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Research review paper

Oxidoreductases on their way to industrial biotransformations

Angel T. Martínez[a,*], Francisco J. Ruiz-Dueñas[a], Susana Camarero[a], Ana Serrano[a], Dolores Linde[a], Henrik Lund[b,1], Jesper Vind[b], Morten Tovborg[b], Owik M. Herold-Majumdar[b], Martin Hofrichter[c], Christiane Liers[c], René Ullrich[c], Katrin Scheibner[d], Giovanni Sannia[e], Alessandra Piscitelli[e], Cinzia Pezzella[e], Mehmet E. Sener[f], Sibel Kılıç[f], Willem J.H. van Berkel[g], Victor Guallar[h,i], Maria Fátima Lucas[h,i], Ralf Zuhse[j], Roland Ludwig[k], Frank Hollmann[l], Elena Fernández-Fueyo[l], Eric Record[m], Craig B. Faulds[m], Marta Tortajada[n], Ib Winckelmann[o], Jo-Anne Rasmussen[p], Mirjana Gelo-Pujic[q], Ana Gutiérrez[r], José C. del Río[r], Jorge Rencoret[r], Miguel Alcalde[s]

[a] Centro de Investigaciones Biológicas, CSIC, Madrid, Spain
[b] Novozymes A/S, Bagsvaerd, Denmark
[c] Technische Universität Dresden, Zittau, Germany
[d] JenaBios GmBH, Jena, Germany
[e] Università degli Studi di Napoli Federico II, Naples, Italy
[f] Setas Kimya Sanayi AS, Tekirdag, Turkey
[g] Wageningen University & Research, The Netherlands
[h] Anaxomics, Barcelona, Spain
[i] Barcelona Supercomputing Center (BSC), Barcelona, Spain
[j] Chiracon GmBH, Luckenwalde, Germany
[k] University of Natural Resources and Life Sciences (BOKU), Vienna, Austria
[l] Department of Biotechnology, Delft University of Technology, Delft, The Netherlands
[m] Aix Marseille University, INRA, UMR 1163 Biodiversité et Biotechnologie Fongiques (BBF), Marseille, France
[n] Biopolis, Valencia, Spain
[o] Cheminova A/S, Lemvig, Denmark
[p] CLEA Technologies BV, Delft, The Netherlands
[q] Solvay, Brussels, Belgium
[r] Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, Seville, Spain
[s] Instituto de Catálisis y Petroleoquímica, CSIC, Madrid, Spain

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ABSTRACT

Fungi produce heme-containing peroxidases and peroxygenases, flavin-containing oxidases and dehydrogenases, and different copper-containing oxidoreductases involved in the biodegradation of lignin and other recalcitrant compounds. Heme peroxidases comprise the classical ligninolytic peroxidases and the new dye-decolorizing peroxidases, while heme peroxygenases belong to a still largely unexplored superfamily of heme-thiolate proteins. Nevertheless, basidiomycete unspecific peroxygenases have the highest biotechnological interest due to their ability to catalyze a variety of regio- and stereo-selective monooxygenation reactions with H_2O_2 as the source of oxygen and final electron acceptor. Flavo-oxidases are involved in both lignin and cellulose decay generating H_2O_2 that activates peroxidases and generates hydroxyl radical. The group of copper oxidoreductases also includes other H_2O_2 generating enzymes - copper-radical oxidases - together with classical laccases that are the oxidoreductases with the largest number of reported applications to date. However, the recently described lytic polysaccharide monooxygenases have attracted the highest attention among copper oxidoreductases, since they are capable of oxidatively breaking down crystalline cellulose, the disintegration of which is still a major

Abbreviations: AAD, aryl-alcohol dehydrogenase; AAO, aryl-alcohol oxidase; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); CDH, cellobiose dehydrogenase; CPK, Corey/Pauling/Koltun (atom coloring convention); CRO, copper-radical oxidase; DFF, 2,5-diformylfuran; DyP, dye-decolorizing peroxidase; FDCA, 2,5-furandicarboxylic acid; FFCA, 2,5-formylfurancarboxylic acid; GDH, glucose dehydrogenase; GMC, glucose-methanol-choline oxidase/dehydrogenase; GOX, glucose oxidase; HMF, 5-hydroxymethylfurfural; HSQC, heteronuclear single quantum correlation (NMR experiment); HTP, heme-thiolate peroxidase; LiP, lignin peroxidase; LPMO, lytic polysaccharide monooxygenase; LRET, long-range electron transfer; MCO, multicopper oxidase; MnP, manganese peroxidase; MOX, methanol oxidase; NMR, nuclear magnetic resonance; P2O, pyranose 2-oxidase; PELE, protein energy landscape exploration (software); QM/MM, mixed quantum mechanics/molecular mechanics; UPO, unspecific peroxygenase; VAO, vanillyl-alcohol oxidase; VP, versatile peroxidase

* Corresponding author.
E-mail address: ATMartinez@cib.csic.es (A.T. Martínez).
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bottleneck in lignocellulose biorefineries, along with lignin degradation. Interestingly, some flavin-containing dehydrogenases also play a key role in cellulose breakdown by directly/indirectly "fueling" electrons for polysaccharide monooxygenase activation. Many of the above oxidoreductases have been engineered, combining rational and computational design with directed evolution, to attain the selectivity, catalytic efficiency and stability properties required for their industrial utilization. Indeed, using *ad hoc* software and current computational capabilities, it is now possible to predict substrate access to the active site in biophysical simulations, and electron transfer efficiency in biochemical simulations, reducing in orders of magnitude the time of experimental work in oxidoreductase screening and engineering. What has been set out above is illustrated by a series of remarkable oxyfunctionalization and oxidation reactions developed in the frame of an intersectorial and multidisciplinary European RTD project. The optimized reactions include enzymatic synthesis of 1-naphthol, 25-hydroxyvitamin D_3, drug metabolites, furandicarboxylic acid, indigo and other dyes, and conductive polyaniline, terminal oxygenation of alkanes, biomass delignification and lignin oxidation, among others. These successful case stories demonstrate the unexploited potential of oxidoreductases in medium and large-scale biotransformations.

1. Fungal oxidoreductases

Oxidoreductases take advantage from the incorporation of different cofactors - such as heme, flavin and metal ions - to catalyze redox reactions. In these reactions, they use a variety of electron acceptors and a large number of electron-donating substrates yielding many products of industrial interest (Gygli and van Berkel, 2015). Fungi, in first place wood-rot basidiomycetes, are involved in the oxidative degradation of lignocellulosic biomass, recycling the carbon fixed by plant photosynthesis through a battery of secreted and robust high redox-potential oxidoreductases (Martínez et al., 2017). Fungal oxidoreductases of biotechnological interest typically include: i) heme-containing peroxidases and peroxygenases, being activated by H_2O_2 as sole electron acceptor; ii) flavin-containing oxidases and dehydrogenases, being activated by O_2 and other oxidants - such as Fe^{3+} and quinones - respectively; and iii) copper-containing oxidases and monooxygenases, being activated by O_2, the latter with a more complicated activation mechanism.

Classical fungal oxidoreductases comprise basidiomycete ligninolytic peroxidases, and ascomycete and basidiomycete multicopper oxidases (MCO, mainly laccases) with different redox potentials and abilities to act on lignin-derived products. Moreover, new heme- and copper-containing oxidoreductases of high biotechnological interest have been recently discovered including: i) unspecific peroxygenases (UPOs) catalyzing a variety of regio- and stereo-selective oxyfunctionalizations with H_2O_2 acting as the oxygen source (peroxygenation reaction) and terminal electron acceptor; ii) other still unexplored peroxidases, such as the so-called dye-decolorizing peroxidases (DyPs); and iii) copper-containing lytic polysaccharide monooxygenases (LPMOs), which turned out to be the "missing" enzymes in the microbial attack of crystalline cellulose and other recalcitrant polysaccharides.

Enzymes of the glucose-methanol-choline oxidase/dehydrogenase (GMC) and copper-radical oxidase (CRO) superfamilies have been typically investigated as the source of H_2O_2 for: i) ligninolytic peroxidases in white-rot (*i.e.* lignin-degrading) basidiomycetes; or ii) hydroxyl radical generated *via* Fenton chemistry in brown-rot (*i.e.* cellulose-degrading) basidiomycetes. However, the preferential or optional use of other electron acceptors by some of them (dehydrogenase activity) has suggested additional functions, *e.g.* preventing lignin re-polymerization or "fueling" electrons to LPMOs. These and other fungal flavin-oxidases are also of emerging industrial relevance.

2. Oxidoreductases as industrial biocatalysts

The above oxidoreductases are biocatalysts of interest for establishing a bio-based economy (Fig. 1) with the highest potential in the production of polymer building blocks, sustainable chemicals and materials from plant biomass within lignocellulose biorefineries. However, the chemical industry, specially bulk chemicals' production, has not yet been embracing enzymatic oxidation reactions to a large extent. This is primarily due to lack of biocatalysts with the required selectivity, commercial availability and compatibility with the rigorous process conditions in terms of high substrate concentrations, use of solvents, and strongly oxidative conditions. Nowadays, oxidoreductases are most often employed in specific segments of the chemical industry and often in the form of whole-cell catalysts (*e.g.* P450 monooxygenases for selective hydroxylations) and not as isolated protein biocatalysts in medium and large scale biotransformations.

The main bottlenecks for implementing oxidative enzymatic biotransformations mentioned above have been addressed through protein engineering and process optimization using state-of-the-art technologies. The work performed comprised: i) recovery of selective oxidative biocatalysts, from the groups of heme-peroxidases and peroxygenases, flavo-oxidases and copper-oxidoreductases, from fungal genomes and other sources; ii) tailoring the catalytic and operational properties of the enzymes to fulfill the industry needs, by enzyme engineering based on structural-functional information, directed evolution or a combination of both, aided by computational simulations to reduce the experimental work; and iii) optimizing the process conditions including enzyme cascade reactions.

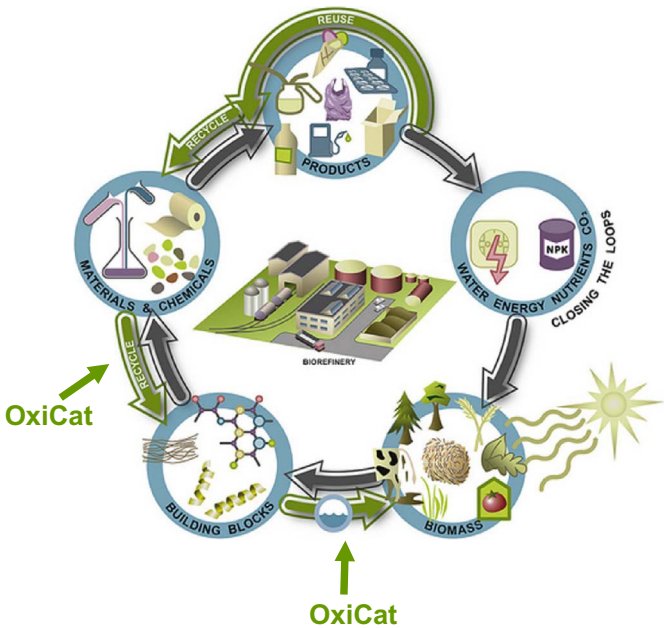

Fig. 1. Oxidative biocatalysts for a bio-based economy. Production of renewable building blocks and manufacture of sustainable chemicals and materials are the steps where oxidative biocatalysts (OxiCats) can exert the most positive impact for greener and more efficient biotransformation routes in a bio-based (and circular) economy.
Adapted from http://biconsortium.eu/news/bioeconomy-circular-nature.