DOI: 10.1002/adsc.200900033

A New Regeneration System for Oxidized Nicotinamide Cofactors

Seda Aksu,[a] Isabel W. C. E. Arends,[a,*] and Frank Hollmann[a,*]

[a] Biocatalysis and Organic Chemistry, Department of Biotechnology, Delft University of Technology, Julianalaan 136, 2628BL, Delft, The Netherlands
Fax: (+31)-(0)15-278-1415; phone: (+31)-(0)15-278-4423 (IWCEA), (+31)-(0)15-278-1957 (FH);
e-mail: i.w.c.e.arends@tudelft.nl or f.hollmann@tudelft.nl

Received: January 16, 2009; Published online: May 28, 2009

Dedicated to Prof. M.T. Reetz on the occasion of his 65th birthday.

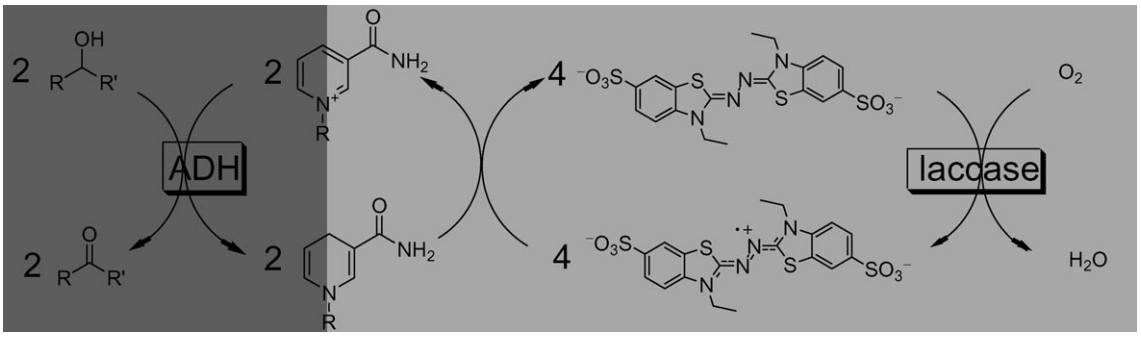
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/adsc.200900033.

Abstract: A novel regeneration system for oxidized nicotinamide cofactors (NAD^+ and $NADP^+$) is presented. By combining 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS)-catalyzed oxidation of NAD(P)H with laccase-catalyzed utilization of molecular oxygen as terminal oxidant, a simple chemo-enzymatic $NAD(P)^+$ regeneration method is achieved. Thus, the advantages of both worlds, chemical oxidation of reduced nicotinamide cofactors and laccase-catalyzed utilization of oxygen from air are combined in a simple and generally applicable new approach for biooxidation catalysis. This new application of the well-known laccase-mediator system (LMS) is successfully used to promote alcohol dehydrogenase-catalyzed oxidation reactions of primary and secondary alcohols. Already under non-optimized conditions, high turnover numbers of >300 and >16000 were obtained for the nicotinamide cofactor and ABTS, respectively. In this communication, we present the proof-of-principle and initial characterization of the proposed new regeneration system.

Keywords: aerobic oxidation; cofactors; enzyme catalysis; laccase mediator system; oxidoreductases

Oxidation of alcohols is one of the most fundamental and important reactions in organic chemistry. Amongst the catalytic procedures[1] such as using metal catalysts[2] or organocatalysts,[3] biocatalytic procedures are gaining more and more importance as they enable environmentally benign and selective oxidations.[4,5] For example, oxidative kinetic resolutions[6] and selective oxidations of polyols[7] have been reported. Alcohol dehydrogenases (ADHs, E.C. 1.1.1.x, also called ketoreductases) are the preferred biocatalysts. Mechanistically, ADH-catalyzed oxidation involves reversible hydride-abstraction from the alcohol C atom to the oxidized nicotinamide cofactor [$NAD(P)^+$] yielding the carbonyl product as well as the reduced cofactor [NAD(P)H]. For economic reasons and to shift the unfavourable thermodynamic equilibrium,[8] regeneration of the catalytically active oxidized form is inevitable. In contrast to the manifold regeneration approaches for NAD(P)H,[9–11] complementary systems for the regeneration of oxidized cofactors $NAD(P)^+$ are scarce.[4] Most commonly, a reducible cosubstrate is coadministered serving as terminal electron sink to drive the desired oxidation reaction. In this biocatalytic variant of the Oppenauer oxidation, the reversible character of the ADH-catalyzed transfer hydrogenation is elegantly exploited in a single-enzyme system. A drawback of this methodology however is that, apart from few exceptions,[12] high molar surpluses of the cosubstrate are necessary to shift the equilibrium to the side of the desired products and so generating additional waste. Furthermore, also enzyme stability often is impaired (even though in recent times, highly solvent-tolerant ADHs have been reported).[13] More elegantly, the high oxidation potential of the O_2/H_2O couple [E^0_{SHE} (pH 7) = 0.82 V] can be exploited to efficiently drive the reoxidation of NAD(P)H [E^0_{SHE} (pH 7) = −0.315 V]. This approach is also attractive from an economic and ecological point of view as molecular oxygen is cheap, easy to handle and only water is formed as by-product. Recently, water- and hydrogen peroxide-forming NAD(P)H oxidases (E.C. 1.6.3.x) have acquired some interest.[14–19]

Chemical oxidants such as quinoid structures,[20–22] transition metals[23,24] are also attractive since they are



Scheme 1. The novel LMS-based NAD(P)$^+$ regeneration approach (light grey) coupled to an ADH-catalyzed alcohol oxidation (dark grey). NAD(P)H (originating from an ADH-catalyzed oxidation) is oxidized by the (formal) ABTS radical cation whose regeneration is accomplished in a laccase-catalyzed, aerobic oxidation yielding water as sole by-product.

robust, inexpensive and do not distinguish between phosphorylated and non-phosphorylated nicotinamide cofactors. However, to avoid the generation of hazardous hydrogen peroxide, molecular oxygen has to be substituted by an anode as terminal electron acceptor, resulting in complicated reaction set-ups.[11] Using such an approach Liese and co-workers recently reported on the fast and efficient combination of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and electrochemical regeneration to promote ADH-catalyzed oxidation reactions.[25]

ABTS is well-known as a spectrophotometric probe for peroxidase and laccase activity determination. Combinations of ABTS and laccases in the so-called laccase-mediator-system (LMS) have been successfully applied by Haltrich and co-workers for the oxidative regeneration of flavin-dependent oxidases.[26,27] We became interested in the question of whether the LMS can be also used to promote ADH-catalyzed oxidation reactions as outlined in Scheme 1. Such a system would combine the advantages of both approaches: laccase-catalyzed regeneration of oxidized ABTS enables the use of O_2 from air while forming water as sole by-product and ABTS as mediator should enable regeneration of both NAD$^+$ and NADP$^+$. Due to its supposed high thermal robustness we chose the commercial laccase from the thermophilic fungus *Myceliophthora thermophila* (*MT*laccase, Novozymes) as a model system.

LMS-catalyzed oxidation of NAD(P)H: Upon addition of laccase to an aerated solution of NADH and ABTS, we were pleased to observe a decrease of the characteristic UV absorption band of reduced nicotinamide at 340 nm (Supporting Information). In the absence of either laccase, mediator, or under anoxic conditions no significant decrease was observed. Hydrogen peroxide was not detectable throughout the course of the reaction. The catalytic mechanism of the ABTS-mediated oxidation should include two sequential one-electron transfer steps from the reduced nicotinamide to the ABTS radical cation. Generally, hydride acceptors are preferred to avoid the occurrence of intermediate NAD radicals which might dimerize and thereby become catalytically inactive.[11] However, using ESR spectroscopy we were unable to detect a putative pyridinium radical suggesting extremely low (<0.1 μM) concentrations of such intermediate radical species. This observation is in accordance with the published redox potentials for the first and second electron transfer steps (see Supporting Information)[28,29] suggesting the first electron transfer to be overall rate-limiting; the second electron transfer step is significantly easier (as expressed by a lower redox potential) which probably can also be attributed to the aromatization energy contribution.

Substituting NADH with its phosphorylated pendant NADPH resulted in essentially identical progression curves albeit at slightly reduced rates (approximately 90% of the rate observed with NADH). We attribute this slightly decreased rate to the additional negative charge present in the phosphorylated NADPH and the resulting additional electrostatic repulsion with the ABTS anion. Thus, we concluded that the regeneration system depicted in Scheme 1 is generally applicable to NAD$^+$- and NADP$^+$-dependent ADH-catalyzed oxidation reactions.

Encouraged by these results, we further characterized the LMS-based NAD(P)$^+$ regeneration system. Expectedly, a linear correlation between laccase and ABTS concentration and NAD(P)H oxidation rate was observed (Supporting Information). The pH range was investigated between pH 5 and 8 showing an exponential decrease of the NAD(P)H oxidation rate with increasing pH (Figure 1). Using syringaldazine as spectrophotometric probe maximum *MT*laccase activity is found at neutral to slightly acidic pH values (Supporting Information). Thus we attribute the decreasing NAD(P)H oxidation activity to the pH-dependency of the ABTS redox potential. Bearing in mind that ADH-catalyzed oxidations generally are most efficient at alkaline pH values, all subsequent experiments were (unless indicated otherwise)

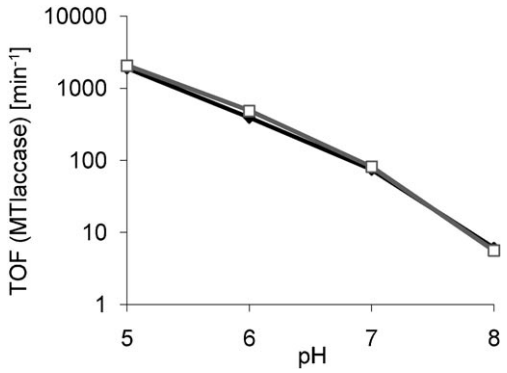


Figure 1. pH-dependence of the rate of the LMS-catalyzed oxidation of NAD(P)H. The efficiency is expressed as turnover frequency (TOF) of *MT*laccase. (closed diamonds: NADH, open squares: NADPH. *Conditions:* buffer: KPi (50 mM adjusted to the appropriate pH value), $T = 25\,^{\circ}C$, $[NAD(P)H]_0 = 0.2$ mM, [ABTS] = 5 μM, [laccase] = 10, 40, 100, and 400 nM (for pH 5, 6, 7, and 8, respectively).

performed at pH 8. It is obvious from Figure 1 that the potentially very high catalytic efficiency of *MT*laccase is not exploited by far. Further studies on identifying more suitable mediators than ABTS such as quinones or phenylenediimines[11] are underway.

The temperature-dependencies of the activity and stability of the LMS at pH8 are shown in Figure 2.

Interestingly, a linear increase of the LMS-catalyzed NAD(P)H oxidation rate was observed. One plausible explanation for this deviation from exponentiality might lie in the decreasing O_2 concentration. *MT*laccase exhibits a comparably low affinity towards O_2 [$K_M(O_2) \approx 50$ μM][30] suggesting *MT*laccase to be increasingly O_2-limited at elevated temperatures thus counteracting the exponential activity increase with temperature. *MT*laccase stability was superb at ambient or slightly elevated temperatures. Thus, we

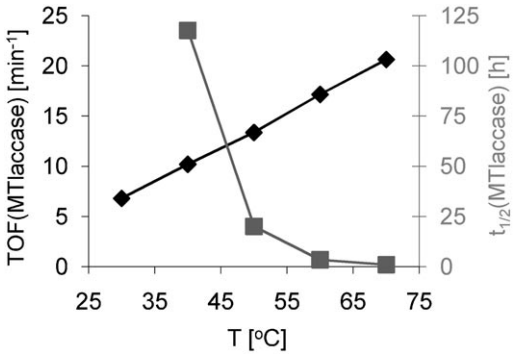


Figure 2. Temperature-dependence of the activity (black diamonds) and stability (grey squares) of the LMS. *Conditions:* KPi (50 mM, pH 8), [*MT*laccase] = 0.4 μM; spectrophotometric detemination of activity was initiated by the simultaneous addition of $[NADH]_0 = 0.2$ mM and [ABTS] = 20 μM.

Table 1. TADH-catalyzed oxidation driven by the LMS-regeneration system.[a]

Substrate	Conv. 5 h [%]	Conv. 24 h [%]
cyclohexanol	48.2	62.8
4-methylcyclohexanol	16.1	33.8
decahydro-2-naphthol	18.0	19.6
citronellol	7.8	13.6
2-octanol	n.d.	19.3

[a] *Conditions:* 100 mM KPi buffer (pH8), $T = 40\,^{\circ}C$, $[substrate]_0 = 10$ mM, [TADH] = 2.96 μM, $[NADH]_0 = 0.1$ mM, [ABTS] = 50 μM, [*MT*laccase] = 10 μM.

concluded that a reaction temperature range of 30–40 °C is optimal considering acceptable catalytic activity and thermal stability of the bioctatalyst.

The coupled LMS-ADH system: As a model system to investigate the feasibility of the envisioned O_2-LMS-driven and ADH-catalyzed oxidation of alcohols, we chose the ADH from *Thermus* sp. (TADH).[31] Especially its facile fermentation/purification procedure, enabling production at scale within 2 days, make it a well-suited model system.[23] In a first set of experiments we compared the productivity of the coupled catalytic system (Scheme 1) with the stoichiometric addition of NAD^+ for a representative selection of TADH substrates (Table 1). While initial rates were hardly influenced (Supporting Information), the conversions observed in the catalytic system were always higher than when stoichiometric amounts of NAD^+ were applied. We attribute this to the decreased competitive inhibition of TADH by the reduced nicotinamide cofactor and shift of the equilibrium.

Interestingly, there was a slight but significant increase of enantioselectivity (E)[32] when the LMS was applied. For example, E(TADH) for the oxidation of racemic 2-octanol increased from 5.8 to 7.1. Currently we are lacking a plausible explanation for this observation.

We further investigated the optimal pH- and T-conditions for the coupled enzyme system in the oxidation of cyclohexanol (Figure 3).

Using the chosen conditions, initial productivities of up to 7.2 $mM\,h^{-1}$ are achievable. The T- and pH-dependence observed reflects the catalytic properties of TADH.[31] Thus, we assume that under these conditions the TADH-catalyzed oxidation was overall rate-limiting. Product inhibition was identified as the major limitation of this set-up (Supporting Information). As a result, maximum conversion in the range of 60–70% (6–7 mM of the ketone product) was achievable thereby also limiting the turnover number (TTN) of the catalysts. To alleviate this inhibition, we

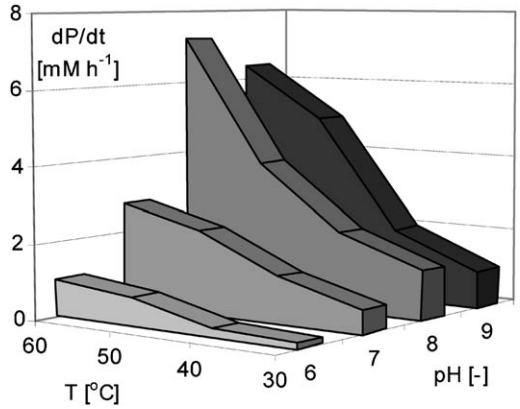


Figure 3. Temperature- and pH-dependence of the coupled system. *Conditions:* $[cyclohexanol]_0 = 10$ mM, [TADH] = 2.96 μM, $[NADH]_0 = 0.1$ mM, [ABTS] = 50 μM, [*MT*laccase] = 10 μM.

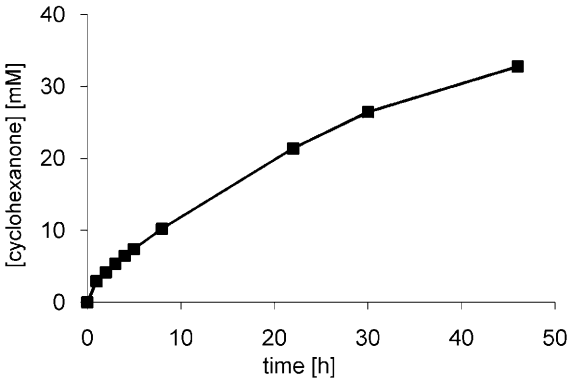


Figure 4. Time course of the two-liquid phase system for the oxidation of cyclohexanol. *Conditions:* $T = 40$ °C, 1:1 ratio of reaction buffer and octane. Organic phase: $[cyclohexanol]_0 = 125$ mM, [dodecane] = 5 mM (internal standard); aqueous phase: KPi buffer (50 mM, pH 8) [TADH] = 3.4 μM, $[NADH]_0 = 0.1$ mM, [ABTS] = 2 μM, [*MT*laccase] = 10 μM.

applied a two-liquid phase system comprising octane as substrate reservoir and product sink.

Indeed, initial experiments using this improved set-up resulted in a significantly prolonged reaction time and product accumulation (Figure 4).

It should be mentioned here that the stability of the combined system ($t_{1/2}$ approx. 25 h) was somewhat lower than expected from the stabilities of the isolated enzymes [at 40 °C: $t_{1/2}$ (*MT*laccase) = 120 h (Figure 2) and $t_{1/2}$ (TADH) ≫ 180 h[23]]. The reaction mixtures exhibited the characteristic colour of the oxidized ABTS radical which is in accordance with the assumption that TADH may be the overall rate-limiting factor. $ABTS^{\cdot+}$ is a known oxidant, for example, for primary alcohols and thereby may oxidatively inactivate one or both enzymes thereby accounting for the lower robustness of the full system. Optimization [i.e., balancing the activities of TADH and the LMS-$NAD(P)^+$ regeneration system] is currently underway.

Nevertheless, more than 30 mM of product were obtained corresponding to a total turnover number of 325 and 16,400 for NAD and ABTS, respectively. A comparison with established $NAD(P)^+$ regeneration approaches demonstrates the potential of our new approach (Table 2). The TTN achieved for NAD with our regeneration system so far is somewhat lower than for the most widely applied enzyme-coupled, glutamate dehydrogenase-catalyzed reductive amination of α-keto acids; but is clearly superior to other state-of-the-art approaches. Furthermore, due to the chemo-enzymatic character both NAD^+- and $NADP^+$-dependent enzymes can be applied. Also, the use of O_2 as terminal oxidant together with the exclu-

Table 2. Comparison of the catalytic performance of the current system with some reported $NAD(P)^+$ regeneration systems.

Regeneration System	Cofactor	Cosubstrate/Coproduct	TTN[a]		Waste Factor[b]
			NAD(P)	Mediator	
LDH[c],[33]	NAD^+	pyruvate/lactate	59	–	90
GluDH[d],[34]	NAD^+	α-ketoglutarate/glutamate	1.600	–	147
ABTS/Anode[25]	$NAD(P)^+$	[e]	32	74	[e]
$[Ru(PDon)_3]$[f],[3,22]	$NAD(P)^+$	O_2/H_2O_2	90	900	34
*Ct*XR/PQ[g],[20]	$NAD(P)^+$	O_2/H_2O_2	125	1,000	34
FMN[35]	$NAD(P)^+$	O_2/H_2O_2	19	1	34
NAD(P)H oxidase[17]	$NAD(P)^+$	O_2/H_2O	110	–	9
ABTS/*MT*laccase	$NAD(P)^+$	O_2/H_2O	325	16,400	9

[a] Total turnover number: mol (product) × mol (cofactor/catalyst)$^{-1}$.
[b] Waste factor: g (by-product) × mol (cofactor)$^{-1}$.
[c] Lactate DH.
[d] Glutamate dehydrogenase.
[e] Depending on the method of power generation.
[f] PDon = 1,10-phenanthroline-5,6-dione.
[g] *Ct*XR = *Candida tenuis* xylose reductase, PQ = 9,10-phenanthrenequinone.

sive formation of water as by-product enables a thermodynamically irreversible and environmentally attractive regeneration system (Table 2).

Further investigations aiming at an in-depth understanding of the inactivation mechanism and preparative scale application of the proposed new oxidation reaction are currently underway in our laboratory. Also studies substituting ABTS by other mediators which do not exhibit a pronounced pH-dependence of their redox potential are underway.

Overall, we are convinced that the LMS-based $NAD(P)^+$ regeneration system will be an attractive alternative to established regeneration approaches.

Experimental Section

Chemicals and Enzymes

Chemicals were purchased from Sigma–Aldrich in the highest purity available and used without further purification. *MTlaccase* (tradename Flavourstar, Novozymes) was obtained as a 0.2 mM solution of the enzyme and used without further purification. *TADH* was produced by recombinant expression of pASZ2 (PET) in *E. coli* BL21(DE3) using the Overnight Express™ autoinduction system (Novagene).[36] Cell lysis and TADH purification were performed simultaneously by incubating the resuspended (50 mM KPi, pH 7.5) *E. coli* cells at 80 °C for 20 min followed by centrifugation (4000 g, 20 min); the resulting light-yellow supernatant contained 1.1 $mg\,mL^{-1}$ (29.6 μM) TADH (<95% purity as judged by SDS-gel electrophoresis).

Activity Assays

TADH activity and the activity of the LMS-$NAD(P)^+$ regeneration system were assayed spectrophotometrically by following the characteristic absorbance of NAD(P)H at 340 nm ($\varepsilon = 6.22\ mM^{-1}\,cm^{-1}$). The LMS stability assays were performed by incubation of *MT*laccase at the temperature indicated in Figure 2; at intervals samples were taken, supplemented with ABTS and NADH and assayed at 30 °C.

Reactions in the Coupled System (Scheme 1)

Reactions in the coupled system were performed on a 1-mL scale. Unless indicated otherwise, reactions were performed in 50 mM potassium phosphate buffer, pH 8 (KPi). Due to the low thermal pK_a-dependence of phosphate ($\Delta pH_{20\rightarrow 60} < 0.1$), pH values were not corrected for thermal effects. Reactions in the two-liquid phase system were performed as above by adding one volume equivalent of octane containing the substrate. Samples were thoroughly extracted with diethyl ether containing 5 mM of dodecane as internal standard and analyzed *via* chiral GC.

Acknowledgements

Evonik Goldschmidt, Essen, Germany and Novozymes Germany, Nieder-Olm, Germany are gratefully acknowledged for generous enzyme donations. Dr. K. Bühler and Prof. A. Schmid are acknowledged for providing the plasmid encoding TADH. This project is financially supported by the Dutch Ministry of Economic Affairs and the B-Basic partner organizations (www.b-basic.nl), a public-private NWO-ACTS programme.

References

[1] R. A. Sheldon, I. W. C. E. Arends, U. Hanefeld, *Green Chemistry and Catalysis*. Wiley-VCH, Weinheim, **2007**.
[2] I. Marko, P. Giles, M. Tsukazaki, I. Chelle-Regnaut, A. Gautier, R. Dumeunier, F. Philippart, K. Doda, J. Mutonkole, S. Brown, C. Urch, *Efficient, ecologically benign, aerobic oxidation of alcohols*, in: *Advances in Inorganic Chemistry*, Elsevier Academic Press, San Diego, **2004**; Vol. 56, pp 211–240.
[3] R. A. Sheldon, I. W. C. E. Arends, *Adv. Synth. Catal.* **2004**, *346*, 1051–1071.
[4] W. Kroutil, H. Mang, K. Edegger, K Faber, *Adv. Synth. Catal.* **2004**, *346*, 125–142.
[5] A. Schmid, F. Hollmann, B. Bühler, *Oxidation of Alcohols, Phenols, Aldehydes, and Carboxylic Acids*, in: *Enzyme Catalysis in Organic Chemistry*, (Eds.: K.-H. Drauz, H. Waldmann), Wiley-VCH, Weinheim, **2002**.
[6] W. Stampfer, B. Kosjek, C. Moitzi, W Kroutil, K. Faber, *Angew. Chem.* **2002**, *114*, 1056–1059; *Angew. Chem. Int. Ed.* **2002**, *41*, 1014–1017.
[7] M. Manzoni, M. Rollini, S. Bergomi, *Proc. Biochem.* **2001**, *36*, 971–977.
[8] H. Chenault, G. Whitesides, *App. Biochem. Biotechnol.* **1987**, *14*, 147–197.
[9] W. A. van der Donk, H. Zhao, *Curr. Opin. Biotechnol.* **2003**, *14*, 421–426.
[10] F. Hollmann, K. Hofstetter, A. Schmid, *Trends Biotechnol.* **2006**, *24*, 163–171.
[11] F. Hollmann, A. Schmid, A. , *Biocatal. Biotransf.* **2004**, *22*, 63–88.
[12] I. Lavandera, A. Kern, V. Resch, B. Ferreira-Silva, A. Glieder, W. M. F. Fabian, S. de Wildeman, W. Kroutil, *Org. Lett.* **2008**, *10*, 2155–2158.
[13] W. Stampfer, B. Kosjek, W. Kroutil, K. Faber, *Biotechnol. Bioeng.* **2003**, *81*, 865–869.
[14] J.-I. Hirano, K. Miyamoto, H. Ohta, *Appl. Microbiol. Biotechnol.* **2008**, *80*, 71–78.
[15] B. Geueke, B. Riebel, W. Hummel, *Enz. Microb. Technol.* **2003**, *32*, 205–211.
[16] B. Riebel, P. R. Gibbs, W. B. Wellborn, A. S. Bommarius, *Adv. Synth. Catal.* **2003**, *345*, 707–712.
[17] B. Riebel, P. R. Gibbs, W. B. Wellborn, A. S. Bommarius, *Adv. Synth. Catal.* **2002**, *344*, 1156–1168.
[18] J.-I. Hirano, K. Miyamoto, H. Ohta, *Tetrahedron Lett.* **2008**, *49*, 1217–1219.
[19] Z. Findrik, I. Simunovic, Đ. Vasic-Racki, *Biochem. Eng. J.* **2008**, *39*, 319–327.
[20] S. L. Pival, M. Klimacek, B. Nidetzky, *Adv. Synth. Catal.* **2008**, *350*, 2305–2312.
[21] G. Hilt, B. Lewall, G. Montero, J. H. P. Utley, E. Steckhan, *Liebigs Ann./Rec.* **1997**, 2289–2296.

[22] G. Hilt, T. Jarbawi, W. R. Heineman, E. Steckhan, *Chem. Eur. J.* **1997**, *3*, 79–88.
[23] F. Hollmann, A. Kleeb, K. Otto, A. Schmid, *Tetrahedron: Asymmetry* **2005**, *16*, 3512–3519.
[24] F. Hollmann, B. Witholt, A. Schmid, *J. Mol. Catal. B: Enzym.* **2002**, *19–20*, 167–176.
[25] I. Schröder, E. Steckhan, A. Liese, *J. Electroanal. Chem.* **2003**, *541*, 109–115.
[26] U. Baminger, R. Ludwig, C. Galhaup, C. Leitner, K. D. Kulbe, D. Haltrich, *J. Mol. Catal. B: Enzym.* **2001**, *11*, 541–550.
[27] R. Ludwig, M. Ozga, M. Zamocky, C. Peterbauer, K. D. Kulbe, D. Haltrich, *Biocatal. Biotransform.* **2004**, *22*, 97–104.
[28] A. Anne, S. Fraoua, V. Grass, J. Moiroux, J. M. Saveant, *J. Am. Chem. Soc.* **1998**, *120*, 2951–2958.
[29] P. Hapiot, J. Moiroux, J. M. Saveant, *J. Am. Chem. Soc.* **1990**, *112 (4)*, 1337–1343.
[30] F. Xu, *App. Biochem. Biotechnol.* **2001**, *95*, 125–133.
[31] V. Höllrigl, F. Hollmann, A. Kleeb, K. Bühler, A. Schmid, *Appl. Microbiol. Biotechnol.* **2008**, *81*, 263–273.
[32] A. J. J. Straathof, J. A. Jongejan, *Enz. Microb. Technol.* **1997**, *21 (8)*, 559–571.
[33] O. Bortolini, E. Casanova, G. Fantin, A. Medici, S. Poli, S. Hanau, *Tetrahedron: Asymmetry* **1998**, *9*, 647–651.
[34] C. H. Wong, J. R. Matos, *J. Org. Chem.* **1985**, *50*, 1992–1994.
[35] A. J. Irwin, J. B. Jones, *J. Am. Chem. Soc.* **1977**, *99*, 1625–1630.
[36] F. W. Studier, *Prot. Express. Purif.* **2005**, *41*, 207–234.

