Oxygenations

International Edition: DOI: 10.1002/anie.201507881
German Edition: DOI: 10.1002/ange.201507881

Peroxygenase-Catalyzed Oxyfunctionalization Reactions Promoted by the Complete Oxidation of Methanol

*Yan Ni[+], Elena Fernández-Fueyo[+], Alvaro Gomez Baraibar, René Ullrich, Martin Hofrichter, Hideshi Yanase, Miguel Alcalde, Willem J. H. van Berkel, and Frank Hollmann**

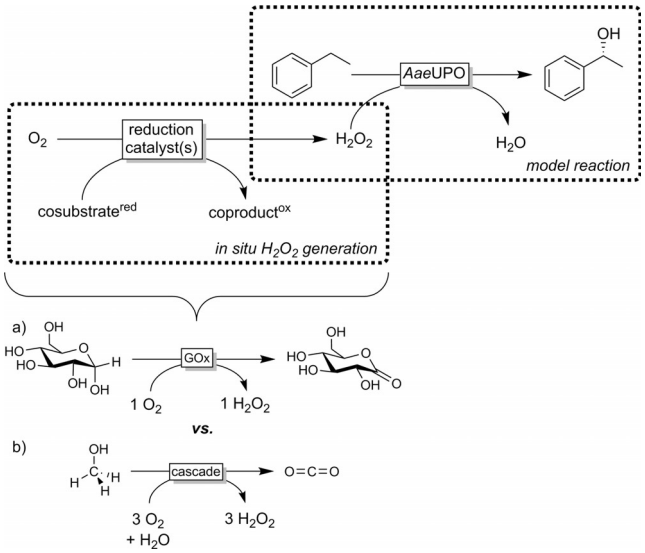
Abstract: *Peroxygenases catalyze a broad range of (stereo)-selective oxyfunctionalization reactions. However, to access their full catalytic potential, peroxygenases need a balanced provision of hydrogen peroxide to achieve high catalytic activity while minimizing oxidative inactivation. Herein, we report an enzymatic cascade process that employs methanol as a sacrificial electron donor for the reductive activation of molecular oxygen. Full oxidation of methanol is achieved, generating three equivalents of hydrogen peroxide that can be used completely for the stereoselective hydroxylation of ethylbenzene as a model reaction. Overall we propose and demonstrate an atom-efficient and easily applicable alternative to established hydrogen peroxide generation methods, which enables the efficient use of peroxygenases for oxyfunctionalization reactions.*

The selective oxyfunctionalization of C–H bonds certainly represents one of the most challenging reactions in organic chemistry.[1] P450 monooxygenases have been investigated for more than two decades as (bio)catalysts for selective oxyfunctionalization reactions.[2] More recently, peroxygenases have emerged as alternatives to P450 monooxygenases.[3] On the one hand, peroxygenases exhibit a similarly rich oxyfunctionalization chemistry as P450 monooxygenases as both rely on a highly reactive oxyferryl species (compound I, conferring reactivity) embedded into the well-defined steric environment of the enzyme active site (conferring selectivity).[4]

On the other hand, peroxygenases do not depend on complicated electron-transport chains as P450 monooxygenases do but rather form compound I directly from H_2O_2. Obviously, this results in significantly simplified reaction schemes, rendering peroxygenases promising catalysts for selective oxyfunctionalization reactions. Furthermore, in the past decade, the portfolio of peroxygenases available to the organic chemist has been extended significantly.[3c,5]

Peroxygenases, however, exhibit only poor robustness against H_2O_2, so that the amount of H_2O_2 present in the reaction mixture needs to be carefully adjusted to balance reactivity and oxidative inactivation of the enzymes. Various enzymatic,[6] electrocatalytic,[7] and photocatalytic methods[8] for the in situ formation of H_2O_2 by the catalytic reduction of O_2 have been reported.

Today, the glucose/glucose oxidase system for in situ H_2O_2 generation prevails (Scheme 1). However, glucose is used very (atom-)inefficiently in this system as only one equivalent



Scheme 1. The new in situ H_2O_2 generation system and the model reaction used in this study. The unspecific peroxygenase from *Agrocybe aegerita* (*Aae*UPO) catalyzes the chemo- and stereospecific conversion of ethylbenzene into enantiopure (*R*)-1-phenylethanol. In situ provision with H_2O_2 is achieved by catalytic reduction of ambient oxygen (O_2). Shown below are a) the established method based on the glucose oxidase (GOx) catalyzed single oxidation of glucose, which generates one equivalent of H_2O_2, and b) the envisioned full oxidation of methanol to CO_2, generating three equivalents of H_2O_2.

[*] Dr. Y. Ni,[+] Dr. E. Fernández-Fueyo,[+] Dr. A. G. Baraibar, Dr. F. Hollmann
Department of Biotechnology
Delft University of Technology
Julianalaan 136, 2628BL Delft (The Netherlands)
E-mail: f.hollmann@tudelft.nl
Dr. R. Ullrich, Prof. Dr. M. Hofrichter
Department of Bio- and Environmental Science
Technical University of Dresden—International Institute Zittau
02763 Zittau (Germany)
Prof. Dr. H. Yanase
Department of Chemistry and Biotechnology
Graduate School of Engineering, Tottori University
4–101 Koyamacho-Minami, Tottori, Tottori 680-8552 (Japan)
Prof. Dr. M. Alcalde
Department of Biocatalysis
Institute of Catalysis, CSIC
28049 Madrid (Spain)
Prof. Dr. W. J. H. van Berkel
Laboratory of Biochemistry, Wageningen University
Dreijenlaan 3, 6703 HA Wageningen (The Netherlands)
[+] These authors contributed equally to this work.

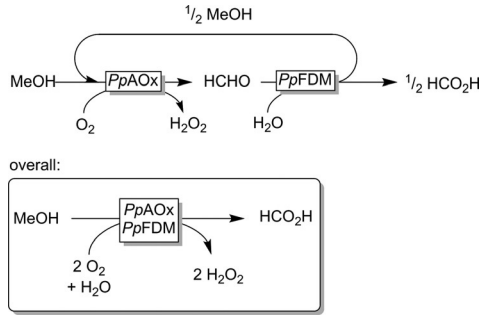
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201507881.

of H_2O_2 can be obtained from one equivalent of glucose. In other words, only two of the 24 electrons theoretically obtainable through the complete oxidation of all C atoms present in glucose are used. To circumvent the issues mentioned above, we envisioned the utilization of methanol as a sacrificial electron donor. Ideally, methanol would be fully mineralized to CO_2, liberating six electrons that could be used for the reductive activation of molecular oxygen, yielding three equivalents of H_2O_2 (Scheme 1).

Early contributions by Therisod and co-workers,[9] who used methanol as a sacrificial electron donor to promote peroxidase reactions, unfortunately had very little impact on the field. Most probably, this is due to the fact that only the first oxidation step (methanol to formaldehyde) was reported. The resulting stoichiometric formation of formaldehyde not only represents a possible environmental hazard and health burden but also severely impairs biocatalyst stability and utilizes the sacrificial electron donor very inefficiently. Therefore, we revisited this approach, combining it with an enzymatic system for formaldehyde dismutation and oxidation of the resulting formic acid.

As a model reaction, we focused on the enantioselective hydroxylation of ethylbenzene into (*R*)-1-phenylethanol (Scheme 1). The model peroxygenase used in this study was *Agrocybe aegerita* (*Aae*UPO, E.C.1.11.2.1) owing to its high activity towards (non-)activated C(sp^3)–H bonds.[3b,5,8b,10] The enantioselectivity of this reaction was invariably very high, yielding > 99 % *ee* in all experiments reported here.

The first step of the methanol mineralization cascade is the aerobic oxidation of methanol to formaldehyde (Scheme 2). Among the two commercially available fungal



Scheme 2. Proposed bioenzymatic cascade featuring alcohol oxidase (AOx) and formaldehyde dismutase (FDM) for the overall double oxidation of methanol to formic acid, generating two equivalents of H_2O_2.

alcohol oxidases (from *Candida boidinii* (*Cb*AOx) and *Pichia pastoris* (*Pp*AOx)) that were evaluated, *Pp*AOx excelled in terms of activity (turnover frequency, TOF(*Cb*AOx) = 7 s^{-1} vs. TOF(*Pp*AOx) = 30 s^{-1}; see the Supporting Information, Figure S2). Therefore, all subsequent reactions were performed with *Pp*AOx as the catalyst of in situ H_2O_2 generation. As *Aae*UPO, just like any other heme enzyme, is prone to H_2O_2-dependent oxidative inactivation of its prosthetic group, the in situ H_2O_2 levels need to be kept as low as possible to maintain activity. Therefore, we systematically investigated the influence of the ratio of *Aae*UPO to *Pp*AOx on the efficiency and robustness of the overall reaction (Figure S3). A molar ratio of one was sufficient to minimize H_2O_2-induced inactivation of the peroxygenase while maximizing the hydroxylation rate.

Next, we evaluated formaldehyde dismutase (FDM, E.C.1.2.99.4) as a co-catalyst to dismutate formaldehyde into formic acid and methanol (Scheme 2). Iteration of this process will eventually lead to a double oxidation of methanol to formic acid, overall enabling the generation of two equivalents of H_2O_2 per one equivalent of methanol. For our studies, we used the FDM from *Pseudomonas putida* F61 (*Pp*FDM) overexpressed in *E. coli*.[11]

In a first set of experiments, we investigated whether the theoretical stoichiometry of two equivalents of H_2O_2 per equivalent of methanol could indeed be found experimentally. Therefore, we performed the overall cascade reaction under methanol-limiting conditions (5 mM) with *Pp*AOX either in the presence or absence of *Pp*FDM. In the absence of *Pp*FDM (Figure 2, ◆), the reaction practically stopped upon complete methanol consumption. In the presence of *Pp*FDM (Figure 2, ■), the overall reaction proceeded smoothly to provide 10 mM of the product, corresponding to an overall double methanol oxidation. Furthermore, we determined the final formate concentration in the reaction mixture to be 4.50 ± 0.03 mM, confirming our hypothesis of double oxidation of methanol. It should be mentioned that *Pp*FDM exhibits a rather poor affinity towards its substrate formaldehyde (K_M = 350 mM).[11d] To avoid the undesired accumulation of formaldehyde, the concentration of *Pp*FDM was therefore comparably high (295 nM).

Encouraged by these results, we scaled up the reaction to compare the performance of the in situ H_2O_2 generation cascade in the absence and presence of *Pp*FDM (Figure 1). The presence of *Pp*FDM had no significant influence on the initial hydroxylation rate but a very distinct influence on the robustness of the overall process: In the absence of *Pp*FDM (◆, Figure 1), the reaction rate decreased significantly already after 24 hours, and the reaction ceased completely after 72 hours. However, in the presence of *Pp*FDM (■), almost linear product accumulation was observed for at least 96 hours with continuous production for at least 120 hours. Overall, ethylbenzene was fully converted into (*R*)-1-phenylethanol in 62 % yield (31 mM), and acetophenone was detected as the sole side product (16 %, 8 mM). The apparent discrepancy in the mass balance is due to partial evaporation of the reactants as confirmed by control experiments. The presence of *Pp*FDM improved the efficiency of the system (in terms of the amount of product formed) by more than 40 %. Consequently, also the catalyst turnover numbers (TON(*Aae*UPO) and TON(*Pp*AOx)) increased from 291 500 and 485 800 in the absence of *Pp*FDM to more than 468 500 and 780 800 in the presence of *Pp*FDM.

At intervals, the residual *Pp*AOx activity in the reaction medium was determined revealing a good correlation with the results shown in Figure 1. In the absence of *Pp*FDM, the oxidase activity dropped by approximately 50 % within the first 24 hours and was practically zero after 72 hours whereas in the presence of *Pp*FDM, the *Pp*AOx activity decreased much more slowly (> 50 % of the initial activity after 72 h).

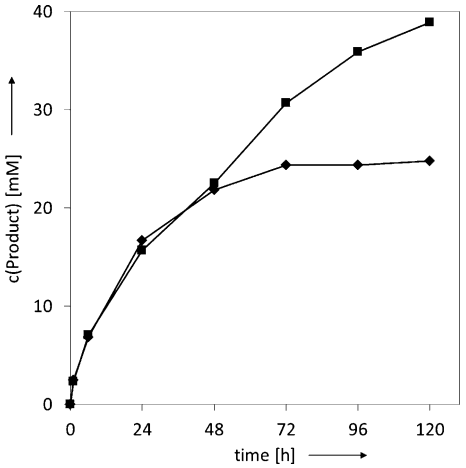
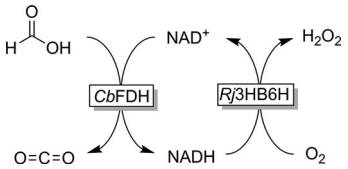


Figure 1. Enzymatic hydroxylation of ethylbenzene in the absence (◆) and presence (■) of *Pp*FDM. Conditions: [ethylbenzene] = 50 mM, [methanol] = 200 mM, [*Aae*UPO] = 100 nM, [*Pp*AOx] = 60 nM, [*Pp*FDM] = 295 nM in 100 mM phosphate buffer (pH 7.0), T = 30 °C. For the sake of clarity, both (*R*)-1-phenylethanol and acetophenone are shown, please see the text for further information.

We therefore assume that mainly the stability of the alcohol oxidase against formaldehyde was overall limiting the robustness of the reaction, and that the addition of *Pp*FDM could efficiently alleviate this inactivation.

It is worth mentioning here that this reaction setup is not limited to the stereospecific hydroxylation of ethylbenzene but could also be extended to a representative range of *Aae*UPO-catalyzed oxyfunctionalization reactions (Table S1).

Finally, we aimed at establishing the entire cascade. Formate oxidases (FOx, E.C.1.2.3.1) appeared to be the catalysts of choice to accomplish the last oxidation step (formic acid to CO_2), yielding the third equivalent of H_2O_2. Unfortunately, the operational windows of the reported formate oxidases do not overlap sufficiently with those of the enzymes used in this study.[12] In particular, the pH optimum of the known formate oxidases of around pH 3–4 and their low activities at more ambient pH values render their application in this context difficult if not impossible. Therefore, we turned our attention to the well-known formate dehydrogenase from *Candida boidinii* (*Cb*FDH), which oxidizes formate to CO_2 while transferring the liberated reduction equivalents to NAD^+. To aerobically reoxidize the resulting NADH and produce H_2O_2, we used the 3-hydroxybenzoate-6-hydroxylase from *Rhodococcus jostii* RHA1 (EC.1.14.13.24, *Rj*3HB6H), which, in the presence of its natural product, acts as an NADH oxidase yielding H_2O_2 (Scheme 3).[13]



Scheme 3. The final oxidation step (formic acid to CO_2) produces H_2O_2 by using a combination of formate dehydrogenase (*Cb*FDH) and 3-hydroxybenzoate-6-hydroxylase (*Rj*3HB6H).

The performance of the three systems for in situ H_2O_2 generation, namely *Pp*AOx alone (◆), *Pp*AOx combined with *Pp*FDM (■), and the entire cascade (▲), in the presence of limiting amounts of methanol (5 mM) is compared in Figure 2. The expected amount of product was found for every cascade (i.e., 5, 10, and 15 mM of (*R*)-1-phenylethanol), confirming the feasibility of the proposed triple oxidation of methanol.

The turnover numbers and frequencies achieved with the different enzymes are summarized in Table 1. Pleasingly, the catalytic performance of the oxyfunctionalization catalyst (*Aae*UPO) was superb, with turnover numbers that are suitable for the economic production of speciality and even bulk chemicals.[14] Similarly, the primary H_2O_2 generation catalyst (*Pp*AOx) performed exceptionally well in the presence of *Pp*FDM. Efficient dismutation of the primarily formed formaldehyde proved to be crucial to maintain *Pp*AOx activity. Still, *Pp*FDM mutants with a higher affinity towards formaldehyde will be highly desirable to decrease its concentration. The final step of the methanol oxidation cascade, which currently comprises two enzymes (*Cb*FDH and *Rj*3HB6H) and one cofactor (NAD), should be seen as

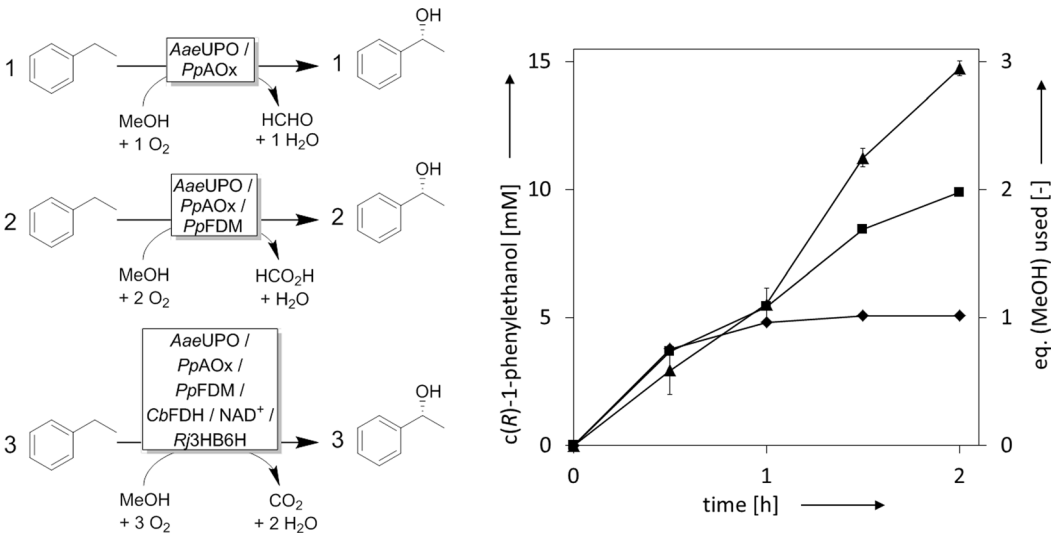


Figure 2. Comparison of the different cascade processes for methanol oxidation to promote the *Aae*UPO-catalyzed hydroxylation of ethylbenzene. ◆: *Pp*AOx only; ■: *Pp*AOx combined with *Pf*FDM; ▲: the entire cascade. General conditions: [ethylbenzene] = 15 mM, [methanol] = 5 mM, [*Aae*UPO] = 50 nM, [*Pp*AOx] = 60 nM in 100 mM phosphate buffer (pH 7.0), T = 30 °C; ■: [*Pp*FDM] = 590 nM; ▲: [*Pp*FDM] = 590 nM, [NAD^+] = 1.6 mM, [2,5-dihydroxybenzoic acid] = 1 mM, [*Rj*3HB6H] = 11 μM, [*Cb*FDH] = 1 μM.

Table 1: Catalytic performance of the enzymes and cofactors used in the MeOH mineralization experiment (Figure 2, ▲).

Catalyst	TON[a] [mol mol^{-1}]	TOF[b] [s^{-1}]
*Aae*UPO	294 700	41
*Pp*AOx	245 580	34
*Pp*FDM	25 400	3.5
*Rj*3HB6H	1330	0.18
*Cb*FDH	14 730	2
NAD	9	0.001

[a] TON = moles of product divided by moles of catalyst used.
[b] TOF = TON divided by the reaction time (here 2 h).

a temporary solution for proof-of-concept studies. An economically attractive (and more elegant) solution would be to substitute these catalysts by just one. Unfortunately, the formate oxidases available thus far are not compatible considering their optimal operational window. New formate oxidase variants with increased activity at ambient pH values are urgently needed. Alternatively, preliminary experiments on the photocatalytic oxidation of formic acid gave promising results (although they necessitated external illumination of the reaction mixture). At present, the system reported here is not ready for practical applications, which is mainly due to the very low *Aae*UPO concentration. Further studies will focus on scale up by using higher enzyme concentrations and will also have to evaluate increased substrate loadings and possibly optimized oxygen supply.

Overall, peroxygenases exhibit an enormous potential for selective oxyfunctionalization chemistry. To unveil this potential, efficient, robust, scalable, and environmentally acceptable methods for in situ H_2O_2 generation will be necessary. However, herein we have already demonstrated that methanol can be used for the atom-efficient in situ generation of H_2O_2.

Acknowledgments

This work was financially supported by the European Union (KBBE-2013-7-613549, "INDOX") and the "Deutsche Bundesstiftung Umwelt" (AZ 13270).

Keywords: heme proteins · hydrogen peroxide · hydroxylation · oxidation · peroxygenases

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 798–801
Angew. Chem. **2016**, *128*, 809–812

[1] a) T. Newhouse, P. S. Baran, *Angew. Chem. Int. Ed.* **2011**, *50*, 3362–3374; *Angew. Chem.* **2011**, *123*, 3422–3435; b) M. C. White, *Science* **2012**, *335*, 807–809; c) S. R. Neufeldt, M. S. Sanford, *Acc. Chem. Res.* **2012**, *45*, 936–946; d) K. Kamata, K. Yonehara, Y. Nakagawa, K. Uehara, N. Mizuno, *Nat. Chem.* **2010**, *2*, 478–483; e) M. Sun, J. Z. Zhang, P. Putaj, V. Caps, F. Lefebvre, J. Pelletier, J. M. Basset, *Chem. Rev.* **2014**, *114*, 981–1019; f) M. T. Reetz, *J. Am. Chem. Soc.* **2013**, *135*, 12480–12496.

[2] a) S. T. Jung, R. Lauchli, F. H. Arnold, *Curr. Opin. Biotechnol.* **2011**, *22*, 809–817; b) E. O'Reilly, V. Kohler, S. L. Flitsch, N. J. Turner, *Chem. Commun.* **2011**, *47*, 2490–2501; c) F. E. Zilly, J. P. Acevedo, W. Augustyniak, A. Deege, U. W. Häusig, M. T. Reetz, *Angew. Chem. Int. Ed.* **2011**, *50*, 2720–2724; *Angew. Chem.* **2011**, *123*, 2772–2776; d) S. Kille, F. E. Zilly, J. P. Acevedo, M. T. Reetz, *Nat. Chem.* **2011**, *3*, 738–743; e) M. Bordeaux, A. Galarneau, F. Fajula, J. Drone, *Angew. Chem. Int. Ed.* **2011**, *50*, 2075–2079; *Angew. Chem.* **2011**, *123*, 2123–2127.

[3] a) S. Bormann, A. Gomez Baraibar, Y. Ni, D. Holtmann, F. Hollmann, *Catal. Sci. Technol.* **2015**, *5*, 2038–2052; b) M. Hofrichter, R. Ullrich, *Curr. Opin. Chem. Biol.* **2014**, *19*, 116–125; c) F. van Rantwijk, R. A. Sheldon, *Curr. Opin. Biotechnol.* **2000**, *11*, 554–564.

[4] D. Holtmann, M. W. Fraaije, D. J. Opperman, I. W. C. E. Arends, F. Hollmann, *Chem. Commun.* **2014**, *50*, 13180–13200.

[5] M. Hofrichter, R. Ullrich, *Appl. Microbiol. Biotechnol.* **2006**, *71*, 276–288.

[6] a) D. Thiel, D. Doknić, J. Deska, *Nat. Commun.* **2014**, *5*, 5278; b) Q. L. Sheng, J. B. Zheng, *Biosens. Bioelectron.* **2009**, *24*, 1621–1628; c) C. López, A. Cavaco-Paulo, *Eng. Life Sci.* **2008**, *8*, 315–323.

[7] a) L. Getrey, T. Krieg, F. Hollmann, J. Schrader, D. Holtmann, *Green Chem.* **2014**, *16*, 1104–1108; b) T. Krieg, S. Huttmann, K.-M. Mangold, J. Schrader, D. Holtmann, *Green Chem.* **2011**, *13*, 2686–2689; c) S. Lütz, E. Steckhan, A. Liese, *Electrochem. Commun.* **2004**, *6*, 583–587.

[8] a) E. Churakova, I. W. C. E. Arends, F. Hollmann, *ChemCatChem* **2013**, *5*, 565–568; b) E. Churakova, M. Kluge, R. Ullrich, I. Arends, M. Hofrichter, F. Hollmann, *Angew. Chem. Int. Ed.* **2011**, *50*, 10716–10719; *Angew. Chem.* **2011**, *123*, 10904–10907; c) D. I. Perez, M. Mifsud Grau, I. W. C. E. Arends, F. Hollmann, *Chem. Commun.* **2009**, 6848–6850; d) S. Gargiulo, I. W. C. E. Arends, F. Hollmann, *ChemCatChem* **2011**, *3*, 338–342.

[9] a) F. Pezzotti, M. Therisod, *Tetrahedron: Asymmetry* **2007**, *18*, 701–704; b) F. Pezzotti, K. Okrasa, M. Therisod, *Tetrahedron: Asymmetry* **2005**, *16*, 2681–2683.

[10] a) S. Peter, A. Karich, R. Ullrich, G. Grobe, K. Scheibner, M. Hofrichter, *J. Mol. Catal. B* **2014**, *103*, 47–51; b) P. Molina-Espeja, E. Garcia-Ruiz, D. Gonzalez-Perez, R. Ullrich, M. Hofrichter, M. Alcalde, *Appl. Environ. Microbiol.* **2014**, *80*, 3496–3507; c) X. Wang, S. Peter, R. Ullrich, M. Hofrichter, J. T. Groves, *Angew. Chem. Int. Ed.* **2013**, *52*, 9238–9241; *Angew. Chem.* **2013**, *125*, 9408–9411; d) K. Piontek, E. Strittmatter, R. Ullrich, G. Gröbe, M. J. Pecyna, M. Kluge, K. Scheibner, M. Hofrichter, D. A. Plattner, *J. Biol. Chem.* **2013**, *288*, 34767–34776; e) M. Kluge, R. Ullrich, K. Scheibner, M. Hofrichter, *Green Chem.* **2012**, *14*, 440–446; f) R. Ullrich, M. Hofrichter, *FEBS Lett.* **2005**, *579*, 6247–6250; g) R. Ullrich, J. Nüske, K. Scheibner, J. Spantzel, M. Hofrichter, *Appl. Environ. Microbiol.* **2004**, *70*, 4575–4581.

[11] a) H. Yanase, K. Moriya, N. Mukai, Y. Kawata, K. Okamoto, N. Kato, *Biosci. Biotechnol. Biochem.* **2002**, *66*, 85–91; b) N. Kato, S. Mizuno, Y. Imada, C. Sakazawa, *Appl. Microbiol. Biotechnol.* **1988**, *27*, 567–571; c) N. Kato, H. Kobayashi, M. Shimao, C. Sakazawa, *Agric. Biol. Chem.* **1984**, *48*, 2017–2023; d) N. Kato, K. Shirakawa, H. Kobayashi, C. Sakazawa, *Agric. Biol. Chem.* **1983**, *47*, 39–46.

[12] A. Chang, M. Scheer, A. Grote, I. Schomburg, D. Schomburg, *Nucleic Acids Res.* **2009**, *37*, D588–D592.

[13] a) J. Sucharitakul, C. Tongsook, D. Pakotiprapha, W. J. H. van Berkel, P. Chaiyen, *J. Biol. Chem.* **2013**, *288*, 35210–35221; b) S. Montersino, W. J. H. van Berkel, *Biochim. Biophys. Acta Proteins Proteomics* **2012**, *1824*, 433–442.

[14] P. Tufvesson, J. Lima-Ramos, M. Nordblad, J. M. Woodley, *Org. Process Res. Dev.* **2011**, *15*, 266–274.

Received: August 23, 2015
Published online: November 26, 2015

