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Electroenzymatic synthesis of chiral alcohols in an aqueous–organic two-phase system

Falk Hildebrand and Stephan Lütz*

Institute of Biotechnology 2, Research Centre Jülich, D-52425 Juelich, Germany

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Abstract—The reduction of acetophenone to (*R*)-phenylethanol catalysed by the alcohol dehydrogenase from *Lactobacillus brevis* in combination with electrochemical regeneration of NADPH mediated by a rhodium complex is reported. The reaction in buffer solution was optimised with regard to high productivity (up to 14 $gL^{-1}d^{-1}$) and enantioselectivity (>99.9%). Enzyme stability under the reaction conditions was increased either by addition of bovine serum albumin as a sacrificial protein or by immobilisation, leading to full conversion and enzyme *ttn*'s of up to 75,000. To improve the utilisation of cofactor and mediator as well as to broaden the substrate spectrum to more hydrophobic substrates, we introduced an organic phase of methyl *tert*-butyl ether. This is the first reported two-phase approach for electrochemical cofactor regeneration, which yielded mediator and cofactor *ttn*'s twice as high as in the one-phase approach. Furthermore, a concentrated product solution of 180 mM enantiopure (*R*)-phenylethanol was obtained, facilitating product work-up.

1. Introduction

Alcohol dehydrogenases (ADH, E.C. 1.1.1.*x*) play an important role in the production of pharmaceuticals and fine chemicals because of their superior chemo-, regio- and enantioselectivity.[1,2] Several processes are already known, which use alcohol dehydrogenases on an industrial scale.[2–4] However, these enzymes use nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$), so-called cofactors, as redox equivalents. In a living cell, these cofactors are linked to several enzyme-catalysed reactions, resulting in a continuous change of redox state. When using isolated enzymes, these cofactors must be regenerated by providing the redox equivalents. These redox equivalents are used to return the cofactors to the desired redox state[5] ($NAD(P)^+$ for oxidation reactions, NAD(P)H for reduction reactions).

There are several approaches for cofactor regeneration.[6,7] For the reduction of prochiral ketones catalysed by alcohol dehydrogenases, a reductive regeneration of NAD(P)H is necessary. One possibility is the addition of a second enzyme and a second substrate,[8] which uses the oxidised cofactor for an oxidation reaction, thus regenerating the cofactor (enzyme-coupled regeneration). For this method, it is crucial to choose reaction conditions which suit both enzymes. In addition, the complex mixture of substrates and products increases the process costs and complicates the prediction of the reactor parameters as well as downstream processing. Alternatively, it is also possible to supply an inexpensive alcohol (e.g., 2-propanol) in excess, which is oxidised by the same enzyme, while a more valuable alcohol is formed from the corresponding ketone[9,10] (substrate-coupled regeneration). It should be noted that because of the thermodynamic equilibria involved, even high excesses of the co-substrate did not lead to full conversion.

A very elegant way to provide redox equivalents is the use of electrochemistry.[11] Basically, only two electrons are needed for the regeneration of NADPH. These can easily be supplied by an electric current, being the cheapest redox equivalent (apart from hydrogen).[12,13] No co-substrates or -enzymes are needed in this approach, making the overall process, in principle, more simple and more cost effective.

Unfortunately, the direct reduction of $NADP^+$ at the cathode suffers from unselective side reactions. Therefore, a

* Corresponding author. Tel.: +49 2461 61 4388; fax: +49 2461 61 3870; e-mail: s.luetz@fz-juelich.de

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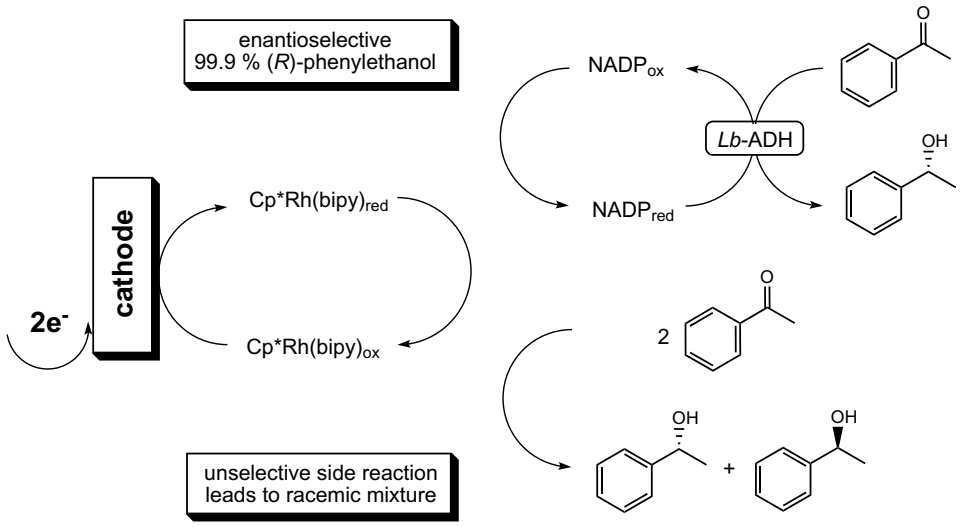

Figure 1. Indirect electrochemical cofactor regeneration and side reaction.

mediator ((2,2′-bipyridyl)(pentamethylcyclopentadienyl)-rhodium, Cp*Rh(bipy)) is used to shuttle the two electrons from the electrode to the oxidised cofactor (see Fig. 1, upper part).

Following Steckhan,[11] this mediator fulfils four requirements:

It transfers two electrons or a hydride ion in one step, avoiding radical formation and is reduced at a potential of less than −900 mV versus Ag|AgCl, above which direct cofactor reduction would occur. Moreover, the two electrons are preferably transferred to the cofactor, not directly to the substrate. In this process, only enzymatically active 1,4-NADPH is formed.

Although the process of indirect electrochemical cofactor regeneration with Cp*Rh(bipy) has been known for some time, it still suffers from lower enantiomeric excesses than possible with enzyme-catalysed reactions. It is our aim to investigate the reasons for these low ee's and find a way to overcome this drawback. Since there are other well established regeneration methods, special consideration must be paid to the practical aspects. A suitable process must find a balance between sufficient utilisation of the reagents and adequate productivity.

For our investigations we chose to use the alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*-ADH), which is known to have a high (*R*)-enantioselectivity and a broad substrate spectrum.[4] As indicated in Figure 1, the redox reaction we investigated was the reduction of acetophenone to (*R*)-phenylethanol.

Both the cofactor and the mediator are valuable reagents, which play an important role when considering economical applicability. Since separating them from the product mixture is not easily done, it would not be economical to use the electrochemical cofactor regeneration in an ordinary batch reaction. Especially for substrates which are barely water-soluble, very low concentrations of both cofactor and mediator would be necessary to reach average turnover numbers. Thus, the reaction velocities are low and the long process times favour enzyme inactivation and cofactor decay.

The use of a second phase of organic solvent decouples the cofactor and mediator concentration from the substrate concentration.[14,15] With this approach, it is possible to use higher concentrations, because the aqueous phase is continuously supplied with fresh substrate while products are extracted. Additionally, it must be considered that working in aqueous solutions limits the final product concentration to the maximum solubility. In contrast, the solubility in organic media may be much higher; the organic phase can thus act as a reservoir in which the product can be accumulated to much higher concentrations. Together with the fact that many organic solvents can more easily be removed in vacuo than water, this facilitates downstream processing.

Thus it is our aim to introduce the concept of two-phase systems to an established reaction setup of indirect electrochemical cofactor regeneration. We characterise the system with respect to the utilisation of the enzyme, the cofactor and mediator and compare it to homogeneous aqueous systems.

2. Results and discussion

2.1. Investigations of the one-phase system

2.1.1. Choice of reaction conditions. The combination of electrochemistry and biocatalysis is always a balancing act between choosing reaction conditions suitable for the enzyme and those which are appropriate for electrochemistry. Generally, enzymes prefer low buffer concentrations, while from an electrochemical point of view, the reaction medium must have sufficient conductivity, that is, high ion concentration to allow good current yields (fraction of current used for the desired reaction) and low cell voltages.

In the reported investigations, we struck a balance between these requirements by using a 100 mM phosphate buffer with a pH of 7. Furthermore, it contained 100 mM of sodium sulfate for higher conductivity and 1 mM of magnesium chloride.

2.1.2. Batch reactions. It has been reported in the literature that the excellent selectivity of the enzyme-catalysed reaction is often diminished by an unselective side reaction, reducing the overall ee.[16–18] Although the electron pair is preferably transferred to the oxidised cofactor, the direct reduction of the substrate also takes place to a minor extent. Since the direct reduction is unselective, both enantiomers are formed. Until now, there have been only a very few publications dealing with applications of electrochemical cofactor regeneration with Cp*Rh(bipy) on a synthetic scale. The processes that have been reported still suffer from low enantiomeric excesses or low conversion, which are still below the synthetical possibilities of the biocatalyst.

We performed batch experiments on a 200 mL scale. With regards to future industrial applications, we focused mainly on the productivities rather than high turnover numbers. We are confident that by making concessions on the productivity, the reported turnover numbers could be increased even more.

In our batch experiments, we encountered a loss in ee after some hours of operation (see Fig. 2). This is caused by a rapid loss of enzyme activity in the reaction mixture. Since the reaction still continues with high enantioselectivity after complete loss of enzyme activity, we discovered that the enzyme showed a high tendency to adsorb on the carbon felt used as the working electrode. The enzyme is still active in its adsorbed state, but unfortunately, the adsorbed enzyme shows only low stability, probably because amino acid residues are reduced by the electric current, thus altering the tertiary structure and therefore reducing the catalytic activity. Once no enzyme activity is left and most of the cofactor is already in its reduced state, the side reaction of direct reduction gains importance, thus diminishing the overall ee (see Fig. 2).

Since it is vital for an economic process to achieve high enzyme utilisation, we investigated two possibilities of avoiding fast enzyme inactivation. These are the addition of bovine serum albumin (BSA) to the reaction mixture and the immobilisation of the enzyme.

The enzyme inactivation occurs only after adsorption onto the carbon felt. By adding BSA as a sacrificial second protein, it should be possible to reduce the amount of adsorbed enzyme. This cheap protein is expected to compete against the alcohol dehydrogenase for the adsorption points on the carbon felt, thus increasing the enzyme concentration in the solution.

Figure 3 shows that the enzyme was effectively shielded from the electrode in the presence of BSA, making it obvious that the process is improved by this simple alteration. The side reaction could be totally suppressed for over 6 h, leading to an enantiopure product (ee >99.9%). The volumetric productivity, defined as weight of product formed per litre and day, based on 90% conversion, reaches a value 14 g L^{-1} d^{-1}, while the current yield ranged about 55%. An amount of 3.5 mmol of product is formed, thus the turnover numbers (mole product per mole of catalyst) resulted in total turnover number (ttn) = 35 for both cofactor and mediator. For the enzyme, a value of ttn = 75,000 is reached, which is an excellent utilisation, considering the fact that after the experiment a part of the enzyme activity still remains.

Another possibility to avoid fast enzyme inactivation is its immobilisation. We recently reported the covalent binding of *Lb*-ADH to porous polymer support under subsequent cross-linking with glutardialdehyde.[19] By this procedure, a stabilising effect was achieved, evident by an increase in storage stability by a factor of 60. We applied this enzyme-support preparation to the standard batch reactor.

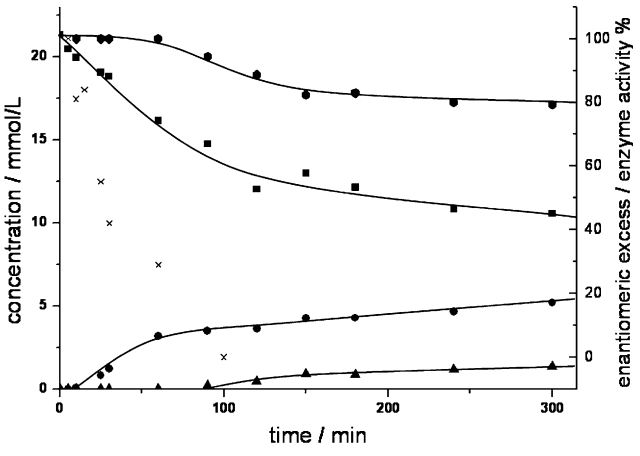

Figure 2. Batch reaction with soluble enzyme (5 U mL^{-1}) 20 mM acetophenone, 0.5 mM NADP; 0.5 mM Cp*Rh(bipy); quadrates: acetophenone, circles: (*R*)-phenylethanol, triangles: (*S*)-phenylethanol, hexagons: enantiomeric excess; crosses: enzymatic activity.

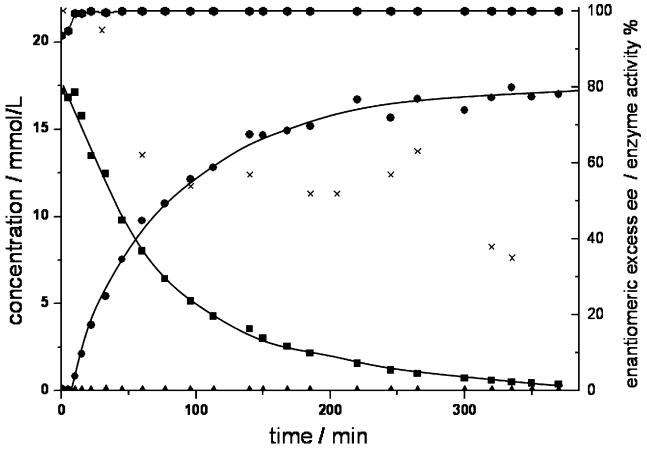

Figure 3. Batch reaction with soluble enzyme (5 U mL^{-1}) and BSA (5 mg mL^{-1}); 17 mM acetophenone, 0.5 mM NADP; 0.5 mM Cp*Rh(bipy); quadrates: acetophenone, circles: (*R*)-phenylethanol, triangles: (*S*)-phenylethanol, hexagons: enantiomeric excess; crosses: enzymatic activity.

The enzyme-support preparation was contained in a porous filter bag, which was sealed and wrapped afterwards around the carbon felt. Since the mediator is formed at the cathode, it was our aim to keep the immobilised enzyme in the direct vicinity of the cathode in order to achieve high reaction velocities. However, the reduced mediator might diffuse into reactor compartments where no enzymatic activity is present, so the side reaction might not be totally suppressed. In order to reduce the effects of this local confinement of the enzyme, the mediator concentration was reduced, while the cofactor concentration was increased, thereby creating a more favourable ratio of mediator to cofactor. During the experiment, a constant ee of over 98% was possible (see Fig. 4). We are confident that in future investigations, this value can further be increased by improving the reactor design.

Nevertheless, the experiment yielded a space time yield of 9 g L^{-1} d^{-1}. This is a high value, considering the fact that the mediator concentration was lower and the heterogeneous reduction of the mediator is expected to be rate limiting. This also raises the turnover number for the mediator to $ttn = 55$. The cofactor ttn is significantly lower with $ttn = 12$. Current yields could be slightly improved upon to an average yield of 61%.

The turnover number of the enzyme is $ttn = 21{,}000$, lower than in the experiment with soluble enzyme. But it must be considered that the enzyme-support preparation could be recovered and used several times, giving a much better utilisation in the long run.

We were able to show that the drawback of the side reaction could be overcome by applying a sufficient enzyme concentration. An ample high enzymatic activity shifts the rate-limiting step to the cofactor regeneration. Nearly all cofactor molecules are now present in their oxidised state, providing a very favourable ratio of mediator to cofactor. Consequently, the mediator reduces the cofactor exclusively. This allowed us to fully exploit the qualities of the enzyme, leading to ee's of over 99.9%.

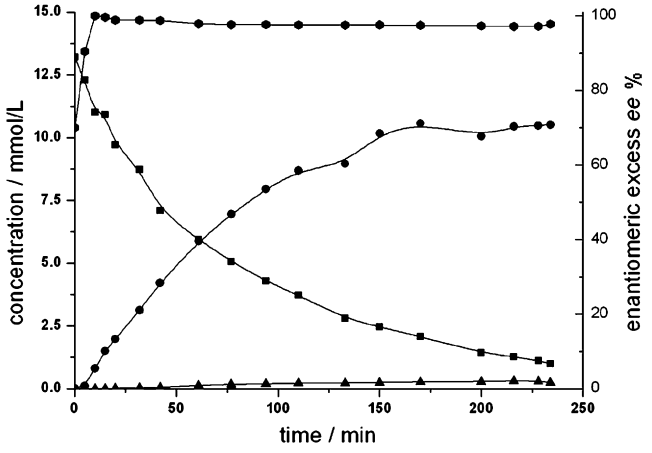

Figure 4. Batch reaction with immobilised enzyme; 13 mM acetophenone, 1.0 mM NADP; 0.2 mM $Cp^*Rh(bipy)$; quadrates: acetophenone, circles: (*R*)-phenylethanol, triangles: (*S*)-phenylethanol, hexagons: enantiomeric excess.

2.2. Investigations of the two-phase system

Under the aspect of substrate solubility, the fact that most enzymatic reactions take place in buffer solution may be seen as a disadvantage. This problem can be overcome by using a two-phase system approach. The added organic phase acts as a reservoir for the substrates and continuously extracts product from the reactive aqueous phase.

This not only results in a continuous substrate supply, but also highly concentrated product solutions and the retention of cofactor and mediator in the aqueous phase are also possible.

When trying to establish an organic phase in a former pure aqueous system, it is necessary to check whether the organic solvent is compatible to the process. Investigations must be conducted to show that the enzyme is able to work in the presence of the organic solvent. The fraction of organic solvent solved in the aqueous phase in particular may have detrimental or beneficial effects on the enzyme.[20] Furthermore, in order to justify the additional effort in establishing a second phase, substrates and products must have suitable partition coefficients, that is, the substrates should predominantly be present in the reaction phase, while already formed product should be extracted into the reservoir phase. Special enzyme properties such as substrate or product inhibition may also have an influence on the choice of organic solvent.[21]

2.2.1. Enzyme stability in a two-phase system. We investigated the enzyme storage stability in water-immiscible organic solvents of different chemical structures, following previous work carried out in our group.[22] For every substance class, a representative solvent was chosen with regards to cost, low toxicity and already known use in industrial processes.

It is obvious that an organic solvent can have both positive and negative effects on enzyme stability (see Fig. 5). While the enzyme shows only very small half-life times in solvents, which are known to be detrimental to protein

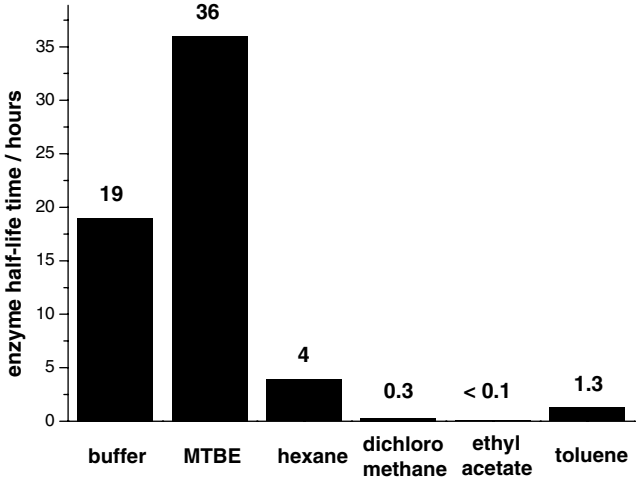

Figure 5. Enzyme stability in organic solvents.

stability, such as dichloromethane and ethyl acetate, the incubation in methyl *tert*-butyl ether (MTBE) nearly doubled the enzyme stability.

2.2.2. Phase partitioning. Partitioning of substrates and products between the phases may have a positive influence on the reaction performance. *Lb*-ADH suffers from substrate inhibition and, to a higher degree, from product inhibition (data not shown). Therefore, medium substrate concentrations and low product concentrations in the aqueous phase are desired. Unfortunately, since the production of an alcohol from a ketone increases the polarity of the molecule, the more polar phenylethanol always shows a higher tendency to remain in the aqueous phase than the non-polar acetophenone (see Fig. 6). Thus, we focused on high product extraction to avoid product inhibition. From the possible solvents with high partitioning coefficients for phenylethanol, we chose to use MTBE to benefit from the stabilising effect.

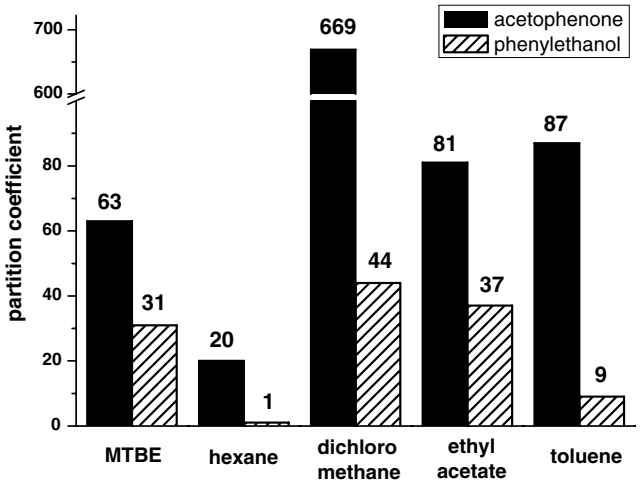

Figure 6. Partition coefficients of acetophenone and phenylethanol.

2.2.3. Two-phase reactor with soluble enzyme in buffer/MTBE. After choosing MTBE as an adequate second phase, we set up a reaction system, where the concentrations of the enzyme, cofactor and mediator were equal to the system employing soluble enzyme and BSA. By using the same conditions, we found it feasible to compare the turnover numbers obtained in both experiments. We chose to use a batch reactor with 150 mL of buffer solution and 30 mL MTBE, adding 9 mmol of acetophenone, which corresponds to twice the amount of substrate soluble in the single aqueous phase. After reaching an equilibrium distribution of substrate in both phases, we started the reaction by applying the potential. The mixture was stirred vigorously to allow for fast phase-transfer of the substrate and the product.

Figure 7 shows the concentrations in both aqueous and organic phase.

It is clearly visible that the two-phase system is working, as (*R*)-phenylethanol is continuously extracted into the ether phase. The concentrations of both the substrate and product in the aqueous phase are constantly kept low, never exceeding a value of 6 mM, effectively avoiding inhibition phenomena. Since the reaction slowed down considerably, the experiment was stopped at a conversion of 65%. The decrease in reaction velocity may be contributed to the depletion of substrate in the reactive phase, which decreased to less than 2 mM.

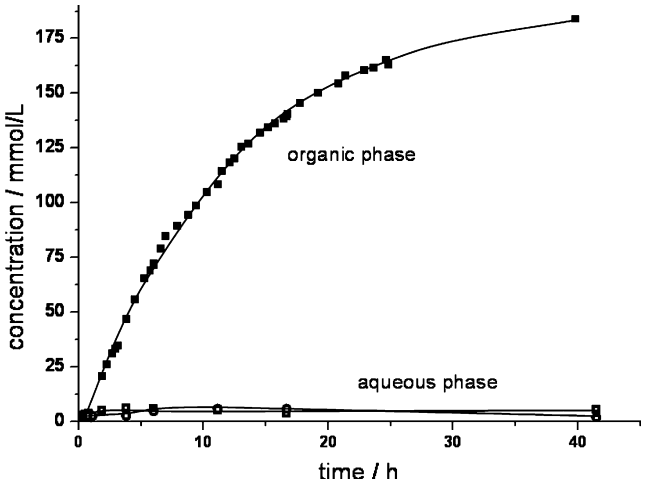

Figure 7. Two-phase reactor; 150 mL buffer solution, 30 mL MTBE, 3 U mL^{-1} enzyme; 5 mg mL^{-1} BSA; 9 mmol acetophenone, 0.5 mM NADP; 0.5 mM Cp*Rh(bipy); quadrates: acetophenone (organic phase); empty quadrates: acetophenone (aqueous phase); open circles: (*R*)-phenylethanol (aqueous phase).

The final product solution contained 180 mM of (*R*)-phenylethanol, which is enantiopure by GC measurements (ee >99.9%). The volumetric productivity referring to 60% conversion yields a value of 3 g L^{-1} d^{-1}, significantly lower than in the one-phase system. This is due to the intense mixing of the phases, which reduces the electric conductivity because of ether solved in the aqueous phase. Moreover, a smaller area of the electrode can effectively be used. This reduces the electric current (ca. 40 mA in aqueous systems vs 20 mA in this approach), while having comparable current yields (45%).

Another benefit of the two-phase system is displayed when looking at the turnover numbers of the mediator and cofactor. With identical concentrations, the *ttn*'s could be increased to $ttn = 64$, nearly doubling the value achieved in the one-phase system. Once again it should be pointed out that the absolute values could be increased even more by reducing the concentrations, this benefit coming at the cost of lower productivity. Considering enzyme utilisation we found that the enzyme showed a tendency to adsorb to the phase interface. In contrast to the adsorption on the carbon felt, this does not have a negative effect on the enzymatic activity, but determining enzyme activity in the aqueous phase yields smaller values than are really present. To exclude the possibility that the enzymatic reaction becomes rate limiting, we added additional enzyme amounts to the reaction mixture twice, after 15 and 30 h. This excess of enzyme reduced the total turnover number to $ttn = 5000$.

3. Conclusion

There are two major obstacles when using indirect electrochemical cofactor regeneration in the way we have described. On the one hand, we observed relatively low enzyme stability under the described reactor conditions. This is due to adsorption on the porous carbon felt and subsequent inactivation. On the other hand, low enzyme activity inevitably leads to an unselective side reaction, diminishing overall ee.

We presented two possibilities to overcome these problems:

When adding a inexpensive second protein such as BSA, the enzyme adsorption on the felt could be significantly reduced. The higher activity present in a solution leads to higher turnover numbers (*ttn*(enzyme) = 75,000). Additionally, the observed productivity of 14 g L^{-1} d^{-1} is one of the highest values reported so far for the electroenzymatic synthesis of chiral alcohols with alcohol dehydrogenases.

Contact between the enzyme and electrode can also be avoided by immobilising the enzyme as described earlier. The advantage of even better enzyme utilisation by the possibility of re-using the immobilisate comes at the cost of a slightly lower ee. This might be overcome by improved reactor design. Moreover, though we reduced the mediator concentration by 60%, we were still able to reach a volumetric productivity of 9 g L^{-1} d^{-1}.

The best experiments in the one-phase batch show an enantioselectivity of over 99.9%, which is higher than any ee ever reported when regenerating NADPH electrochemically with Cp*Rh(bipy) for an ADH-catalysed reaction. When trying to take advantage of the superior selectivity of oxidoreductases, the electrochemical cofactor regeneration is now on the same level as other regeneration methods reported.[23]

Table 1 summarises the different approaches:

One possibility to improve several reaction aspects is the addition of an organic second phase. The substrate supply, as well as the extraction of the product keeps the concentrations of substrate and product in the reactive phase low to avoid inhibition effects. Furthermore, the product solution is much more concentrated and down-stream processing is significantly facilitated. The third and most important aspect is the retention of the cofactor, mediator and enzyme in the aqueous phase. It is now possible to recover the product from the reservoir phase without losing the catalyst in the process. This decoupling of the catalyst from the product allows for a much better utilisation of these reagents.

To the best of our knowledge, we have reported herein the first combination of Cp*Rh(bipy)-mediated electrochemical cofactor regeneration with a two-phase approach. These first experiments, in which the cofactor and mediator could be utilised to almost twice the extent compared to a similar one-phase experiment, already give a hint at the potential two-phase systems offer to electroenzymatic synthesis.

Although the reported productivities increase the values known before, there are already other methods of cofactor regeneration, which show higher productivities as well as better cofactor utilisation. To be really competitive to these processes and to fully exploit the benefits of electrochemical cofactor regeneration, more work has to be done to overcome these drawbacks. Especially intelligent reactor design and further investigations on the field of two-phase systems will be aspects that can further improve electroenzymatic synthesises.

4. Experimental

4.1. Materials

Amino-epoxy Sepabeads® (EC-HFA) were kindly donated by Resindion S.R.L., Milan, Italy, from Mitsubishi Chemical Corporation. Alcohol dehydrogenase from *Lactobacillus brevis* (MW = 107 kDa) was purchased from Julich Chiral Solutions, Jülich, Germany. All other reagents, as well as the dialysis sacks, were purchased from Sigma, Germany, and were of analytical grade or better. The mediator Cp*Rh(bipy) was synthesised according to Spika.[24] The buffer used, was a 100 mM phosphate buffer (pH 7) with the addition of 100 mM Na_2SO_4 and 1 mM $MgCl_2$.

4.1.1. Enzyme activity assay. Enzymatic activity of both soluble and immobilised enzyme was determined spectrophotometrically as previously described.[19]

4.1.2. Protein determination. The protein content was determined according to Bradford.[25] For calibration, bovine serum albumin was used.

Table 1. Comparison of different reactor setups

	One-phase system	One-phase system	Two-phase system
Reactor	Soluble enzyme	Immobilised enzyme	Soluble enzyme
ee (%)	>99.9	>98.0	>99.9
Productivity	14 g L^{-1} d^{-1} (90%)	9 g L^{-1} d^{-1} (90%)	3 g L^{-1} d^{-1} (60%)
Current yield (%)	55	61	45
ttn (NADP)	35	12	64
ttn (Cp*Rh(bipy))	35	55	64
ttn (ADH)	75,000	21,000	5,000
Conversion (%)	>98	93	65

4.1.3. Gas chromatography (GC). Analytical measurements were carried out on an Agilent 6890N system (Agilent Technologies, CA, USA) with a Cyclodex β-1/P column of 25 m × 250 μm and a hydrogen gas flow of 0.2 mL min^{-1}. A 0.2 μL sample solution were injected at 250 °C, the initial column temperature was 100 °C, and kept for 11.5 min; afterwards the temperature was increased to 180 °C within three minutes. Acetophenone was detected after 5.7 min, (*R*)-phenylethanol after 9.8 min and (*S*)-phenylethanol after 10.5 min.

4.1.4. Immobilisation of alcohol dehydrogenase on amino-epoxy Sepabeads®. The enzyme-support preparation was prepared as previously described.[19]

4.1.5. Thermal stability. The thermal stabilities of the soluble (1 U/mL) and immobilised enzymes were determined by incubating the samples at 20 °C in buffer solution or in a 1:1 mixture of buffer and organic solvent. Periodically withdrawn samples were assayed for activity as described earlier. The thermal stability is given as a half-life time, the time after which half the original activity remains, assuming exponential decay.

4.1.6. Determination of partition coefficients. Partition coefficients were determined by adding 10 mL of organic solvent to 10 mL of phosphate buffer solution (pH 7) containing either 5 or 15 mM of acetophenone or phenylethanol, respectively. After 3 h of strong stirring, samples from both phases were taken and measured by GC. The partition coefficient is calculated by the quotient of the concentration in the organic phase divided by the concentration in the aqueous phase.

4.1.7. Operation of batch reactions. Reactions were conducted at 20 °C in a 250 mL vessel stirred by a magnetic stirrer. As a working electrode, a carbon felt (17 × 5 cm) fixed on a frame of stainless steel was used. The counter electrode consisted of a platinum grid in a dialysis sack filled with buffer solution. An Ag|AgCl reference electrode from Cypress Systems, Chelmsford, MA, was used. In all experiments, a potential of −700 mV versus Ag|AgCl was applied.

Acknowledgements

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