Towards electroenzymatic processes involving old yellow enzymes and mediated cofactor regeneration

Tosstorff, Andreas; Kroner, Cora; Opperman, Diederik J.; Hollmann, Frank; Holtmann, Dirk

DOI
10.1002/elsc.201600158

Publication date
2017

Document Version
Accepted author manuscript

Published in
Engineering in Life Sciences

Towards electro enzymatic processes involving old yellow enzymes and mediated cofactor regeneration

Andreas Tosstorff[1,†]

Cora Kroner[1,†]

Diederik J. Opperman[2]

Frank Hollmann[3]

Dirk Holtmann[1]

† Both authors equally contributed to this work

[1]DECHEMA Research Institute, Theodor-Heuss-Allee 25, 60486 Frankfurt, Germany

[2]University of the Free State, Department of Biotechnology, 9300 Bloemfontein, South Africa

[3]TU Delft, Department of Biotechnology, Julianalaan 136, 2628 BL Delft, The Netherlands

Correspondence: Dr. Dirk Holtmann, [1]DECHEMA Research Institute, Theodor-Heuss-Allee 25, 60486 Frankfurt, Germany

E-mail: holtmann@dechema.de

Keywords: C=C reductions, Design of experiments, electro enzymatic syntheses, mediated electron transfer, old yellow enzyme

Abbreviations: **CE**, current efficiency; **DoE**, design of experiments; **OYE**, old yellow enzyme; **TF**, turn over frequency; ***Ts*OYE**, old yellow enzyme from Thermus scotoductus

Practical application

New production routes for fine and bulk chemicals are important to establish further sustainable processes in industry. Besides the identification of new biocatalysts and substrates the optimization of existing processes in regard to an improved utilization of resources such as cofactors is needed. In this paper we describe the successful development of a mediated electro enzymatic process to regenerate the NADPH as reducing agent for old yellow enzymes. The enzyme family of old yellow enzyme (OYE) is now receiving an increased interest by the academic and industrial community because of their broad applicability. The prohibitive high cost of the needed NADPH necessitates their use in catalytic amounts together with a suitable in situ regeneration approach. Due to the fact that the overall process was affected by a broad set of parameters, a DoE approach was chosen to identify suitable process conditions. Our investigations resulted in a process with high productivities in combination with high electron transfer efficiencies. In comparison with other electro-enzymatic processes as well as with established reaction systems with OYE, our process design allows high space-time yields with a reduced demand on energy and chemicals.

Abstract

Old yellow enzymes (OYEs) are able to catalyze asymmetric C=C reductions. A mediated electro enzymatic process to regenerate the NADPH in combination with an OYE was investigated. Due to the fact that the overall process was affected by a broad set of parameters a DoE approach was chosen to identify suitable process conditions. Process conditions with high productivities of up to 2.27 mM h^{-1} in combination with approx. 90 % electron transfer efficiency were identified.

1 Introduction

First found in yeast in 1932 by Warburg and Christian [1] the family of so called old yellow enzymes (OYE) is now receiving an increased interest by the academic and industrial community because of their potential application in the production of fine chemicals, pharmaceuticals, and agrochemical products as well as in the decontamination of areas polluted with the trinitrotoluene [2-4]. Their substrate scope spans a broad range of compounds containing conjugated C=C-double bonds [2]. The catalytic mechanism of OYE entails a Michael-type hydride transfer to the β-C-atom of the conjugated C=C-double bond. The hydride is transferred from a reduced, enzyme-bound flavin (FMN) cofactor which had previously been generated from NAD(P)H (scheme 1) [5]. The prohibitive high cost of the latter necessitates their use in catalytic amounts together with a suitable *in situ* regeneration approach. In the last decades several co-factor regeneration systems have been investigated. One method of choice is electrochemistry. In general, electrochemical methods can be used to generate hydrogen peroxide [6-9], regenerate cofactors [9-13] or even to substitute cofactors [14, 15]. One recent study has shown the interactions of electrode reactions as well as reduced electrochemical mediators with an oxygen-dependent reduction by monooxygenases [14]. From this work it can be concluded that the combination of an oxygen-independent enzyme reaction with an electrochemical reaction is more advisable. Therefore, a mediated electro enzymatic process to regenerate the NADPH in combination with a reaction catalyzed by OYE was investigated. As model enzyme we chose the OYE

homologue from *Thermus scotoductus* (*Ts*OYE) [4, 16] due to its high degree of stability, ability to function over a wide-temperature range and relative ease of purification [17, 18]. The enzyme was optimally active at 65°C but was also active over a wide temperature range, retaining 70% of its activity at 80 °C [19]. Carvone has served as a substrate for OYEs in several publications [17, 20]. When carvone is reduced by OYE, the resulting reaction product is dihydrocarvone, which serves as a precursor for insect antifeedants [21].

2 Materials and methods

*Ts*OYE (NCBI Accession No. AM902709.1) was synthesized and cloned into pET28b(+) via the *Nde*I and *Eco*RI restriction sites (Genscript). Protein expression was performed in *E. coli* BL21(DE3). Transformed strains were grown in LB-medium (10 g L^{-1} Peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl) containing 35 mg L^{-1} kanamycin to an OD600 of approximately 0.8-1.0 (30 °C, 200 rpm). Protein expression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were harvested by centrifugation (4000 rpm, 10 min, 4 °C) after 4 hours, washed with MOPS-NaOH buffer (50 mM, 10 mM $CaCl_2$, pH 7) and stored at -20 °C. The cells were disrupted by sonication (pause on: 1s, pause off: 2s, overall time: 2 min, amplitude: 10 %) where after *E. coli* proteins were denatured by a heat precipitation step (90 min, 70 °C). After centrifugation (4000 rpm, 10 min, 4 °C) the supernatant was used for the further experiments. Enzyme

concentrations were determined using BCA assay. [Cp*Rh(bpy)Cl]Cl was synthesized following the procedure described in the literature [22]. All electrochemical experiments were conducted with the potentiostat PCI4 300 (Gamry Instruments, Warminster, USA). Before each experiment the mediator containing solution was purged with nitrogen in order to remove oxygen. After the addition of enzyme and substrate to the solution, only the head space of the reactor was purged with nitrogen to avoid enzyme deactivation and reduce substrate and product evaporation. Temperature was kept constant using a water recirculator. Enzyme concentration in the reaction mixture was always 1 µM.

The mediators (scheme 2) were tested in an 18 ml reactor with a commercial 3D-glassy carbon electrode in a bulk electrolysis cell (ALS Co. Ltd., Tokyo, Japan) under continuous stirring. A platinum wire counter electrode and an Ag/AgCl (3M KCl) reference electrode were used. A design of experiment tool (Design Expert 9, Stat-Ease Inc., Minneapolis, USA) was applied in order to identify the optimal $NADP^+$ and mediator concentrations, temperature and applied potential (table 1). These experiments were conducted in an 85 ml reactor with a 3D-glassy carbon foam (ALS Co. Ltd, Tokyo, Japan) working electrode. The Ag/AgCl counter electrode was inserted into the anodic chamber. A platinum wire as counter electrode was separated by a glass frit, the anodic chamber was filled with buffer solution. A TRIS buffer was used (100 mM, pH 7, 10 mM $CaCl_2$) as electrolyte/buffer in combination with Cp*Rh(bpy) as mediator. MOPS buffer (50 mM, pH 7, 10 mM $CaCl_2$) was used for the other mediator experiments. 500 µl samples

were taken after 0, 30 and 60 minutes. Samples were extracted with 500 µl of EtOAc containing 5 mM cyclohexanol as internal standard. (-)-Carvone and reaction products were detected via gas chromatography using an Agilent J&W DB-WAXetr column and GC 17A (Shimadzu Deutschland GmbH, Duisburg, Germany).

3 Results and Discussion

In an attempt to identify an effective mediator for the direct regeneration of the active site of *Ts*OYE, a mediator screening in the 18 mL reactor was performed. Cobalt sepulchrate, safranin T and [Cp*Rh(bpy)Cl]Cl were used as electron transfer mediators. Although small product amounts were detected in all three experiments, in no experiment was there a significant increase compared to a negative control (without enzyme and mediator, fig. 1). Therefore, it can be concluded that the small amount of product resulted from impurities in the substrate or a non-enzymatic product formation. In order to ensure the activity of the enzyme in the reactions mixtures, NADPH was added after 18 hours to the reaction with safranine T, resulting in a fast eightfold increase of product concentration (data not shown). Given that there was no significant increase in product concentration compared to the negative control, it can be ruled out that there was any electron transfer to the enzyme, neither from the electrode nor from the mediator.

As [Cp*Rh(bpy)Cl]Cl is known to regenerate NADPH and FMN [11, 12, 17], these systems were used to investigate the cofactor regeneration in combination with the OYE. The reaction system is shown in scheme 3. A

significant increase in product concentration compared to the negative control could be measured in these investigations. Due to the fact that the combination of [Cp*Rh(bpy)Cl]Cl and $NADP^+$ showed the highest productivity, this system was further investigated in order to scale-up the reaction up to 85 mL scale and to optimize it with a DOE approach. Table 1 shows the reaction conditions for 16 runs to optimize the reaction conditions and figure 2 the resulting product formation rates.

Several interactions were observed to have an effect on the product formation rate. Increasing the mediator concentration would lead to a higher product formation rate at high temperatures rather than at lower ones. Increasing the mediator concentration at higher $NADP^+$ concentrations leads to a stronger increase of the reaction rate than at lower $NADP^+$ levels. Obviously, there is an interaction between $NADP^+$ concentrations and temperature. Increasing temperature has a more positive effect at high $NADP^+$ levels. Finally, it can be concluded, that the highest product formation rate will be obtained when both concentrations are at the highest level. The highest productivity was measured with 0.05 mM mediator, 0.2 mM $NADP^+$, 80 °C and a potential of -760 mV vs. Ag/AgCl as reference electrode (entry 3). The second highest productivity was found by using similar conditions (entry 15), only the potential was changed to -860 mV vs. Ag/AgCl instead of -760 mV vs Ag/AgCl. So far, one of the major issues with electro enzymatic processes comes from an insufficient energy and mediator efficiency, causing, especially under aerobic conditions, very low electron efficiencies (maximum value of 21 %, [13-15]) and turn over frequencies (between 0.125 – 291 h^{-1} [11, 13, 23]).

Therefore, these parameters where investigated in detail. Figure 3 shows the calculated current efficiency and turn over frequency in the different runs. Current efficiency or electron transfer efficiencies were calculated according to Eq. 1, where CE is the current efficiency, z is the equivalent of electrons transferred in each reaction, F is the Faraday constant, $n_{Product}$ is the amount of product formed and $\int I\,dt$ is the overall amount of electrons transferred.

Eq. 1: $$CE = \frac{z \cdot F \cdot n_{Product}}{\int I dt}$$

The highest current efficiency was detected under the conditions (entry 3) that lead to the above mentioned highest productivity. From the DoE approach only one general interaction was observed for the electron efficiency. $NADP^+$ concentration and temperature show an antagonistic interaction. At low $NADP^+$ levels, increasing the temperature will reduce the electron efficiency, while at high $NADP^+$ levels it will lead to an increase of the electron efficiency. Most probably interactions between reduced cofactor and the mediator takes place and are more pronounced at low $NADP^+$-concentrations. At higher $NADP^+$-concentration mainly the desired reaction took place. Furthermore, it was concluded that an increased reaction rate shifts the ratio of carvone conversion and the unwanted reaction in the direction of the desired reaction. Mediator turn over frequencies (TF) varied between 10 and 45 h^{-1}. Several interactions with effect on the turn over frequency can be observed. While at high mediator levels, an increase of the potential has almost no effect, it causes a decreased TF at low mediator concentrations. Another interaction occurs between the potential and the temperature. While at 80 °C, a change in potential has little effect, increasing the potential at 60 °C will cause a reduced turn over

frequency. An additional interaction can be observed between the temperature and the mediator concentration. The TF increases with rising mediator levels only at 80 °C. At 60 °C, the opposite effect is observed. Independently of the mediator concentration, the TF is always higher at 80 °C than at 60 °C. Temperature interacts also with $NADP^+$ concentrations. While the TF is higher at 0.2 mM $NADP^+$ and 60 °C than at 0.1 mM and 60 °C, it can be further increased by raising the temperature to 80 °C. At low $NADP^+$ levels on the other hand, changing the temperature has no significant effect on the turn over frequency of the mediator. The DoE in regard to the turn over frequency indicates that the highest TF values can be achieved by working at high mediator, temperature and $NADP^+$ levels. Compared to values given in the literature, we have observed not only the second highest TF [13] relative to the mediator reported for an electro enzymatic processes, but also the highest electron efficiency in these processes. It must be mentioned that a current efficiency higher than 100 % is not reasonable, therefore the value of entry 3 must be reagrarded as approx. 100 % current efficiency. The most likely causes for the discrepancy are sampling errors or fluctuation in the calculation of the current efficiencies. By comparing the results of the above mentioned entries 3 and 15, the calculated current efficiencies vary between 106 % and 88 %. Therefore, it can be concluded that the application of 0.05 mM mediator, 0.2 mM $NADP^+$ and a reaction temperature of 80 °C resulted in current efficiencies of approx. 90 %.

In general, more negative potential leads to a higher amount of side reactions and therefore to a reduced current efficiency. Nevertheless, the observed high current efficiencies enable processes with high energy efficiency and a minor formation of

side products. The values for the enantiomeric excess varied, except for one case, between 70 and 90 % (data not shown). A similar enantiomeric excess of approx. 90% was also reported for the natural NADPH driven carvone reduction [17]. An interaction between mediator concentration and temperature appeared to be relevant for the outcome of the enantiomeric excess. The two parameters show an antagonistic interaction: at high mediator concentrations, it does not seem relevant whether the temperature is at a high or low level, while at low mediator concentrations, lower temperatures favor a higher enantiomeric excess.

Finally, it must be mentioned that a long term experiment under the chosen electrochemical reaction conditions in combination with an increased amount of substrate did not result in the expected increase in the product concentration. Most probably the reaction was limited by inhibitory high concentrations of the substrate and/or the product. Recently, it was shown that biphasic reaction can be used to overcome these limitations [4, 23, 24]. Therefore, further optimisation in regard to the combination of the electrochemical system and a biphasic system are currently ongoing in our laboratory.

4 Concluding remarks

The development of different systems for regeneration and substitution of NADPH as natural OYE cofactor may help granting access to technical in vitro OYE applications in the future. Clearly, enzymatic cofactor regeneration systems are already of high industrial potential and have already proven to be well suited for commercial applications, as is the case, for instance, with alcohol dehydrogenase-

catalyzed syntheses. In each case, only catalytic levels of $NAD(P)^+$ are required but stoichiometric levels of a co-substrate are needed to drive the recycling enzyme. When driving an enzymatically catalyzed reaction by an electrode, instead of using an enzymatic cofactor regeneration system, process costs could be reduced drastically. Electrochemical cofactor regeneration would result in a cosubstrate- and coproduct-free reaction setup. As this system does not need a cosubstrate, no by-products are produced, which facilitates the recovery of the desired product. Our results show that high productivities and high current efficiencies of approx. 90 % can be achieved by using enzymes working at anaerobic conditions like OYE in combination with electrochemical cofactor regeneration. By using oxygen-depended enzymes (e.g. P450s) in similar approaches, only current efficiencies up to 21 % can be measured [13-15]. The cathode potentials required for efficient reduction of the mediators are more negative than the O_2 reduction potential. Hence, direct cathodic O_2 reduction occurs during the electrolyses, reducing the current yield [25].

Fisher et al investigated an electrochemical cofactor substitution system in combination with OYE and measured product formation rates up to 1.25 mM h^{-1} [23]. Here we investigated the cofactor regeneration and identified conditions for a productivity of up to 2.27 mM h^{-1} (corresponding to 38 µM s^{-1}). Both approaches show clearly the high potential of the electro enzymatic processes with OYE. Which cofactor regeneration or substitution system is finally "greener" must be evaluated in detail. For this evaluation the long term stability of all components, the cost for the production of the enzymes and the synthesis of the different mediators as well

as the impact of the mediators on the environment must be considered. Certainly, the recently investigated photochemical approaches [16, 24, 26] as well as electrochemical systems to substitute or regenerate cofactors in combination with OYE have a high potential to develop efficient processes with a low impact on the environment and small E-factor values [27] due to the avoidance of high amounts of side products. Finally, it can be concluded that electro enzymatic processes under anaerobic conditions can lead to more sustainable processes compared to processes in the presence of oxygen and using oxygenases [13-15]. The presented productivities are in the same range as in electro enzymatic processes to produce hydrogen peroxide [6-8, 28] or the regeneration of oxidized nicotinamide cofactors [10, 29]. It can be concluded that anaerobic regeneration of oxidized cofactors, anaerobic regeneration or substitution of cofactors as well as the electrochemical production of hydrogen peroxide can be successfully implemented in relevant industrial processes.

Conflict of interest

The authors have to declare no conflict of interest.

5 References

[1] Warburg, O., Christian, W., Uber ein neues Oxydationsferment und sein Absorptionsspektrum. *Biochem. Z* 1932, *254*, 53.

[2] Williams, R. E., Bruce, N. C., 'New uses for an Old Enzyme'--the Old Yellow Enzyme family of flavoenzymes. *Microbiology (Reading, England)* 2002, *148*, 1607-1614.

[3] Toogood, H. S., Gardiner, J. M., Scrutton, N. S., Biocatalytic Reductions and Chemical Versatility of the Old Yellow Enzyme Family of Flavoprotein Oxidoreductases. *ChemCatChem* 2010, *2*, 892-914.

[4] Toogood, H. S., Knaus, T., Scrutton, N. S., Alternative Hydride Sources for Ene-Reductases: Current Trends. *ChemCatChem* 2014, *6*, 951-954.

[5] Vaz, A. D. N., Chakraborty, S., Massey, V., Old yellow enzyme: Aromatization of cyclic enones and the mechanism of a novel dismutation reaction. *Biochemistry* 1995, *34*, 4246-4256.

[6] Holtmann, D., Krieg, T., Getrey, L., Schrader, J., Electroenzymatic process to overcome enzyme instabilities. *Catalysis Communications* 2014, *51*, 82-85.

[7] Krieg, T., Hüttmann, S., Mangold, K.-M., Schrader, J., Holtmann, D., Gas diffusion electrode as novel reaction system for an electro-enzymatic process with chloroperoxidase. *Green Chemistry* 2011, *13*, 2686-2689.

[8] Lütz, S., Vuorilehto, K., Liese, A., Process development for the electroenzymatic synthesis of R-methylphenylsulfoxide by use of a 3-dimensional electrode. *Biotechnology and Bioengineering* 2007, *98*, 525-534.

[9] Varničić, M., Vidaković-Koch, T., Sundmacher, K., Gluconic Acid Synthesis in an Electroenzymatic Reactor. *Electrochimica Acta* 2015, *174*, 480-487.

[10] Kochius, S., Park, J. B., Ley, C., Könst, P.*, et al.*, Electrochemical regeneration of oxidised nicotinamide cofactors in a scalable reactor. *Journal of Molecular Catalysis B: Enzymatic* 2014, *103*, 94-99.

[11] Hollmann, F., Schmid, A., Steckhan, E., The first synthetic application of a monooxygenase employing indirect electrochemical NADH regeneration. *Angewandte Chemie International Edition* 2001, *40*, 169-171.

[12] Hollmann, F., Witholt, B., Schmid, A., [Cp∗ Rh (bpy)(H 2 O)]$^{2+}$: a versatile tool for efficient and non-enzymatic regeneration of nicotinamide and flavin coenzymes. *Journal of Molecular Catalysis B: Enzymatic* 2002, *19*, 167-176.

[13] Ruinatscha, R., Dusny, C., Buehler, K., Schmid, A., Productive Asymmetric Styrene Epoxidation Based on a Next Generation Electroenzymatic Methodology. *Advanced Synthesis & Catalysis* 2009, *351*, 2505-2515.

[14] Tosstorff, A., Dennig, A., Ruff, A. J., Schwaneberg, U.*, et al.*, Mediated electron transfer with monooxygenases—Insight in interactions between reduced mediators and the co-substrate oxygen. *Journal of Molecular Catalysis B: Enzymatic* 2014, *108*, 51-58.

[15] Çekiç, S. Z., Holtmann, D., Güven, G., Mangold, K.-M.*, et al.*, Mediated electron transfer with P450cin. *Electrochemistry Communications* 2010, *12*, 1547-1550.

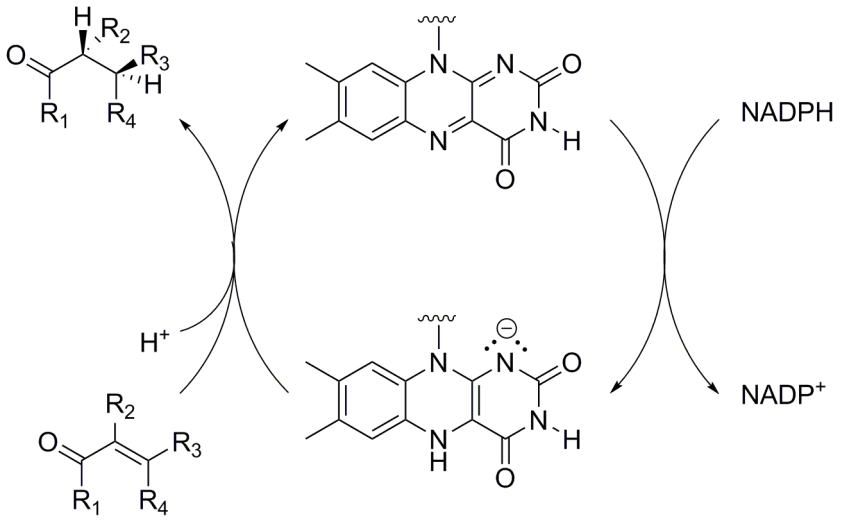
[16] Grau, M. M., van der Toorn, J. C., Otten, L. G., Macheroux, P.*, et al.*, Photoenzymatic Reduction of C=C Double Bonds. *Advanced Synthesis & Catalysis* 2009, *351*, 3279-3286.

[17] Bernard, J., van Heerden, E., Arends, I. W. C. E., Opperman, D. J., Hollmann, F., Chemoenzymatic Reduction of Conjugated C=C Double Bonds. *ChemCatChem* 2012, *4*, 196-199.
[18] Opperman, D. J., Sewell, B. T., Litthauer, D., Isupov, M. N.*, et al.*, Crystal structure of a thermostable old yellow enzyme from Thermus scotoductus SA-01. *Biochemical and biophysical research communications* 2010, *393*, 426-431.
[19] Opperman, D. J., Piater, L. A., van Heerden, E., A novel chromate reductase from Thermus scotoductus SA-01 related to old yellow enzyme. *Journal of bacteriology* 2008, *190*, 3076-3082.
[20] Goretti, M., Ponzoni, C., Caselli, E., Marchigiani, E.*, et al.*, Biotransformation of electron-poor alkenes by yeasts: Asymmetric reduction of (4S)-(+)-carvone by yeast enoate reductases. *Enzyme and Microbial Technology* 2009, *45*, 463-468.
[21] Jansen, B. J. M., Kreuger, J. A., De Groot, A., The conversion of (−)- and (+)-dihydrocarvone into chiral intermediates for the synthesis of (−)-polygodial, (−)-warburganal and (−)-muzigadial. *Tetrahedron* 1989, *45*, 1447-1452.
[22] Kölle, U., Grützel, M., Organometallic Rhodium(III) Complexes as Catalysts for the Photoreduction of Protons to Hydrogen on Colloidal TiO_2. *Angewandte Chemie International Edition in English* 1987, *26*, 567-570.
[23] Fisher, K., Mohr, S., Mansell, D., Goddard, N. J.*, et al.*, Electro-enzymatic viologen-mediated substrate reduction using pentaerythritol tetranitrate reductase and a parallel, segmented fluid flow system. *Catalysis Science & Technology* 2013, *3*, 1505-1511.
[24] Peers, M. K., Toogood, H. S., Heyes, D. J., Mansell, D.*, et al.*, Light-driven biocatalytic reduction of α, β-unsaturated compounds by ene reductases employing transition metal complexes as photosensitizers. *Catalysis Science & Technology* 2016, *6*, 169-177.
[25] Holtmann, D., Hollmann, F., The Oxygen Dilemma: A Severe Challenge for the Application of Monooxygenases? *ChemBioChem* 2016.
[26] Taglieber, A., Schulz, F., Hollmann, F., Rusek, M., Reetz, M. T., Light-Driven Biocatalytic Oxidation and Reduction Reactions: Scope and Limitations. *ChemBioChem* 2008, *9*, 565-572.
[27] Ni, Y., Holtmann, D., Hollmann, F., How green is biocatalysis? To calculate is to know. *ChemCatChem* 2014, *6*, 930-943.
[28] Getrey, L., Krieg, T., Hollmann, F., Schrader, J., Holtmann, D., Enzymatic halogenation of the phenolic monoterpenes thymol and carvacrol with chloroperoxidase. *Green Chemistry* 2014, *16*, 1104-1108.
[29] Kochius, S., Paetzold, M., Scholz, A., Merkens, H.*, et al.*, Enantioselective enzymatic synthesis of the α-hydroxy ketone (R)-acetoin from meso-2, 3-butanediol. *Journal of Molecular Catalysis B: Enzymatic* 2014, *103*, 61-66.

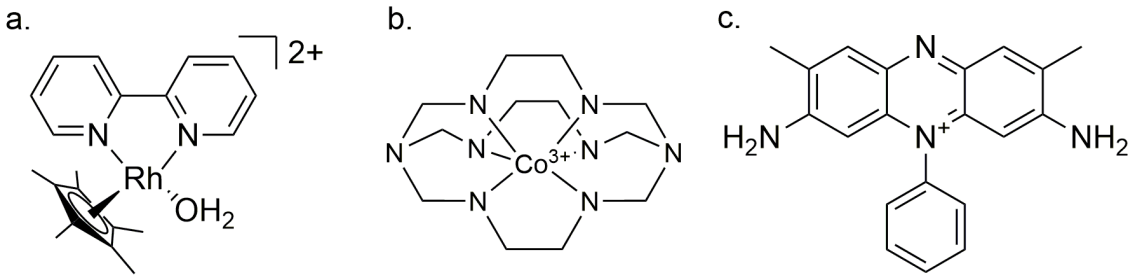
Table 1. Reaction conditions to optimize mediated electron transfer between electrodes and an OYE

Entry	c (Mediator) [mM]	c ($NADP^+$) [mM]	T [°C]	U [mV]
1	0.01	0.2	80	-860
2	0.05	0.2	60	-860
3	0.05	0.2	80	-760
4	0.01	0.1	80	-860
5	0.05	0.1	80	-760
6	0.05	0.1	60	-860
7	0.05	0.1	60	-760
8	0.01	0.2	60	-860
9	0.01	0.1	60	-860
10	0.01	0.2	60	-760
11	0.01	0.2	80	-760
12	0.05	0.2	60	-760
13	0.01	0.1	80	-760
14	0.05	0.1	80	-860
15	0.05	0.2	80	-860
16	0.01	0.1	60	-760

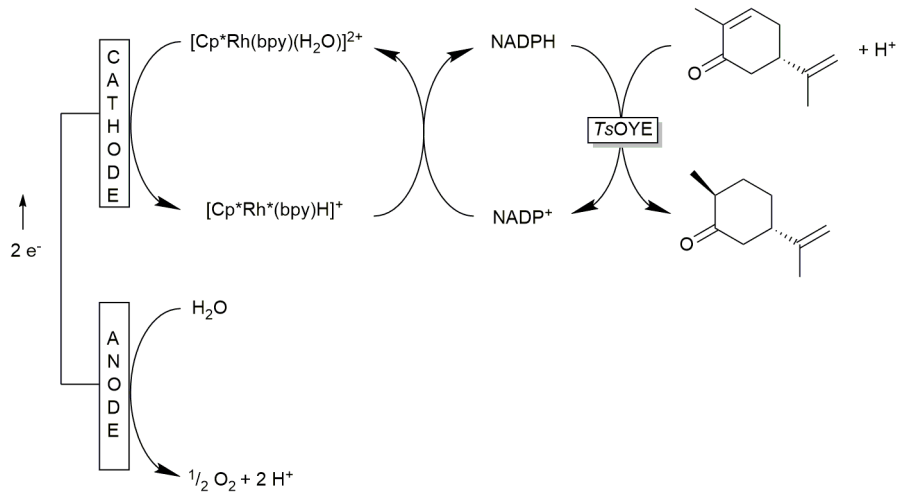
Figure and scheme legends



Scheme 1: Catalytic cycle of the asymmetric reduction of activated double bonds by OYE. The active reductant within OYEs is a reduced flavin. The catalytic cycle is initiated by nicotinamide dependent reduction of the flavin.



Scheme 2: Structures of the used mediators (a: [Cp*Rh(bpy)Cl]Cl; b: cobalt sepulchrate, c. Safranin T)



Scheme 3: Electro enzymatic process involving old yellow enzymes and mediated cofactor regeneration, the substrate (-)-carvone was converted into (+)-dihydrocarvone

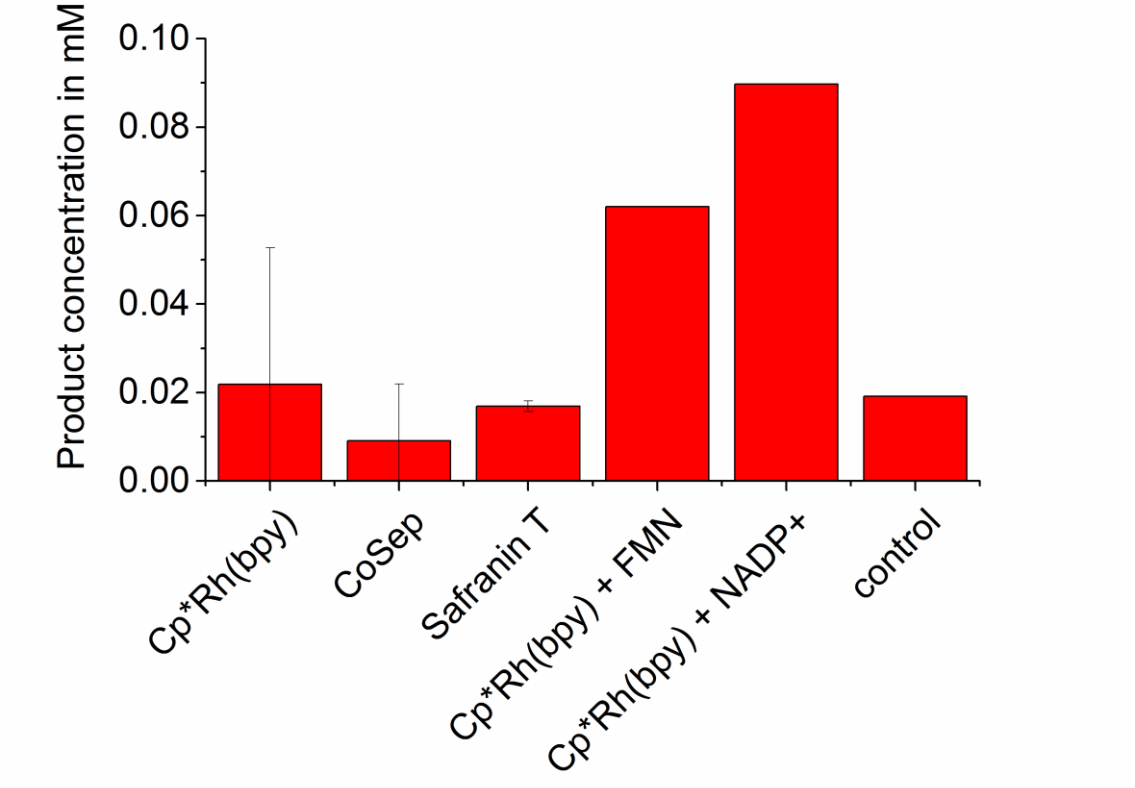


Figure 1: Product formation of dihydrocarvone at different reactions for a mediated cofactor substitution or regeneration (reaction conditions: 5 mM *R*-(-)-

carvone, experiments with Cp*Rh(bpy) were performed in TRIS buffer (100 mM, pH 7, 10 mM $CaCl_2$), all other experiments in MOPS buffer (50 mM, pH 7, 10 mM $CaCl_2$), Cp*Rh(bpy), CoSep and Safranin T: 250 µM mediator; Cp*Rh(bpy) + FMN: 100 µM mediator and 100 µM FMN; Cp*Rh(bpy) + $NADP^+$: 50 µM mediator and 100 µM $NADP^+$ (n = 2). An additional experiment with safranine T as mediator in the Tris/HCl-buffer was performed. Again, no product concentration could be measured. Therefore it can be concluded that the buffer has no significant influence on the results.

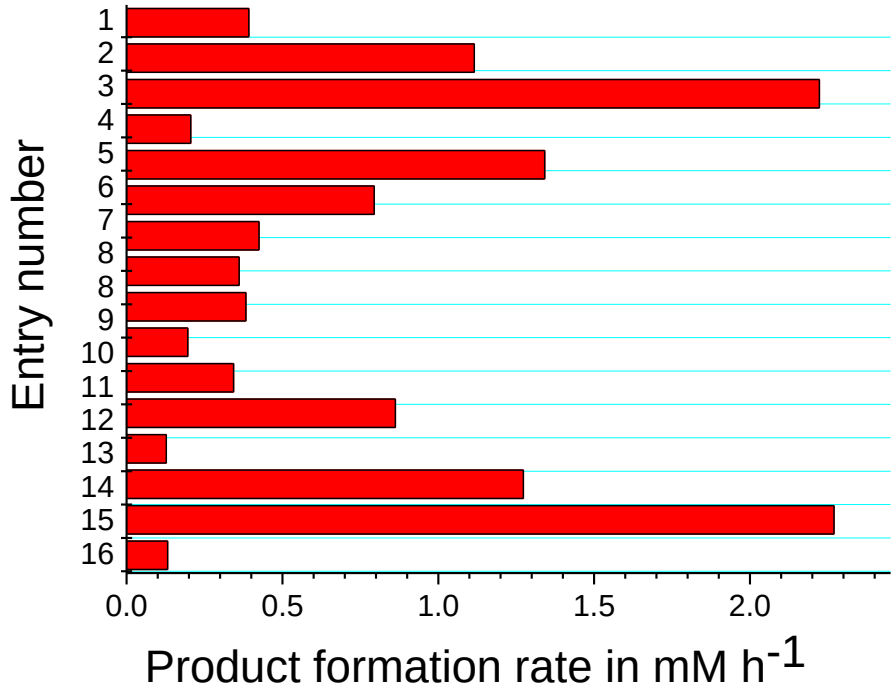


Figure 2: Product formation rate at different reaction conditions in order to optimize the electro enzymatic conversion of carvone by using an OYE (n = 1).

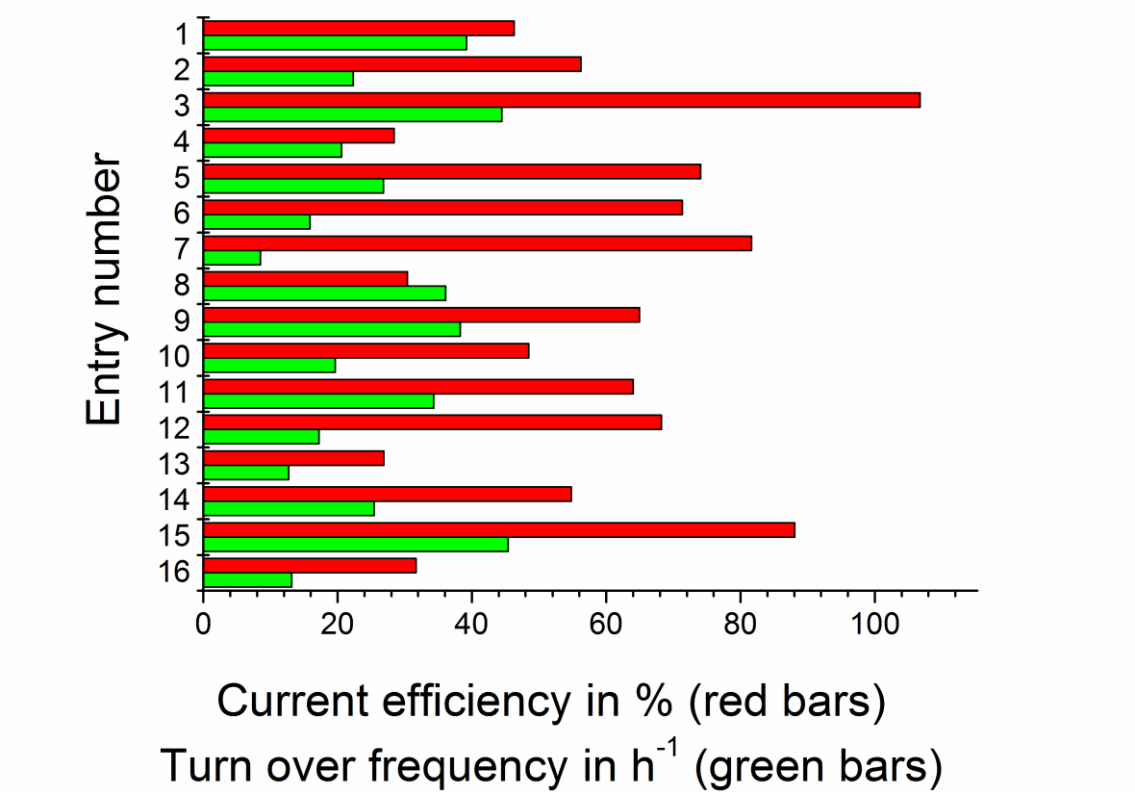


Figure 3: Current efficiency and turn over frequency at different reaction conditions in order to optimize the electro enzymatic conversion of carvone by using an OYE (n = 1).