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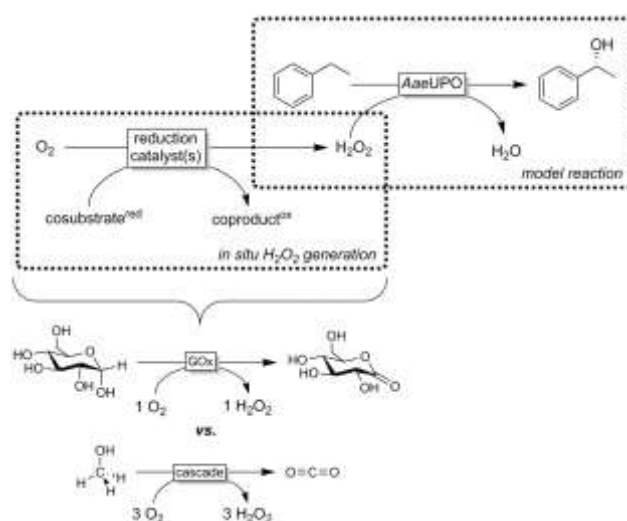
Complete oxidation of methanol to promote peroxygenase-catalysed oxyfunctionalisation reactions

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Abstract: Peroxygenases enable a broad range of (stereo-)selective oxyfunctionalisation reactions. To access their full catalytic potential, however, peroxygenases need balanced provision with hydrogen peroxide to allow for high catalytic activity while minimising oxidative inactivation. Here we report an enzymatic cascade using methanol as sacrificial electron donor for reductive activation of molecular oxygen. Full oxidation of methanol is achieved generating three equivalents of hydrogen peroxide, which can be used completely for the stereoselective hydroxylation of ethyl benzene as a model reaction. Overall we propose and demonstrate an atom-efficient and easily applicable alternative to the established hydrogen peroxide generation methods allowing for efficient use of peroxygenases for organic oxyfunctionalisation chemistry.

Selective oxyfunctionalisation 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 oxyfunctionalisation reactions.^[2] More recently, peroxygenases have emerged as alternatives to the aforementioned P450 monooxygenases.^[3] On the one hand, peroxygenases exhibit a similarly rich oxyfunctionalisation chemistry as P450 monooxygenases as both rely on a highly reactive oxoferryl species (Compound I, conferring reactivity) embedded into the well-defined steric environment of an enzyme active site (conferring selectivity).^[4] On the other hand, peroxygenases do not depend on complicated electron transport chains as P450 monooxygenases but rather form Compound I directly from H₂O₂. Obviously, this results in significantly simplified reaction schemes making peroxygenases promising catalysts for selective oxyfunctionalisation reactions. Furthermore, in the past decade the portfolio of peroxygenases available for the organic chemist has been extended significantly.^[3c, 5]

Peroxygenases, however, exhibit only poor robustness against H₂O₂, necessitating provision with suitable amounts of H₂O₂ to balance reactivity and oxidative inactivation of the enzymes (both inversely depending on the *in situ* concentration of H₂O₂). Catalytic reduction of O₂ using enzymatic,^[6] electrocatalytic^[7] or photocatalytic methods^[8] are under investigation.



Scheme 1. Schematic representation of the new *in situ* H₂O₂ generation system and the model reaction used in this study. The unspecific peroxygenase from *Agroclybe aegerita* (AaeUPO) catalyses the chemo- and stereospecific conversion of ethyl benzene to enantiopure (*R*)-1-phenylethanol (model reaction). *In situ* provision with H₂O₂ is achieved by catalytic reduction of ambient oxygen (O₂) (regeneration reaction). Shown below are: (1) the established method using glucose oxidase (GOx)-catalysed single oxidation of glucose generating one equivalent of H₂O₂ and (2) the envisioned 'through oxidation' of methanol to CO₂ generating 3 equivalent of H₂O₂.

Today, the glucose/glucose oxidase-system for *in situ* H₂O₂ generation prevails (Scheme 1). However, glucose is used very (atom-)inefficiently in this system as only one equivalent of H₂O₂ can be obtained from one equivalent of glucose. In other words, only 2 electrons of the 24 electrons (as theoretically obtainable from complete oxidation of all C-atoms present in glucose) are used. To circumvent the issues mentioned above we envisioned utilising methanol as sacrificial electron donor. Ideally, methanol would be fully mineralised to CO₂ liberating six electrons used for the reductive activation of molecular oxygen yielding three equivalents of H₂O₂ (Scheme 1).

Early contributions by Therisod and co-workers^[9] using methanol as 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 and health burden but also severely

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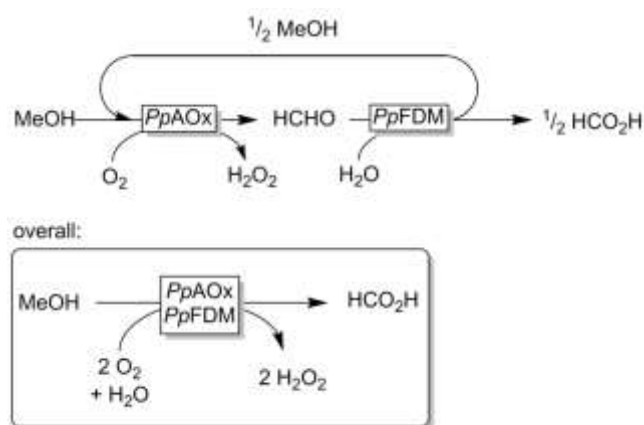
impairs biocatalyst stability and utilises the sacrificial electron donor very inefficiently. Therefore, we revisited this approach extending it with an enzymatic system for formaldehyde dismutation and oxidation of the resulting formic acid.

The model peroxygenase used in this study was *AaeUPO* (E.C. 1.11.2.1) due to its high activity towards (non-)activated sp^3 -C-H-bonds.^[3b, 5, 8b, 10] As model reaction we focussed on the enantioselective hydroxylation of ethyl benzene into (*R*)-1-phenylethanol (Scheme 1). It is worth mentioning here that the enantioselectivity of this reaction was invariably very high yielding >99% ee in all experiments reported here.

The first step of the methanol mineralisation cascade consists of the aerobic oxidation of methanol (Scheme 2, first half). From the two commercially available fungal alcohol oxidases (from *Candida boidinii* (*CbAOx*) and *Pichia pastoris* (*PpAOx*)) evaluated *PpAOx* excelled in terms of activity (i.e. turnover frequency $TF(CbAOx) = 7\text{ s}^{-1}$ vs. $TF(PpAOx) = 30\text{ s}^{-1}$, Supplementary Figure 2). Therefore, all subsequent reactions were performed using *PpAOx* as *in situ* H_2O_2 -generation catalyst.

AaeUPO, just like any other heme-enzyme, is prone to H_2O_2 -dependent oxidative inactivation of its prosthetic group necessitating *in situ* H_2O_2 levels as low as possible to maintain activity. Therefore, we systematically investigated the influence of the ratio of *AaeUPO* to *PpAOx* on the efficiency and robustness of the overall reaction (Supplementary Figure 3). A molar ratio of *AaeUPO* to *PpAOx* of one or higher was sufficient to minimise H_2O_2 -caused inactivation of the peroxygenase while maximising the hydroxylation rate.

Next we evaluated formaldehyde dismutase (FDM, E.C. 1.2.99.4) as 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 generation of two equivalents of H_2O_2 per equivalent of methanol. For our studies we used the FDM from *Pseudomonas putida* F61 (*PpFDM*) overexpressed in *E. coli*.^[11]



Scheme 2. The proposed bienzymatic cascade comprising alcohol oxidase (AOx) and formaldehyde dismutase (FDM) for the overall double oxidation of methanol to formic acid generating two equivalents of H_2O_2 .

In a first set of experiments we investigated whether the theoretical stoichiometry of two equivalents of H_2O_2 per mol of

methanol could indeed be found experimentally. Therefore, we performed the overall cascade reaction under methanol-limiting conditions (5 mM) either in the presence or absence of *PpFDM*. In the absence of *PpFDM* (Figure 2, \blacklozenge), the reaction practically stopped upon completion of methanol. In the presence of *PpFDM* (Figure 2, \blacksquare), the overall reaction proceeded smoothly to 10 mM of product corresponding to an overall double oxidation of methanol. In addition, we determined the final formate concentration in the reaction mixture to be 4.50 ± 0.03 mM further confirming our assumption of double oxidation of methanol. It should be mentioned here that *PpFDM* exhibits a rather poor affinity towards its substrate formaldehyde ($K_M=350$ mM).^[11d] To avoid undesired accumulation of formaldehyde the concentration of *PpFDM* was therefore comparably high (295 nM).

Encouraged by these results, we further scaled the reaction up to compare the performance of the *in situ* H_2O_2 generation cascade with and without *PpFDM* (Figure 1).

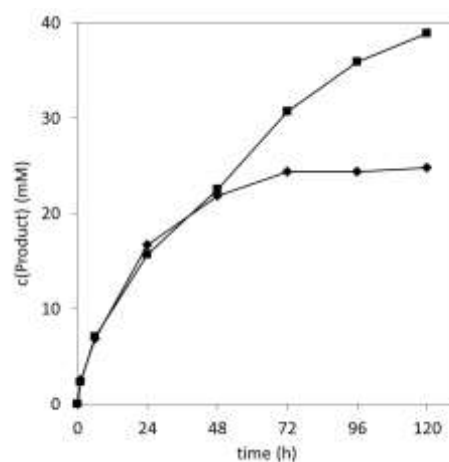


Figure 1. Enzymatic hydroxylation of ethylbenzene in the absence (\blacklozenge) and presence (\blacksquare) of *PpFDM*. Conditions: [ethyl benzene] = 50 mM, [methanol] = 200 mM, [*AaeUPO*] = 100 nM, [*PpAOx*] = 60 nM, [*PpFDM*] = 295 nM in 100 mM phosphate buffer (pH 7.0), $T=30^\circ\text{C}$. For the sake of clarity, both (*R*)-1-phenylethanol and acetophenone are shown, please see the text for further information.

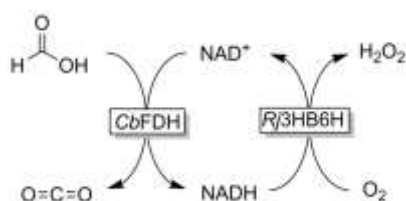
The presence of *PpFDM* had no significant influence on the initial hydroxylation rate but a very distinct influence on the robustness of the overall reaction: In the absence of *PpFDM* (Figure 1 \blacklozenge), the reaction rate decreased significantly already after 24 h and ceased completely after 72 h. However, in the presence of *PpFDM* (Figure 1 \blacksquare) almost linear product accumulation was observed for at least 96 h with continuous production for at least 120 h. Overall, the starting material (ethyl benzene) was fully converted giving the desired (*R*)-1-phenylethanol in 62% yield (31 mM) and acetophenone as the sole detectable side-product (16%, 8 mM). The apparent discrepancy in mass-balance is due to partial evaporation of the reactants as confirmed in control experiments. The presence of *PpFDM* improved the efficiency of the system (in terms of product formed) by more than 40%. Consequently also the

catalyst turnover numbers (TN(*AaeUPO* & *PpAOx*)) increased from 291500 & 485800 in the absence of *PpFDM* to more than 468500 & 780800 in the presence of *PpFDM*.

At intervals, the residual *PpAOx* activity in the reaction medium was determined revealing a good correlation with the results shown in Figure 1: In the absence of *PpFDM*, the oxidase activity dropped by ca. 50 % within the first 24 h and was practically zero after 72 h whereas in the presence of *PpFDM* the *PpAOx* activity decreased much slower (>50% of the initial activity after 72 h). We therefore assume that mainly the stability of the alcohol oxidase against formaldehyde was overall limiting the robustness of the reaction and that addition of *PpFDM* could efficiently alleviate this inactivation.

It is worth mentioning here that this reaction setup is not limited to the stereospecific hydroxylation of ethyl benzene but could be extended to a representative range of *AaeUPO*-catalysed oxyfunctionalisation reactions with good success (Supplementary Table 1).

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 CO₂) yielding the third equivalent of H₂O₂. Unfortunately, the operational windows of the reported formate oxidases do not overlap sufficiently with the enzymes used in this study.^[12] In particular the pH optimum of the known formate oxidases around pH 3-4 and their low activities at more ambient pH values makes their application in this context difficult if not impossible. Therefore, we turned our attention to the well-known formate dehydrogenase from *Candida boidinii* (*CbFDH*) oxidising formate to CO₂ while transferring the reduction equivalents liberated to NAD⁺. To aerobically re-oxidise the resulting NADH and produce H₂O₂ we used 3-hydroxybenzoate-6-hydroxylase from *Rhodococcus jostii* RHA1 (EC 1.14.13.24, *R3HB6H*), which in the presence of its natural product acts as NADH oxidase yielding H₂O₂ (Scheme 3).^[13]



Scheme 3. The final oxidation step (formic acid to CO₂) producing H₂O₂ using a combination of formate dehydrogenase (*CbFDH*) and 3-hydroxybenzoate-6-hydroxylase (*R3HB6H*).

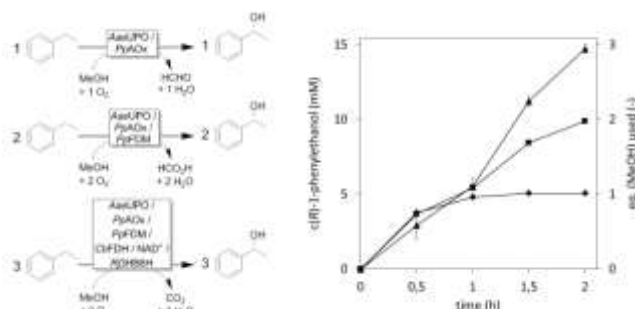


Figure 2. Comparison of the different cascades for methanol oxidation to promote the *AaeUPO*-catalysed hydroxylation of ethyl benzene. Diamonds (◆): using *PpAOx* only; Squares (■): *PpAOx* combined with *PpFDM* and Triangles (▲): the entire cascade. General conditions: [ethylbenzene] = 15 mM, [methanol] = 5 mM, [*AaeUPO*] = 50 nM, [*PpAOx*] = 60 nM in 100 mM phosphate buffer (pH 7.0), T = 30°C; ■: [*PpFDM*] = 590 nM; ▲: [*PpFDM*] = 590 nM, [NAD⁺] = 1.6 mM, [2,5-dihydroxybenzoic acid] = 1 mM, [*R3HB6H*] = 11 μM, [*CbFDH*] = 1 μM.

Figure 2 compares the product formation of the three *in situ* H₂O₂ generation systems comprising *PpAOx* alone (◆), *PpAOx* combined with *PpFDM* (■), and the entire cascade (▲) in the presence of limiting amounts of methanol (5 mM). 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 to optimally utilise it as sacrificial electron donor. Table 1 summarises the catalytic performance of the enzymes used (Figure 2, ▲).

Table 1. Summary of the catalytic performance of enzymes and cofactor used in the complete MeOH mineralisation experiment (Figure 2, ▲).

Catalyst	TN [mol mol ⁻¹] ^[a]	TF [s ⁻¹] ^[a]
<i>AaeUPO</i>	294700	41
<i>PpAOx</i>	245580	34
<i>PpFDM</i>	25400	3.5
<i>R3HB6H</i>	1330	0.18
<i>CbFDH</i>	14730	2
NAD	9	0.001

[a] TN: turnover number = moles of product divided by moles of catalyst used; TF: turnover frequency = TN divided by reaction time (here 2 h).

Pleasingly, the catalytic performance of the oxyfunctionalisation catalyst (*AaeUPO*) is superb reaching values turnover numbers required for economic production of speciality and even bulk chemicals.^[14] Similarly, the primary H₂O₂ generation catalyst (*PpAOx*) performs exceptionally well in the presence of *PpFDM*. Efficient dismutation of the primarily formed formaldehyde proved to be crucial to maintain *PpAOx* activity. Still, *PpFDM* mutants with higher affinity towards formaldehyde will be highly desirable to decrease its concentration. The final step of the methanol oxidation cascade comprising two enzymes (*CbFDH* and *R3HB6H*) and one cofactor (NAD) should be seen as a temporary solution to attain the proof-of-concept. An economically attractive (and more elegant) solution would be to substitute these catalysts by only one. Unfortunately, the formate oxidases available so far are not compatible with respect to their optimal operational window. New formate oxidase variants with increased activity at ambient pH are highly desirable. Alternatively, preliminary experiments

using photocatalytic oxidation of formic acid gave promising results (albeit necessitating external illumination of the reaction mixture). At present stage the system reported here is not ready for practical application, which is mainly due to the very low AaeUPO concentration applied. Further works focussing on scale-up using higher enzyme concentrations will also have to focus on increased substrate loadings and possibly on optimized oxygen supply.

Overall, peroxygenases exhibit an enormous potential for selective oxyfunctionalisation chemistry. To unveil this potential, efficient, robust, scalable and environmentally acceptable *in situ* provision with H₂O₂ is necessary. Here, we have demonstrated that methanol can be used for atom-efficient *in situ* generation of H₂O₂.

Acknowledgements

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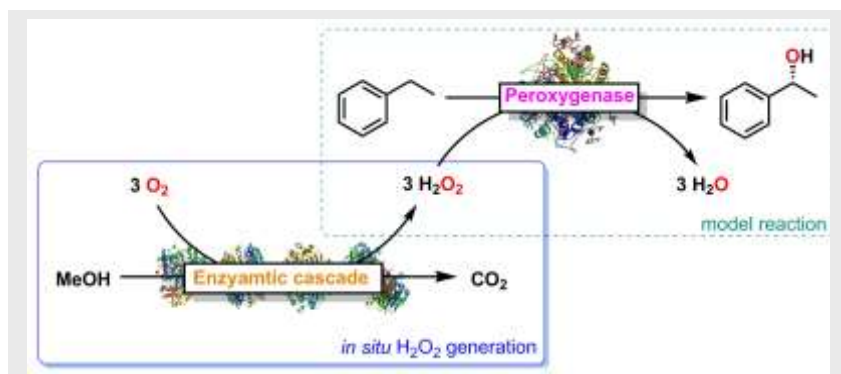
Keywords: heme proteins • peroxygenase • hydrogen peroxide • oxidation • hydroxylation

References

- [1] a) T. Newhouse, P. S. Baran, *Angew. Chem.* **2011**, *123*, 3422–3435; *Angew. Chem. Int. Edit.* **2011**, *50*, 3362–3374; 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. Comm.* **2011**, *47*, 2490–2501; c) F. E. Zilly, J. P. Acevedo, W. Augustyniak, A. Deege, U. W. Häusig, M. T. Reetz, *Angew. Chem.* **2011**, *123*, 2772–2776; *Angew. Chem. Int. Ed.* **2011**, *50*, 2720–2724; d) S. Kille, F. E. Zilly, J. P. Acevedo, M. T. Reetz, *Nat. Chem.* **2011**, *3*, 738–743; e) M. Bordeaux, A. Galarnreau, F. Fajula, J. Drone, *Angew. Chem.* **2011**, *123*, 2123–2127; *Angew. Chem. Int. Ed.*, **2011**, *50*, 2075–2079.
- [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*; 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. Lutz, 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.* **2011**, *123*, 10904–10907; *Angew. Chem. Int. Ed.* **2011**, *50*, 10716–10719; c) D. I. Perez, M. Mifsud Grau, I. W. C. E. Arends, F. Hollmann, *Chem. Comm.* **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 Asymm.* **2007**, *18*, 701–704; b) F. Pezzotti, K. Okrasa, M. Therisod, *Tetrahedron Asymm.* **2005**, *16*, 2681–2683.
- [10] a) S. Peter, A. Karich, R. Ullrich, G. Grobe, K. Scheibner, M. Hofrichter, *J. Mol. Catal. B. Enzym.* **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.* **2013**, *125*, 9408–9411; *Angew. Chem. Int. Ed.* **2013**, *52*, 9238–9241; 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. Shima, C. Sakazawa, *Agri. Biol. Chem.* **1984**, *48*, 2017–2023; d) N. Kato, K. Shirakawa, H. Kobayashi, C. Sakazawa, *Agri. 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, *BBA-Proteins Proteomics* **2012**, *1824*, 433–442.
- [14] P. Tufvesson, J. Lima-Ramos, M. Nordblad, J. M. Woodley, *Org. Proc. Res. Dev.* **2010**, *15*, 266–274.

Entry for the Table of Contents

COMMUNICATION



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Page No. – Page No.

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