Bioengineering

Efficient Whole-Cell Biotransformation in a Biphasic Ionic Liquid/Water System**

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Whole-cell biocatalysis represents an elegant way of producing fine chemicals.[1] Industrial processes benefit from the high product selectivity, simple catalyst preparation, and, in the case of redox reactions, the recycling system inherent to the cells. Problems arise from substrates and products that are poorly soluble in water and/or display inhibitory or toxic effects on the biocatalyst. Multiphase processes have been established to address these limitations,[2] but the conventional organic solvents used are often, themselves, toxic for the cells, possibly explosive, and environmentally harmful. Green solvents such as supercritical fluids and ionic liquids (ILs) are promising alternatives.[1i,3]

Here we demonstrate the application of ILs as a substrate reservoir and in situ extracting agent in an efficient multiphase process for the whole-cell-catalyzed synthesis of fine chemicals exemplified by the asymmetric reduction of 4-chloroacetophenone to (*R*)-1-(4-chlorophenyl)ethanol with *Lactobacillus kefir*.[4] We have shown for the first time that a cellular cofactor regeneration system is active in the presence of ILs, and consequently high product concentrations can be achieved without cofactor supplements.

Economically interesting whole-cell-catalyzed redox reactions require a solvent that is nontoxic for the biocatalyst, a prerequisite for efficient cofactor regeneration,[1e] and that dissolves poorly water-soluble substrates. We focused on water-immiscible ILs as the second liquid phase because with a virtually nonexistent vapor pressure they are safe and easy to handle, and first reports indicated whole-cell biocatalytic activity in their presence.[3a,b,5] The ILs used in this study were 1-*n*-butyl-3-methylimidazolium hexafluorophosphate (BMIM[PF_6]), BMIM-bis(trifluoromethanesulfonyl)imide (BMIM[Tf_2N]), and methyltrioctylammonium-[Tf_2N] (OMA[Tf_2N]).

In the absence of substrate, the effect of different ILs present at 20% by volume on the *L. kefir* cell membrane, the primary target of solvent toxicity, was determined.[6] The results were compared to those of a spectrum of organic solvents, among which *tert*-butyl methyl ether (MTBE) and diisopropyl ether are known to help stabilize alcohol dehydrogenase from *Lactobacillus brevis*.[7] For organic solvents, membrane integrity clearly depends on their log*P* values (Figure 1), as found earlier.[8,9] For *n*-decane (log*P* = 5.0) membrane integrity decreased only to 52.7%. With MTBE (log*P* = 0.9), diisopropylether (1.5), *n*-octanol (3.0), and *n*-decanol (4.6) membrane integrity was 10% or less.

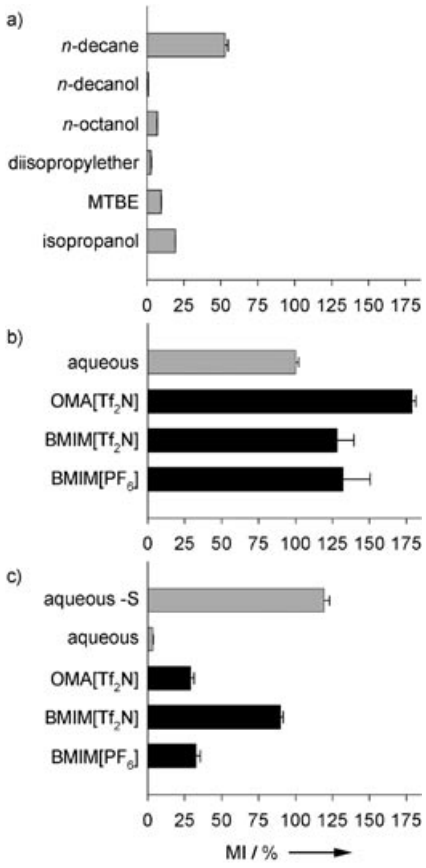

Figure 1. Membrane integrity (MI) of *Lactobacillus kefir* cells after 5 h exposure to biphasic systems consisting of: a) buffer and organic solvents, b) buffer and ionic liquids compared to pure buffer system (aqueous), c) buffer containing glucose and ionic liquids containing 4-chloroacetophenone compared to pure buffer systems containing glucose with ("aqueous") and without ("aqueous -S") dispersed 4-chloroacetophenone.

ILs alone do not damage the cell membrane of *L. kefir* (Figure 1b). Membrane integrity rather increases, an effect that can be explained by a change in morphology similar to that observed in growing cells (data not shown). We also investigated the ability of ILs to circumvent substrate and product toxicity. Without the addition of a solvent phase the presence of 150 mM substrate and product led to nearly complete membrane destruction (Figure 1c), whereas whereas with BMIM[Tf_2N] the membrane remained almost intact (89.8%) (Figure 1c). BMIM[PF_6] and OMA[Tf_2N] reduced toxicity to only around 30% although distribution coefficients for the substrate and the product are nearly the

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Table 1: Distribution coefficients log *D* of 4-chloroacetophenone and 1-(4-chlorophenyl)ethanol between the aqueous phase and different solvents, chemical yields *Y* of biotransformation reactions in such biphasic systems with different cell densities and product purity (as enantiomeric excess).

	Aqueous	BMIM[PF_6]	BMIM[Tf_2N]	OMA[Tf_2N]	MTBE	*n*-Decane[a]
log *D* (4-Cl-AP)	solubility ≈ 7 mM	2.65 (±0.17)	2.71 (±0.10)	2.77 (±0,.7)	2.73 (±0.38)	2.38 (±0.16)
log *D* (1-4-Cl-PE)	solubility ≈ 25 mM	1.93 (±0.08)	2.03 (±0.09)	1.98 (±0.12)	2.54 (±0.37)	1.54 (±0.10)
Y [%] (25 g L^{-1} cells)	42.1 (±6.5)	82.7 (±2.1)	89.0 (±6.2)	80.1 (±17.1)	2.4 (±0.0)	n.d.
Y [%] (50 g L^{-1} cells)	46.2 (±3.7)	88.2 (±4.7)	92.8 (±3.4)	88.4 (±6.0)	4.2 (±0.1)	n.d.
ee [%] (50 g L^{-1} cells)	98.1 (±0.2)	99.8 (±0.0)	99.7 (±0.1)	99.4 (± n.d.)	96.3 (±0.2)	n.d.

[a] n.d. = not determined.

same (Table 1). Hence, suitability of ILs for whole-cell biocatalytic processes cannot be deduced by their distribution coefficients and biocompatibility in the absence of toxic reactants alone. Tests under the actual process conditions are necessary to choose the right IL for a given system of biocatalyst and reactants.

Equilibrium distribution coefficients of substrate and product are nevertheless interesting with regard to process efficiency. Inter alia, they determine the potential process yield. Due to its low log *D* value for 1-4-Cl-PE (1.54) decane, for example, would reduce the process yield in a 20% solvent setup by about 11.5%, which is the fraction remaining in the aqueous phase (Table 1). With BMIM[Tf_2N] this loss only accounts for 3.7%.

Biotransformation reactions with different solvents were conducted on the 2.8-mL scale to determine the chemical yield and product purity. With 25 g L^{-1} *L. kefir* (dry weight) in the aqueous phase, the reaction proceeded for 6 h and yielded a little less product than reactions with 50 g L^{-1} that were finished after 3 h (Table 1). Employing a pH control did not significantly increase the yield (data not shown). The advantage of ILs used as solvent phase was clearly demonstrated. With BMIM[Tf_2N] the chemical yield of 1-4-Cl-PE was doubled from 46.2% to 92.8% relative to the process with dispersed substrate and product, and the product purity was pushed to an excellent 99.7% *ee*.

Despite the affected cell membranes, the results with BMIM[PF_6] and OMA[Tf_2N] were also very good (Table 1). With MTBE the yield is very low. Cells obviously disintegrate even faster in the presence of MTBE than in the absence of a solvent so that the cofactor regeneration breaks down before significant amounts of product can be formed.

We conducted the reaction as a 200-mL batch process with BMIM[Tf_2N] in a stirred-tank reactor (600 rpm, 2.3 W L^{-1}).[10] The reaction kinetics and process parameters were monitored (Figure 2). The chemical yield (93.8% ± 1.0) and product purity (99.6% *ee* ± 0.1) are identical to those obtained on the 2.8-mL scale. The process yield (88.3% ± 1.4), volumetric productivity (20.4 g L^{-1} h^{-1} ± 0.4), and final product concentration (81.6 g L^{-1} ± 1.6) are much higher than reported so far for whole-cell-catalyzed reductions of ketones in IL.[5d] Furthermore, these key figures are also very good compared to industrial biocatalytic processes.[1b,g] No emulsification of the two-phase medium was observed. Therefore, the phases could be separated easily by sedimentation or centrifugation. For GC analysis, the product was extracted from the ILs with hexane. Quantitative and environmentally benign recovery of product from the IL should be possible by distillation,

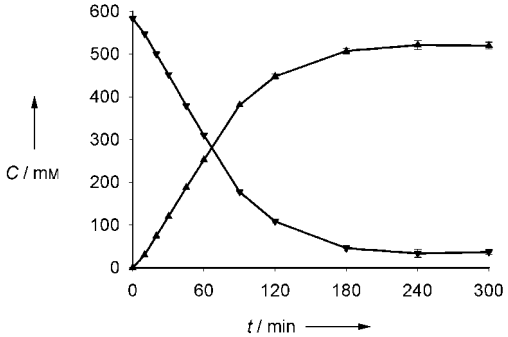

Figure 2. Concentrations of 4-chloroacetophenone (▼) and (*R*)-1-(4-chlorophenyl)ethanol (▲) in BMIM[Tf_2N] during biotransformation with *Lactobacillus kefir* in a biphasic system.

pervaporation, nanofiltration, or extraction with supercritical fluids.[11] Practically no interfering by-products accumulated in the IL (data not shown). The IL may consequently be recycled. Membrane integrity of the cells at the end of the reaction was 101.7% (±5.8), which indicates that the biocatalyst could potentially be reused. The cofactor turnover number of this process is infinite, as the reaction did not require additional NADPH or other redox equivalents. Thus, overall costs of such a process are estimated to be very competitive.

In summary, it could be shown that the ionic liquid BMIM[Tf_2N] exhibits very good solvent properties for 4-chloroacetophenone and its benzyl alcohol reduction product without destructive effects on the cell membranes of *L. kefir*. Therefore, BMIM[Tf_2N] could be used successfully as a substrate reservoir and in situ extracting agent for an efficient whole-cell biocatalytic process with inherent cofactor regeneration. We are currently testing this process design with other reactions in order to evaluate its application range. If positive, it could possibly boost the industrial use of whole-cell catalysts for the production of fine chemicals.

Experimental Section

For biotransformations the IL or organic solvent (20% by volume) containing 600 mM 4-chloroacetophenone, the buffer (0.2 M KP_i, pH 6.5, 0.2 M glucose, 5 mM $MgCl_2$) and *Lactobacillus kefir* DSM20587 cells were combined and stirred in vials (2.8 mL) or in a 200-mL bioreactor, resulting in IL droplets with diameters < 1 mm dispersed in buffer.[10] Reference batches without a solvent phase contained the same amount of substrate dispersed in buffer. Samples were quenched with HCl if necessary, extracted with ethyl acetate or

hexane, and analyzed by GC on a CP-3800 GC system (Varian) fitted with a chiral BGB-174 column (BGB Analytik AG).

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