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Water immiscible ionic liquids as solvents for whole cell biocatalysis

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Abstract

Whole cell biocatalysis can effectively be used for the production of enantiomerically pure compounds, but efficiency is often low. Toxicity and poor solubility of substrates and products are the main obstacles. In this study, water immiscible ionic liquids are shown to have no damaging effects on the cell membranes of *Escherichia coli* and *Saccharomyces cerevisiae*. Thus, they can be used as biocompatible solvents for microbial biotransformations exemplified by an increase in yield of chiral alcohol synthesis. As key point to the success of these processes, the distribution ratio of the reactants between the ionic liquid and the aqueous phase was identified. The use of ionic liquids as substrate reservoir and in situ extracting agent for the asymmetric reduction of various ketones resulted in an increase of chemical yield from <50% to 80–90% in simple batch processes. (*R*)-1-(4-chlorophenyl)ethanol was produced at a higher initial reaction rate in the biphasic system (>50 $\mu M\,s^{-1}\,L^{-1}$) compared to the aqueous system. This result demonstrates that good mass transfer rates can be obtained despite the relatively high viscosity of ionic liquids.

Keywords: Ionic liquid; Biocatalysis; Whole cell; Multiphase system; Asymmetric reduction; Chiral alcohol

1. Introduction

Biocatalytic synthesis of enantiomerically pure pharmaceutical, agrochemical and nutritional intermediates is attracting much attention (Bommarius and Riebel, 2004; Liese et al., 2000; Schmid et al., 2001). Key advantages of using enzymes as catalysts are their high selectivity without the need for blocking and deblocking steps and their great diversity which can either be found in nature or generated with evolutionary methods or rational design (Robertson and Steer, 2004). Whole cell biocatalysis combines these benefits with simple catalyst preparation and the possibility to develop efficient processes for cofactor requiring or multi step conversions.

Unfortunately, many preferred intermediates and their precursors are poorly soluble in the aqueous reaction medium and/or display toxic effects on the

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biocatalyst. Multiphase processes have been developed in an attempt to restore process efficiency in such a case, but commonly used organic solvents often damage the microbial cells themselves (Leon et al., 1998). Moreover, they are explosive and environmentally harmful. All this points to the need for alternative solvents.

Ionic liquids are a promising new class of solvents as they are nonvolatile and nonflammable and dissolve a wide range of chemical compounds (Wasserscheid and Welton, 2003). Besides multiple encouraging results in isolated enzyme catalysis such as the increase in enantioselectivity and stability (Yang and Pan, 2005) ionic liquids were shown to be biocompatible solvents that support a highly efficient whole cell biocatalytic process in a biphasic ionic liquid/water system (Pfruender et al., 2004). In contrast to organic solvents, the ionic liquids studied did not impair membrane integrity of *Lactobacillus kefir*. Furthermore, they were able to reduce the toxicity of the substrate and product during the reduction of a prochiral ketone. Consequently, yield and enantiomeric excess of the process could be increased without the addition of external cofactor. Studies on the activity of lactic acid-producing and xenobiotic-degrading bacteria and the hydrogenation of C–C double bonds using *Sporomusa termitida* confirmed the activity of whole cells in the presence of ionic liquids due to the good solvent biocompatibility (Matsumoto et al., 2004; Baumann et al., 2005; Lenourry et al., 2005). Nevertheless, the activity is often reduced compared to the purely aqueous environment.

Based on the use of a biphasic ionic liquid/water system for the asymmetric synthesis of alcohols key requirements for the successful development of such processes were studied in this work.

2. Material and methods

2.1. Chemicals

4-Chloro acetoacetate (CAAE, >98%), 4-chloroacetophenone (4-Cl-AP, >98%) and 1-(4-chlorophenyl)ethanol (1-4-Cl-PE, >97%) were purchased from Merck (Darmstadt, Germany). (*R*)- and (*S*)-4-chloro-3-hydroxybutanoate (CHBE, ∼96%) were obtained from Aldrich (Schnelldorf, Germany). *tert*-Butyl 6-chloro-3,5-dioxohexanoate (CDOH), *tert*-butyl (*S*)-6-chloro-5-hydroxy-3-oxohexanoate (CHOH) and different dihydroxyhexanoate products (CDHH) were kindly provided by M. Mueller (Research Center, Juelich, Germany). $NADP^+$ and $NADPH/H^+$ (99.9%) were purchased from Calbiochem (Darmstadt, Germany). The ionic liquids 1-*n*-butyl-3-methylimidazolium hexafluorophosphate (BMIM[PF_6]), BMIM-bis(trifluoromethanesulfonyl)imide (BMIM[Tf_2N]) and methyltrioctylammonium-[Tf_2N] (OMA[Tf_2N]) were kindly provided by Solvent Innovation (Cologne, Germany). All other chemicals were of analytical grade from various suppliers.

2.2. Microorganisms and cultivation conditions

Saccharomyces cerevisiae FasB His6 was provided by Consortium für elektrochemische Industrie (Munich, Germany). The strain originates from *S. cerevisiae* CM3260 and contains a homologous fatty acid synthase under the phosphoglycerate kinase promotor and a thermostable variant of the *Bacillus subtilis* glucose dehydrogenase under the *S. cerevisiae* alcohol dehydrogenase promotor (Engelking et al., 2005). The cells were cultured in a complex medium consisting of 20 g L^{-1} glucose·H_2O, 20 g L^{-1} peptone, 2% (v/v) corn steep liquor and 10 g L^{-1} yeast extract. Shaking flasks without baffles were filled with 20% complex medium, inoculated and incubated at 28 °C, 250 rpm and 5 cm excentricity for 3 days.

Escherichia coli K12 (DSM 498) was grown in a complex medium containing 6 g L^{-1} glucose·H_2O, 10 g L^{-1} yeast extract, 5 g L^{-1} NaCl and 5 g L^{-1} peptone. Shaking flasks without baffles were filled with 20% complex medium, inoculated and incubated at 37 °C, 200 rpm and 5 cm excentricity for 17 h.

L. kefir DSM 20587 used for 4-chloroacetophenone reduction was purchased from Julich Fine Chemicals (Julich, Germany).

L. kefir DSM 20587 used for *tert*-butyl 6-chloro-3,5-dioxohexanoate reduction was produced as described in Pfruender et al. (2005).

2.3. Biotransformations

Biotransformation reactions and incubations of the cells under reaction conditions for determination of solvent biocompatibility were conducted in 4 ml vials,

which were stirred with a magnetic bar. Cells were washed once and suspended in buffer at an appropriate concentration before use. For *S. cerevisiae*, potassium phosphate buffer (100 mM, pH 7.0) with 100 mM NaCl was used, for *E. coli* potassium phosphate buffer (50 mM, pH 7.0) and for *L. kefir* potassium phosphate buffer (200 mM, pH 6.5) with 5 mM $MgCl_2$.

For biocompatibility experiments, the cell suspensions were combined with 20% (v/v) water immiscible organic solvents or ionic liquids. For biotransformation reactions, glucose was added to the cell suspensions (200 mM final aqueous concentration) prior to combination with 20% (v/v) solvent phase containing the reaction substrate. *n*-Butyl acetate for CAAE reduction was used in 7% (v/v) according to Engelking et al. (2005). For one phase aqueous processes, the same amount of substrate was added directly to the cell suspension. Ethyl acetate containing acetophenone as internal standard was used to extract substrate and product. Samples with ionic liquids were centrifuged prior to extraction from the aqueous phase.

2.4. Viability assays

S. cerevisiae viability was assayed with Methylene Blue. After incubation of the cells with dye solution (1%, v/v saturated ethanol in buffer) for 15 min light microscopy pictures were taken and analysed for blue dead cells and colourless viable ones.

E. coli viability was assayed with the LIVE/DEAD® *Bac*light test kit from Molecular Probes (Invitrogen, Karlsruhe, Germany). The cells were incubated with the fluorescent dyes for 15 min. Then the read out ratio of both wavelengths was taken in a micro titer plate fluorescence photometer and compared to a scale obtained with fresh cells, isopropanol damaged cells and mixtures thereof.

2.5. Distribution coefficients

In order to determine the distribution coefficients for substrates and products between ionic liquids and buffer ($\log D$), 20% (v/v) ionic liquid containing 300 mM substrate and 300 mM product were combined with 80% (v/v) buffer in a gas tight vial and vigorously shaken in a mixer mill mM200 (Retsch, Haan, Germany) at 1800 min^{-1} for 1 h. After centrifugation the aqueous phase was extracted with ethyl acetate and the substrate and product concentrations were determined by gas chromatography. Subsequently the $\log D$ was calculated.

Values for the partitioning coefficients between 1-octanol and water ($\log P$) were obtained from www.syrres.com/esc/ (experimental data).

2.6. Analytics

Quantitative gas chromatographic analysis was performed on a CP-3800 system (Varian, Darmstadt, Germany) equipped with a flame ionisation detector (FID). CAAE and CHBE were separated on a Lipodex E column (Macherey Nagel, Düren, Germany). Typical retention times with 5.0 ml min^{-1} helium and a temperature of 110 °C were 3.68 min for CAAE, 4.91 min for (*R*)-CHBE and 5.22 min for (*S*)-CHBE.

CDOH, CHOH and CDHH were separated on a CP-Sil 8 CB-MS Chrompack column (Varian). Typical retention times with 3.0 ml min^{-1} helium and a temperature gradient of 100–160 °C (5 °C min^{-1}) and a hold at 160 °C were 15.3 for CDOH, 14.2 min for CHOH and 21.7 min for CDHH.

4-Cl-AP and 1-4-Cl-PE were separated on a BGB-174 column (BGB Analytik, Schlossboeckelheim, Germany). Typical retention times with 9.0 ml min^{-1} helium and a temperature gradient from 75 °C to 150 °C (2.5 °C min^{-1}) were 20.75 min for 4-Cl-AP, 25.98 min for (*R*)-1-4-Cl-PE and 26.33 min for (*S*)-1-4-Cl-PE.

3. Results and discussion

3.1. Biocompatibility of ionic liquids

In order to broaden the applicability of biphasic ionic liquid/water systems beyond *L. kefir* the effects of the ionic liquids BMIM[PF_6], BMIM[Tf_2N] and OMA[Tf_2N] on the cell membrane of *E. coli* and *S. cerevisiae*, commonly used microorganisms for whole cell biocatalysis, were determined. Viability assays conducted after the incubation of 20 g L^{-1} (in the aqueous phase) *E. coli* K12 dry cell weight (DCW) with 20% (v/v) solvent at 27 °C and 350 rpm for 5 h show that most organic solvents damage membrane integrity nearly completely, except for *n*-decane, a very unpolar and poor solvent (Fig. 1a). Whereas, the three ionic

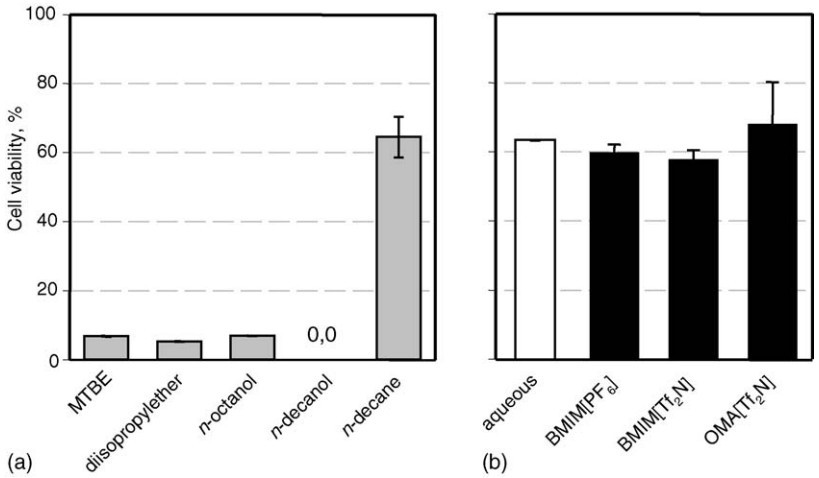

Fig. 1. Cell viability of *E. coli* K12 after 5 h exposure to biphasic systems consisting of: (a) buffer and 20% (v/v) organic solvents and (b) buffer and 20% (v/v) ionic liquids compared to pure buffer system (aqueous).

liquids are totally biocompatible with *E. coli* (Fig. 1b). The same experiments with 20 $g_{DCW}\,L^{-1}$ *S. cerevisiae* FasB His6 at 27 °C and 300 rpm for 20 h showed a less pronounced but still significant toxicity of organic solvents (Fig. 2a). Again, the ionic liquids exhibit complete biocompatibility (Fig. 2b).

Together with previously published work on Gram positive *L. kefir* (Pfruender et al., 2004) the results shown here demonstrate that water immiscible ionic liquids BMIM[PF_6], BMIM[Tf_2N] and OMA[Tf_2N] support cell viability of a selected Gram positive and Gram negative bacterium as well as of a yeast. Therefore, these microorganisms and possibly many others can be efficiently used as whole cell biocatalysts in a biphasic ionic liquid/water system as the cells stay intact and cofactor leaching is prevented. For BMIM[PF_6] biocompatibility is confirmed for lactic acid-producing bacteria and *S. termitida* (Matsumoto et al., 2004; Lenourry et al., 2005). The biocompatibility of water immiscible phosphonium ionic liquids

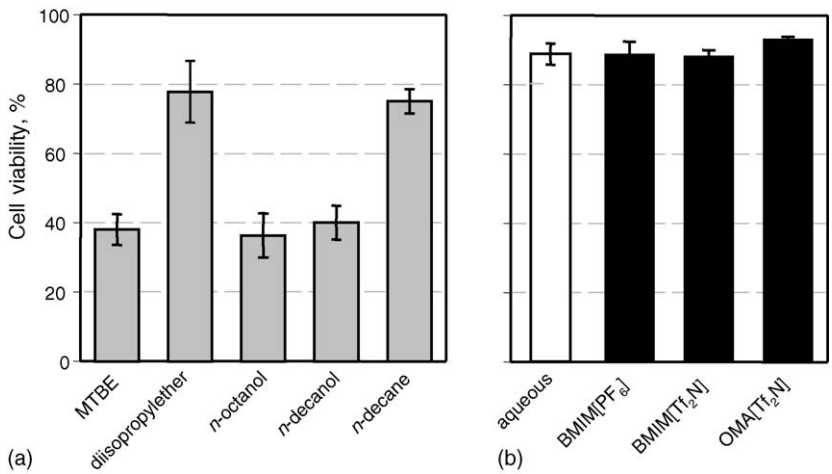

Fig. 2. Cell viability of *S. cerevisiae* FasB His6 after 20 h exposure to biphasic systems consisting of: (a) buffer and 20% (v/v) organic solvents and (b) buffer and 20% (v/v) ionic liquids compared to pure buffer system (aqueous).

was demonstrated with xenobiotic-degrading bacteria (Baumann et al., 2005).

3.2. Distribution coefficients of substrates and products

Due to their good biocompatibility, the ionic liquids BMIM[PF_6] and BMIM[Tf_2N] were used as substrate reservoir and in situ product extracting agent for the biocatalytic synthesis of (*S*)-CHBE, a key intermediate for HMG-CoA reductase inhibitors. 0.68 M substrate was dissolved in the ionic liquid which then was dispersed in a cell suspension containing 20 $g_{DCW}\,L^{-1}$ *S. cerevisiae* FasB His6 and 200 mM glucose. After incubation at 27 °C and stirring at 300 rpm for 20 h (*S*)-CHBE was obtained in 28.7% yield with BMIM[PF_6] compared to 12.4% with BMIM[Tf_2N] and 16.8% with the typically used organic solvent *n*-butyl acetate (Fig. 3a). The same experiments conducted with 100 μM $NADP^+$ in the aqueous phase resulted in significantly higher yields. Thus, differences in yield could originate from damaged cells. To confirm this hypothesis, cell viability was assessed after the process and found to correlate with the obtained yield (Fig. 3b). As the ionic liquids are biocompatible, cell damage must be due to toxic concentrations of the substrate or product in the aqueous phase despite the presence of a solvent phase. This indicates the importance of sufficiently high distribution coefficients of these compounds in favour of the solvent phase in order to effectively reduce their aqueous concentrations.

In order to benefit from the reduced toxicity in the ionic liquid/water system, asymmetric synthesis of (*S*)-CHBE with 50 $g_{DCW}\,L^{-1}$ *S. cerevisiae* FasB His6 and 100 μM $NADP^+$ was performed with 20% (v/v) BMIM[PF_6]. This process resulted in an increase in enantiomeric excess from 61.7% to 83.9% and in chemical yield from 44.4% to 80.2% compared to the use of *n*-butyl acetate as solvent phase (Fig. 4a). For the BMIM[PF_6]/water system the process yield, that is the percentage of product found in the extracting agent compared to the initially dissolved substrate is 46.9%. This again is due to the low distribution coefficient of the product between the two phases ($\log D = 0.75$) which demonstrates the need for higher distribution coefficients if a simple batch process with one step in situ extraction is to be applied.

Another example for the increase of chemical yield by means of an ionic liquid two phase system is the asymmetric synthesis of *tert*-butyl (3*R*,5*S*)-6-chloro-dihydroxyhexanoate, a chiral building block for HMG-CoA reductase inhibitor, with *L. kefir* in a batch process at 30 °C and 2000 rpm. The use of 20% (v/v) BMIM[Tf_2N] as a reservoir of 0.4 M substrate resulted in a chemical yield of 81.3% (Fig. 4b) compared to 47.5% in a purely aqueous system (Pfruender et al., 2005). But with a distribution coefficient of 1.21 the process yield drops again to 65.3%.

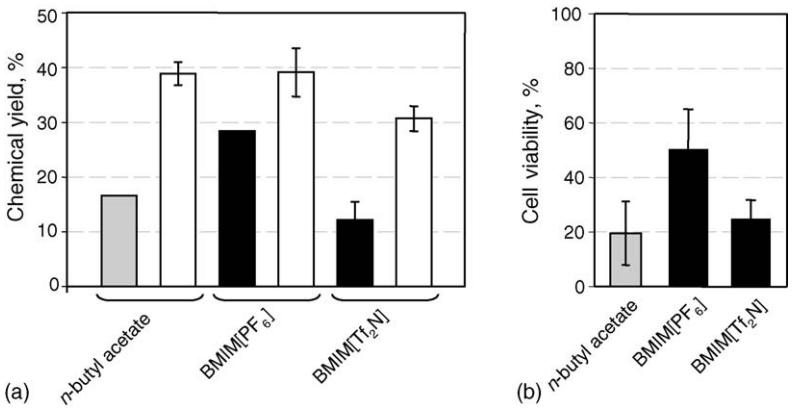

Fig. 3. (a) Chemical yield of (*S*)-4-chloro-3-hydroxybutanoate after reduction of 4-chloro acetoacetate (0.68 mM in 20% (v/v) ionic liquid and 1.94 M in 7% (v/v) butyl acetate, respectively) with *S. cerevisiae* FasB His6 (20 $g_{DCW}\,L^{-1}$ in 80% (v/v) buffer, 200 mM glucose, without $NADP^+$ and with 100 μM $NADP^+$) at 27 °C, 300 rpm for 20 h and (b) cell viability of *S. cerevisiae* FasB His6 after reduction of 4-chloro acetoacetate.

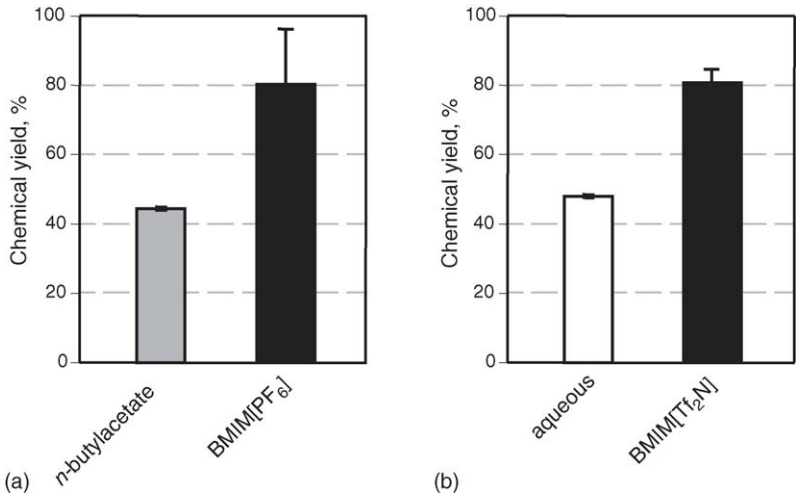

Fig. 4. Chemical yield of (a) (*S*)-4-chloro-3-hydroxybutanoate after reduction of 4-chloro acetoacetate (0.68 M in 20%, v/v BMIM[PF_6] and 1.94 M in 7%, v/v butyl acetate, respectively) with *S. cerevisiae* FasB His6 (50 g_{DCW} L^{-1} in 80%, v/v buffer, 200 mM glucose, 100 μM $NADP^+$) at 27 °C, 300 rpm for 20 h and (b) *tert*-butyl (3*R*,5*S*)-6-chloro-dihydroxyhexanoate after reduction of *tert*-butyl 6-chloro-3,5-dioxohexanoate (0.4 M in 20%, v/v BMIM[Tf_2N] and the same amount in purely aqueous medium) with *L. kefir* (50 g_{DCW} L^{-1} in 80%, v/v or 100%, v/v buffer, respectively, 200 mM glucose) at 27 °C, 300 rpm for 20 h.

Aforementioned examples indicate the great potential of ionic liquids for increasing the efficiency of whole cell biocatalytic processes but also point to the necessity of sufficiently high distribution coefficients in order to reduce substrate and product toxicity and augment process yield. Therefore, possible substrates and products of chiral alcohol syntheses were examined. The plot against their partitioning coefficients log *P* in the 1-octanol/water system shows that more hydrophilic compounds like diones, diols and β-ketoesters without big lipophilic residues and their corresponding alcohols exhibit rather poor log *D* in the studied biphasic ionic liquid/water systems (Fig. 5). Thus, for these syntheses other ionic liquids with better extraction characteristics have to be applied.

For monoketones, monoalcohols and β-ketoesters with a big lipophilic side chain and their corresponding alcohols good to very good distribution coefficients have been found between the aqueous phase and BMIM[PF_6], BMIM[Tf_2N] and OMA[Tf_2N], respectively. For example, log *D* of 4-chloroacetophenone between buffer and BMIM[Tf_2N] is 2.71 and of 1-(4-chlorophenyl)ethanol 2.03 which for the substrate is enough to prevent toxic effect on *L. kefir* nearly completely and for the product reduces process yield

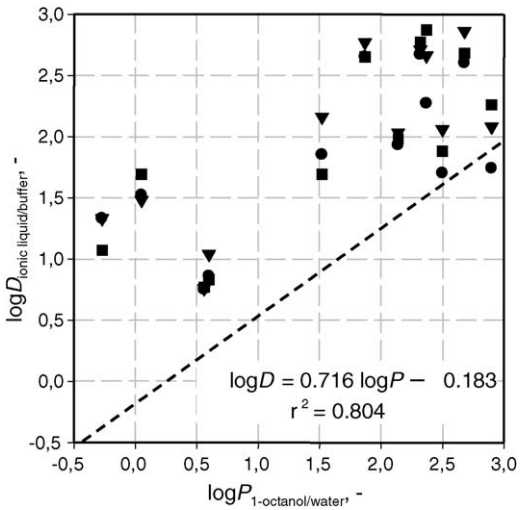

Fig. 5. Distribution coefficient log *D* of various compounds between the ionic liquids BMIM[PF_6], BMIM[Tf_2N], OMA[Tf_2N] and buffer vs. their partitioning coefficient log *P* between 1-octanol and water. 2,5-hexanedione (log *P*−0.27), 4-chloro acetoacetate (0.05), 4-chloro-3-hydroxybutanoate (0.56), 2,5-hexanediol (0.62), ethyl-3-hydroxy-3-phenylpropionate (1.52), ethyl benzoylacetate (1.87), 1-(4-chlorophenyl)ethanol (2.14), 4-chloroacetophenone (2.32), 2-octanone (2.37) 1-(pentafluorophenyl)ethanol (2.50), pentafluoroacetophenone (2.68), 2-octanol (2.90). The dashed line represents the correlation determined by Holbrey et al. (2003) upon data from Huddleston et al. (2001).

compared to chemical yield by only 3.7% in a 20% (v/v) solvent setup (Pfruender et al., 2004).

The generally higher log *D* values in the present study compared to data presented by Huddleston et al. (2001), probably are due to the use of buffer as the aqueous phase rather than pure water which drives the equilibrium to the ionic liquid. For compounds with log *P* in the range of 1.5–3.0 the distribution coefficient between ionic liquid and aqueous phase do not follow the log *P* as suggested by Holbrey et al. (2003). But as the data presented by Holbrey also scatters quite a bit in this narrow log *P* range the presented results might not contradict with the hypothesis of a linear correlation established for a wider log *P* range.

3.3. Mass transfer

High viscosity and low mass transfer rates between the two phases are common preconceptions of an ionic liquid/water system. Asymmetric reduction of 4-chloroacetophenone (0.6 M in the ionic liquid, 20% (v/v) ionic liquid) with 50 $g_{DCW}\,L^{-1}$ *L. kefir* (in the aqueous phase together with 0.2 M glucose) shows that the initial reaction rate integrated over the first 15 min is 75 $\mu M\,s^{-1}\,L^{-1}$ for the two phase process with BMIM[PF_6], 62 $\mu M\,s^{-1}\,L^{-1}$ for BMIM[Tf_2N] and 55 $\mu M\,s^{-1}\,L^{-1}$ for OMA[Tf_2N] (Fig. 6). Therefore, they are higher than the initial reaction rate of the aqueous system with dispersed substrate (43 $\mu M\,s^{-1}\,L^{-1}$). As here the mass transfer rate is the same as the reaction rate, this result demonstrates the possibility to attain sufficiently high mass transfer rates in the biphasic ionic liquid/water system for efficient processes.

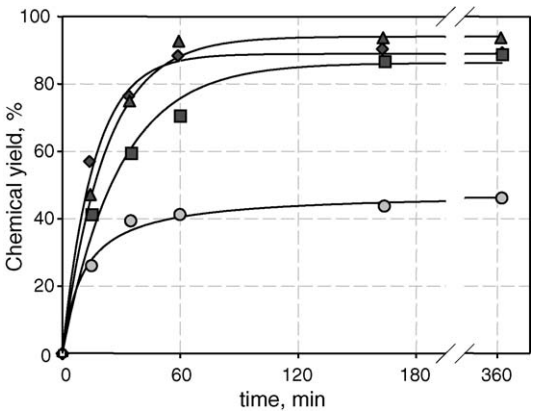

Fig. 6. Chemical yield of (*R*)-1-(4-chlorophenyl)ethanol ((*R*)-1-4-Cl-PE) during biotransformation of 4-chloroacetophenone with *L. kefir* in biphasic ionic liquid/water systems with 20% (v/v) BMIM[PF_6], BMIM[Tf_2N] and OMA[Tf_2N] compared to the reaction in a pure aqueous system. Lines are drawn as a visual aid.

The dynamic viscosity of the water equilibrated ionic liquids are 397 mPa s (BMIM[PF_6]) and 27 mPa s (BMIM[Tf_2N]) (Huddleston et al., 2001). For OMA[Tf_2N] only the dynamic viscosity of the dried ionic liquid, which is generally significantly higher, is known to be 897 mPa s according to Merck KGaA (Darmstadt, Germany). As high mass transfer rates are observed, the simple stirring mechanism is sufficient for the formation of small ionic liquid droplets. This proves that viscosity is not an issue in this system.

In the two phase system, the enantiomeric excess (>99.5%) and the chemical yield (>90%) is much higher than in the aqueous system and the total reaction time (3 h) is equal. This confirms results that have been presented earlier (Pfruender et al., 2004).

3.4. Guidelines for efficient whole cell biocatalysis in biphasic ionic liquid/water systems

Results obtained in this study suggest a procedure to be followed for the use of biphasic ionic liquid/water systems in whole cell biocatalysis (Fig. 7). Such processes are of great interest for the fine chemicals production where substrates and products are poorly water soluble or toxic to the microbial cells and where common organic solvents are not able to solve these problems or need to be replaced by more environmentally benign ones. In this case, ionic liquids can lead to higher efficiency and sustainability.

For a given system of biocatalyst and substrate/product, the choice of the appropriate ionic liquid is crucial. The availability of the specific ionic liquid without impurities that compromise the process as well as cost, stability, corrosive effects and toxicological data are important. Biocompatibility of the ionic liquid was shown for selected Gram positive and Gram negative bacteria as well as for a yeast, but need to be examined for other organisms to achieve optimum results. This can be done by applying a viability test to the cells that have been incubated under process conditions in the biphasic ionic liquid/water system lacking substrate. Furthermore, suitability for methods of product isolation like supercritical CO_2 extraction

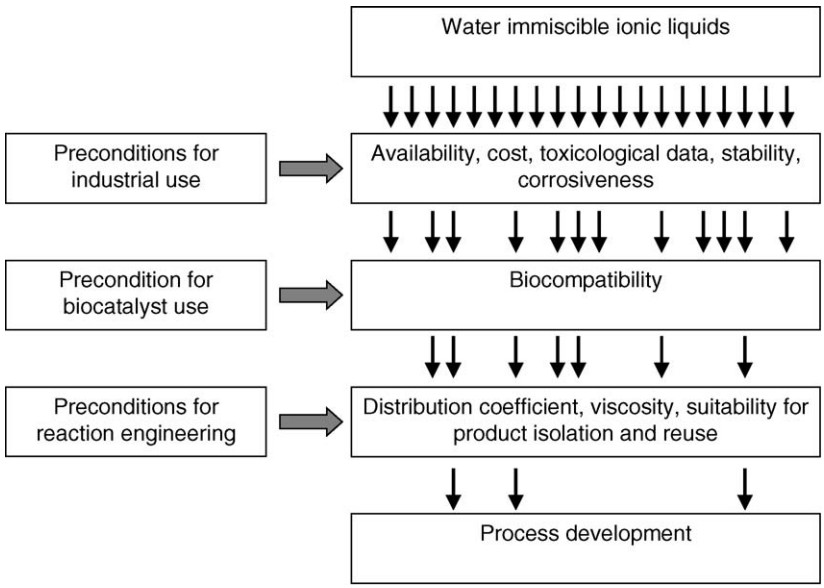

Fig. 7. Scheme for the procedure to select the appropriate water immiscible ionic liquid for the use as substrate reservoir and in situ extracting agent in whole cell biocatalysis.

or distillation (Blanchard et al., 1999; Schofer et al., 2001) and ionic liquid reuse is necessary. Viscosity has to be considered although the examples given demonstrated support of good mass transfer rates during the reaction.

The ionic liquids that have been selected upon the aforementioned criteria must then be characterised for their ability to dissolve the substrate and product as well as for the distribution ratios of the substrate and product between the two phases. The ionic liquids can only be used efficiently if they dissolve the reactants at least up to several hundred millimolar and if log *D* are greater than 2.0. The latter is important not only for extraction efficiency but also for the reduction of aqueous concentrations of the substrate and product and hence their toxic effects on the biocatalytically active cells. Thereby, a small number of ionic liquids should be identified for further process development.

Despite the positive example of 4-chloroacetophenone reduction, one should be aware that low aqueous concentrations and other effects of biphasic systems can result in kinetic and mass transport limitations of the reaction. But better exploitation of the catalytic capacity of microbial cells as promoted by ionic liquids generally leads to more efficient processes.

4. Conclusion

The ionic liquids BMIM[PF_6], BMIM[Tf_2N] and OMA[Tf_2N] were tested for their biocompatibility towards *E. coli* and *S. cerevisiae*. Together with previously published results, it is shown that these water immiscible ionic liquids do not damage microbial cells. Thus, they can be used as substrate reservoir and in situ product extracting agent for biphasic whole cell biocatalytic processes replacing organic solvents and thereby increasing process efficiency. Appropriate distribution coefficients of the substrate and product between the two phases are essential prerequisites for optimum results. Exemplified by the asymmetric reduction of 4-chloroacetophenone with *L. kefir* the possibility to develop an efficient process with high reaction rate could be shown.

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