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Ionic liquid facilitates biocatalytic conversion of hardly water soluble ketones

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ABSTRACT

Ionic liquids represent a promising alternative to conventional cosolvents as biocompatible solubilisers for biocatalysis. This was shown using water miscible ionic liquids to facilitate the stereoselective reduction of hardly water soluble, aliphatic ketones catalysed by the alcohol dehydrogenase from *Lactobacillus brevis*. Ten ionic liquids were screened for activity and solubility. Improved storage stabilities besides improved enzyme activities, as well as reduced substrate surplus and product inhibitions were found, while applying the most promising AMMOENG™ 101 in more detailed investigations. Batch reactions with cofactor regeneration via a glucose dehydrogenase showed increased reaction rates; thus underlining the positive influence of AMMOENG™ 101. For (*R*)-3-octanol, (*R*)-2-nonanol, (*R*)-2-decanol, and (*R*)-2-octanol space time yields between 250 and 350 mmol L^{-1} d^{-1} were achieved.

1. Introduction

Due to superior regio- and enantioselectivity, along with higher product purities more and more biocatalytic processes are established in industry [1]. Unfortunately, without strategies to overcome the frequently poor aqueous solubility of industrially attractive substrates, the application of biocatalysts is limited. In consequence, the potential of aqueous organic one phase and two phase systems has been explored to overcome this problem [2]. Besides conventional organic solvents, ionic liquids (ILs) are discussed as promising alternative as improved activity, stability, and selectivity of biocatalysts were found [3–9]. However, this field of research is still expanding and by now mainly focused on hydrolytic enzymes [3]. Only few examples of applications with oxidoreductases in the presence of IL are published [10–17]. Moreover, it was shown that IL may have a stabilising effect on nicotinamide cofactors, which are used as redox equivalents by dehydrogenases [2,18].

We focused on the application of water miscible IL as performance additives for ADH catalysed reductions of hardly water soluble aliphatic ketones (Fig. 1). The reduction is either impractical or impossible in aqueous buffer alone due to low solubility. For the longer chain ketones, a two phase approach is not possible as the low solubility would lead to low rates and conversion [19]. Ketones of interest were 3-octanone, 2-octanone, 2-nonanone and 2-decanone with high added value of the enantiopure alcohols (Fig. 2).

2. Experimental

Alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*ADH) and glucose dehydrogenase from *Bacillus spec.* (GDH) are available from X-Zyme GmbH (Düsseldorf, Germany). AMMOENG™ 101 (CAS 61791-10-4) of at least 95% purity was kindly supplied by Solvent Innovation (Cologne, Germany). NADPH and $NADP^+$ were purchased from Carl Roth GmbH (Karlsruhe, Germany) and N-methyl-N-trimethylsilyl-trifluoracetamide (N-MSTFA) from CS-Chromatographie Service GmbH (Langerwehe, Germany). All other reagents were purchased from Sigma–Aldrich (Schnelldorf, Germany), and were at least of analytical grade. Deionised water was obtained by reverse osmosis. All experiments were conducted in potassium phosphate buffer (100 mmol, pH 7) if not mentioned otherwise.

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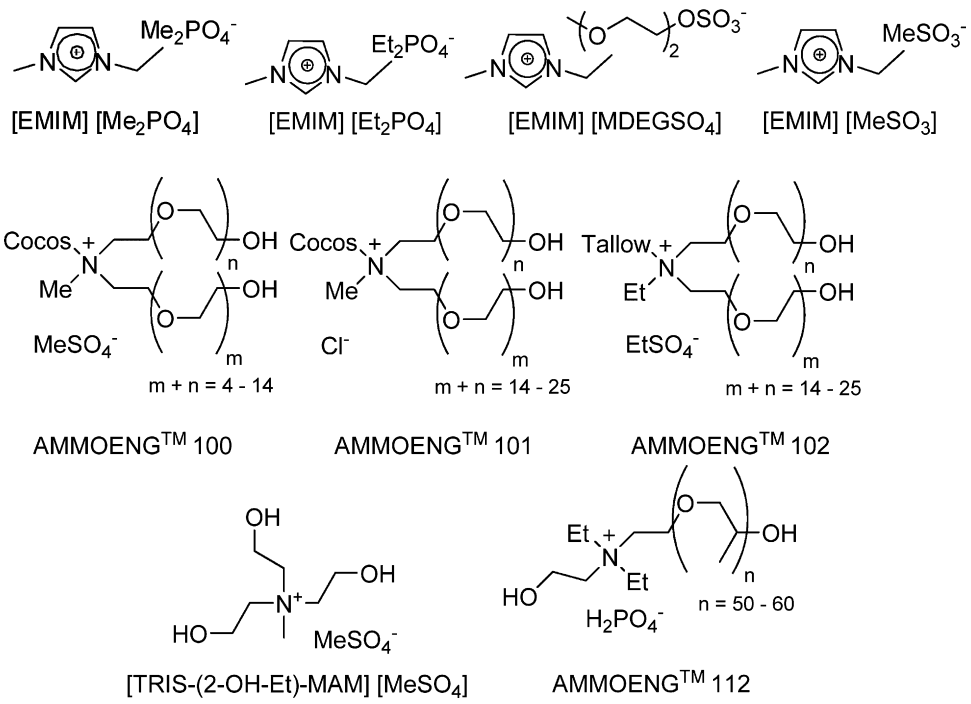

Fig. 1. Structures of the water miscible IL.

2.1. Preparation of IL buffer mixtures

For the preparation of one litre IL buffer mixture (100 mmol, pH 7), K_2HPO_4 (12.63 g, 72.5 mmol) and KH_2PO_4 (3.76 g, 27.6 mmol) were dissolved in 500 mL water. Taking water content of the IL into account, the required amount of IL and $MgCl_2$ (238 mg, 2.5 mmol) was added and fixed to pH 7 by addition of concentrated phosphoric acid and filled up to one litre volume with water. The IL buffer mixture did not show blind activity for all with both reduction and oxidation with either *Lb*ADH or GDH.

2.2. Determination of IL water contents

The water content of the IL was determined by Karl Fischer titration. The measurements were carried out by directly injecting IL into the titration solution of a 756 KF coulometer equipped with a diaphragm electrode from Metrohm.

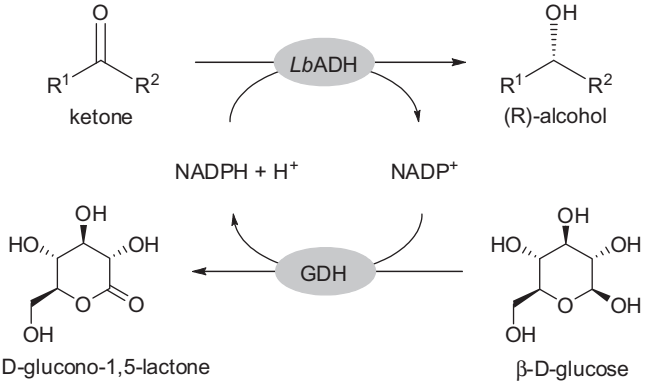

Fig. 2. *Lactobacillus brevis* ADH catalysed reduction of prochiral ketones to the corresponding (*R*)-alcohols with glucose dehydrogenase catalysed cofactor regeneration.

2.3. LbADH activity

Enzyme activity was determined by spectrophotometry measuring absorption at 340 nm for 2 min reaction time. The extinction coefficient of NADPH was determined for each IL. As a model reaction for the activity measurements, the reduction of 2-octanone was conducted. In a 96-well quartz plate, ketone solution (7.8 mmol L^{-1},180 μL) and NADPH solution (10 mmol L^{-1}, 10 μL) were mixed. The reaction was started by the addition of the *Lb*ADH solution (0.25 mg mL^{-1}, 10 μL).

2.4. Gas chromatography

Aqueous samples were extracted with *n*-hexane and the organic phase was analysed via gas chromatography (GC) (column: Chirasil-Dex (25 m × 0.25 mm ID) from Varian GC Capillary Columns, carrier gas: H_2, 0.4 bar).

3-Octanone/3-octanol: 90 °C (2 min), 5 °C min^{-1} to 130 °C (2 min), 40 °C min^{-1} to 180 °C (2 min). Retention times: 3-octanone (6.2 min), 3-octanol (10.3 min), internal standard 1-octanol (10.3 min). *2-Octanone/2-octanol*: 80 °C (3 min), 10 °C min^{-1} to 120 °C (6 min), 40 °C min^{-1} to 180 °C (2 min). Retention times: 2-octanone (7.3 min), 2-octanol (9.4 min), internal standard 1-octanol (11.8 min). *2-Nonanon/2-nonanol*: 110 °C (0 min), 2 °C min^{-1} to 120 °C (0 min), 40 °C min^{-1} to 180 °C (2 min). Retention times: 2-nonanone (5.5 min), 2-nonanol (6.4 min), internal standard 1-octanol (6.3 min). *2-Decanon/2-decanol*: 120 °C (2 min), 3 °C min^{-1} to 150 °C (0 min), 40 °C min^{-1} to 180 °C (2 min). Retention times: 2-decanone (6.7 min), 2-decanol (8.7 min), internal standard 1-octanol (5.9 min).

2.5. Solubility of ketones in IL buffer mixtures

To determine the solubility of 3-octanone, 2-octanone, 2-nonanone and 2-decanone in buffer and IL buffer mixtures, saturated solutions of the ketones in the reaction media were prepared by shaking excess ketone with aqueous buffer at room

temperature for 60 h. Subsequently, aqueous samples were taken and analysed by GC. Throughout, measurements were carried out in triplicate and standard deviation was below 5%.

2.6. Determination of enantiomeric excess

To determine enantiomeric excess (*ee*), aqueous samples were extracted with *n*-hexane (250 μL), subsequently mixed with N-MSTFA (50 μL), and heated to 80 °C for 30 min and analysed by GC.

(R)-/(S)-3-Octanol: 60 °C (51 min), 40 °C min^{-1} to 180 °C (2 min). Retention times: (*R*)-3-octanol (47.1 min), (*S*)-3-octanol (48.4 min). *(R)-/(S)-2-Octanol*: 80 °C (3 min), 1 °C min^{-1} to 100 °C (5 min), 40 °C min^{-1} to 180 °C (2 min). Retention times: (*R*)-2-octanol (14.9 min), (*S*)-2-octanol (15.2 min). *(R)-/(S)-2-Nonanol*: 75 °C (60 min), 40 °C min^{-1} to 180 °C (2 min). Retention times: (*R*)-2-nonanol (55.0 min), (*S*)-2-nonanol (56.6 min). *(R)-/(S)-2-Decanol*: 80 °C (92 min), 40 °C min^{-1} to 180 °C (2 min). Retention times: (*R*)-2-decanol (86.0 min), (*S*)-2-decanol (87.7 min).

2.7. Activity of GDH

In a 96-well plate, glucose solution (0.0156–1.0 mol L^{-1},180 μL) and $NADP^+$ solution (10 mmol L^{-1}, 10 μL) were mixed in buffer. The reaction was started by addition of GDH solution (10 μL). Enzymatic activity was determined by following the absorption at 340 nm for 2 min reaction time.

2.8. Stability of enzymes in pure buffer and AMMOENG™ 101 buffer mixtures

The enzymes were stored in the corresponding IL-buffer mixtures at 25 °C; samples were withdrawn and analysed for activity. The half life was determined assuming first order exponential decay of activity versus time.

2.9. Batch experiments in buffer and AMMOENG™ 101 buffer mixtures

Procedure 1: 3-octanone (6.7 mg, 52 μmol) or 2-octanone (6.7 mg, 52 μmol), glucose (190 mg, 960 μmol), *Lb*ADH (0.1 mg lyophilisate) and GDH (2 mg lyophilisate) were dissolved in pure buffer or AMMOENG™ 101 buffer mixture (7.9 mL). With the addition of $NADP^+$ solution (0.63 mg, 0.8 μmol in 100 μL corresponding buffer mixture), the reaction was started. The reaction vessel was kept at 30 °C and shaken at 150 rpm. Samples were extracted with hexane and analysed by GC.

Procedure 2: 3-octanone (41.0 mg, 320 μmol or 82.1 mg, 640 μmol), 2-octanone (41.0 mg, 320 μmol or 82.1 mg, 640 μmol), 2-nonanone (45.5 mg, 320 μmol or 91.0 mg, 640 μmol) or 2-decanone (50.0 mg, 320 μmol or 100 mg, 640 μmol), glucose (238 mg, 1.20 mmol or 301 mg, 1.52 mmol), *Lb*ADH (0.5 mg lyophilisate, 0.079 mg protein) and GDH (5 mg lyophilisate, 1.36 mg protein) were dissolved in AMMOENG™ 101 buffer mixtures leading to a volume of 7.9 mL. To maintain constant pH, $CaCO_3$ (128 mg, 1.28 mmol or 256 mg, 2.56 mmol) was added. With the addition of $NADP^+$ solution (0.63 mg, 0.8 μmol in 100 μL AMMOENG™ 101 buffer mixture), the reaction was started. The reaction vessel was kept at 30 °C and shaken at 150 rpm. Samples were extracted with hexane and analysed by GC.

3. Results and discussion

The number of potential IL as performance additives for biocatalysis is large and to date neither a rational preselection process is available for a given synthetic problem nor sufficient empirical data is available to facilitate the choice without experimental screening. In order to minimise the experimental effort and need of materials for a given synthetic challenge, a generic strategy was devised to narrow down the number of IL in a few convenient steps [20]:

1. Choose a set of IL candidates on the basis of pre-existing knowledge and market availability.
2. Test activity of the enzymes. Discard strongly inhibiting or deactivating IL.
3. Determine solubilisation properties. Discard IL with low solubilisation power.
4. Perform storage stability tests. Discard IL that destabilise the enzyme.
5. Perform batch experiments to validate results.

Table 1
Influence of AMMOENG™ 101 on the solubility of different prochiral ketones (standard deviation below 5% throughout).

Substrate	Maximum solubility/mmol L^{-1}		
	Buffer	100 g IL L^{-1}	200 g IL L^{-1}
3-Octanone	7.4	67	122
2-Octanone	7.9	94	145
2-Nonanone	2.1	77	172
2-Decanone	0.6	52	116

Ideally, with a robust and straightforward activity assay, the number of ILs chosen may be narrowed down with minimum resources. In the case of oxidoreductases monitoring of the reduced cofactor by UV can be carried out after recalibration. The next steps become more and more laborious or require more sophisticated analysis methods.

The initial set of IL was selected on the basis of previous results and market availability (Fig. 1). At first, the influence of 10 vol% of these ILs on the initial activity of the *Lb*ADH was tested with 2-octanone as substrate. Fig. 3 depicts the results of these measurements.

All of the selected ILs, except from [EMIM] [Et_2PO_4], led to high residual activities of at least 80% compared to buffer. With AMMOENG™ 101 activity was improved by factor 1.8. Though, in AMMOENG™ 140 and AMMOENG™ 112 an unidentified precipitate was observed. Therefore, these ILs were not considered for further investigations. The biocompatibility of the *Lb*ADH with hydroxyfunctionalised IL (AMMOENG™ series and [TRIS-(2-OH-Et)-MAM] [$MeSO_4$]) is in general accordance with the findings for an ADH from *Rhodococcus ruber* by de Gonzalo et al. [12]. In contrast to our work they did not find improved activities, which might be due to higher IL contents, as they applied 50 vol% and more.

All ILs which led to improved activities relative to the buffer were tested as solubiliser for 2-octanone at 10 vol%. In buffer the solubility of 2-octanone is 7.9 mmol L^{-1}. [TRIS-(2-OH-Et)-MAM] [$MeSO_4$] showed no increase in solubility. With [EMIM] [$MeSO_3$] a slight increase by factor 1.5 (11.5 mmol L^{-1}) was measured. Both, the AMMOENG™ IL 100 and 102, led to an emulsion. This, after settling for 5 days, resulted in a three phase system [21]. Best results were obtained by applying AMMOENG™ 101 for which a 15-fold increased solubility to 123 mmol L^{-1} was obtained. As this IL already showed best results in the activity measurements, it was chosen for further characterisation of solubility and influence on kinetics.

Overall, the solubility can be increased up to 190-fold depending on the ketone and the amount of IL (Table 1). The highest increase can be obtained for the almost water unsoluble 2-decanone. Surprisingly, the results for 2- and 3-octanone differ considerably.

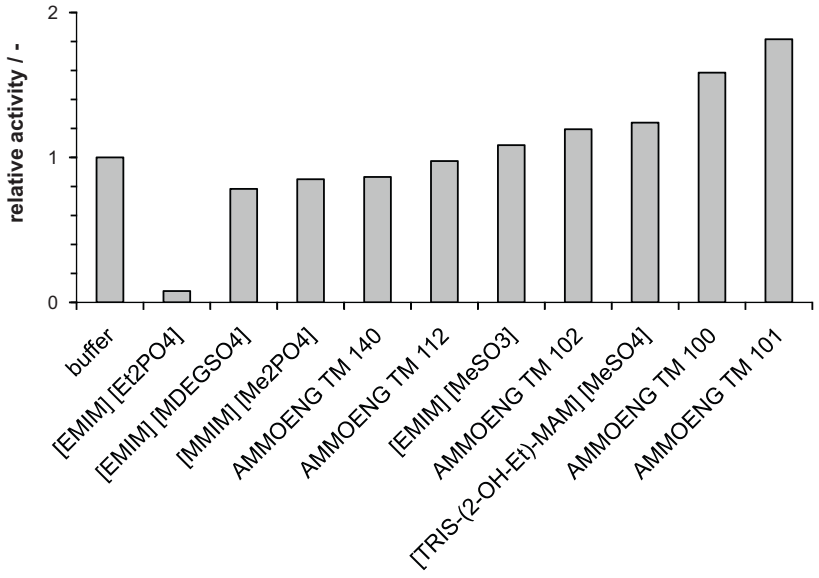

Fig. 3. Influence of 10 vol% of different IL on the *Lb*ADH activity for the reduction of 2-octanone.

Table 2
Model equations for the calculation of kinetic constants [23].

*Lb*ADH catalysed reduction of 2-octanone

$$v = v_{max} \cdot \frac{[NADPH]}{K_{NADPH} + [NADPH]} \cdot \frac{[ketone]}{K_{ketone} \cdot (1 + ([alcohol]/K_{alcohol\ inhibition})) + [ketone] \cdot (1 + ([ketone]/K_{ketone\ inhibition}))}$$

(two substrate kinetics and inhibition by substrate and product)

*Lb*ADH catalysed reduction of 3-octanone, 2-nonanone, 2-decanol

$$v = v_{max} \times \frac{[NADPH]}{K_{NADPH} + [NADPH]} \cdot \frac{[ketone]}{K_{ketone} \times (1 + ([alcohol]/K_{alcohol\ inhibition})) + [ketone]}$$

(two substrate kinetics and inhibition by the product)

GDH catalysed oxidation of glucose

$$v = v_{max} \cdot \frac{[glucose]}{K_{glucose} + [glucose] \cdot (1 + ([glucose]/K_{glucose\ inhibition}))}$$

(single substrate kinetics and inhibition by the substrate)

In view of storage stability, the addition of AMMOENG™ 101 led to prolonged half life of both *Lb*ADH and GHD. Whereas, the *Lb*ADH showed a half life of 49 h in buffer, the values were improved to 91 h in 100 g IL L^{-1} and 158 h in 200 g IL L^{-1}. Stability of GDH in buffer (8.4 h) and 100 g IL L^{-1} (8.5 h) was unaffected but with 200 g IL L^{-1} the half life was increased to 29 h. Both increased and decreased stabilities have been reported for IL [22]. In particular, this finding confirms similar previous investigations [21] of *Lb*ADH stability in the presence of AMMOENG™ 101. Investigations concerning the stability of a *Rhodococcus erythropolis* ADH with other IL from the AMMOENG™ series showed both decreased and increased stabilities [22].

To gain a more detailed insight into the effect of AMMOENG™ 101 on *Lb*ADH and GDH, detailed kinetic investigations were carried out. Hence, photometric measurements with varied concentrations of substrate, cofactor or product were conducted. For 2-nonanone and 2-decanone, no experiments in pure buffer were conducted as the solubility of these ketones is too low. The results of the experiments were used to estimate kinetic parameters using simple multiplicative Michaelis–Menten type expressions [23] (Table 2). Alternatively, an ordered bi-bi mechanism as proposed in the literature could have been used [24]. However, no experimental evidence is available to approve one over the other. (Detailed experimental data can be found in the supplementary information.)

The kinetic parameters were estimated for all substrates from initial rate experiments. It turned out that the rates cannot be described by a single set of parameters (Table 3). Apparently, in case of *Lb*ADH the IL alters affinity to the alcohol. This is especially

Table 3
Estimated kinetic parameters for the *Lb*ADH catalysed reductions.

Substrate	IL content/g L^{-1}	v_{max}/U mg^{-1}	K_{NADPH}/mmol L^{-1}	K_{ketone}/mmol L^{-1}	$K_{alcohol\ inhibition}$/mmol L^{-1}	$K_{ketone\ inhibition}$/mmol L^{-1}
3-Octanone	0	24.2 ± 0.6	0.11 ± 0.01	0.45 ± 0.04	0.40 ± 0.05	
	100	21.2 ± 0.8	0.10 ± 0.02	0.55 ± 0.09	0.45 ± 0.09	
	200	28.6 ± 1.7	0.14 ± 0.03	2.71 ± 0.05	6.5 ± 2.2	
2-Octanone	0	66.4 ± 5.4	0.18 ± 0.03	0.42 ± 0.06	0.18 ± 0.02	4.6 ± 0.6
	100	98.0 ± 6.2	0.32 ± 0.04	0.44 ± 0.06	0.32 ± 0.05	37 ± 3
	200	95 ± 12	0.28 ± 0.07	2.45 ± 0.49	3.39 ± 1.4	49 ± 8
2-Nonanone	100	58.6 ± 3.7	0.31 ± 0.05	0.40 ± 0.07	0.6 ± 0.1	
	200	61.5 ± 3.9	0.28 ± 0.05	1.61 ± 0.21	2.0 ± 0.3	
2-Decanone	100	55.8 ± 4.2	0.29 ± 0.06	0.76 ± 0.15	1.0 ± 0.3	
	200	59.4 ± 4.3	0.26 ± 0.05	2.63 ± 0.39	15.7 ± 7.6	

Table 4
Estimated kinetic parameter for the GDH catalysed oxidation of glucose.

IL content/g L^{-1}	v_{max}/U mg^{-1}	$K_{glucose}$/mmol L^{-1}	$K_{glucose\ inhibition}$/mol L^{-1}
0	9.0 ± 0.2	2.9 ± 0.6	7.7 ± 3.5
100	8.0 ± 0.2	3.7 ± 0.5	4.0 ± 0.9
200	8.1 ± 0.3	2.1 ± 0.7	1.8 ± 0.4

Table 5
Apparent rates[a] and space time yield (STY)[b] for the batch experiments with AMMOENG™ 101 (*ee* > 99% in all cases).

Substrate	@100 g L^{-1}		@200 g L^{-1}	
	Rate/mmol L^{-1} min^{-1}	STY/mol L^{-1} d^{-1}	Rate/mmol L^{-1} min^{-1}	STY/mol L^{-1} d^{-1}
3-Octanone	42	0.25	41	0.1
2-Octanone	56	0.35	72	0.1
2-Nonanone	46	0.30	82	0.1
2-Decanone	51	0.30	73	0.1

[a] Calculated for initial 30 min of product formation.
[b] For conversion >99%.

pronounced for inhibition by the product alcohol. For all four alcohols the inhibition constant $K_{alcohol\ inhibition}$ is increased by at least an order of magnitude when the IL content is increased from 100 to 200 g L^{-1}. Whereas, the addition of 100 g L^{-1} for 2- and 3-octanone does not lead to a significant increase of the inhibition constant. The change in inhibition is also mirrored by the 3–5-fold increases in K_{ketone}. Noteworthy, the substrate inhibition found for 2-octanone is already decreased 8-fold by 100 g L^{-1}. Affinity towards NADPH as reflected by K_{NADPH} remains unchanged. The GDH activity is also affected by IL addition as the inhibition by glucose is increased (Table 4). Whether these findings can be explained by an interaction of the IL with the enzyme or the alteration of reactant availability cannot be deduced from the kinetic measurements alone. For the synthetic purpose of producing the enantiopure alcohols, the substantially lowered inhibition is beneficial as higher substrate concentrations are possible without bargaining a decrease in overall performance.

To test the overall reaction system, batch experiments were carried out in IL buffer mixtures with different IL contents and compared on the basis of reaction rates, space time yields (STY = amount of product produced per litre reaction volume and day), and turnover numbers (TON = amount of product per amount of catalyst or cofactor). To enable the isolated influence of the IL alone, the reactions were deliberately carried out within the solubility constraints imposed by aqueous buffer.

The time course of the batch experiments is given in Fig. 4. For all reactions, quantitative conversion of the respective ketone and *ee* > 99.9% was achieved (data not shown). Furthermore, in buffer the synthesis of (*R*)-2-octanol proceeds slightly faster than the synthesis of (*R*)-3-octanol with ca. 80% conversion after 3.5 h and 4.6 h, respectively. The addition of AMMOENG™ 101 led to an increase in reaction rates for the conversion of 2-octanone with more than 80% conversion already after 1 h while the increase in reaction rates for 3-octanone is less pronounced (3.5 h for 80% conversion). Reaction rates (calculated for the first 30 min reaction time) showed an increase by factor 1.8 with addition of 100 g IL L^{-1} and by factor 2.0 with 200 g IL L^{-1} for 2-octanone, whereas for 3-octanone an increase by 1.2 with 100 g IL L^{-1} and 1.3 with 200 g IL L^{-1} was observed. Hence, the productivity of an *Lb*ADH catalysed ketone reduction can be improved by careful addition of AMMOENG™ 101.

Consequently, batch experiments with higher starting concentration were conducted to assess the potential of AMMOENG™ 101 as a solubiliser (Fig. 5). With initial concentrations of 40 and 80 mmol L^{-1} at 100 and 200 g L^{-1} AMMOENG™ 101, the solubility limit was not fully exploited to avoid the formation of biphasic mixtures. As above, all batches led to quantitative conversions with *ee* > 99.9%. Thereby, turnover numbers of $TON_{LbADH} = 421 \times 10^3$, $TON_{GDH} = 9.6 \times 10^3$, and $TON_{cofactor} = 400$ could be achieved at 100 g IL L^{-1}. With 200 g IL L^{-1} and the simultaneous doubled substrate concentration, the TON doubled to $TON_{LbADH} = 842 \times 10^3$, $TON_{GDH} = 19 \times 10^3$, and $TON_{cofactor} = 800$. Apparent initial product formation rates indicate acceleration of the overall reaction with increased IL loading for the 2-ketones (Table 5, columns 2 and 4). Merely, for the conversion of 3-octanone no apparent rate increase was observed. The space time yields (STYs) were conservatively estimated at conversion higher than 99% (Table 5). For the model compound 2-octanone they are lower than the

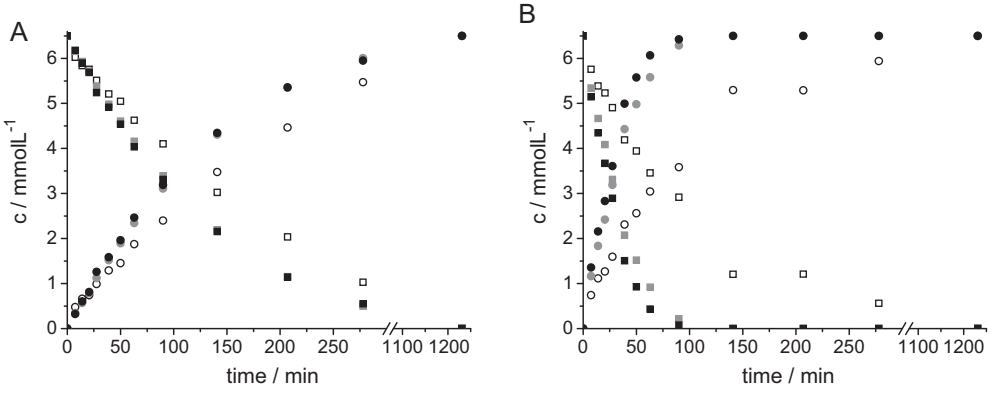

Fig. 4. Concentration as a function of time for batch experiments: (A) 3-Octanon to (*R*)-3-octanol; (B) 2-octanon to (*R*)-2-octanol ((□) ketone (buffer), (■) ketone (100 g IL L^{-1}), (■) ketone (200 g IL L^{-1}), (○) alcohol (buffer), (●) alcohol (100 g IL L^{-1}), (●) alcohol (200 g IL L^{-1})).

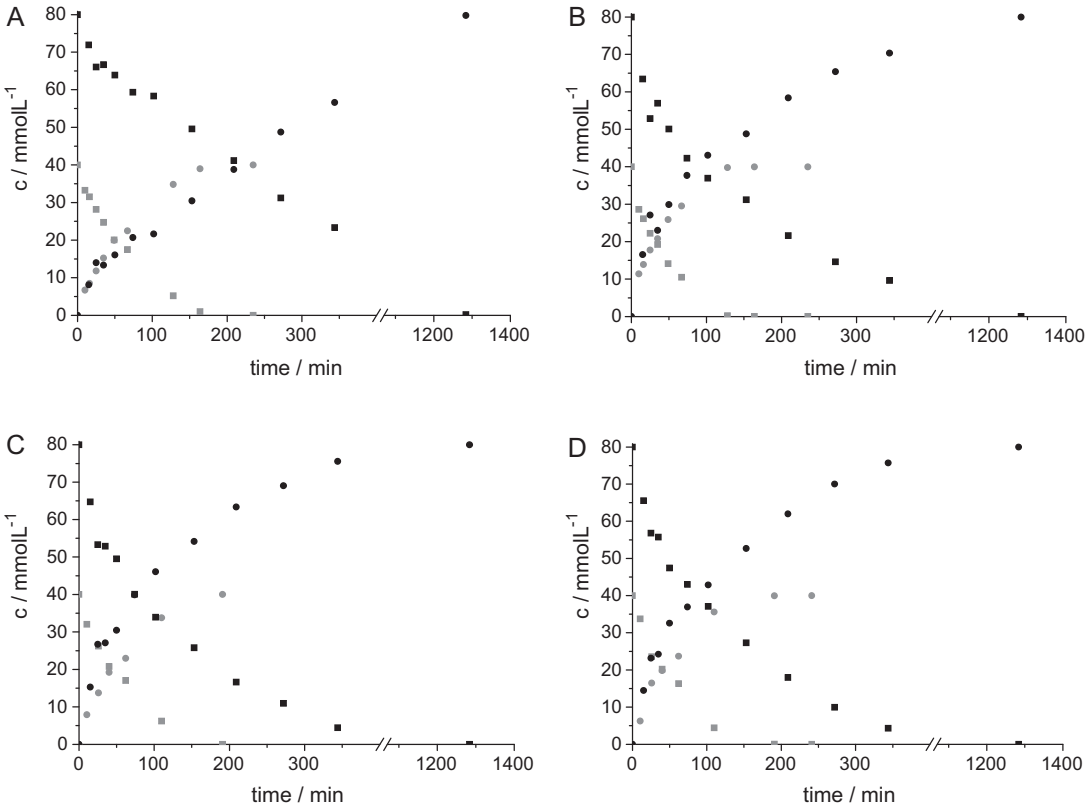

Fig. 5. Time course of the enantioselective batch reactions with AMMOENG™ 101: (A) 3-octane to (*R*)-3-octanol; (B) 2-octane to (*R*)-2-octanol; (C) 2-nonanone to (*R*)-2-nonanol; (D) 2-decanone to (*R*)-2-decanol ((■) ketone (100 g IL L^{-1}), (■) ketone (200 g IL L^{-1}), (●) alcohol (100 g IL L^{-1}), (●) alcohol (200 g IL L^{-1})).

1.3 mol L^{-1} d^{-1} reported for whole cell applications [25] but substantially higher than the 0.02 given for the biphasic approach using water/2-methoxy-2-methyl-propane (MTBE) [26]. In view of the enantioselective reduction of 2-nonanone and 2-decanone, to the best of our knowledge no comparison is available.

4. Conclusion

Within this work, we identified the IL AMMOENG™ 101 among 10 in total as performance additive for the biocatalytic conversion of hardly water soluble, aliphatic ketones. The detailed investigation of solubilising properties, as well as enzyme stabilities and kinetics of the enzymes *Lb*ADH and GDH revealed the high potential of the approach. Especially, the kinetic investigations indicate specific interactions of the biocatalysts and the IL. In batch reactions, the overall performance could be increased with the so far not accessible reduction of 2-decanone to enantiopure (*R*)-2-decanol by biocatalysis. In summary, the AMMOENG™ 101 is a promising performance additive for biocatalytic syntheses also in view of its commercial availability [27]. Further optimisation including downstream processing and recycling of the IL is currently under investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.10.003.

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