▌ **Biocatalysis**

Fatty Acid Chain Shortening by a Fungal Peroxygenase

Andrés Olmedo,[a] José C. del Río,[a] Jan Kiebist,[b] René Ullrich,[c] Martin Hofrichter,[c] Katrin Scheibner,[b] Angel T. Martínez,[d] and Ana Gutiérrez*[a]
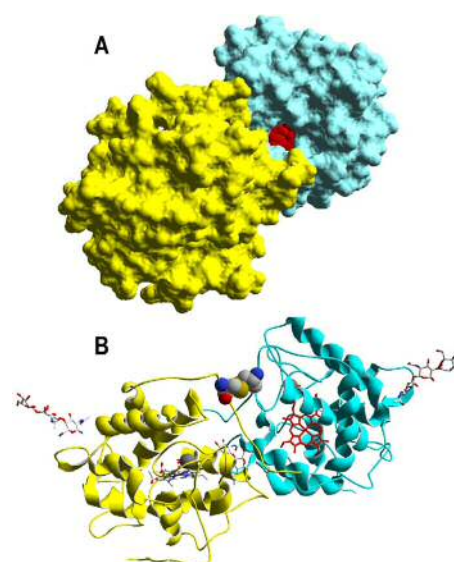
Abstract: A recently discovered peroxygenase from the fungus *Marasmius rotula* (*Mro*UPO) is able to catalyze the progressive one-carbon shortening of medium and long-chain mono- and dicarboxylic acids by itself alone, in the presence of H_2O_2. The mechanism, analyzed using $H_2^{18}O_2$, starts with an α-oxidation catalyzed by *Mro*UPO generating an α-hydroxy acid, which is further oxidized by the enzyme to a reactive α-keto intermediate whose decarboxylation yields the one-carbon shorter fatty acid. Compared with the previously characterized peroxygenase of *Agrocybe aegerita*, a wider heme access channel, enabling fatty acid positioning with the carboxylic end near the heme cofactor (as seen in one of the crystal structures available) could be at the origin of the unique ability of *Mro*UPO shortening carboxylic acid chains.

The use of biocatalysts for organic synthesis replacing traditional metal catalysts has several advantages, such as better regio- and stereoselectivity, fewer side products, and potentially lower environmental impact. Enzymes that catalyze the transfer of an oxygen atom from peroxide to substrates are classified as peroxygenases (EC.1.11.2). Unspecific peroxygenase (UPO, EC 1.11.2.1) is the most prominent member of this group because of its versatility for oxygen transfer reactions[1] that makes it highly attractive as industrial biocatalyst.[2–4]

The first UPO was described in the basidiomycetous fungus *Agrocybe aegerita* (*Aae*UPO)[5] catalyzing reactions formerly assigned only to P450 monooxygenases (P450s). However, unlike P450s that are intracellular enzymes and often require a flavin-containing auxiliary enzyme or protein domain and a source of reducing power [NAD(P)H], UPO is a secreted protein, therefore far more stable, and only requires H_2O_2 for activation.[2] *Aae*UPO was shown to catalyze oxygenation reactions on aromatic compounds,[6] and later, the action on aliphatic compounds was demonstrated,[7,8] expanding its biotechnological relevance.

Since then, similar UPO proteins have been purified from other basidiomycetes and ascomycetes such as *Coprinellus radians*,[9] *Marasmius rotula*[10] and *Chaetomium globosum*;[11] and almost 3000 related sequences (from sequenced genomes and environmental samples) are currently available in databases.[1,11] The peroxygenase from *M. rotula* (*Mro*UPO) shows several special features compared to other UPOs, for example, the inability to oxidize halides, less pronounced capacity to oxygenate aromatics,[10] and the unique ability for terminal hydroxylation of *n*-alkanes.[12]

Despite the widespread occurrence of peroxygenases and related heme–thiolate peroxidases, only two molecular structures have been reported to date, corresponding to the classic

[a] A. Olmedo, Prof. J. C. d. . Río, Prof. A. Gutiérrez
Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC
Reina Mercedes 10, 41012 Seville (Spain)
E-mail: anagu@irnase.csic.es

[b] J. Kiebist, Prof. K. Scheibner
JenaBios GmbH
Löbstedter Str. 80, 07749 Jena (Germany)

[c] R. Ullrich, Prof. M. Hofrichter
TU Dresden
Markt 23, 02763 Zittau (Germany)

[d] Prof. A. T. Martínez
Centro de Investigaciones Biológicas, CSIC
Ramiro de Maeztu 9, 28040 Madrid (Spain)

Figure 1. Dimeric *Mro*UPO. A) Solvent-access surface with one of the heme cofactors (red spheres) visible through an access channel. B) Ribbon-like model including the C227-C′227 disulfide bond (CPK-colored spheres), the two cofactors (red and CPK sticks), and surface glycosylation (CPK sticks). From 5FUJ.

ascomycete chloroperoxidase (CPO),[13] and *Aae*UPO.[14] Fortunately, the crystal structure of *Mro*UPO has just been made available (PDB entries 5FUJ and 5FUK). Interestingly, although *Mro*UPO is a dimeric protein due to an inter-monomer disulfide bond (Figure 1 B, whereas *Aae*UPO and CPO are monomers), this fact does not affect the accessibility to the heme channel and cofactor (Figure 1 A).

Here, the one-carbon shortening of carboxylic acids, a fascinating reaction catalyzed by *Mro*UPO, is revealed. This reaction was first evidenced when a dicarboxylic acid (tetradecanedioic acid) was tested as substrate of the enzyme, and the products were analyzed by gas chromatography–mass spectrometry (GC-MS) (Figures 2 A,B, S1 and S2). High (93%) substrate
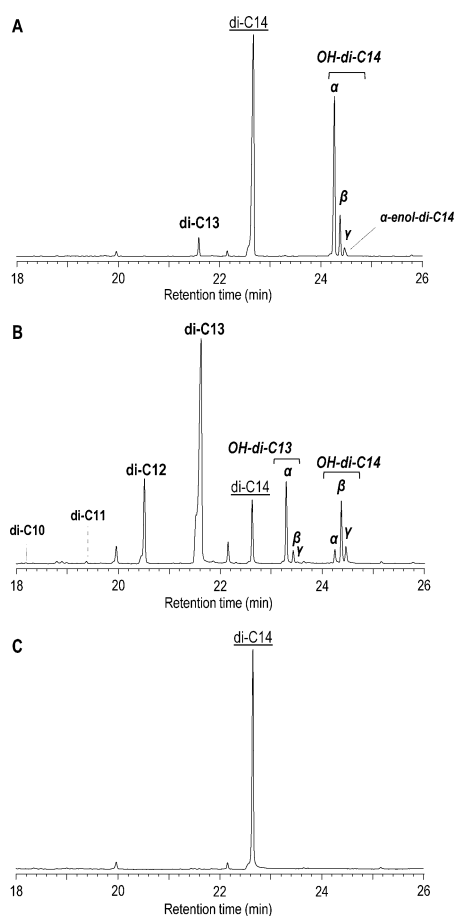
Figure 2. GC-MS of 1 h (**A**) and 24 h (**B**) reactions of tetradecanedioic acid (underlined) with *Mro*UPO, and with AaeUPO after 24 h (**C**), showing the shortened dicarboxylic acids, and the α-, β-, and γ-hydroxylated and α-enol derivatives (italics) (see mass spectra in Figures S1 and S2).

(0.1 mM) conversion by *Mro*UPO (≈2 μM) was attained in 24 h, yielding a series of chain-shortened dicarboxylic acids, such as tridecanedioic (63% of products) and dodecanedioic (13%) acids, together with α-, β- and γ-hydroxyderivatives. The reaction was also studied with *Aae*UPO (Figure 2 C), which failed to convert the substrate.

When monocarboxylic fatty acids were studied as *Mro*UPO substrates, the shortening reaction seemed to be chain-length dependent. With tetradecanoic acid, terminal and subterminal
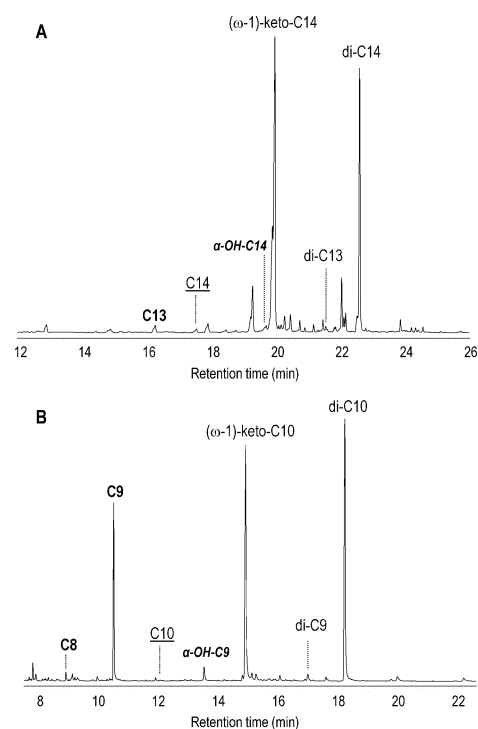
Figure 3. Comparison of tetradecanoic acid (**A**) and decanoic acid (**B**) reactions (2 h) with *Mro*UPO showing the remaining substrate (underlined), the shortened monocarboxylic acids, and the α-hydroxy, (ω-1)-keto and dicarboxylic derivatives.

oxygenations (forming the dicarboxylic and [ω-1]-keto derivatives, respectively) were predominant (Figure 3 A). However, with decanoic acid, a relevant amount of nonanoic acid was generated (Figure 3 B), although the reaction was less selective than with dicarboxylic acids.

To explore the role of α-, β- and γ-hydroxy-derivatives as intermediates in the chain-shortening mechanism, we studied the reactions of α- and β-hydroxytetradecanoic acids with *Mro*UPO. With the α-hydroxy-derivative, tridecanoic acid was one of the main products (Figure S3 A), but no chain shortening was accomplished with the β-hydroxy-derivative (Figure S3 B). This confirms that both even and odd chain fatty acids are produced by successive removal of one carbon atom after α-oxidation (although, under some forced reaction conditions, some two-carbon shortenings of β-hydroxy acid could be observed as well; Figure S4).

Table 1. Apparent kinetic constants for tetradecanedioic acid (di-C14) and decanoic acid (C10) reactions with *Mro*UPO.

|	k_{cat} [min^{-1}]	K_m [μM]	k_{cat}/K_m [min^{-1} mM^{-1}]
Chain shortening[a]			
di-C14	32±9	557±295	58±4
C10	293±98	703±549	420±350
Other oxygenations[b]			
di-C14	7±1	239±42	28±5
C10	337±92	649±430	520±370
[a] After oxygenation at α position. [b] At β, γ, ω and ω-1 positions.			
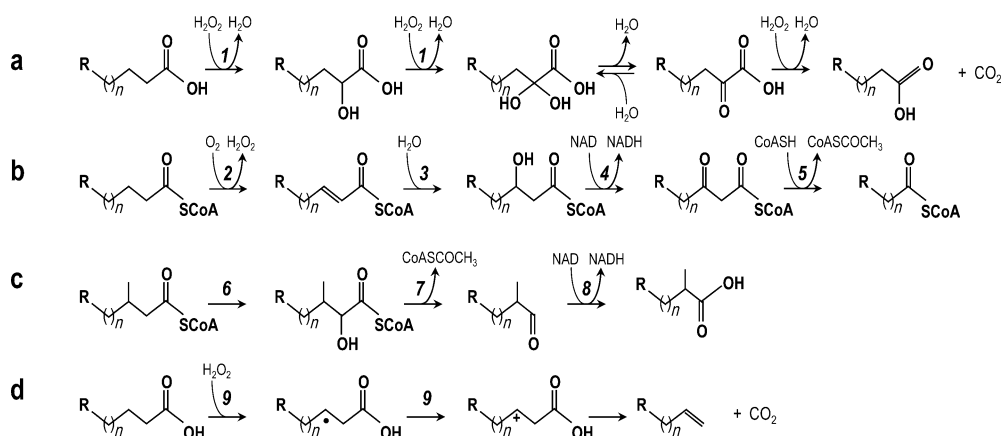
Scheme 1. Comparison of fatty-acid shortening reactions: a) α-oxidation and decarboxylation by *Mro*UPO; b) usual β-oxidation (multienzymatic) pathway; c) α-oxidation (multienzymatic) pathway for β-methylated acids; and d) decarboxylation and alkene formation by P450; (R, H or COOH; enzymes: *1*, *Mro*UPO *2*, acyl-CoA oxidase; *3*, enoyl-CoA hydratase; *4*, 3-hydroxyacyl-CoA dehydrogenase; *5*, 3-ketoacyl-CoA thiolase; *6*, phytanoyl-CoA hydroxylase; *7*, 2-hydroxy-phytanoyl-CoA lyase; *8*, aldehyde dehydrogenase; and *9*, P450 fatty-acid decarboxylase).

Despite the difficulties for GC-MS estimation of initial rates in the above *Mro*UPO reactions, apparent kinetic constants could be obtained for the products: i) resulting in chain-shortening; and ii) of other oxygenation reactions (Table 1). Concerning shortening, the *Mro*UPO had higher catalytic efficiency (k_{cat}/K_m) on C10 than on di-C14, due to the almost 10-fold higher catalytic constant (k_{cat}), although it was less selective as shown by the ratios (0.8 and 2.0, respectively) between the catalytic efficiencies of shortening and other reactions. The chain-shortening of both mono- and dicarboxylic acids, and the α-hydroxylation of carboxylic acids by a peroxygenase are reported here for the first time.

To get additional insight into the chain shortening mechanism, ^{18}O-labeling studies with $H_2^{18}O_2$ (90% isotopic purity) were performed using tetradecanedioic acid as target substrate (Figures S1 and S2). Overall, our data led to the chain-shortening mechanism depicted in Scheme 1a. The initial product of *Mro*UPO reaction will be the α-hydroxy acid, as demonstrated by incorporation of $H_2^{18}O_2$ oxygen to form α-hydroxytetradecanedioic acid, whose diagnostic fragment (m/z 373, Figure S1A, top) appeared fully (90%) ^{18}O-labeled (m/z 375, Figure S1A, bottom). Its oxidation will yield a *gem*-diol (ketone hydrate) from a second C_α hydroxylation by *Mro*UPO that will be in equilibrium with the ketone by dehydration, and then will react with excess H_2O_2 decarboxylating and forming a new carboxyl group (chain shortening) as explained below.

Direct evidence for involvement of $H_2^{18}O_2$-borne oxygen into the *gem*-diol/ketone formation yielding α-ketotetradecanedioic could not be obtained, since α-keto acids rapidly decarboxylate in the presence of oxidizing agents including H_2O_2.[15] However, evidence of their transient formation was obtained, as the enolic form was detected (Figures 2A, S3A and S5A). ^{18}O-labeling also illuminated the formation of tridecanedioic acid after incorporation of two or one ^{18}O-atoms (Figure S1B, bottom). The co-existence of single and double ^{18}O-labeling in the carboxylic group suggests that a *gem*-diol, with some hy-

droxyl exchange with the water (labelling loss), may occur prior to decarboxylation and chain shortening. This second α-hydroxylation is clearly provoked by the enzyme and not by the H_2O_2, as revealed by the negative control with α-hydroxy-tetradecanoic acid and H_2O_2 in the absence of enzyme (Figure S6). However, the final reaction step can directly be produced by the H_2O_2 present in the UPO reaction set-up, mediated by a hydroperoxide intermediate, as reported for other α-ketoacids.[16]
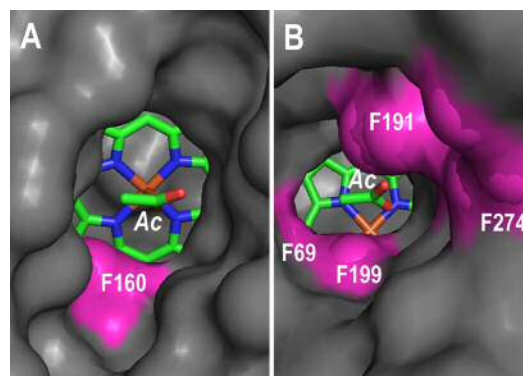
Figure 4. Different sizes of the heme-access channel in *Mro*UPO (**A**) and *Aae*UPO (**B**), and bulky phenylalanine residues (magenta) (an acetate occupies the substrate-binding site). From 5FUJ (**A**) and 2YP1 (**B**).

The different reactivity of *Mro*UPO and *Aae*UPO regarding α-hydroxylation could be explained by the only recently available crystal structure of *Mro*UPO (PDB entries 5FUJ and 5FUK) compared with the previously reported *Aae*UPO structure.[14] *Mro*UPO is a smaller protein but it has a wider heme-access channel (Figure 4A) than *Aae*UPO (Figure 4B), the channel of which is flanked by several bulky phenylalanine residues (a narrow access channel also exists in CPO). This wider heme channel directly exposes the reactive Fe=O of H_2O_2-activated
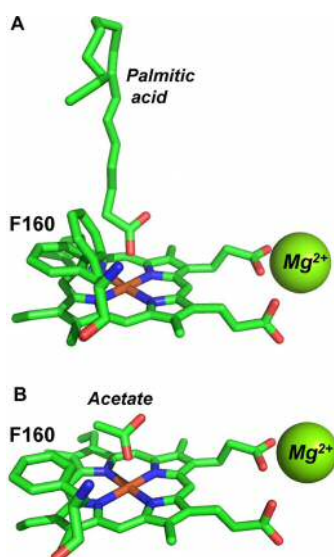
Figure 5. Palmitic acid (**A**) and acetate (**B**) ligands in *Mro*UPO crystal structures. Ligands, heme cofactor and neighbor Phe160 are shown as sticks (CPK colors) while Mg^{2+} cations are shown as spheres (when palmitic acid is present in the crystal, the Phe160 side-chain adopts two different conformations). From PDB entries 5FUK (A) and 5FUJ (B).

*Mro*UPO (compound I) to the entering substrate, enabling oxygenation at the α-position of carboxylic acids.

Interestingly, one of the *Mro*UPO crystal structures available (5FUK) includes a bound palmitic acid molecule along the heme access channel with one of the carboxylate oxygens at coordination distance of the heme iron (Figure 5 A) (while an acetate occupies this position in 5FUJ, Figure 5 B). Sub-terminal oxygenation by most UPOs implies fatty-acid binding with the carboxyl located at the channel entrance. However, the palmitic acid position found in the 5FUK crystal is in agreement with the unique chain-shortening ability reported here for *Mro*UPO.

In summary, we show the ability of *Mro*UPO to catalyze the stepwise chain shortening of carboxylic acids through a chemo-enzymatic reaction cascade (Scheme 1 a). In plants, fungi and animals, the general β-oxidation pathway, leads to two-C shorter acids (Scheme 1 b)[17] and the alternative α-oxidation pathway (Scheme 1 c), leading to one-C shorter fatty acids, typically includes several steps (hydroxylation, activation, cleavage of the C1–C2 bond and aldehyde dehydrogenation) with several enzymes involved.[18] However, *Mro*UPO is capable of catalyzing all these reactions self-sufficiently (i.e. alone), in the presence of H_2O_2. Bacterial P450s are also known to decarboxylate fatty acids, but in this case $n-1$ terminal alkenes (Scheme 1 d), instead of chain-shortened fatty acids, are formed.[19]

This carbon-by-carbon chain-shortening reaction represents a novel chemistry that may be used in biotechnological applications including the obtainment of tailor-made acids such as odd-numbered dicarboxylic or monocarboxylic fatty acids (less abundant in nature than the even-numbered ones). The "odd-even" effect on the aqueous solubility of dicarboxylic acids[20] could be used for product isolation, and in the synthesis of ad hoc polymers.[21]

The chain-shortening reaction described here must be added to the repertoire of reactions that versatile fungal peroxygenases catalyze on linear[12, 22] and cyclic aliphatic compounds,[23, 24] in addition to aromatic compounds.[1, 2] The availability of a heterologous expression system for *Mro*UPO will permit to improve the catalytic properties of this promising enzyme, for example, in chain-shortening and/or alkane terminal hydroxylation reactions,[12] using the protein engineering tools recently applied to *Aae*UPO.[25]

Conflict of interest

The authors declare no conflict of interest.

Keywords: carboxylic acids · chain shortening · enzymes · hydrogen peroxide · oxidation

[1] M. Hofrichter, H. Kellner, M. J. Pecyna, R. Ullrich, *Adv. Exp. Med. Biol.* **2015**, *851*, 341–368.

[2] M. Hofrichter, R. Ullrich, *Curr. Opin. Chem. Biol.* **2014**, *19*, 116–125.

[3] S. Bormann, A. G. Baraibar, Y. Ni, D. Holtmann, F. Hollmann, *Catal. Sci. Technol.* **2015**, *5*, 2038–2052.

[4] A. T. Martínez, F. J. Ruiz-Dueñas, S. Camarero, A. Serrano, D. Linde, H. Lund, J. Vind, M. Tovborg, O. M. Herold-Majumdar, M. Hofrichter, et al., *Biotechnol. Adv.* **2017**, *35*, 815–831.

[5] R. Ullrich, J. Nuske, K. Scheibner, J. Spantzel, M. Hofrichter, *Appl. Environ. Microbiol.* **2004**, *70*, 4575–4581.

[6] M. Hofrichter, R. Ullrich, M. J. Pecyna, C. Liers, T. Lundell, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 871–897.

[7] A. Gutiérrez, E. D. Babot, R. Ullrich, M. Hofrichter, A. T. Martínez, J. C. del Río, *Arch. Biochem. Biophys.* **2011**, *514*, 33–43.

[8] S. Peter, M. Kinne, X. Wang, R. Ulrich, G. Kayser, J. T. Groves, M. Hofrichter, *FEBS J.* **2011**, *278*, 3667–3675.

[9] D. H. Anh, R. Ullrich, D. Benndorf, A. Svatos, A. Muck, M. Hofrichter, *Appl. Environ. Microbiol.* **2007**, *73*, 5477–5485.

[10] G. Gröbe, M. Ullrich, M. Pecyna, D. Kapturska, S. Friedrich, M. Hofrichter, K. Scheibner, *AMB Express* **2011**, *1*, 31–42.

[11] J. Kiebist, K. U. Schmidtke, J. Zimmermann, H. Kellner, N. Jehmlich, R. Ullrich, D. Zänder, M. Hofrichter, K. Scheibner, *ChemBioChem* **2017**, *18*, 563–569.

[12] A. Olmedo, C. Aranda, J. C. del Río, J. Kiebist, K. Scheibner, A. T. Martínez, A. Gutiérrez, *Angew. Chem. Int. Ed.* **2016**, *55*, 12248–12251; *Angew. Chem.* **2016**, *128*, 12436–12439.

[13] M. Sundaramoorthy, J. Terner, T. L. Poulos, *Structure* **1995**, *3*, 1367–1377.

[14] 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.

[15] A. J. L. Cooper, J. Z. Ginos, A. Meister, *Chem. Rev.* **1983**, *83*, 321–358.

[16] A. A. Vlessis, D. Bartos, D. Trunkey, *Biochem. Biophys. Res. Commun.* **1990**, *170*, 1281–1287.

[17] H. Schulz in *Biochemistry of Lipids, Lipoproteins and Membranes, Vol. 5*, (Eds.: D. E. Vance, J. Vance), Elsevier, Amsterdam **2008**, 131–154.

[18] G. A. Jansen, R. J. A. Wanders, *Biochim. Biophys. Acta Mol. Cell Res.* **2006**, *1763*, 1403–1412.

[19] A. Dennig, M. Kuhn, S. Tassoti, A. Thiessenhusen, S. Gilch, T. Bülter, T. Haas, M. Hall, K. Faber, *Angew. Chem. Int. Ed.* **2015**, *54*, 8819–8822; *Angew. Chem.* **2015**, *127*, 8943–8946.

[20] H. Zhang, C. Xie, Z. Liu, J. Gong, Y. Bao, M. Zhang, H. Hao, B. Hou, Q. Yin, *Ind. Eng. Chem. Res.* **2013**, *52*, 18458–18465.

[21] S. Huf, S. Krugener, T. Hirth, S. Rupp, S. Zibek, *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 548–561.

[22] E. D. Babot, J. C. del Río, L. Kalum, A. T. Martínez, A. Gutiérrez, *Biotechnol. Bioeng.* **2013**, *110*, 2323.

[23] E. D. Babot, J. C. del Río, M. Cañellas, F. Sancho, F. Lucas, V. Guallar, L. Kalum, H. Lund, G. Gröbe, K. Scheibner, R. Ullrich, M. Hofrichter, A. T. Martínez, A. Gutiérrez, *Appl. Environ. Microbiol.* **2015**, *81*, 4130–4142.

[24] F. Lucas, E. D. Babot, J. C. del Río, L. Kalum, R. Ullrich, M. Hofrichter, V. Guallar, A. T. Martínez, A. Gutiérrez, *Catal. Sci. Technol.* **2016**, *6*, 288–295.

[25] P. Molina-Espeja, M. Canellas, F. J. Plou, M. Hofrichter, F. Lucas, V. Guallar, M. Alcalde, *ChemBioChem* **2016**, *17*, 341–349.