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Electroenzymatic Asymmetric Reduction of *rac*-3-Methylcyclohexanone to (1*S*,3*S*)-3-Methylcyclohexanol in Organic/Aqueous Media Catalyzed by a Thermophilic Alcohol Dehydrogenase

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Abstract: Electrochemical regeneration of nicotinamide cofactors has been discussed as a promising, clean, and sustainable technology since the 1980s. However, most concepts for the coupling of this technology to enzymes suffer from low productivities, insufficient stabilities, or are difficult to scale up. We have developed an electrochemical cell for the efficient regeneration of NAD(P)H, which can be coupled to a reduction reaction catalyzed by the thermophilic alcohol dehydrogenase from *Thermus* sp. Octane, as second organic phase avoided product inhibition and allowed for the production of (1*S*,3*S*)-3-methylcyclohexanol at a diastereomeric excess of 96% from the corresponding racemic ketone with a productivity of 0.13 $g L^{-1} h^{-1}$ and a current efficiency of 85%. After 10 h, the experiment was actively terminated and the final product concentration reached was 1.32 $g L^{-1}$. In our opinion this concept defines a new state of the art in electroenzymology and provides a strong basis for applications in organic synthesis.

Keywords: alcohol dehydrogenase; electrochemistry; enantioselectivity; enzyme catalysis; *in situ* product extraction; reactor design

Electroenzymology, the combination of electrochemistry and biocatalysis, is a highly interesting alternative for the production of chiral compounds and has been discussed as a promising and sustainable technology since the 1980s.[1] Electrochemistry provides a cheap and clean source of reduction equivalents over a very broad range of reaction conditions and has already been used for the production of enzymatically active NAD(P)H at high productivities (41.6 $g L^{-1} h^{-1}$).[2] However, the production of chiral compounds is fairly difficult if only electrochemical means are employed as no intrinsic stereoselective selection pressure is present.[3] Due to their high stereoselectivity, broad versatility, and high specificity, oxidoreductases, like dehydrogenases have a high potential for efficient, asymmetric synthesis reactions.[4] The main drawback for industrial application of these enzymes is their dependence on reduction equivalents like NAD(P)H in stoichiometric amounts, which makes the introduction of regeneration systems necessary.[5] Thus, a combination of electrochemical cofactor regeneration coupled to cofactor-dependent enzymatic catalysis should reveal strong synergistic effects.

However, many problems arise at the interface between the biological and the electrochemical system. Enzymes often get inactivated at the electrode surface, the rate of the cofactor regeneration is too low, compared to the reaction rate of the biocatalyst, or the coupling efficiency is insufficient. This became evident in a recent survey by Ruinatscha et al., in which electroenzymatic processes were evaluated with regard to their application potential.[6] Out of thirteen reviewed reactions, only three showed sufficient process performance to be of industrial relevance, while the others displayed insufficient productivity or process stability.

Here, we describe the electroenzymatic production of enantiomerically-pure (1*S*,3*S*)-3-methylcyclohexanol [(1*S*,3*S*)-**2**] from *rac*-3-methylcyclohexanone (*rac*-**1**) as outlined in Figure 1, which fulfills the minimal requirements for a preparative process, as defined by Ruinatscha et al. The thermophilic NAD-dependent alcohol dehydrogenase from *Thermus* sp. (TADH) served as a biocatalyst. The reduced cofactor NADH was regenerated *via* $[Cp^*Rh(bpy)(H_2O)]^{2+}$, a very selective electrochemical mediator for the regeneration of nicotinamide cofactors.[2,7,8] This prevented unspe-

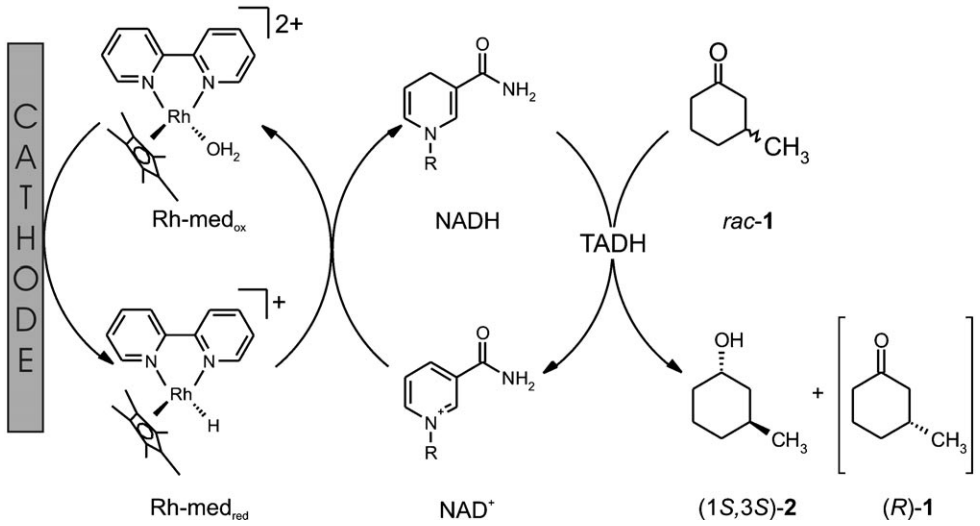

Figure 1. Reaction scheme of the electroenzymatic reduction reaction.

cific reduction of NAD^+ at the electrode, which would have lead to enzymatically inactive cofactor species.[9] For the reaction system, an electrochemical cell was designed as shown in Figure 2. The cathodic and anodic compartments were separated by a Nafion 1110 anion exchanger membrane. Both electrodes were made of reticulated vitreous carbon, a glassy carbon-like material, which provides a very large surface to volume ratio of 66 $cm^2 cm^{-3}$, resulting in an active surface area of 3300 cm^2 available for the electrochemical reaction. The remaining void volume is almost 97%, which makes this material very suitable for scale-up, as no significant backpressure is generated.[10] The biotransformation took place in the cathodic compartment filled with 100 mM Bis-Tris buffer, pH 6 at 60 °C, which provides optimal reaction conditions for TADH-catalyzed reductions.[11] In addition, the decomposition rate of NADH in the presence of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ at 60 °C is lower in Bis-Tris buffer (0.0137 mM h^{-1}) than in other buffer systems for example, sodium phosphate buffer (0.024 mM h^{-1}). Furthermore, by using Bis-Tris buffer interactions between the enzyme and $[Cp^*Rh(bpy)(H_2O)]^{2+}$ were prevented so that no loss of enzyme activity was detected over a period of 7 h (data not shown).

For system investigations, initially, 40 mM *rac*-**1** were applied as substrate (Table 1). The maximal productivity for the formation of (1*S*,3*S*)-**2** was achieved within the first hours reaching 0.27 $g L^{-1} h^{-1}$, while at a product concentration of 3.7 mM the rate decreased and after 6.3 h no further product was formed and thus an overall productivity of 0.07 $g L^{-1} h^{-1}$ was reached. The product was almost entirely the desired (1*S*,3*S*)-**2** at a diastereomeric excess (*de*) of 96% (see Supporting Information) with a current efficiency of 74%. With 10 mM *rac*-**1** as substrate under otherwise

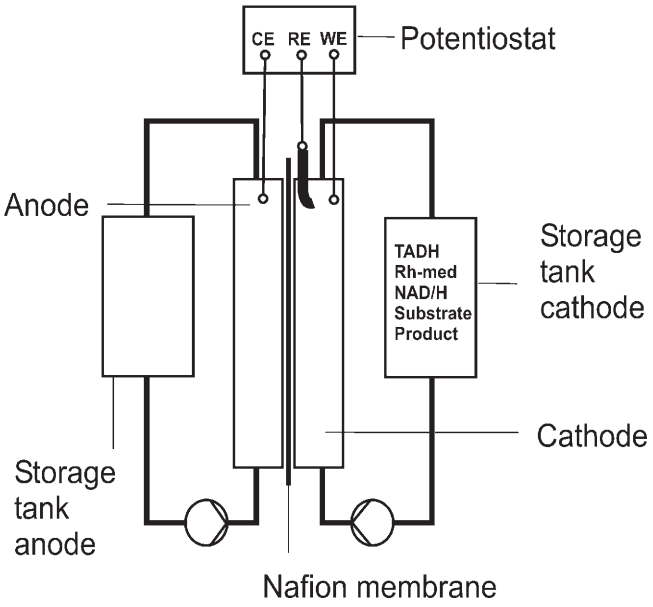

Figure 2. Set-up of the electroenzymatic reactor. CE: counter electrode, WE: working electrode, RE: reference electrode.

Table 1. Summary of electroenzymatic conversions.

rac-**1** [mM]	Product extraction	(1*S*,3*S*)-**2** [mM]	Time [h]	*de*[a] [%]	Productivity [$g L^{-1} h^{-1}$]	Current yield [%]
40	-	3.7	6.3	96	0.07	74
10	-	3.2	5.85	96	0.06	67
150[b]	octane[c]	11.6[b]	10.4	96	0.13[b]	85

[a] Determined *via* GC. For further details see Supporting Information.
[b] Based on organic phase.
[c] Ratio octane:aqueous phase of 1:1.5 was applied.

similar conditions, almost the same final product concentration of 3.2 mM was reached (Table 1 and Supporting Information). From these findings it can be concluded that TADH exhibits product inhibition with respect to **2.**

In order to prevent product inhibition and to reach higher final product concentrations, octane was used as second phase for *in situ* product extraction. This concept has already been applied in electrochemistry to prevent the formation of by-products.[12] The partitioning coefficients K_p [defined as c(octane)/c-(aqueous)] for substrate and product were determined previously ($K_p = 13$ for *rac*-**1** and 10 for *rac*-**2**).[11] Except for the addition of octane as organic phase to the cathode side (1:1.5 octane:aqueous) the two-liquid phase reactions were performed as described above. Under these conditions, the overall productivity was 0.13 $g L^{-1} h^{-1}$ (based on organic phase) reaching a final product concentration of 11.6 mM in the octane phase (*de* = 96%) with a current efficiency of 85% (Figure 3 and Table 1). After 4.5 h the product formation rate as well as the regeneration rate of the NADH declined. After addition of the rhodium mediator, the rates recovered, although the initial high values were not reached again. An explanation for this apparent deactivation of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ is its adsorption to the Nafion membrane. This was already noticed in control experiments without enzyme, where only cofactor regeneration was investigated. Here, the initial high NADH production rate of 13 $mM h^{-1}$ could not be maintained throughout the entire electrolysis, as there was a loss of regeneration power. This was also indicated by a drop in catalytic current, which could be partly restored by adding rhodium mediator to the reaction solution. Currently investigations are on the way to elucidate this loss of regeneration power. A solution could be the application of a different membrane material for the separation of the anodic and cathodic compartments to avoid adsorption of the mediator.

In summary, a concept for a scalable reactor to couple electrochemical cofactor regeneration efficiently to the enzymatic production of chiral alcohols with *in situ* product extraction was developed. The process performance of this system is already in an interesting range even though the potential is not yet fully exploited. Overall, the key advantage of this hybrid approach is its flexibility towards reaction parameters, organic and other solvents, and its scalability. This characterizes it as a suitable platform technology for the application of a wide range of oxidoreductases for fine and specialty chemical production.

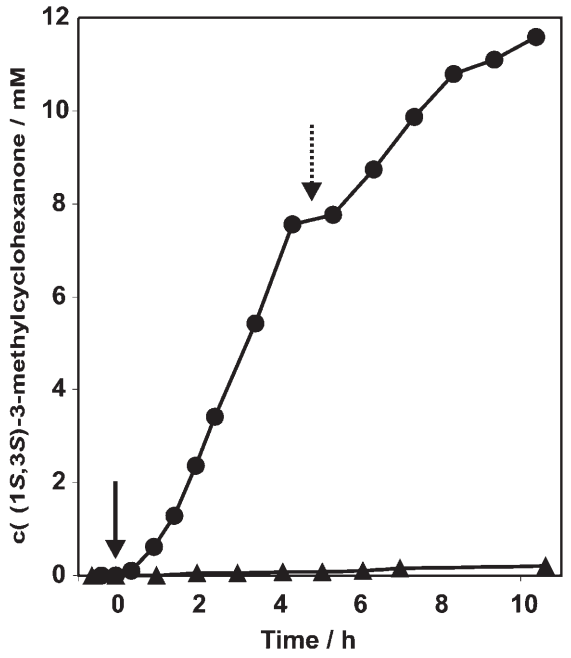

Figure 3. (1*S*,3*S*)-**2** formation during electroenzymatic reduction of *rac*-**1**. *General conditions:* 100 mM Bis-Tris, $pH_{60°C}$ 6.0, $V_{aq} = 150$ mL, $V_{octane} = 100$ mL, $T = 60$ °C, flow rate = 6.75 $mL min^{-1}$, $c([Cp^*Rh(bpy)(H_2O)]^{2+}) = 100$ μM, $c(NAD^+) = 0.5$ mM, $c(rac\text{-}1)_{octane} = 150$ mM. (●) c(TADH) = 0.216 μM; (▲) control experiment without TADH. Arrow indicates the start of the electrolysis by application of −800 mV *vs.* Ag/AgCl. Dotted arrow indicates the addition of 50 μM $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

Experimental Section

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (Buchs, Switzerland) at the highest grade available and were used without further purification. $[Cp^*Rh(bpy)(H_2O)]^{2+}$ was synthesized as described previously.[7,13]

Biocatalyst Preparation

TADH was recombinantly produced in *E. coli* BL21 (DE3) pLysS pASZ2[11] in a KLF2000 bioreactor (Bioengineering, Wald, Switzerland) using 1 L Terrific Broth[14] as medium supplemented with ampicillin (100 $\mu g mL^{-1}$) and chloramphenicol (20 $\mu g mL^{-1}$). The cells were grown to a cell dry weight of 0.5 $g L^{-1}$ and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside. After 6 h cells were harvested by centrifugation at 8200 × *g*, 4 °C, and 20 min in a Sorvall RC-5B centrifuge (Thermo Electron Corporation, Langenselbold, Germany). The cell pellet was resuspended in 20 mM Tris pH 7.5 and directly heat treated at 75 °C for 20 min. Insoluble fraction and precipitated proteins were separated by ultracentrifugation in a Sorvall Discovery 90SE centrifuge (Kendro Laboratory Products GmbH, Langenselbold, Germany) at 91500 × g, 4 °C, 1 h and the supernatant was applied to a Fractogel EMD TMAE (Merck KGaA, Darmstadt, Germany) anion exchanger column equilibrated with 20 mM Tris, pH 7.5. TADH was eluted with a linear gradient of 20 mM Tris, pH 7.5 and 2 M NaCl. Fractions containing TADH were pooled, sterile filtered and stored at −80 °C until use.

Electrochemical Cell

The electrochemical cell was made entirely of glass. The anodic and cathodic compartments (50 mL each) were separated by means of a Nafion 1110 membrane (DuPont de Nemours, Bad Homburg, Germany). Both electrodes consist of reticulated vitreous carbon (ERG Materials and Aerospace Corporation, Oakland, USA) at a porosity of 100 ppi. 100 mM Bis-Tris, $pH_{60°C}$ 6.0 and 200 mM Na-phosphate, $pH_{60°C}$ 7.0 were used for the cathodic and anodic compartments, respectively. The cell was constructed as a three-electrode set-up with an RE-5B Ag/AgCl reference electrode (Bioanalytical Systems Inc., W. Lafayette, USA). The reaction was controlled by an Autolab PGSTAT302 potentiostat (Deutsche Metrohm GmbH & Co. KG, Filderstadt, Germany) to maintain the cathodic potential at −800 mV *vs.* Ag/AgCl. Both cathodic and anodic storage vessels were degassed with nitrogen and heated to 60 °C. For *in situ* product extraction octane was used as second organic phase at a ratio of 1:1.5 with respect to the aqueous phase.

Analysis

Protein concentration was determined using the Bradford assay[15] (Biorad) with a standard curve made from BSA. The course of electroenzymatic reductions was followed by analyzing samples *via* GC on a chiral Rt-βDex-sm (Restek) column.

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