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# Recycling of the ionic liquid phase in process integrated biphasic whole-cell biocatalysis

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ABSTRACT

Ionic liquids (IL) show a large set of interesting physicochemical properties, which make them an interesting alternative to commonly used organic solvents for applications in biphasic whole-cell biocatalysis. However, the currently still large cost of this class of solvents makes their use in industrial processes only competitive if the ionic liquid phase is recycled without loss of productivity during the process. Exemplarily for such an application, the asymmetric reduction of 2-octanone to (R)-2-octanol by a recombinant *Escherichia coli* overexpressing the *Lactobacillus brevis* alcohol dehydrogenase (ADH) and the *Candida boidinii* formate dehydrogenase (FDH) in biphasic ionic liquid/water systems was considered in this work. The repeated use of the ionic liquid phase was studied in a reaction system containing 20% (v/v) 1-hexyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide ([HMPL][NTF]) and a substrate concentration of 300 mM 2-octanone in the ionic liquid phase. 25 batch biotransformations were performed followed by phase separation, product isolation through distillation and reuse of the ionic liquid. No decrease in conversion was experienced during the 25 subsequent batch biotransformations. The average conversion was 98.5 (±0.7)%, and enantiomeric excesses were constant at values ≥99.5% (R). Over all cycles, a total of 999 (±6) g (R)-2-octanol $L_{IL}^{-1}$ was produced.



## 1. Introduction

In order to meet the high standards of enantioselectivity imposed in the production of pharmaceuticals and fine chemicals, the chiral building blocks needed are frequently produced using biocatalytic methods rather than traditional chemical pathways [1,2]. Here, the use of oxido-reductases for the asymmetric transformation of prochiral substrates has become a standard approach to yield chiral intermediates at high purity. These enzymes require the presence of cofactors, which are consumed stoichiometrically during the reaction, to perform the transformation of interest. In whole-cell biocatalysis, this cofactor is regenerated inside the cell. In contrast to isolated enzymes, this approach thus constitutes a good solution to avoid the addition of very costly cofactors like NADH or NADPH during the process.

Organic compounds presenting low water solubility and/or toxicity towards the biocatalyst make the application of whole-cell biocatalysis in some cases difficult, because the substrate cannot be added into the reaction system at high concentrations. These limitations can be overcome by choosing a biphasic reaction setup: the aqueous phase is formed by a buffer and contains the biocatalyst. The second phase is a water immiscible phase, for which the substrate and the product show a strong preference. This second phase will then act as a substrate reservoir during the reaction and as *in situ*-extractant for the product [3,4]. The presence of this second phase thus makes it possible to increase the availability of the substrate in the reaction system, while still limiting the inhibition of the enzymes or the damage of the biocatalyst, because the concentrations of toxic substrate and/or product near the biocatalyst (in the aqueous phase) will be comparatively low. In addition, this setup facilitates the product isolation after the biotransformation.

Traditionally, the second phases used in biphasic reaction systems were organic solvents. However, their very volatile nature, the low flash point and the often toxic character make their use inconvenient [5]. A good alternative to organic solvents is ionic liquids (IL). Ionic liquids are salts that have a melting point <100 °C due to the large and asymmetric structure of the composing anion and cation [6]. These solvents show an interesting set of properties: they have very low vapour pressure, they are non-flammable and show high chemical and thermal stability [7,8]. In addition to this, it has been shown that some ionic liquids show particularly good biocompatibility [9]. This makes them even more suitable as second phase in whole-cell biocatalysis.

After first experiments using ionic liquids in biphasic whole-cell biocatalysis were performed in 2000 by Cull et al. [10], several other applications followed in this field [11–16]. The reaction systems

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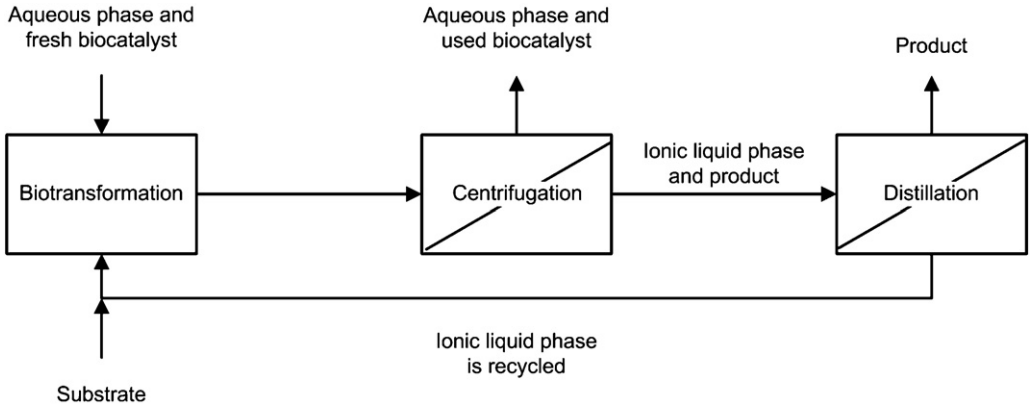


**Fig. 1.** Scheme of the process integrated whole-cell biocatalysis in biphasic ionic liquid/water systems with recycling of the ionic liquid phase.

studied showed that good conversions of up to 80–99%, sometimes with excellent enantiomeric excesses (>99%), could be achieved using ionic liquids in a biphasic reaction setup.

The disadvantage when using ionic liquids is that ionic liquids are still very expensive. A process involving these solvents is thus only competitive if the ionic liquid phase is recycled and reused as many times as possible. To the authors' knowledge no data on the repeated use of ionic liquids in whole-cell biocatalysis over a large number of cycles has been published so far.

Exemplarily for a biotransformation necessitating a biphasic reaction setup, the production of (R)-2-octanol by asymmetric reduction of 2-octanone using a recombinant *Escherichia coli* was considered here. First, a parameter study analysing the effect of varying initial substrate concentrations and ionic liquid volume fractions was performed to identify reaction conditions that lead to conversions >99%. Reaching maximum conversion during the biotransformation is necessary to directly recover the product at sufficient purity, and thus avoid costly product purification steps at the end of the process.

Then, the recycling of the ionic liquid phase over a large number of cycles was studied in these improved reaction conditions, using the same ionic liquid batch during 25 subsequent biotransformations. The scheme of this process integrated whole-cell biocatalysis in biphasic ionic liquid/water systems with recycling of the ionic liquid phase is depicted in Fig. 1.

## 2. Materials and methods

### 2.1. Chemicals

2-Octanone (>98%), 2-octanol (>97%), (R)- and (S)-2-octanol (99%) were purchased from Sigma–Aldrich (Schnelldorf, Germany). $NAD^+$ (~98%) and NADH (>98%) were obtained from Carl Roth (Karlsruhe, Germany). The ionic liquids were kindly provided by Merck KGaA (Darmstadt, Germany). All other chemicals were of analytical grade from various suppliers.

### 2.2. Bacterial strain and cultivation

*E. coli* BL21 (DE3) T1r(pET24a-adh$_{L.brevis}$-fdh$_{C.boidinii}$), subsequently referred to as *E. coli* (ADH, FDH), was kindly provided by Julich Chiral Solutions (Jülich, Germany). It contains the *Lactobacillus brevis* alcohol dehydrogenase (ADH) for the reduction of the ketone and the *Candida boidinii* formate dehydrogenase (FDH) for the internal cofactor regeneration (NADH). The cultivation of *E. coli* (ADH, FDH) was performed in fed-batch mode in a stirred-tank reactor of 7.5 L nominal volume (Labfors, Infors AG, Bottmingen, Switzerland) as described previously [17]. The batch medium used was a complex medium. During the cultivation, two different feeds were applied: first, a glucose feed was added to permit the further growth of the biocatalyst after the batch phase. Then, a glycerol feed was used during the protein expression after induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Once harvested, the cells were stored at 4 °C without loss of activity until their further use.

### 2.3. Enzyme activity assay

The ADH and FDH activity of the biocatalyst was determined by photometrical measurement of the decrease and the increase of NADH, respectively, at 340 nm, as described previously [17]. One unit of ADH or FDH enzyme activity (U) was defined as the amount of enzyme catalyzing the conversion of 1 μmol 2-octanone or formate, respectively, per min at 30 °C. The biocatalyst used typically showed ADH activities of ~250 U $g_{CDW}^{-1}$ and FDH activities of ~200 U $g_{CDW}^{-1}$.

### 2.4. Whole-cell biotransformations

The biotransformations were carried out at 20 °C in a stirred-tank bioreactor (modified bubble column Profors, Infors AG, Bottmingen, Switzerland) of nominal volume 400 mL [18]. The biocatalyst was suspended in the aqueous phase at a concentration of 50 $g_{CDW}$ $L^{-1}$, except stated otherwise. The aqueous phase was composed of a potassium phosphate buffer (0.5 M, pH 6.5), and contained the cosubstrate (1 M sodium formate). The total substrate quantity was dissolved in the ionic liquid phase at varying concentrations. Substrate concentrations indicated in the following as "initial substrate concentration in the ionic liquid phase" refer to the concentrations of 2-octanone found in the ionic liquid before equilibration of the loaded ionic liquid with the aqueous phase. Varying volume ratios of aqueous phase to ionic liquid were used. The experiments were started by mixing the loaded ionic liquid phase with the aqueous phase containing the biocatalyst. The stirrer speed was fixed to 600 rpm throughout the whole batch process (volumetric power input of 0.4 W $L^{-1}$). Experiments at different stirrer speeds and evaluations of the corresponding interfacial area showed that the reaction system was not limited by the mass transfer between the dispersed ionic liquid and the continuous buffer phase in these conditions (data not shown).

Samples of the ionic liquid phase were taken before starting each biotransformation to determine the exact amount of substrate initially present in the system. Samples of the emulsion formed after starting the reaction were continuously taken to monitor the evolution of conversion and enantiomeric excess during the biotransformation. The data shown represent the average of two samples analysed per time point.

### 2.5. Recycling experiments

The recycling of the ionic liquid phase was evaluated through the following procedure: the first biotransformation was executed under fixed conditions, i.e. an initial substrate concentration of 300 mM 2-octanone in the ionic liquid phase (i.e. 1.34 g 2-octanone for a total reaction volume of 200 mL), 50 $g_{CDW}$ $L^{-1}$ biocatalyst and 0.3 M sodium formate in the aqueous phase, and at an ionic liquid volume fraction of 20%. After the biotransformation, the emulsion formed during the reaction by mixing the aqueous phase containing the biocatalyst with the ionic liquid was completely separated into its individual components by centrifugation (5 min at 4500 × *g*, room temperature). The ionic liquid phase was isolated from the other components and then introduced in a rotary evaporator, where the product, as well as any non converted substrate was withdrawn by distillation. The distillation was performed at a temperature of 130 °C under minimal pressure ($P < 5$ mbar) over a duration of 5 h. This step constitutes both the product recovery and the purification of the ionic liquid phase.

After the distillation, the purified ionic liquid was reused in a subsequent biotransformation. The sampling and the transfer of ionic liquid between the different recipients resulted in a loss of ionic liquid in each cycle, which was completed before each new biotransformation in order to guarantee a constant volume ratio between the two phases. New substrate was added at the beginning of each cycle to a concentration of 300 mM 2-octanone in the ionic liquid phase. Fresh aqueous phase containing fresh biocatalyst was introduced into the stirred-tank bioreactor and

mixed to the ionic liquid containing the substrate. The cosubstrate (sodium formate) necessary for the cofactor regeneration was contained in the aqueous phase and was thus supplied simultaneously with the latter. Samples were taken before starting the reaction, during the biotransformation, as well as before and after the distillation.

### 2.6. Purity of the product recovered at each cycle

The purity of the product recovered at the end of each cycle was determined by gas chromatography. The values indicated were calculated by comparison of the concentration of (R)-2-octanol in the distillate to the concentration of (R)-2-octanol determined in purchased samples of known analytical degree.

### 2.7. Analytics

To determine the amounts of substrate and product(s) in the aqueous phase and in the ionic liquid phase, the samples taken during the biotransformation were extracted in one step with ethyl acetate (volume ratio 1:1) and hexane (volume ratio 1:4), respectively. The concentrations of 2-octanone, (R)-2-octanol and (S)-2-octanol in the extract were then determined by chiral gas chromatography (CP-3800, Varian, Palo Alto, USA), using a flame ionization detector (FID) and helium as carrier gas. The separation and quantification of the different compounds were performed using a BGB-175 column (BGB Analytik, Schlossboeckelheim, Germany), a flow rate of 2.5 mL $min^{-1}$ helium, and a temperature of 50 °C. Typical retention times were 31.1 min for 2-octanone, 34.0 min for (S)-2-octanol and 35.3 min for (R)-2-octanol.

Samples of the ionic liquid phase were analysed by $^1$H NMR to detect compounds resulting from the possible degradation of the cation due to the repeated use of the solvent. Accumulation of potentially harmful substances in the ionic liquid phase through contamination during the recycling procedure or through degradation of either the anion or the cation was also verified by ion chromatography. These analyses were carried out on a Metrohm system (Deutsche METROHM GmbH & Co. KG, Filderstadt, Germany), equipped with a model 820 IC separation center, a 819 IC detector, and a Metrosepp A Supp 5 column. The eluent was composed of an aqueous mixture containing $3.2 \times 10^{-3}$ mol $L^{-1}$ $Na_2CO_3$, $1.0 \times 10^{-3}$ mol $L^{-1}$ $NaHCO_3$ and 5% acetonitrile.

## 3. Results and discussion

Guidelines for selecting ionic liquids that are suitable for applications in whole-cell biotransformations in biphasic ionic liquid/water systems have been presented previously [9,19]. The methodology is based on physicochemical properties of the ionic liquids, such as melting point (≤30 °C) or density (≥1.2 g $cm^{-3}$), but it also includes biochemical and industrial rating criteria, e.g. biocompatibility, chemical yield, toxicity, stability and water solubility of the ionic liquid, and costs. On the basis of these criteria, 1-hexyl-1-methylpyrrolidinium bis(trifluoromethylsulfonyl)imide ([HMPL][NTF]) was chosen as the second phase for the production of (R)-2-octanol by asymmetric reduction of 2-octanone using the recombinant *E. coli* (ADH, FDH) in biphasic ionic liquid/water systems [17]. Using this solvent, good conversions of 95% were reached after 6 h. However, the remaining substrate present after the biotransformation constitutes a contamination of the end product (Fig. 1) and it should thus be reduced to a minimum to avoid costly product purification steps after the biotransformation. To this end, a parameter study varying the ionic liquid volume fraction and the initial substrate concentration was performed to further increase the conversion up to values >99%.

### 3.1. Varying ionic liquid volume fractions

Different phase ratios ranging from 10% to 70% ionic liquid were analysed, while keeping the initial substrate quantity added into the ionic liquid phase, as well as the biocatalyst quantity in the aqueous phase constant. The biocatalyst and substrate quantity added to each system, regardless of the ionic liquid volume fraction used, were 8 $g_{CDW}$ and 3.83 mL 2-octanone. This corresponds to a biocatalyst concentration of 50 $g_{CDW}$ $L^{-1}$ in the aqueous phase and a substrate concentration of 600 mM in the ionic liquid phase for a reaction system with an ionic liquid volume fraction of 20%. Due to the defined partitioning of the substrate between the ionic liquid and the aqueous phase, this procedure results in different initial substrate concentrations in the aqueous phases of the various systems (Table 1), and consequently, the initial reaction rates will be affected. Still, only this approach permits comparison of the conversions reached in the different setups.

**Table 1**
Initial substrate concentrations in the ionic liquid resulting from the variation of the phase ratio while keeping the substrate quantity constant.

| Ionic liquid volume fraction | Resulting substrate concentration, mM |
|---|---|
| 10% | 1200 |
| 20% | 600 |
| 30% | 400 |
| 40% | 300 |
| 50% | 240 |
| 60% | 200 |
| 70% | 171 |

As mentioned above, the initial reaction rates observed were indeed larger for systems with lower ionic liquid volume fractions, due to the larger substrate concentrations found within them (Fig. 2). However, increased concentrations of substrate – and later also of product – are also detrimental for the process, as both substances show severe toxicity towards the biocatalyst [20–22]. In fact, as was observed for the reaction system containing only 10% [HMPL][NTF], reaction systems with low ionic liquid fractions – and thus large substrate and product concentrations in the aqueous phase (up to ~1 mM) – reach only relatively low final conversions.

Systems with larger ionic liquid volume fractions show lower initial reaction rates, because the initial substrate concentrations in the aqueous phase are lower. However, they also reach larger final conversions, because the exposure of the biocatalyst to toxic substrate and product is reduced. This was observed for reaction systems with ionic liquid volume fractions of 20–40%, with final conversions of 95–98% (Fig. 2). These three systems reach very similar final conversions, indicating that a further increase of the phase ratio above 20% does not lead to a considerable improvement in terms of productivity.

On the contrary, when a given phase ratio is exceeded, a second effect is observed due to the interaction between the ionic liquid and the biocatalyst. This diminishes or even completely removes the advantage created by decreased substrate and product concentrations in systems with larger ionic liquid volume fractions. Even if some ionic liquids show better biocompatibility than commonly used organic solvents [12], their large presence does influence the biocatalyst and decrease its activity. Here, this limit is reached at an ionic liquid volume fraction between 40% and 50%. Above this value, the final conversion decreases with increasing phase ratio.

The enantiomeric excesses observed did not differ significantly in the different reaction setups. They were ≥99.5% (R) over the whole duration of the reaction for each biotransformation performed.

### 3.2. Varying initial substrate concentrations

Another important parameter in the process design is the initial substrate concentration. The initial substrate concentrations in the ionic liquid phase were varied in the range of 150–1200 mM 2-octanone. This corresponds to a range of initially 0.12–0.97 mM 2-octanone in the aqueous phase after equilibration with the loaded ionic liquid phase (logarithmic distribution coefficient ionic liquid – aqueous phase for 2-octanone: $\log D = 3.1$).

As expected, reaction systems initially containing larger concentrations of substrate in the ionic liquid phase show larger initial reaction rates than those with lower initial substrate concentrations (Fig. 3). Due to the constant equilibrium between both phases, larger substrate concentrations in the ionic liquid phase also mean

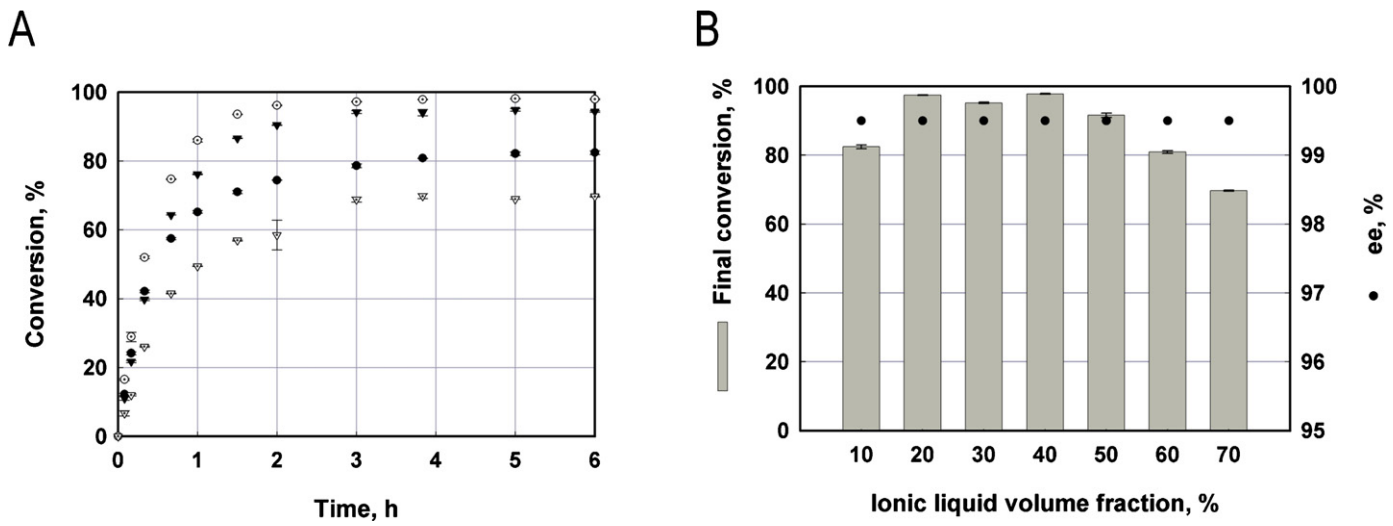


**Fig. 2.** Conversion and enantiomeric excess (ee) for the asymmetric reduction of 2-octanone in biphasic reaction systems with varying volume fractions of [HMPL][NTF]. A: Evolution of conversion with time for systems with 10% (●), 20% (○), 50% (▼), or 70% (▽) [HMPL][NTF]. For purposes of clarity not all the reaction setups analysed are shown. B: Final conversion and enantiomeric excess reached after 6 h. Enantiomeric excesses were constantly ≥99.5% (R) in each setup.

larger substrate concentrations in the aqueous phase containing the biocatalyst, resulting in larger initial reaction rates. These systems also produce larger final quantities of (R)-2-octanol.

However, when considering the final conversion reached, the systems with lower initial substrate concentrations show more satisfying results (Fig. 3). In reaction systems with initial substrate concentrations between 150 mM and 450 mM final conversions ≥99% were observed. Larger initial substrate concentrations provoke a more pronounced reduction of the reaction rate before reaching complete conversion due to inactivation of the biocatalyst. The final conversion reached in these systems thus stays comparatively low. This effect becomes particularly intense in systems with initial substrate concentrations larger than 600 mM 2-octanone in the ionic liquid phase, while below this concentration, an increase of the initial substrate concentration does not affect the final conversion as noticeably. The enantiomeric excesses observed did not differ significantly in the different reaction setups. They were ≥99.5% (R) over the whole duration of the reaction for each biotransformation performed.

### 3.3. Choice of reaction conditions for the recycling process

Only if the conversion reached during the biotransformation is >99%, the purity of the final product will be sufficient to use it directly as it is recovered at the end of the process, avoiding any subsequent costly purification steps. Accordingly, of the different sets of reaction conditions tested above, only those reaching a conversion >99% were considered for further experiments. The reaction systems satisfying this requirement were setups with 20% [HMPL][NTF] and an initial substrate concentration of either 150 mM, 300 mM, or 450 mM 2-octanone in the ionic liquid. Among these, it was evaluated which of the systems leads to the largest space–time yield of (R)-2-octanol (Fig. 4). This showed that the most favourable system would be a reaction setup with an initial substrate concentration of 300 mM 2-octanone in the ionic liquid phase (20% ionic liquid), leading to a space–time yield of 33.5 (±0.9) g (R)-2-octanol $L_{IL}^{-1}$ $h^{-1}$ after a reaction time of 1 h 30 min. This setup was thus chosen for further experiments.

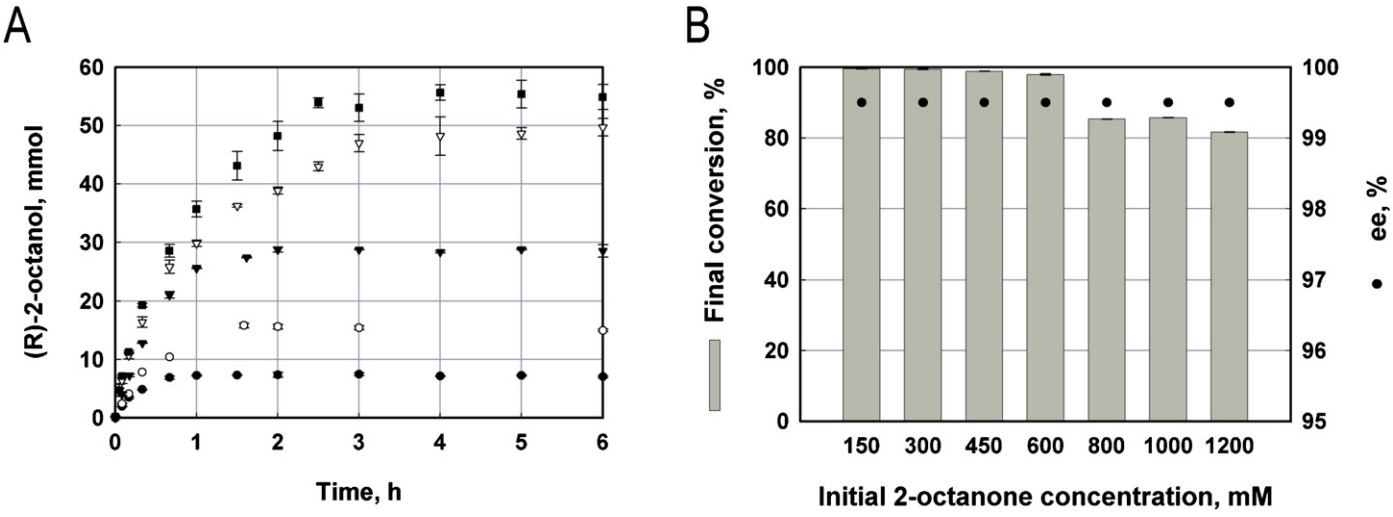


**Fig. 3.** Conversion and enantiomeric excess (ee) for the asymmetric reduction of 2-octanone in biphasic reaction systems with varying initial substrate concentrations. A: (R)-2-octanol production in biphasic systems with initial substrate concentrations of 150 mM (●), 300 mM (○), 600 mM (▼), 1000 mM (▽), and 1200 mM (■). For purposes of clarity not all the reaction setups analysed are shown. B: Final conversion and enantiomeric excess reached after 6 h. Enantiomeric excesses were constantly ≥99.5% (R) in each setup.

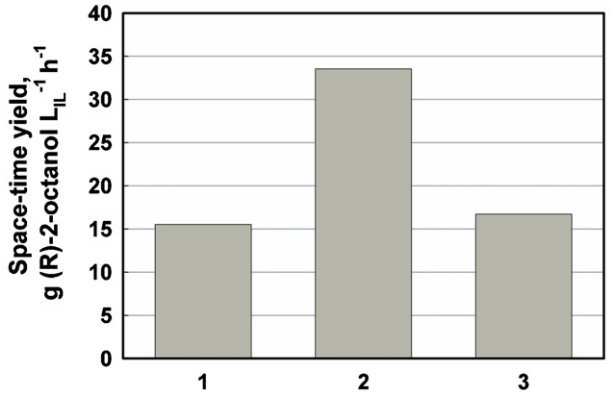


**Fig. 4.** Maximal space–time yield reached in different reaction setups at conversion >99.0%. Initial reaction conditions used: 1:20% [HMPL][NTF], 150 mM 2-octanone, 2:20% [HMPL][NTF], 300 mM 2-octanone, 3:20% [HMPL][NTF], 450 mM 2-octanone.

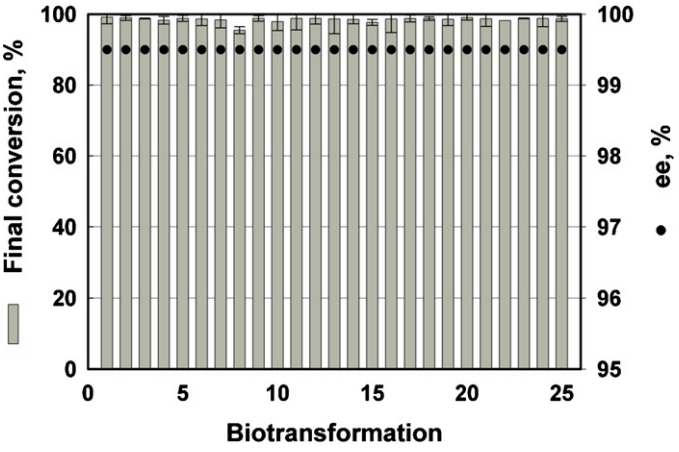


**Fig. 5.** Final conversion reached after 6 h reaction time by each biotransformation throughout 25 cycles. The enantiomeric excess was ≥99.5% (R) over the whole duration of each biotransformation.

### 3.4. Recycling of the ionic liquid

The recycling of the ionic liquid can only be considered successful if two conditions are satisfied: (1) it must be guaranteed that the productivity of the reaction involved is not affected by the recycling procedure; (2) it is essential that no substances harming or inhibiting the biocatalyst are accumulating over time and that the ionic liquid is not degraded during the process. Repeated cycles of biotransformation followed by product isolation and ionic liquid purification were performed to test the recyclability of the ionic liquid used here. After each cycle, samples of the ionic liquid phase were taken to control the quality of the material. Based on the previous results, the reaction conditions were set to a volume fraction of 20% [HMPL][NTF] and an initial substrate concentration of 300 mM 2-octanone in the ionic liquid phase. Fig. 5 shows the conversion reached at the end of each biotransformation, as well as the enantiomeric excess observed. It can be seen that no significant loss of productivity occurred due to the repeated use of the same ionic liquid batch. There are in fact no substantial variations of conversion and selectivity throughout the 25 cycles.

The phase separation through centrifugation resulted very efficiently in a complete separation of the emulsion into its three composing parts: the aqueous phase, the cells, and the ionic liquid. The distillation enabled the recovery of 97% of the product contained in the ionic liquid phase at the end of the biotransformation, at each cycle.

Over the 25 cycles, a total of 999 (±6) g (R)-2-octanol $L_{IL}^{-1}$ was formed, with an average product purity of 96.79 (±2.94)%. The recovery yield of ionic liquid was on average 82.78 (±0.83)%. The loss of ionic liquid occurred mainly due to sampling, as well as due to the transfer of the solvent from one recipient to the other. The volume lost is therefore strongly dependent on the scale of the process and could be significantly reduced at industrial scale. The ionic liquid effectively lost in the aqueous phase constitutes only 0.57%.

The analyses of the ionic liquid samples taken after each cycle by ion chromatography and $^1$H NMR did not show any signs of decomposition, neither for the anion nor for the cation constituting the ionic liquid, and no accumulation of other substances in the ionic liquid was detected (data not shown). A degradation of the ionic liquid during the process can thus be excluded and it can be confirmed that [HMPL][NTF] is recyclable over at least 25 cycles.

## 4. Conclusion

The work presented determined reaction conditions in which the biotransformation of 2-octanone to (R)-2-octanol could be performed at almost complete final conversion and at very satisfying enantiomeric excess (≥99.5% (R)). The setup led to a space–time yield of 33.5 (±0.9) g (R)-2-octanol $L_{IL}^{-1}$ $h^{-1}$ after a reaction time of 1 h 30 min, and the product was recovered at the highest product purity published to date for the direct production of (R)-2-octanol by chemical or biochemical means.

The repeated use of the same ionic liquid phase in subsequent biotransformations showed that the ionic liquid [HMPL][NTF] could be reused over 25 process cycles without loss of productivity during the process and without degradation of the solvent. This knowledge now makes the process attractive for industrial purposes. As no signs of degradation were observed at all, it is strongly supposed that [HMPL][NTF] could be reused over an even larger number of cycles.

Estimating the material costs per reaction batch, the ionic liquid accounts for ~90% of the material costs for the process considered here. Recycling the ionic liquid over 25 cycles would therefore save ~70% of the total material costs incurred, while performing 100 subsequent biotransformations with reuse of the same ionic liquid batch would potentially reduce the material costs by almost a factor 9. The main argument against the use of ionic liquids – the large costs they incur – could thus be refuted.

The same recyclability as shown for [HMPL][NTF] is expected for any ionic liquid presenting the same thermal and chemical stability as the ionic liquid used here. The present process setup could thus be applied to other asymmetric whole-cell transformations limited by the low solubility and/or the toxicity of the substrate and/or of the product of interest, and therefore requiring a biphasic setup. The sole condition is that the ionic liquid must present sufficiently large partition coefficients for the substrate and the product, good biocompatibility and non-water miscibility.

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