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Productivity of Selective Electroenzymatic Reduction and Oxidation Reactions: Theoretical and Practical Considerations

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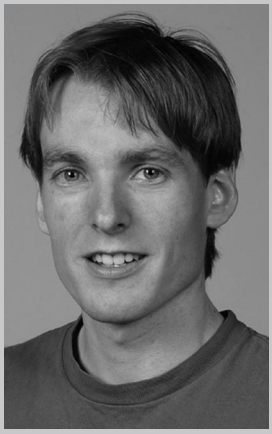
Abstract: The volumetric productivities, final product concentration and total process times, of electroenzymatic processes comprising reductive electrochemical cofactor regeneration coupled to dehydrogenase or oxygenase catalyzed redox reactions determine process performance. Exemplified for the production of fine chemicals, operational windows were defined to consider these parameters. This theoretical approach allows the identification of limiting process parameters and promising process developments. Several biocatalytic processes for syntheses of specialty chemicals are already in a performance range of technical relevance. Electroenzymology thus evolved to a strong additional technology in the toolbox of biocatalysis and organic chemistry.

Keywords: cofactor regeneration; dehydrogenases; electrochemistry; enzyme catalysis; operational window; oxygenases

1 Introduction

In (bio)catalysis " (…) selectivity is only one important issue among others, which determines the usefulness of catalysts. (…) Organic chemists should pay more attention to catalyst productivity, activity and recycling. These are key factors for a general application, too."[1] This is especially true for energy-demanding processes like those based on enzymatic oxidation and reduction reactions, catalyzed by the versatile class of oxidoreductases (EC 1.x.x.x.). Reductions carried out by dehydrogenases as well as oxidations accomplished by oxygenases enable direct and environmentally friendly routes from readily available substrates to functionalized building blocks that are difficult to achieve with chemical reaction counterparts.[2] The use of these enzymes for the synthesis of high value-added products is therefore very attractive, but requires the presence of reduced cofactors. Dehydrogenases use reduced cofactors as hydride donors for the reduction of substrates, whereas oxygenases employ them to reductively activate molecular oxygen for subsequent oxidation reactions. As a stoichiometric use of these expensive cofactors is economically not feasible for *in vitro* applications,[3] cofactors are rather supplied in the reduced form *via* regeneration systems using only catalytic amounts of the generally less expensive oxidized form. The reducing power may then originate from reduction equivalents, which are far cheaper than the cofactor itself. This decreases high cofactor costs with increasing regeneration cycles (total turnover numbers) so that they are not cost-determining any more.[4]

To date, established *in vitro* cofactor regeneration systems employ either a second enzyme that performs cofactor regeneration (enzyme-coupled regeneration) or use the production enzyme to accomplish the biotransformation as well as the regeneration of the cofactors (substrate-coupled regeneration).[5,6] The enzymatic transfer of reduction equivalents from substrates to cofactors is often highly selective and thus favors efficient usage of the employed reduction equivalents with respect to the formation of enzymatically active cofactors. The most widely applied enzymatic cofactor regeneration system employs the formate dehydrogenase (FDH, EC 1.2.1.2) catalyzed re-



Reto Ruinatscha (1978) is a biochemist and graduated in 2004 at the Swiss Federal Institute of Technology (ETH) in Zurich, with a thesis in biotechnology on the selective hydrolysis of the nitrile group of *cis*-dihydrodiols derived from aromatic nitriles. He then joined the Bioprocess Laboratory (Prof. S. Panke, ETH Zurich) as staff researcher. There he worked on the *in silico* genome-scale reconstruction and validation of the *Staphylococcus aureus* metabolic network. In May 2005 he started his PhD thesis in the Enzyme Technology Group of Dr. K. Otto at the Chair of Chemical Biotechnology (University of Dortmund) under the supervision of Prof. A. Schmid on electroenzymatic oxidation reactions.

Katja Otto (1973) graduated as a biologist at the University of Hamburg. During her PhD she worked on the microbiology and biochemistry of alkane biodegradation in *Thermus* sp. under the guidance of Prof. R. Müller (Hamburg University of Technology). After she received her PhD in 2001 she went to Switzerland to work as a postdoc at the ETH Zurich in the Institute of Biotechnology. There she focused on the biochemistry of isolated flavin-monooxygenases. In 2004 she joined Prof. A. Schmid to establish the Chair of Chemical Biotechnology at the University of Dortmund, where she is now heading a research group in the field of enzyme technology.

Volker Höllrigl (1977) studied chemical engineering at the Hamburg University of Technology, Germany and at the Royal Institute of Technology, Stockholm, Sweden. He did his diploma work in the group of Prof. D. S. Clark at the University of California, Berkeley on the purification and characterization of the thermosome from *Methanococcus jannaschii* and graduated in August 2004. He then started his PhD in the Enzyme Technology Group of Dr. K. Otto at the Chair of Chemical Biotechnology at the University of Dortmund headed by Prof. A. Schmid and is currently working on process development of electroenzymatic reduction reactions.

Andreas Schmid (1966) graduated as a biologist (microbiology and biochemistry) at the University of Stuttgart (Germany) in 1992 and received his PhD with a topic on environmental microbiology at the University of Stuttgart in February 1997. After joining the Laboratory of Waste Air Purification (Department of Civil Engineering, University of Stuttgart, 1995 to 1996) and an internship at the Swiss Federal Institute for Environmental Science and Technology (EAWAG, Dübendorf, Switzerland), he joined the Institute of Biotechnology (ETH Zurich) as a postdoc where he was head of the Technical Enzymology Laboratory from 1998 until 2004. He received his venia legendi for biotechnology and microbiology in 2004 from ETH Zurich. Since October 2004 he holds the Chair of Chemical Biotechnology at the University of Dortmund and since January 2005 the "Systems Biotechnology" laboratory at the Institute for Analytical Sciences (ISAS, a member of the Leibniz Association).

duction of nicotinamide cofactors with formic acid as substrate.[7,8] Here the highest performances are reported with dehydrogenases as production enzymes achieving productivities of 640 $g L^{-1} d^{-1}$,[9] process life times of more than 90 days,[4] and total turnover numbers of over 600,000.[10] Oxygenases have been coupled to these regeneration systems as well. For example, styrene-type epoxidations catalyzed by styrene monooxygenase (StyAB) were performed with productivities of 1 $g L^{-1} h^{-1}$ and process life times of over 10 h.[11] Similarly 2,3-dihydroxybiphenyl was produced using 2-hydroxybiphenyl-3-monooxygenase (HbpA)

with process performances of 0.45 $g L^{-1} h^{-1}$ for at least 7 h, which is comparable to the corresponding whole-cell process.[12] The predominant use of the FDH/formic acid regeneration system arises from the fact that formic acid is a cheap co-substrate and its oxidation leads to the formation of CO_2, which is not only favorable for the thermodynamic equilibrium of the reaction but is also easily separated from the reaction solution and hence simplifies downstream processing. The drawbacks of FDH as regeneration enzyme are the rather high production costs[8] and the relatively low specific activity (up to 10 $U mg^{-1}$).[13] Furthermore, diverging optimal reaction conditions with respect to the production enzymes are often critical for process performances.

Chemical cofactor regeneration systems, based on mediators instead of regeneration enzymes, have also been developed, using low-cost and non-by-product forming agents. Bis(phosphine)rhodium complexes were applied for the dihydrogen (H_2) driven, indirect regeneration of NADH. Using this regeneration system, the coupled horse liver alcohol dehydrogenase (HLADH) was able to asymmetrically reduce norbornanone with an average productivity of 0.34 $g L^{-1} h^{-1}$ during 8 days, yet only low turnover frequencies (up to 10 h^{-1}) of the mediators were achieved.[14] In another approach H_2 served to directly regenerate NADPH using $[RuCl_2(TPPTS)_2]_2$ [TPPTS = tris(*m*-sulfonatophenyl)phosphine] as mediator. Within 4 h the applied alcohol dehydrogenase (TbADH) was able to convert 18% 2-heptanone into the corresponding alcohol. Here, the mediator performed 2.5 turnovers per hour.[15] Oxidation reactions have also been coupled to chemical regeneration systems. For example, the oxidase component of styrene monooxygenase (StyA) was coupled to the formic acid/(2,2′-bipyridyl)(pentamethylcyclopentadienyl)rhodium $\{[Cp^*Rh(bpy)(H_2O)]^{2+}\}$ driven regeneration of $FADH_2$. Interestingly, epoxidation rates in the range of 70% of the full enzymatic activity were achieved with turnover frequencies of up to 17.6 h^{-1} for the mediator.[16] In general, the disadvantages of chemical regeneration systems are not only the low turnover frequencies of the mediators (only several turnovers per hour), but also their low total turnover numbers.[17] In most cases this is attributed to the inactivation of the mediators.[18] As a consequence, rather high mediator concentrations are required to reach satisfactory process performances.[16]

Enzymatic and chemical regeneration systems have in common that reducing reagents have to be employed in at least stoichiometric amounts to drive the cofactor regeneration reaction. An economically and ecologically very interesting alternative is given by electrochemical cofactor regeneration where electrons are used as a reagent-free source of reduction equivalents.[19] This becomes evident when considering atom efficiencies in terms of gram co-substrate used per electron delivered.[20] Additionally, in enzymatic or chemical cofactor regeneration, typically one chemical bond of the co-substrate stores the required electrons. The remaining part of the molecule is important for molecular interactions with the regeneration enzyme or mediator and forms the oxidized by-product after the subsequent redox reaction. In contrast, electrochemical regeneration strategies do not result in associated by-product formation since electrons as a reagent-free source of reduction equivalents are used. In electrochemical regeneration systems not only co-substrates are replaced by electrons but also regeneration enzymes or mediators are in some cases not necessary any more; for example, during direct cofactor regeneration at electrodes. For efficient usage of the reduction equivalents it is possible to influence the selectivity of the electron transfer by adjusting the electrode potential or by changing the chemical nature of the electrode surface. The selectivity of the regeneration reaction may be adapted to a given process by the choice of the mediator that shuttles reduction equivalents from electrode to cofactor using indirect cofactor regeneration strategies.[19] Electrochemical cofactor regeneration is applicable to a broad range of reaction conditions in terms of buffers, temperature, pH, etc., allowing optimal reaction parameters for the production enzyme.[20]

Even though electrochemical cofactor regeneration appears to offer many advantages, and electricity is also one of the cheapest sources of reduction equivalents,[21] the technology has to be put into perspective with the performance of electroenzymatic processes. As productivities ($g L^{-1} h^{-1}$) cannot be taken as a sole measure of process performance final product concentrations ($g L^{-1}$) and total process times (h) have to be taken into account, too. The scope of this article is to evaluate process performances of various electroenzymatic reactions for the first time by quantifying key parameters with respect to industrial relevance. Operational windows for electroenzymatic reactions are defined on the basis of minimal process requirements and maximum achievable performances exemplified for the production of fine and speciality chemicals. Promising reactions based on reductive electrochemical regeneration of cofactors for dehydrogenases and oxygenases are reviewed and limiting process parameters are identified.

2 Operational Windows of Electroenzymatic Processes

A possibility to illustrate reaction performance under process conditions is to plot productivity as a function of final product concentration and total process time.

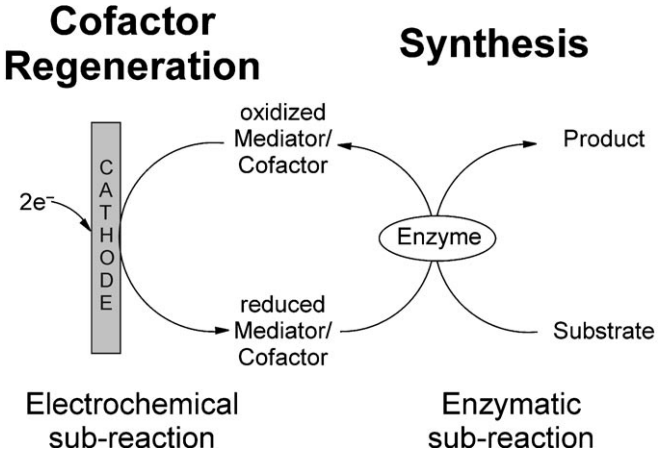


Figure 1. General scheme for electroenzymatic processes.

According to Woodley et al.,[22] an operational window can be defined within such a diagram using minimal process requirements and maximum achievable process performance as constraints. Processes that are located within this operational window are assigned to be of synthetic, technical and possibly industrial relevance with respect to their performance.

Minimal requirements of electroenzymatic processes are determined by economical constraints, which, for our considerations, are deduced from a survey by Straathof et al.[23] Industrially relevant processes employing enzymes to produce fine chemicals require productivities of at least $0.1\,g\,L^{-1}\,h^{-1}$ and minimal final product concentrations of $1\,g\,L^{-1}$. In addition, we assume a minimal total process time of 1 h. Maximum achievable process performances of electroenzymatic reactions are determined by quantifying the productivity and the total process time of the individual electrochemical and enzymatic sub-processes (Figure 1). The intersection of the resulting individual windows determines the overall operational window for electroenzymatic processes.

A theoretical operational window is defined based on an ideal electrochemical and enzymatic sub-reaction with maximized reaction parameters in order to estimate absolute upper process performances. For electrochemical reactions, the theoretical productivity in terms of space time yield ($STY_{e,th}$) is generally given by Eq. (1):

$$STY_{e,th} = M_p \times j \times \varepsilon \times A_v \times (n \times F)^{-1} \quad (1)$$

where STY_e is the space-time yield of electrochemical processes ($g\,L^{-1}\,h^{-1}$), M_p is the molecular weight of product ($g\,mol^{-1}$), j is current density ($A\,cm^{-2}$), ε is current efficiency (fraction of the current passing through an electrode that accomplishes the desired electrochemical reaction), A_v is ratio of electrode surface to cell volume (cm^{-1}), n is number of electrons transferred in the half-reaction, and F is the Faraday constant (96494 As per mole of product).

To calculate the theoretical $STY_{e,th}$ of the ideal electrochemical sub-process, we assume a redox half-reaction, where two electrons are exchanged and only the product of interest is formed ($M_p = 100\,g\,mol^{-1}$), without any side reactions. In contrast to the stoichiometry of the redox reaction, the following assumptions are made for the process parameters: as upper limit of the current density we propose $j = 3\,A\,cm^{-2}$. This has been reported previously using electrochemical catalysts on the basis of iridium oxide nanoparticles for the electrolysis of water.[24] The maximum current efficiency is defined as 98%. This is one of the highest values known and it has been reported for the chloralkali electrolysis, the production of chlorine gas and sodium hydroxide from aqueous sodium chloride brine.[24] A maximized ratio of electrode surface area to cell volume (A_v) is applied, too. To date, the highest A_v values are achieved using three-dimensional electrodes with A_v values of up to $200\,cm^{-1}$.[25] According to Eq. (1), this results in a theoretical $STY_{e,th}$ of $1.1 \times 10^6\,g\,L^{-1}\,h^{-1}$. The maximum achievable total process time of the ideal electrochemical process is also obtained from the chloralkali electrolysis process, which can be operated for at least 4 years.[24]

For the ideal enzymatic sub-process we assume that the volumetric productivity ($STY_{b,th}$) is only dependent on the specific enzyme activity and enzyme concentration as sole process parameters. $STY_{b,th}$ is then given by Eq. (2):

$$STY_{b,th} = a_{sp} \times c_{enz} \quad (2)$$

where STY_b is the space-time yield of an enzymatic reaction ($g\,L^{-1}\,h^{-1}$), a_{sp} is the specific enzyme activity ($U\,mg^{-1}$), c_{enz} is the enzyme concentration ($g\,L^{-1}$), and U is the international unit, amount of enzyme forming 1 μmol of product per minute

The theoretical $STY_{b,th}$ of the ideal enzymatic sub-process is determined by considering the highest reported specific enzyme activities and concentrations. The peptidylamidoglycolate lyase (EC 4.3.2.5) is one of the most active enzymes described. A specific activity of 1.6×10^7 U mg^{-1} towards α-hydroxyglycine-extended peptides is reported for this enzyme[26] and thus sets the upper theoretical limit regarding this parameter. The maximum applicable enzyme concentration is obtained from the high-fructose corn syrup process that is carried out in a packed-bed reactor. A typical industrial column for the production of high-fructose corn syrup has a volume of 8800 L[27] and is packed with 1800 kg of immobilized xylose isomerase resulting in a catalyst density of $200\,g\,L^{-1}$.[28] According to Eq. (2), the theoretical $STY_{b,th}$ is therefore $1.9 \times 10^{10}\,g\,L^{-1}\,h^{-1}$. As a theoretical upper limit for the total process time of enzymatic processes, the stabilities

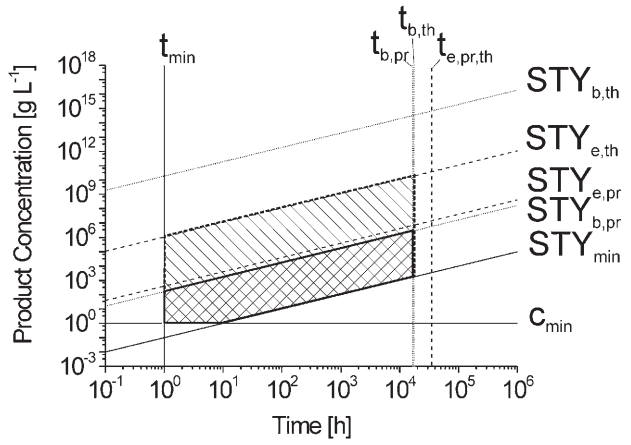


Figure 2. Operational window for electroenzymatic processes. Left hatched area shows the theoretical operational window for an electroenzymatic process; Right hatched area shows the practical operational window; (—): minimal requirements; (···): upper boundaries for enzymatic processes; (-----): upper boundaries for electrochemical processes; t: total process time; c: final product concentration; STY: productivity; $_{min}$: minimal requirement; $_{b}$: enzymatic; $_{e}$: electrochemical; $_{th}$: theoretical; $_{pr}$: practical

achieved with biosensors are considered where enzymes have been shown to be active for two years.[29] Together with the data for the ideal electrochemical process, the theoretical operational window can be drawn as shown in Figure 2. These calculated performance parameters are of course only theoretical maxima to define the absolute framework for electroenzymatic processes.

Real world reaction and process performance is always a compromise between the individual parameters, so that the theoretical maximum performance is basically not achievable. Reaction and process limitations might comprise, e.g., enzyme inhibition, side reactions, or mass transfer limitations. In order to account for the limitations of the individual sub-processes, a practical operational window is defined using productivities and total reaction times of existing electrochemical and enzymatic processes. These constraints allow a rough evaluation of electroenzymatic processes.

The maximum productivity of an electrochemical sub-process considers the Monsanto process, where acrylonitrile is electrohydrodimerized to adiponitrile at productivities of 400 $g L^{-1} h^{-1}$.[30] The maximum total process time is deduced from the chloralkali electrolysis with a total process time of at least 4 years.[24] The performance of enzymatic processes is obtained from the high-fructose corn syrup process. This process with immobilized xylose isomerase has a productivity of 164 $g L^{-1} h^{-1}$ and a life time of 678 days.[28]

The definition of the theoretical and practical operational windows (Figure 2) allows an assessment of the current status of processes based on their performance parameters and reveals potentials for process improvements.

3 Evaluation of Promising Electroenzymatic Processes with Synthetic Potential

Employing the above defined operational windows, the synthetic potential of electroenzymatic processes using dehydrogenases and oxygenases coupled to cathodic regeneration of cofactors is evaluated regarding STY, final product concentration, and total process time. Furthermore, process performance influencing parameters such as current density, current efficiency, and reaction engineering aspects are investigated (Table 1).

Specifically, the process with the highest productivity was developed by Cantet and co-workers[31] using a glutamate dehydrogenase to catalyze the reaction of α-ketoglutarate to L-glutamate (Figure 3). The reaction was established in a divided batch cell with a platinum working electrode, where the cofactor NADH was regenerated electrochemically by a hydrogenase. This process had a productivity of 3.04 $g L^{-1} h^{-1}$ with a current efficiency of nearly 100 % yielding a final product concentration of 15.2 $g L^{-1}$ after 5 h. The main limitation of this process was the instability of the regeneration enzyme which lost almost 70 % of its initial activity after 5 h. A more stable process with sufficiently high productivity (0.14 $g L^{-1} h^{-1}$) was developed using an electroenzymatic membrane reactor for the conversion of cyclohexanone to cyclohexanol by horse liver alcohol dehydrogenase (HLADH) (Figure 4).[32]

The enzyme was confined close to a carbon felt working electrode by means of an ultrafilration mem-

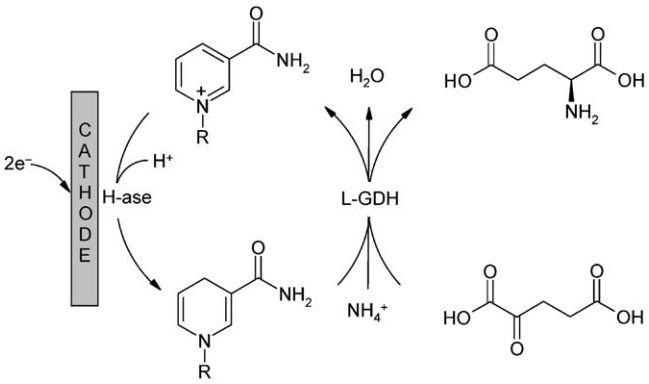


Figure 3. Electroenzymatic production of L-glutamate. L-glutamate dehydrogenase (L-GDH) is used to produce L-glutamate from α- ketoglutarate. NADH is regenerated at a platinum electrode using a hydrogenase (H-ase).

Table 1. Summary of electroenzymatic processes employing dehydrogenases and oxygenases.

No.	Substrate	Product	Enzyme	Regenerated cofactor	Cofactor regeneration	Scale [mL]	Electrode material	Productivity (STY) [$g L^{-1} h^{-1}$]	Total process time [h]	Current density [$A m^{-2}$]	Current efficiency (%)	Ref.
Electroenzymatic reduction reactions												
1	α-Ketoglutarate	L-glutamate	L-GDH 1.4.1.3 20 U	$NADH^D$	Hydrogenase	5	Platinum	3.04	5	n.d.	< 100	[31]
2	Pyruvate	D-lactate	D-LDH 1.1.1.28 1300 U	$NADH^D$	$[Cp^*Rh(bpy)(H_2O)]^{2+}$	50	Carbon felt	0.42	3	n.d.	67	[33]
3	GSH	GSSG	GR 1.6.4.1 n.d.	$NADPH^D$	Alg-V, FRD	10	PAA-modified carbon plate	0.15	2	n.d.	n.d.	[31]
4	Cyclohexanone	Cyclohexanol	HLADH 1.1.1.1 73 U	$NADH^D$	$[Cp^*Rh(bpy)(H_2O)]^{2+}$	100	Carbon felt	0.14	70	n.d.	n.d.	[32]
5	GSH	GSSG	GR 1.6.4.1 0,5 U	$NADPH^D$	FRD/MV^+	25	Carbon plate	0.09	3.5	n.d.	n.d.	[37]
6	Pyruvate	D-lactate	D-LDH 1.1.1.28 50 U	$NADH^D$	Direct regeneration at electrode	50	Cholesterol-modified gold amalgam	0.08	21	n.d.	n.d.	[35]
7	Pyruvate	D-lactate	D-LDH 1.1.1.28 150 U	$NADH^D$	Diphoarase/MV^+	50	Gold amalgam	0.03	120	n.d.	n.d.	[34]
8	Pyruvate	D-lactate	D-LDH 1.1.1.28 30 U	$NADH^D$	FDR/MV^+	600	Tungsten	0.03	336	n.d.	< 100	[55]

Table 1. (Continued)

No.	Substrate	Product	Enzyme	Regenerated cofactor	Cofactor regeneration	Scale [mL]	Electrode material	Productivity (STY) [$g L^{-1} h^{-1}$]	Total process time [h]	Current density [$A m^{-2}$]	Current efficiency (%)	Ref.
9	Pyruvate	L-lactate	L-LDH 1.1.1.27 350 U	$NADH^D$	Direct regeneration at electrode	15	Poly[Rh-(III)Cp*(L)(Cl)]$^+$ modified graphite foil	0.003	18	0.1	73	[38]
10	Cyclohexanone	cyclohexanol	HLADH 1.1.1.1 5 mg	$NADH^D$	Direct regeneration at electrode	25	Rh-$(tterpy)_2^{3+}$ modified RVC	0.002	31	0.5	17	[39]
Electroenzymatic oxidation reactions												
a	2-Hydroxybi-phenyl	2,3-dihydroxybi-phenyl	HbpA 1.14.13.44 19 U	$NADH^D$	$[Cp^*Rh(bpy)(H_2O)]^{2+}$	100	Carbon felt	0.2	2	n.d.	n.d.	[40]
b	*trans*-β-Methyl-styrene	(1*S*,2*S*)-1-phenyl-propylene oxide	StyA n.d. 2,13 μM	$FADH_2{}^D$	Direct regeneration at electrode	10	Carbon felt	0.03	0.3	n.d.	n.d.	[50]
c	Camphor	5-*exo*-hydroxy-camphor	CYP101 (P450cam) 1.14.15.1 0.13 μM	$Heme^P$	Putidaredoxin	20	Tin oxide	0.005	4.5	0.1	22	[41]

^{D}: diffusible cofactor.
^{P}: prosthetic group.
n.d.: not determined.
L-GDH: L-glutamate dehydrogenase; D-LDH: D-lactate dehydrogenase; $[Cp^*Rh(bpy)(H_2O)]^{2+}$: (2,2′-bipyridyl)(pentamethylcyclopentadienyl)rhodium; GSH: L-glutathione, reduced; GSSG: L-glutathione, oxidized; GR: glutathione reductase; Alg-V: polymerized viologens; FRD: ferrodoxin $NADP^+$ reductase; PAA-modified carbon plate: polyaminoaniline modified carbon plate; HLADH: horse liver alcohol dehydrogenase; MV^+: methyl viologen; FDR: ferrodoxin reductase; L-LDH: L-lactate dehydrogenase; poly[Rh(III)Cp*(L)(Cl)]$^+$ modified graphite foil: polypyrrole-(2,2′-bipyridyl)(pentamethylcyclopentadienyl)rhodium film-modified graphite foil; Rh$(tterpy)_2^{3+}$ modified RVC: polypyrrole-rhodium(III) bis-terpyridine complex modified reticulated vitreous carbon; HbpA: 2- hydroxybiphenyl-3-mono-oxygenase; StyA: styrene monooxygenase; CYP101 (P450cam): cytochrome CYP101 (P450cam) monooxygenase

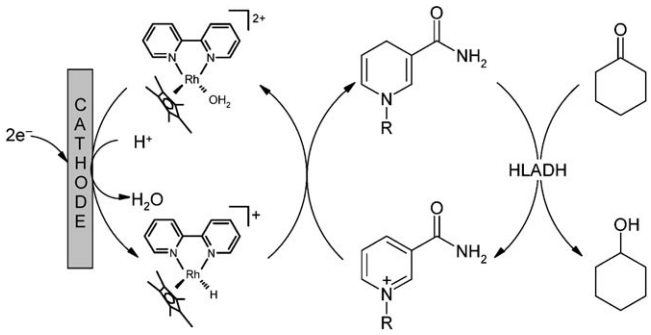


Figure 4. Electroenzymatic production of cyclohexanol. Horse liver alcohol dehydrogenase (HLADH) is used to produce cyclohexanol from cyclohexanone in an electrochemical membrane reactor. The cofactor NADH is regenerated at a carbon felt electrode using the electrochemical mediator $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

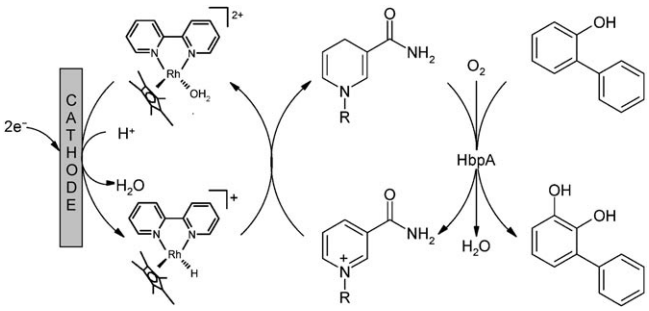


Figure 6. Electroenzymatic production of 2,3-dihydroxybiphenyl. Hydroxybiphenyl monooxygenase (HbpA) is used for the conversion of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl in a divided batch cell. NADH is regenerated at a carbon felt electrode using the electrochemical mediator $[Cp^*Rh(bpy)(H_2O)]^{2+}$. The co-substrate dioxygen (O_2) is supplied by sparging with air.

brane, while substrate, product, cofactor and the electrochemical mediator $[Cp^*Rh(bpy)(H_2O)]^{2+}$ were continuously recycled. The process was running for 70 h, until all cyclohexanone was converted. Another process fulfilling minimal process requirements was developed by Ruppert et al.,[33] where D-lactate dehydrogenase was used to convert pyruvate to D-lactate. Here, a divided electrochemical batch cell with a carbon felt electrode and $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as electron mediator was used to regenerate NADH (Figure 5). The productivity achieved was $0.42\ g\,L^{-1}\,h^{-1}$. The drawback of this process was that 1300 U of enzyme had to be supplied to achieve a final product concentration of $1.26\ g\,L^{-1}$ after 3 h.

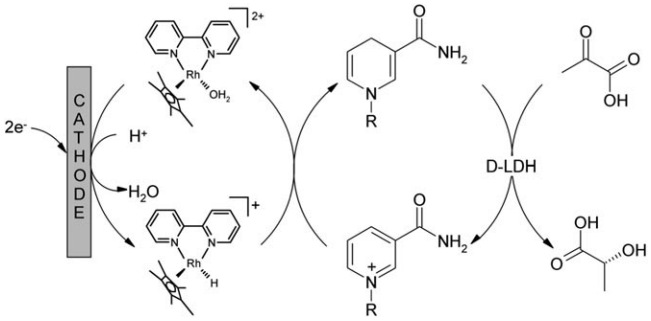


Figure 5. Electroenzymatic production of D-lactate: D-lactate dehydrogenase (D-LDH) is used to transform pyruvate to D-lactate in a divided batch cell. The cofactor NADH is regenerated at a carbon felt electrode using the electrochemical mediator $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

All other evaluated dehydrogenase-based processes did not fulfill the defined minimal requirements for efficiency, either because of insufficient productivities[34,35] or because of too low final product concentrations[36] or both.[37–39]

The reaction with the highest STY employing an oxygenase coupled to cathodic cofactor regeneration was developed by Hollmann et al.[40] 2-Hydroxybiphenyl-3-monooxygenase (HbpA) was employed to catalyze the reaction from 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl (Figure 6). The cofactor NADH was regenerated electrochemically in a divided cell at a carbon felt working electrode using $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as electrochemical mediator. Molecular oxygen, necessary for the enzymatic reaction, was supplied by sparging with air. The productivity had a maximum of $0.2\ g\,L^{-1}\,h^{-1}$ and was sufficiently high to meet the minimal process requirements. However, the process could not be continued for more than 2 h due to inactivation of the enzyme and thus the final product concentration reached only $0.4\ g\,L^{-1}$.

A reaction for the conversion of camphor to 5-*exo*-hydroxycamphor catalyzed by cytochrome CYP101 (P450cam) was developed by Reipa et al.[41] (Figure 7). The natural redox partner putidaredoxin that was reduced at the tin oxide working electrode was used to supply the enzyme with reduction equivalents. Molecular oxygen was supplied by anodic oxidation of water at a platinum anode under otherwise anaerobic conditions. In spite of the increased and sufficiently high reaction stability (4.5 h), the productivity ($0.005\ g\,L^{-1}\,h^{-1}$) as well as the final product concentration ($0.02\ g\,L^{-1}$) did not meet the minimal economical requirements.

Overall, the process performance of the evaluated electroenzymatic processes can be visualized using the defined operational windows (Figure 8). Although most processes do not yet meet the minimal requirements, some are already located in the operational window for technical-scale applications. In general, dehydrogenase-based processes show a better performance compared to processes employing oxygenases, which is reflected by the fact that all processes within the operational window make use of dehydrogenases as production enzymes and reported current efficiencies are mostly higher for these processes.

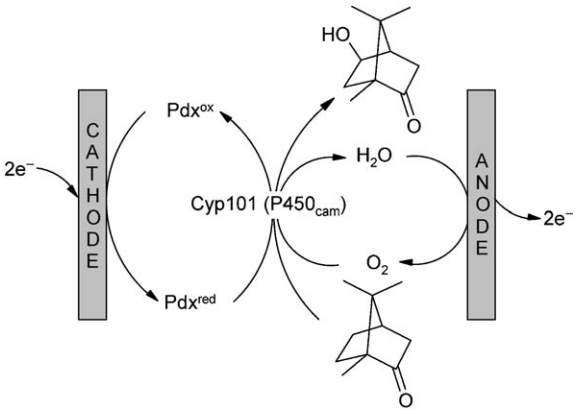


Figure 7. Electroenzymatic production of 5-*exo*-hydroxycamphor. CYP101 (P450cam) is used to produce 5-*exo*-hydroxycamphor from camphor in a batch cell. The natural redox partner putidaredoxin (Pdx) is regenerated at a tin oxide electrode. The co-substrate dioxygen (O_2) is produced at the anode *via* oxidation of water under otherwise anaerobic conditions.

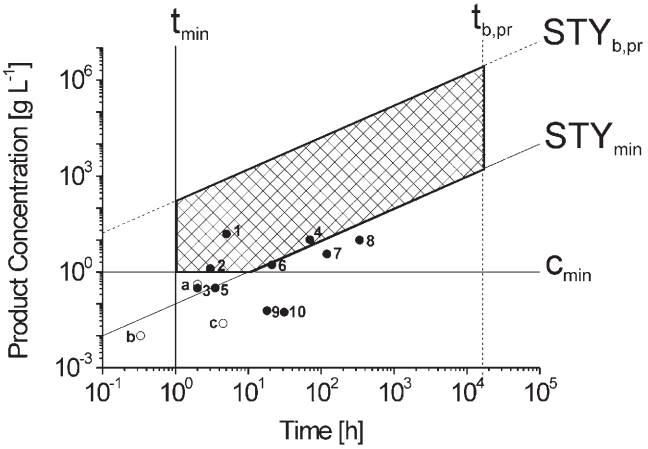


Figure 8. Evaluation of current electroenzymatic processes employing oxidoreductases. Cross hatched area shows the practical operational window; (○): corresponds to processes using oxygenases; (●): corresponds to processes using dehydrogenases; t: total process time; c: final product concentration; STY: productivity; $_{min}$: minimal requirement; $_{b}$: enzymatic; $_{pr}$: practical; detailed information on the processes is given in Table 1.

4 Parameters that Limit the Performance of Electroenzymatic Processes and Promising Developments in Reaction Engineering

Most reviewed processes could be moved into the operational window if their final product concentration (Figure 8) and hence their production rates would be significantly higher. A main bottleneck limiting the performance of the reviewed electroenzymatic processes can therefore be assigned to low productivities. This may be attributed to a relatively slow electrochemical regeneration of cofactors compared to the enzymatic synthesis reaction. Even though electrochemical regeneration reactions in electroenzymatic processes have been shown to reach turnover frequencies of up to 200 h^{-1},[31] this is still far below the catalytic potential of oxidoreductases. Oxygenases display turnover rates of up to 280 s^{-1},[42] whereas for dehydrogenase-catalyzed reductions turnover frequencies of even 110,000 s^{-1} are reported.[43] Due to the heterogeneous character of the electrochemical cofactor regeneration, it is possible to increase the productivity of electroenzymatic processes by enlarging the electrode surface because the reaction rate is directly proportional to the electrode surface.[44] This is especially true for cofactors, e.g., flavins that can be reduced to the enzymatic active form directly at bare electrodes.[45] Here the limitation is mainly determined by reactor design, which has to maximize the electrode surface to volume ratio.[6] For NAD(P)H regeneration, direct reduction at bare electrodes is not feasible because only small amounts of enzymatically active nicotinamide cofactors are formed.[46] Indirect electron transfer is necessary to allow selective regeneration of NAD(P)H. At least one further component has to be included in the process. In the simplest case this is only a mediator which can alternatively be used in solution or immobilized onto the electrode, simplifying downstream process steps. However, immobilization approaches do not yet reach the same turnover frequencies and stabilities as achieved with soluble mediators or electrochemical regeneration enzymes (Table 1). This is why soluble mediators are used most frequently. Often high concentrations of the electrochemical mediator are employed besides the use of enlarged electrode surfaces to compensate for low cofactor regeneration rates. This may result in interactions between the mediator and the production enzyme and may impede the overall reaction. Such unwanted side reactions have been observed for $[Cp^*Rh(bpy)(H_2O)]^{2+}$. $[Cp^*Rh(bpy)(H_2O)]^{2+}$ presumably coordinates lysine residues present at the enzyme's surface leading to enzyme inactivation as well as to withdrawal of the mediator from the reaction mixture, which reduces overall process performance. Nevertheless, there is good evidence that interactions between electrochemical mediator and production enzyme can be entirely prevented by reaction medium engineering.[47]

For oxygenases, a main limiting parameter arises from the requirement of molecular oxygen as a co-substrate which at the same time is prone to cathodic reduction. The reduction potential of oxygen (−300 mV *vs.* SCE at glassy carbon electrodes)[48] is higher than common working potentials of electroenzymatic processes and thus direct oxygen reduction

will always occur as a side reaction. This not only results in decreased current efficiencies and less efficient energy utilization but also in the formation of reactive oxygen species and associated local pH shifts, which subsequently hamper enzyme activity and stability. A solution may be dismutation of hydrogen peroxide to water and oxygen by catalase, which was already shown to improve the stability of P450 monooxygenases under process conditions.[49] This, however, not only increases downstream efforts because an additional enzyme is present in the reaction mixture, but enzyme deactivation cannot be entirely prevented either. This occurs especially close to the electrode, where high concentrations of these reactive oxygen species evolve.[40] Furthermore, the overall productivity may be reduced by reoxidation of reduced cofactors or mediators by molecular oxygen before the reaction with the enzyme in the bulk medium. This is especially critical for cofactors and mediators showing a fast reaction with oxygen, like flavins and methyl viologens.[50,51] The oxygen dilemma can be reduced by controlling oxygen supply *via* the anodic oxidation of water instead of sparging with air.[41] A side effect of this technique is that stripping out of the reactants from the medium is reduced, which occurs during extensive aeration combined with agitation. This is in particular significant for volatile substrates and products.[50] Furthermore, Reipa et al.[41] shielded their tin oxide cathode by a platinum mesh to decompose traces of hydrogen peroxide that originated from the direct reduction of oxygen at the cathode. This set-up resulted in product formation rates which could not be achieved under oxygen-saturated conditions or without the shielding of the electrode.

Such intelligent reactor design concepts also include electrochemical membrane reactors, which confine enzymes close to the electrode. In a theoretical study this was shown to be beneficial, especially if the regeneration step of the cofactor was rate limiting.[52] Using such a set-up, significantly higher reaction rates can be achieved compared to batch systems.[32] For a hydrogenase, used as an electrochemical regeneration enzyme, it could even be shown that the electrode provides a stabilizing effect to the enzyme which further increases the process lifetime.[53]

5 Conclusions

Here, electroenzymatic processes have been reviewed with respect to their process performance. The presented evaluation is based on the concept of operational windows, developed for hybrid processes considering productivity, final product concentration, and total process time. Surprisingly, some processes employing dehydrogenases already show performances comparable to commercial reactions that employ enzymes for the synthesis of fine chemicals and thus are already located within the operational window, yet the majority is not. Rather low volumetric productivities are identified as one of the main limiting factors of the evaluated processes. With an increase of this parameter, the majority of the processes could fulfill the minimal process requirements and thus shift into the operational window. The main reason for the generally low productivities is the relatively slow electrochemical cofactor regeneration rate. In the case of oxygenase-based reactions, the presence of molecular oxygen may further impair process performances, as it gives rise to reactive oxygen species and the reoxidation of reduced cofactors. Several concepts have been developed to address these limitations and numerous improvements in process performance are technically feasible which clearly emphasize the progress in the field of electroenzymology.

For enzymatic reduction and oxidation reactions, the overall process can be significantly simplified by applying electrochemical regeneration approaches instead of enzymatic or chemical *in vitro* regeneration concepts. In the best case, this was shown for the selective epoxidations by styrene monooxygenase StyA. The former regeneration approach employed FDH, the reductase StyB, the cofactors NADH and $FADH_2$, as well as the sacrificial substrate formate,[11] whereas the electrochemical approach directly regenerated $FADH_2$ at the carbon felt cathode thus making all other components obsolete.[50] Even though the process performance is not yet in the range of alternative cofactor regeneration systems it demonstrates the great potential of simplifying processes by using electrochemical approaches.

Many electroenzymatic processes only need minor improvements in process performance to become of synthetic, technical or industrial relevance. This can be best exemplified by comparing current electroenzymatic processes with processes employing the FDH/formic acid regeneration system. The production of 2,3-dihydroxybiphenyl using HbpA *via* electrochemical cofactor regeneration reached productivities of $0.2\,g\,L^{-1}\,h^{-1}$ for 2 h, whereas cofactor regeneration using the FDH/formic acid system resulted in productivities of $0.45\,g\,L^{-1}\,h^{-1}$ (7 h). Processes employing dehydrogenases are already located in the operational window and thus are already of synthetic and technical interest. This is an interesting alternative because these systems can be applied to a broad range of enzymes without any adaptations especially for small scale production of high value-added products.

6 Outlook

The field of electroenzymology has exciting and interesting future perspectives. "Oxidation catalysis brings

together the interests of academic and industrial researchers perhaps to a greater extent than any other reaction class. (…) More generally, the selective catalysis of oxidation of olefins, alcohols, sulfides, and other key functional groups represents some of the most challenging, fascinating, and potentially useful reactions in chemistry today."[54] What can electroenzymology add to these challenges? In the foreseeable future, the biocatalytic production of fine chemicals will be simpler and economically more attractive using whole cell catalysis or enzyme based cofactor regeneration systems, e.g., in membrane reactors or in aqueous/organic emulsions. Yet, recent achievements in electroenzymology for redox catalysis open the door for advanced applications for toxicity tests (possibly replacing microsomal assays in pharma R&D), syntheses of high priced specialties (gram to maybe kg amounts of products), lead compound derivatizations, and high throughput, parallel biocatalytic organic syntheses.

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