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Asymmetric whole cell biotransformations in biphasic ionic liquid/water-systems by use of recombinant *Escherichia coli* with intracellular cofactor regeneration

Stefan Bräutigam,[a,*] Stephanie Bringer-Meyer[b] and Dirk Weuster-Botz[a,*]

[a]*Lehrstuhl für Bioverfahrenstechnik, Technsiche Universität München, Boltzmannstrasse 15, 85748 Garching, Germany*
[b]*Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany*

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Abstract—Ionic liquids such as [BMIM][PF_6] and [BMIM][NTF] are already known as good alternatives to organic solvents in biphasic biotransformation. Herein, we report about a systematic procedure based on physical properties to identify more commercially available ionic liquids exhibiting the potential to improve the efficiency of whole cell biocatalyses. This approach resulted in the identification of seven other water immiscible ionic liquids. These ionic liquids were rated by their biocompatibility, their substrate- and product-specific distribution coefficients and by for example performed asymmetric reductions of several prochiral ketones. With the use of a recombinant *Escherichia coli* as biocatalyst, overproducing a *Lactobacillus brevis* alcohol dehydrogenase and a *Mycobacterium vaccae* N10 formate dehydrogenase for cofactor regeneration, the great potential of asymmetric whole cell biotransformations in biphasic ionic liquid/water-systems were demonstrated in simple batch processes.

1. Introduction

Due to high product selectivities, the biocatalytic production of non-racemic chiral fine chemicals with isolated enzymes or whole cells has gained importance.[1–4] Comparing these two alternatives, the use of whole microbial cells avoids the cost-intensive enzyme-purification and profits from intracellular cofactor regeneration, rendering redundant the supplementary addition of cofactors (NAD(P)H) and cofactor regeneration enzymes.[5,6] In the case of low water solubility or high toxicity of substrate and product a biphasic process design is often applied, in which an additional organic solvent functions as a substrate reservoir and an in situ-extractant.[7,8] However, organic solvents are often toxic to the whole cell biocatalyst, as well.[9] To avoid this disadvantage, water immiscible ionic liquids (IL's) are recommended as interesting alternatives.[10–15] Criteria for the rating of ionic liquids have already been proposed,[15] but a systematic approach for the identification of IL's for whole cell biotransformation still does not exist.

Regarding the specific biocatalyst performance, biotechnologically designed *Escherichia coli* strains overproducing a desired enzyme are superior to the corresponding wild-types. A further improvement of biocatalytic activity can be achieved by implementing an additional suitable intracellular cofactor regeneration system.[16–19] To determine the possible negative impacts on a biocatalyst with cofactor regeneration, we applied a recombinant *E. coli* coexpressing a *Lactobacillus brevis* alcohol dehydrogenase gene ($adh_{L.\ brevis}$) for asymmetric syntheses and a *Mycobacterium vaccae* N10 formate dehydrogenase gene ($fdh_{M.\ vaccae}$) for NADH regeneration with formate.[20]

The performance of a recombinant *E. coli* whole cell biocatalyst with cofactor regeneration in combination with a biphasic process design using different ionic liquids as the second liquid phase was evaluated for the asymmetric reductions of 4-chloroacetophenone (4-Cl-AP) to (*R*)-1-(4-chlorophenyl)ethanol [(*R*)-4-Cl-PE], of ethyl 4-chloroacetoacetate (4-Cl-ACE) to ethyl (*S*)-4-chloro-3-hydroxybutyrate [(*S*)-4-Cl-HBE] and of phenacyl chloride

* Corresponding authors. Tel.: +49 89 28915712; fax: +49 89 28915714 (S.B.); e-mail addresses: S.Braeutigam@lrz.tu-muenchen.de; D.Weuster-Botz@lrz.tu-muenchen.de

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(α-Cl-AP) to (*S*)-α-chloro-1-phenylethanol [(*S*)-α-Cl-PE], respectively.

2. Results and discussion

In order to identify IL's suitable for biphasic biotransformation, the total number of commercially available ionic liquids was narrowed down by their physical properties. Obviously, important preconditions for the application as a second liquid phase in a biphasic process are immiscibility with water and a melting point below 30 °C. An IL-density above 1.2 g/cm^3 is necessary for a simple and efficient phase separation after biotransformation. The viscosity of ionic liquids saturated with water should be below 400 mm^2/s, due to its major impact on dispersion quality and mass transfer limitations. Finally, regarding these exclusion criteria, the total number of currently commercially available ionic liquids was reduced to the nine ILs listed in Table 1.

Subsequently, these ionic liquids had to be rated with respect to their applicability for whole cell biotransformation. An essential criterion for this purpose was their biocompatibility.[14,15] Due to the fact, that toxic solvents' main target is the cell membrane, experiments in continuously stirred 4 ml reactors were performed to determine the influence of respective ILs on biocatalyst's membrane integrity (MI). The results are illustrated in comparison to a pure aqueous system (Fig. 1a). As the investigations show, [PF6]-anions affected the cell membrane of *E. coli* (*fdh*, *adh*) marginally: After a 5 h incubation in a biphasic system consisting of buffer and 20% (v/v) IL, membrane integrity decreased only to 70%, as compared to 95% for the pure aqueous system. In contrast, [NTF]-anions seemed to be more toxic to the cell membrane. The most negative effect was measured for the ILs with [E3FAP]-anions. The influence of the anions on membrane integrity of *E. coli* (*fdh*, *adh*) were neither counteracted nor amplified by the concomitant cations within the observation variance.

Another criterion for the rating of ILs, regarding their ability for biotransformation, is the decadic logarithm of the distribution coefficient between the IL and aqueous phase for substrate and product. The objective of providing substrate concentrations in the ionic liquid of several 100 mM, without exceeding a toxic substrate- and product-concentration in the aqueous phase, states the demand of a log *D* above 2.0.[10–13] As displayed for the 4-Cl-AP/(*R*)-4-Cl-PE reaction system in Figure 1b, all the tested ionic liquids with the exception of [BMPL][E3FAP] and [HMIM][E3FAP] agree with this directive. [NTF]-ILs exhibited the highest product-related distribution coefficients. Furthermore, regarding the first six ionic liquids in Figure 1b, it was obvious, that ILs with hexyl-cation possessed generally higher distribution coefficients than the corresponding cations with a butyl-chain. This effect could be explained by the increasing surfactant character of ILs with increasing alkyl chain length.[21]

The results for the biocompatibility of ILs and their distribution coefficients implicated, that [PF6]-ILs possessed, in comparison to the other ionic liquids, the best qualifications for utilization in biphasic whole cell biotransformation. However, in exemplarily performed conversions of 600 mM 4-Cl-AP on a 1.4 ml scale, [NTF]-ILs show likewise high yields as [PF6]-ILs (Fig. 1c). As this example indicated, the interactions between IL and biocatalyst (MI) and between IL and substrate/product (log *D*) were not sufficient for an estimation of the best suited IL for an entire reaction system.

Due to the unsatisfactory conversion results, [E3FAP]-ILs seemed to be unsuitable for the biphasic biotransformation under study. The remaining six ionic liquids show good performances in simple batch processes. Hexyl-ILs resulted in slightly improved conversions, compared to the corresponding butyl-ILs. The enantiomeric excess for the reactions with ionic liquids was, in all cases, $\geqslant$99.5%. Thus the ee was improved, compared to the aqueous system (ee = 96%). The major advantage of a biphasic process design with the selected ionic liquids was the much higher chemical yield in the batch processes with *E. coli* (*fdh*, *adh*) (~60% compared to 8%).

The toxicity of substrate and product can have a significant influence on chemical yield. Therefore, the results obtained with 4-Cl-AP were also verified for other reaction systems, for example, 4-Cl-ACE and α-Cl-AP, as shown in Table 2. However, [E3FAP]-ILs were neglected in this investigations due to the negative results for their biocompatibilities, product-related distribution coefficients, and the so far performed biocatalytic conversions of 4-Cl-AP. The identified distribution coefficients and chemical yields for the other six ionic liquids show that hexyl-ILs were better qualified than the corresponding ILs with butyl-cations. Furthermore, for the 4-Cl-ACE reaction system, it could be stated, that the target of a nearly complete conversion was not

Table 1. Potential ionic liquids suitable for whole cell biotransformation in biphasic systems

IL-abbreviation	IL-name
[BMIM][PF6]	1-Butyl-3-methylimidazolium hexafluorophosphate
[HMIM][PF6]	1-Hexyl-3-methylimidazolium hexafluorophosphate
[BMIM][NTF]	1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide
[HMIM][NTF]	1-Hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide
[BMPL][NTF]	1-Butyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide
[HMPL][NTF]	1-Hexyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide
[BMPL][E3FAP]	1-Butyl-1-methylpyrrolidinium tris(pentafluoroethyl)trifluorophosphate
[HMIM][E3FAP]	1-Hexyl-3-methylimidazolium tris(pentafluoroethyl)trifluorophosphate
[EWTMG][E3FAP]	*N*,*N*,*N*′,*N*′-Tetramethyl-*N*″-ethylguanidinium tris(pentafluoroethyl)trifluorophosphate

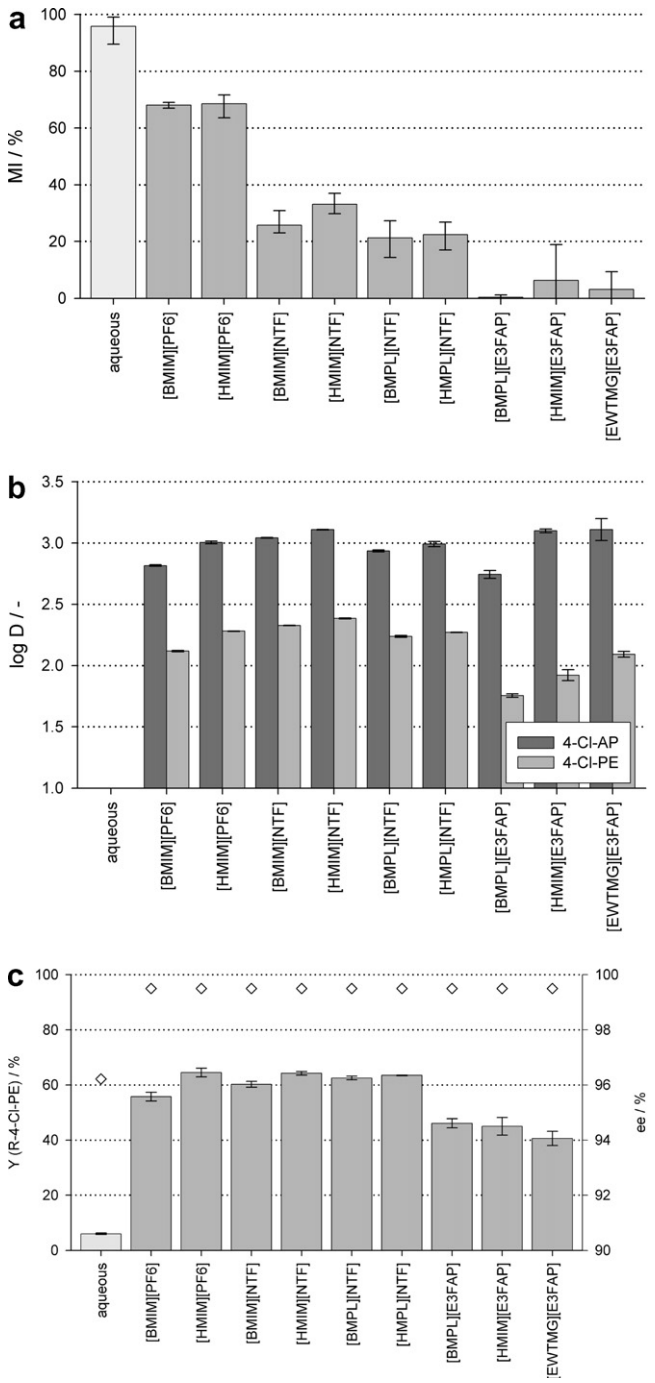

Figure 1. Criteria for the rating of ionic liquids: (a) membrane integrity MI of *E. coli* (*fdh*, *adh*) after 5 h incubation in a biphasic system with 20% (v/v) IL in comparison to buffer; (b) logarithm of distribution coefficients for 4-Cl-AP and 4-Cl-PE between IL and buffer; (c) chemical yield *Y* of (*R*)-4-Cl-PE after 1 h biotransformation in a biphasic system with 20% IL and 600 mM substrate (related to IL volume) at a cell density of 50 $g_{CDW}\,l^{-1}$ in comparison to a biotransformation without IL.

affected negatively by the relatively low distribution coefficients. [BMPL]- and [HMPL][NTF] seemed to be particularly suitable due to comparatively high distribution coefficients, which resulted in the highest chemical yield for (*S*)-4-Cl-HBE. In comparison to that, the phenacyl chloride reaction system offered significantly higher distribution coefficients, but, most probably as a result of a kinetic limitation, the chemical yields were reduced. However, nearly complete conversion of α-Cl-AP was obtained in the following 60 min (results not shown). Another indication for a kinetic limitation could be seen in the fact that [PF6]-ILs offered in these cases the highest conversions, although distribution coefficients recommended [BMIM]- and [HMIM][NTF] for biphasic biotransformation.

3. Conclusion

In conclusion, we have demonstrated that a proceeding analogous to the one displayed in Figure 2 is an appropriate strategy for the identification and rating of ionic liquids suitable for biphasic whole cell biotransformation. In combination with a recombinant *E. coli* biocatalyst, and on the basis of three reaction systems, we have shown for the first time that [HMIM][PF6] and -[NTF], as well as [BMPL]- and [HMPL][NTF], could improve the efficiency of whole cell biocatalyses in a similar way as [BMIM][PF6] and -[NTF]. These results also show that ionic liquids do not negatively affect the intracellular NADH-regeneration ensured by formate dehydrogenase. Accordingly, in comparison to the aqueous system, space–time-yield as well as chemical yield of, for example, (*S*)-4-Cl-HBE were increased 13-fold to 20 $g\,l^{-1}\,h^{-1}$, and 99% conversion, respectively. Moreover, using ionic liquids as a second phase slightly improved the enantiomeric excess to 99.7%, compared to 99.1% without IL.

4. Experimental

E. coli BL21 (DE3) (pBBR1MCS2-$fdh_{M.\ vaccae}$; pBtac-$adh_{L.\ brevis}$), designated in this work *E. coli* (*fdh*, *adh*), was constructed as described in the literature.[20] Precultures of *E. coli* (*fdh*, *adh*) were grown overnight in shaking flasks without baffles filled with 20% complex medium containing 10 $g\,l^{-1}$ yeast extract, 5 $g\,l^{-1}$ peptone, 5 $g\,l^{-1}$ NaCl, 6 $g\,l^{-1}$ glucose·H_2O and 50 $mg\,l^{-1}$ kanamycin and carbenicillin, respectively, at 30 °C, 250 rpm and 5 cm excentricity. Cells were concentrated by centrifugation at $OD_{600} \sim 2$ and were used for inoculation of 4 l medium (same medium as described above, but containing 15 $g\,l^{-1}$ instead of 6 $g\,l^{-1}$ glucose·H_2O) in a 7.5 Labfors stirred tank reactor (Infors, Switzerland). Batch cultivations were performed. After glucose was completely depleted, 0.7 mM IPTG was added. Cells were harvested by centrifugation (4.500*g*, 20 min, 4 °C) when measurements of the in vitro activities of ADH and FDH at time intervals show constant values. Afterwards, the biocatalyst was stored at 4 °C.

To determine the specific ADH- and FDH-activity cell suspension was diluted to an OD_{600} of ~1 and disrupted by adding 10% (v/v) PopCulture (Novagen). The activity of alcohol dehydrogenase was determined photometrically in an assay mixture of 20 µl acetophenone solution (50 mM), 20 µl NADH solution (6.4 $mg\,ml^{-1}$), 140 µl potassium phosphate buffer (100 mM, pH 7.0) and 20 µl disrupted cells. The assay for formate dehydrogenase contained 20 µl sodium formate solution (800 mM), 20 µl

Table 2. Logarithm of distribution coefficient ($\log D$) for 4-Cl-ACE, 4-Cl-HBE, α-Cl-AP and α-Cl-PE between IL and buffer, chemical yield Y after 1 h in a biphasic system with 20% (v/v) IL and 600 mM substrate for a cell density of 50 $g_{CDW}\,l^{-1}$ in comparison to the biotransformations without IL and enantiomeric excess

	Without IL	[BMIM][PF6]	[HMIM][PF6]	[BMIM][NTF]	[HMIM][NTF]	[BMPL][NTF]	[HMPL][NTF]
$\log D$ (4-Cl-ACE)	—	1.92 (±0.02)	1.97 (±0.00)	1.88 (±0.01)	1.88 (±0.02)	1.80 (±0.01)	1.81 (±0.00)
$\log D$ (4-Cl-HBE)	—	1.03 (±0.05)	1.12 (±0.03)	1.14 (±0.03)	1.15 (±0.00)	1.53 (±0.02)	1.55 (±0.00)
Y [%] ((*S*)-4-Cl-HBE)	7.5 (±0.4)	95.8 (±2.2)	98.2 (±1.8)	97.1 (±0.7)	97.5 (±1.7)	99.3 (±1.0)	99.1 (±1.4)
ee [%] ((*S*)-4-Cl-HBE)	99.1 (±0.2)	99.7 (±0.3)	99.6 (±0.4)	99.7 (±0.0)[a]	99.7 (±0.0)[a]	99.7 (±0.0)[a]	99.6 (±0.4)
$\log D$ (α-Cl-AP)	—	2.97 (±0.01)	3.05 (±0.02)	3.01 (±0.00)	3.01 (±0.02)	2.87 (±0.03)	2.93 (±0.01)
$\log D$ (α-Cl-PE)	—	1.94 (±0.01)	2.02 (±0.03)	2.05 (±0.00)	2.07 (±0.05)	1.85 (±0.01)	1.88 (±0.07)
Y [%] ((*S*)-α-Cl-PE)	3.3 (±0.2)	69.5 (n.b.)[b]	89.0 (n.b.)[b]	51.1 (n.b.)[b]	69.8 (n.b.)[b]	66.6 (±3.9)	74.2 (n.b.)[b]
ee [%] ((*S*)-α-Cl-PE)	99.7 (±0.0)[a]	99.7 (±0.0)[a]	99.7 (±0.0)[a]	99.7 (±0.0)[a]	99.7 (±0.0)[a]	99.7 (±0.0)[a]	99.7 (±0.0)[a]

[a] No (*R*)-enantiomer detectable.
[b] Not determined.

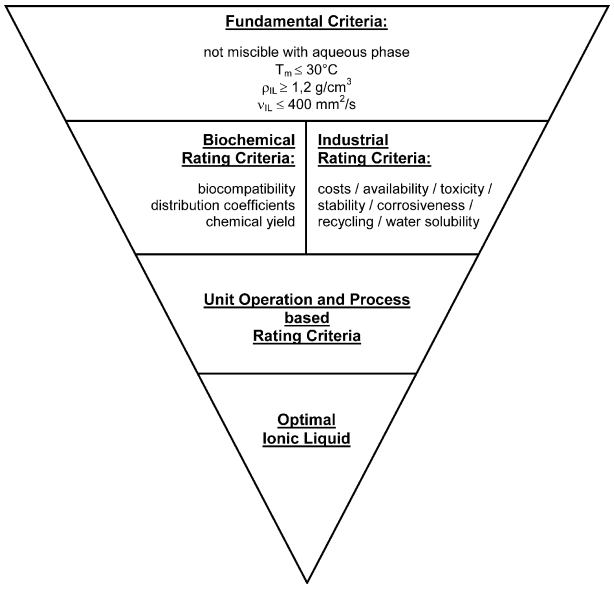

Figure 2. Criteria pyramid for the identification and rating of ionic liquids concerning their applicability in biphasic whole cell biotransformation.

NAD solution (6.4 mg ml^{-1}), 140 µl potassium phosphate buffer (100 mM, pH 8.0) and 20 µl disrupted cells. One unit of enzyme activity (U) was defined as the amount of enzyme catalyzing the conversion of 1 µmol acetophenone per min at 30 °C.

Membrane integrity was determined by the use of the viability test kit LIFE/DEAD *Bac*light (Molecular Probes). In contrast to the 1 h—biotransformation assays, the incubation of *E. coli* (*fdh*, *adh*) with 20% ionic liquid was performed for 5 h, under consideration of a prospective long-term application of the biocatalyst.

To determine the concentrations of the substrates and products in aqueous phase and ionic liquid, extractions were performed with ethylacetate and hexane, respectively. Extracts were analyzed afterwards with gas chromatography (CP-3800, Varian) equipped with a flame ionization detector (FID). Standards of all pure ketones and all pure chiral (*R*)- or (*S*)-alcohols under study were delivered by Sigma–Aldrich and Julich Chiral Solutions with purum grade. 4-Cl-AP, 4-Cl-PE, α-Cl-AP, and α-Cl-PE were separated on a chiral BGB-174 column (BGB Analytik AG). Typical retention times with a flow-rate of 9 ml min^{-1} helium and a temperature gradient from 75 °C to 140 °C (2.5 °C min^{-1}) were 20.8 min with 4-Cl-AP, 24.4 min with α-Cl-AP, 25.2 min with (*R*)-α-Cl-PE, 25.5 min with (*S*)-α-Cl-PE, 26.1 min with (*R*)-4-Cl-PE and 26.5 min with (*S*)-4-Cl-PE. 4-Cl-ACE and 4-Cl-HBE were separated on a chiral Lipodex-E column (Macherey Nagel). Typical retention times with a flow-rate of 4 ml min^{-1} helium and a temperature of 105 °C were 4.5 min with 4-Cl-ACE, 6.2 min with (*R*)-4-Cl-HBE and 6.7 min with (*S*)-4-Cl-HBE.

With an initial substrate concentration of 600 mM (related to IL volume), the biotransformation was performed in 4 ml glass vials equipped with magnetic stirrers by the use of *E. coli* (*fdh*, *adh*)-cells, resuspended in buffer (0.5 M KP_i, pH 6.5, 1 M sodium formate) exhibiting an in vitro NAD(H)-specific ADH-activity of 500 U g_{CDW}^{-1} and an in vitro FDH-activity of 180 U g_{CDW}^{-1}.

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