imidazolium] hexafluorophosphate ([PMIM]PF<sub>6</sub>) [7-12]. Commonly, the partition coefficients  $p$  are near unity for both acetone and 2-propanol [1, 13, 14]. [PMIM]PF<sub>6</sub> was chosen after screening of non-

water miscible IL as it shows full miscibility with acetone as the product of substrate coupled cofactor regeneration but not with the reducing agent 2-propanol. Thus, a favourable shift of equilibrium conversion towards the products should result. In practice, better conversion and enantioselectivity for batch reactions using MTBE were obtained with comparable selectivity (Table 1). This can be attributed to the non-ideal, i.e. concentration dependent, partitioning in the multi-component, non-diluted system (data not shown). In addition, traces of HF in the IL cannot be ruled out as a reason. Hydrolysis of PF<sub>6</sub> can occur [15].

**Table 1. Enantiomeric Excess (*ee*) and Conversion for the Substrate Coupled Reduction of Butanone to (*R*)-2-Butanol (Initial NADP<sup>+</sup> Concentration 0.1 mmol L<sup>-1</sup>)**

Entry	[Butanone] /mmol L <sup>-1</sup>	[2-Propanol] /mol L <sup>-1</sup>	Conversion <sup>a</sup> /-	<i>ee</i> <sup>a</sup> /-	Non-Reactive Phase
1	20	0.2	0.31	0.92	MTBE
2	20	2.0	0.32	0.96	
3	50	0.2	0.19	0.90	
4	50	2.0	0.46	0.98	
5	100	0.2	0.22	0.70	
6	100	2.0	0.33	0.91	
7	20	0.2	0.08	0.99	[PMIM] PF <sub>6</sub>
8	20	2.0	0.12	0.99	
9	50	0.2	0.07	0.65	
10	50	2.0	0.15	0.96	
11	100	0.2	0.04	0.97	
12	100	2.0	0.34	0.76	

<sup>a</sup>Conversion and *ee* after 2 h reaction time.

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To allow assessment of the regeneration method for the nicotine amide cofactors NADPH or NADP<sup>+</sup> in biphasic conditions, batch reactions were carried out utilising substrate coupled and enzyme coupled regeneration methods. Surprisingly, the regeneration method had a large impact on selectivity. The use of malate dehydrogenase (MDH) with *L*-malic acid as the reducing agent and with MTBE as the non-reactive phase showed only moderate *ee* with less than 0.70 (*er*=5.5) (Table 2). Furthermore, enantioselectivity and conversion each showed a nonlinear correlation to the relative amounts of *Lb*ADH and MDH. For conversion a maximum of 0.40 was reached. Both conversion and *ee* maximum values were obtained at an ADH:MDH ratio of 0.13. Exceeding this ratio led to a decrease of conversion as well as *ee*.

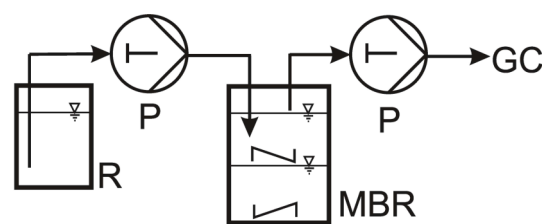
However, the transfer hydrogenation using the substrate coupled approach with 2-propanol as the reducing agent allowed for *ee* of up to 0.98 (*er*=53) and conversion of up to 0.46 using MTBE as the non-reactive phase (Table 1). With the IL *ee* of 0.99 (*er*=4370) and conversion of up to 0.15 were achieved. Besides ADH:MDH ratio the initial butanone and the initial 2-propanol concentration influence conversion and selectivity. At constant 2-propanol concentration higher butanone concentrations cause lower conversion. In contrast, at constant butanone concentration a higher 2-propanol concentration leads to better conversion. Selectivity generally decreases with increasing butanone concentration, but is positively influenced by increasing 2-propanol concentration.

**Table 2.** Enantiomeric Excess (*ee*) and Conversion for the Enzyme Coupled Reduction of Butanone to (*R*)-2-Butanol (Initial NADP<sup>+</sup> Concentration 0.1 mmol L<sup>-1</sup>) Depending on the ADH:MDH Ratio (Non-Reactive Phase: MTBE)

Entry	ADH:MDH	[Butanone] / mol L <sup>-1</sup>	conversion <sup>a</sup> /-	<i>ee</i> <sup>a</sup> /-
1	0.08	50	0.32	0.64
2	0.10	50	0.38	0.66
3	0.13	20	0.35	0.53
4	0.13	50	0.41	0.69
5	0.13	100	0.25	0.67
6	0.17	50	0.40	0.62
7	0.25	50	0.33	0.65
8	0.50	50	0.17	0.50
9	1.00	50	0.05	0.32

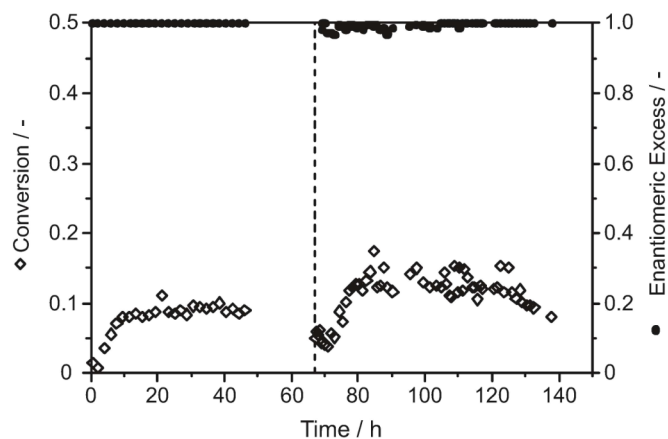
<sup>a</sup>Conversion and *ee* after 2 h reaction time.

To fully exploit the beneficial use of the biphasic system a continuous reaction was carried out. Using the miniaturised biphasic reactor (MBR) [16] the aqueous reactive phase saturated with MTBE was stationary and remained in the reactor. As the non-reactive phase water saturated MTBE was continuously added and withdrawn using one continuously operated piston pump (Micromechatronic Technologies MMT, Germany) (Fig. 1).



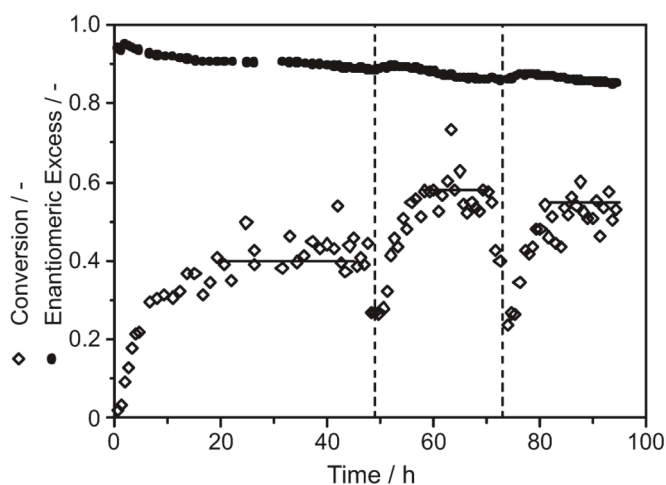
**Fig. (1).** Simplified flow scheme of the miniaturised biphasic reactor setup for the continuous enzymatic reduction (R: reservoir, P: piston pump, MBR: miniaturised biphasic reactor, GC: online gas chromatography).

For comparison with previous results the reactor was first employed for the continuous reduction of acetophenone (AP) (Fig. 2). The reaction could be run for 140 h in total with high selectivity at rather low conversion averaging 0.10. Nonetheless, a total turnover of the cofactor NADP<sup>+</sup> of 1900 far exceeds expectations from comparable batch experiments. After 120h a decrease of conversion was apparent. Either deactivation of the *Lb*ADH under process conditions or deactivation of the nicotine amide cofactors are the most likely reasons.



**Fig. (2).** Conversion and *ee* as a function of time for the continuous biphasic aqueous buffer/MTBE reduction of acetophenone to (*R*)-1-phenylethanol (inlet: MTBE [AP] = 50 mmol L<sup>-1</sup>, [2-propanol] = 2.0 mol L<sup>-1</sup>; aqueous: V = 5 mL, 5 mg *Lb*ADH, potassium phosphate buffer pH = 6.5, [NADP<sup>+</sup>] = 0.1 mmol L<sup>-1</sup>; the perpendicular dashed line indicates change of residence time from 125 to 250 min).

For the continuous reduction of the more challenging butanone a range of conditions were evaluated (Fig. 3). Initially, at 0.40 conversion *ee*=0.95 could be obtained at 85 min residence time. Increasing residence time by 40 min the maximum conversion was obtained at 0.58 giving a space time yield of 0.62 g L<sup>-1</sup>. Surprisingly, further increase of the residence time led to a slight decrease of conversion although the thermodynamically limiting conversion was estimated as 0.93 [2]. Apparently, *ee* decreases with time and is only slightly affected by residence time. The reasons for this are e.g. enzyme deactivation or cofactor availability which are currently under investigation. A total turnover number (TTN) for NADP<sup>+</sup> of 15400 was reached reflecting excellent cofactor and enzyme stability. In contrast, for the corresponding batch experiment (Table 1, entry 4) a TTN of 230 was obtained using MTBE. Assuming the protein in the technical preparation to be pure enzyme a productivity of 26 kmol (mol enzyme)<sup>-1</sup> is reached.



**Fig. (3).** Conversion and *ee* as a function of time for the continuous biphasic aqueous buffer/MTBE reduction of butanone to (*R*)-2-butanol (inlet: MTBE [butanone] = 50 mmol L<sup>-1</sup>, [2-propanol] = 2.0 mol L<sup>-1</sup>; aqueous: V = 5 mL, 5 mg *LbADH*, potassium phosphate buffer pH = 7, [NADP<sup>+</sup>] = 0.1 mmol L<sup>-1</sup>; the perpendicular dashed lines indicate changes of residence time from 85 to 125 to 156 min)

In conclusion continuous stable operation of the *LbADH* in the biphasic system aqueous buffer/MTBE was successfully demonstrated and outperforms the previously obtained results [3, 13, 17, 18]. Both the high operational stability with no apparent loss of activity within 140 h of operation of the catalytic system of *LbADH* and the cofactor NADP<sup>+</sup> under these conditions are promising for further research and industrial applications. In the steady state, i.e. at constant conversion and *ee*, the MBR allows for a more detailed investigation of the factors which influence enzyme deactivation and limit conversion and selectivity. The batch experiments (Tables 1 and 2) have indicated that cofactor regeneration is the rate limiting step [19]. Therefore, the MBR is an appropriate tool to optimize the underlying reactions with respect to economic efficiency for industrial applications.

## 1. MATERIALS AND METHODS

### 1.1. Batch Experiments

Solutions of all reagents were always freshly prepared. The potassium phosphate buffer was used at a concentration of 50 mmol L<sup>-1</sup>, its pH was set to 6.5. Concentrations of butanone and 2-propanol are given in Tables 1 and 2. For the experiments using substrate coupled cofactor regeneration (Table 1) initial NADP<sup>+</sup> concentration was 0.1 mmol L<sup>-1</sup>, concentration of *LbADH* was 1.0 mg mL<sup>-1</sup>. For the experiments using enzyme dependent cofactor regeneration the L-malic acid concentration was 60 mmol L<sup>-1</sup> at an initial NADP<sup>+</sup> concentration of 0.1 mmol L<sup>-1</sup>. ADH:MDH ratios are given in Table 2. The reaction volume was 5 mL for all experiments. Samples of 50  $\mu$ L were taken at intermittent time intervals, mixed with 100  $\mu$ L of a 100 mmol L<sup>-1</sup> 1-butanol standard solution and analyzed *via* GC. Reaction solutions were stirred at 200 rpm.

### 1.2. Continuous Experiments

The MBR [16] has a total volume of 10 mL. The reactive and the non-reactive phase are stirred independently, so the composition of each 5.0 mL phase is uniform. The phase

boundary has to be preserved to allow for continuous removal of the MTBE phase. To avoid depletion of the aqueous phase due to continuous solution of water in the mobile organic phase, MTBE was saturated with water. To avoid initial adverse mixing processes the aqueous phase was presaturated with MTBE prior to the addition of *LbADH* and cofactor. Using a thermostat the reactor is constantly held at 30°C. The substrate reservoir R contains the feed substrate solution which is substrate dissolved in water saturated MTBE (Fig. 1). A cryostat is employed to maintain it at 4.0°C. A piston pump P feeds the substrate solution into the reactive phase of the reactor while it transfers the identical volume out of the reactor through the GC flow cell and into a the product reservoir. Thereby, the piston pump holds the volume of the mobile phase constantly at 5 mL. At time intervals of 40 min, the GC autosampler injects 1  $\mu$ L of a standard 100 mmol L<sup>-1</sup> 1-butanol solution, and 1  $\mu$ L of product solution from the flow cell. Sample and standard are injected together into the GC for analysis. The potassium phosphate buffer was used at a concentration of 50 mmol L<sup>-1</sup>, pH=6.5. Concentrations of butanone and acetophenone were chosen as 50 mmol L<sup>-1</sup> at a 2-propanol concentration of 2.0 mol L<sup>-1</sup>. Initial NADP<sup>+</sup> concentration was 0.1 mmol L<sup>-1</sup>. The *LbADH* was used at a concentration of 5.0 mg mL<sup>-1</sup>. Concentrations of butanone, acetophenone, and 2-propanol refer to a volume of 5 mL non-reactive phase, while concentrations of NADP<sup>+</sup> and *LbADH* refer to a volume of 5 mL reactive phase.

### 1.3. Gas Chromatography

Continuous GC analysis was performed using an Agilent Technologies HP 6890 with a JAS-Unis Inlet. Column specifications: CP-Chirasil-DEX CB (Varian, 25 m x 0.25 mm inner diameter, film thickness 0.25  $\mu$ m). As carrier gas H<sub>2</sub> was used at a constant pressure of 0.5 bar. A temperature program was used as follows: initial temperature 40°C (3 min), 1°C min<sup>-1</sup> to 45°C (5 min), 10°C min<sup>-1</sup> to 60°C (1 min). Retention times: butanone 2.7 min, (*R*)-2-butanol 6.6 min, (*S*)-2-butanol 6.9 min, 1-butanol standard 11.8 min.

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