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Unspecific peroxygenases: The pot of gold at the end of the oxyfunctionalization rainbow?

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Abstract

Fungal unspecific peroxygenases (UPOs) are among the most promising biocatalysts in synthetic chemistry. The kaleidoscope of oxyfunctionalization reactions that are of industrial and environmental relevance offer UPOs the opportunity to provide solutions to many processes. However, there are important shortcomings that must first be overcome in order to convert UPOs into applied biocatalysts, which include their general poor levels of heterologous functional expression, the presence of unwanted peroxidase activity and overoxidation reactions, as well as the oxidative inactivation provoked by the co-substrate, hydrogen peroxide. The specific engineering of these proteins, and that of the reactions in which they participate, is being used to tackle these principal challenges, with a view to generate a portfolio of highly active, selective and stable variants. In this review, we will discuss some of the most recent developments that will convert UPOs into true industrial biocatalysts, focusing on the different trends currently being followed in this fascinating field of research.

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Fungal unspecific peroxygenases, Oxyfunctionalization, Protein engineering, Reaction engineering, Synthetic chemistry.

Abbreviations

UPOs, Unspecific peroxygenases; P450s, Cytochrome P450 monooxygenase; AaeUPO, UPO from Agrocybe aegerita; PabUPO, UPO from Psatyrella aberdarensis; GmaUPO, UPO from Galerina marginata; HspUPO, UPO from Hypoxylon sp. EC38; TanUPO, UPO from Truncatella angustata; rAaeUPO, Evolved UPO variant (PaDa-I mutant) from Agrocybe aegerita; MthUPO, UPO from Myceliophthora termophila; TteUPO, UPO from Thielavia terrestris; MroUPO, UPO from Marasmius rotula; CviUPO, UPO from Collariella virescens; SDR, Site directed recombination in vivo; LRET, Long-range electron transfer pathway; FDCA, Furan-2,5-dicarboxylic acid; GFP, Green fluorescent protein; NNBT, N, N-bis(2-hydroxypropyl)-p-toluidine; TTN, Total turnover number; Thermoelectric bismuth telluride, Bi2Te3; OXO, Oxalate oxidase; CLEA, Crossed linked enzyme aggregate; APIs, Active pharmaceutical ingredients.

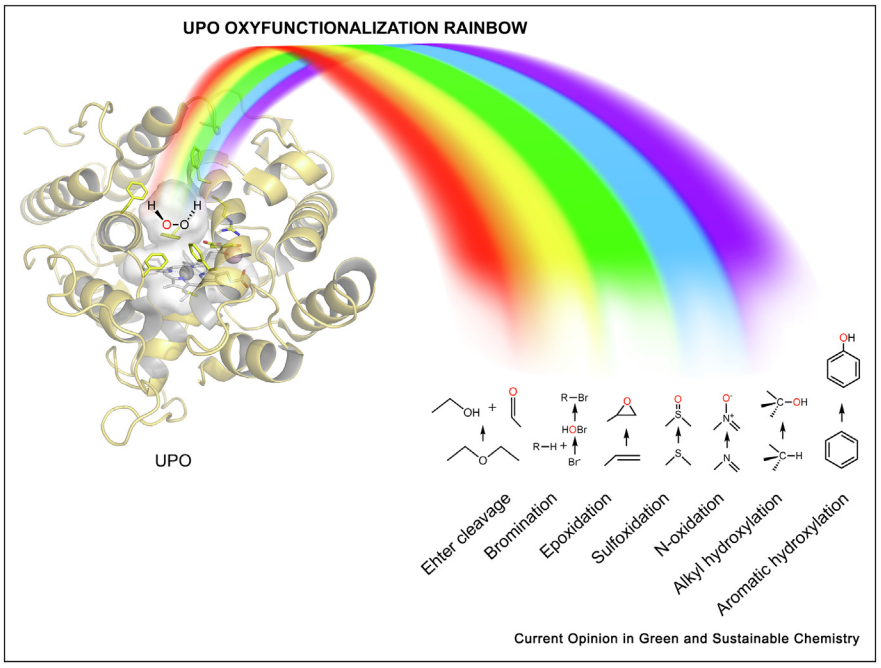
Introduction

Selective oxyfunctionalization of non-activated C—H, C—C and C═C bonds is a much sought-after chemical transformation with huge implications in the synthesis of a multitude of products, ranging from fine chemicals and pharmaceuticals to commodities. However, introducing functionalities into the inert skeletons of organic molecules is quite a challenge in contemporary chemistry. These reactions have yet to be widely incorporated into synthetic routes due to the difficulties in achieving a balance between selectivity and reactivity. Biocatalysis has proven to be a potent tool to surmount these obstacles, since enzymes possess remarkable substrate specificity, chemo-, enantio-, and regioselectivity, and they can provide tens of thousands of turnovers working in mild conditions [1—4].

Peroxygenases are particularly promiscuous and versatile biocatalysts that are starting to change the rules of engagement in relation to oxidation reactions of industrial, pharmaceutical and environmental interest [5]. These enzymes can be found in various organisms, yet the biocatalyst making peroxygenase headlines is referred to as unspecific peroxygenase (UPO, EC 1.11.2.1), found exclusively in the fungi kingdom [6]. Extracellular, stable and with high activity, UPOs are heme-thiolate-containing enzymes that catalyze a range of C—H oxyfunctionalization reactions, triggered simply by H_2O_2 as the main oxygen donor and the final electron acceptor, Figure 1. Even though cytochrome P450 monooxygenases (P450s) perform the same oxyfunctionalization reactions as UPOs, they are much more complex systems. Indeed, P450s require auxiliary flavoproteins, expensive nicotinamide cofactors, suffer from oxygen uncoupling and are not that scalable in industry. By contrast, UPOs are much more simple and robust enzymes, which may lead them to rule the new wave of oxyfunctionalization chemistry [7,8].

Despite the undeniable industrial potential of UPOs, their general poor heterologous expression -a requisite for protein engineering- and the desire to have UPO variants with marked enantio- and regioselectivity

Figure 1



Drifting through the rainbow of oxyfunctionalization reactions driven by UPO. As a highly versatile and promiscuous biocatalysts, UPOs perform a variety of C−H oxyfunctionalizations. Such portfolio of reactions will be widened in the near future by combining directed evolution and computational design.

towards specific targets represent important bottlenecks to their incorporation into industrial processes. In addition, overoxidation of the products released may occur to some extent, decreasing the efficiency of these enzymes and their yields. Furthermore, the combination of peroxygenase activity (a two-electron oxidation reaction responsible for oxygen insertion) with unwanted peroxidase activity (one-electron oxidation) is problematic, especially when considering the hydroxylation of aromatic compounds: The phenolic products of peroxygenase activity are substrates for the peroxidase activity of UPOs, being transformed into phenoxyl radicals and quinones that polymerize, reducing the final yields. Finally, inactivation by the H_2O_2 co-substrate is an issue that has to be overcome to ensure UPO's long-term operational stability in large-scale applications.

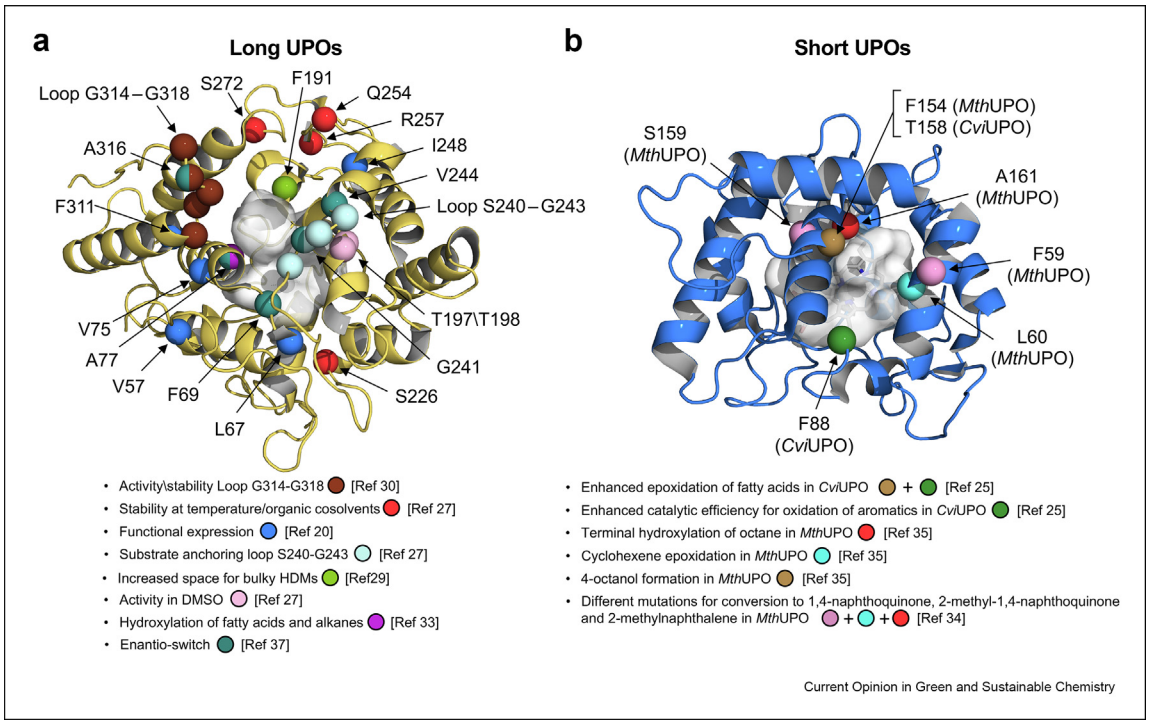
Given their unique capacities, UPOs are among the most important biocatalysts in modern biotechnology, as reflected in recent publications dealing with their engineering [8,9], oxyfunctionalization reactions [10—12], and gene discovery [6,13]. In this review, we shall address the advances over the last two years in protein and reaction engineering relative to these enzymes.

Discovering UPOs

Since the first UPO was discovered in the edible mushroom *Agrocybe (Cyclocybe) aegerita* (*Aae*UPO) in 2004, 22 more UPOs have been isolated, purified and characterized biochemically (12 from ascomycetous and 10 from basidiomycetous), with six UPO crystal structures now currently available [8]. Based on phylogeny, UPOs can be classified into two families with different structural features and substrate profiles: Family I of short UPOs and Family II of long UPOs [6], Figure 2. The strategies used to find new long and short UPOs have evolved rapidly, from searching for candidates in their natural environments to genome mining approaches, including the creation of a vast online database of UPOs (UPObase) with over 1900 putative UPO protein sequences [14]. As such, the identification of new UPOs currently follows three basic steps [13]: (i) genome mining for new candidates; (ii) functional expression of newly identified UPOs, either as recombinants in yeast (*Saccharomyces cerevisiae* or *Komagataella phaffii*/*Pichia pastoris*), filamentous fungi (*Aspergillus niger* and *Aspergillus oryzae*), bacteria (*Escherichia coli*), or in their original host (homologously); and (iii) confirmation of their peroxygenase nature through activity base screening to assign the characteristic UPOs' activity profile.

By following this scheme, and after testing 10 putative short UPO genes selected by comparative alignment and homology modeling, a new set of 3 UPOs was recently reported: one short UPO from *A. niger* (*An*UPO) and two long UPOs from *Psatyrella -Candolleomyces-*

Figure 2



Summary of protein engineering studies carried out during the last two of years for long and short UPOs. Long UPOs have an average size of 44 kDa, differing from short UPOs family, whose size is around 32 kDa (Clade I.1) and 29 kDa (Clade I.2), and have no signal peptide in their majority [6]. Long UPOs are monomeric proteins, have internal disulphide bridges and arginine as charge stabilizer. Short UPOs are mostly dimeric (with the exception of *Hypoxylon sp* (*Hsp*UPO) [17]), they have external disulphide bridges and histidine as charge stabilizer. Although both families showed similar conserved structural motifs, they substantially differ in the heme channel organization (both in topology and dimension), which translates to a different substrate scope, *i.e.* short UPOs accept bulky substrates while long UPOs have enhanced activity towards rather smaller compounds. (**a**) Crystal structure of PaDa-I mutant (PDB ID: 5OXU) highlighting the mutated positions in different protein engineering campaigns for long UPOs (*Agrocybe aegerita -Aae*UPO- and *Psatyrella aberdarensis -Pab*UPO-). Numbering according to *Aae*UPO. Directed evolution campaigns in *Aae*UPO before year 2018 are summarized in Ref. [8]. (**b**) Crystal structure of short UPO from *Hypoxylon* sp. EC38 (*Hsp*UPO, PDB ID: 7O2D) to illustrate the mutagenesis studies carried out in *Marasmius rotula* UPO (*Mro*UPO), *Collariella virescens* UPO (*Cvi*UPO) and *Myceliophthora thermophila* UPO (*Mth*UPO). Numbering according to *Hsp*UPO. The heme access channel is shown as white surface mode.

aberdarensis (*Pab*UPO), which were functionally expressed in *P. pastoris* at yields ranging from 0.14 to 0.3 g/L [15]. A long UPO from *Galerina marginata* (*Gma*UPO) was also identified by genome mining, expressed in *P. pastoris* at high titers, and characterized in epoxidation, sulfoxidation and hydroxylation reactions [16]. Similarly, an active *Hypoxylon* sp. EC38 UPO (*Hsp*UPO) was produced recombinantly in *P. pastoris* (0.2 g/L) [17], and it was characterized at the biochemical and structural levels. *Hsp*UPO is the first short UPO reported that is monomeric, as confirmed by thorough soaking crystallography studies, and it is very stable in organic solvents and in the presence of H_2O_2. A new long UPO from the ascomycetus *Truncatella angustata* (*Tan*UPO) was produced homologously in the original host and it was benchmarked with other well-known UPOs for the synthesis of lipid mediators from arachidonic acid. Remarkably, *Tan*UPO was able to epoxidize ω-6 fatty acids with high selectivity, transforming over 90% of arachidonic acid into a single metabolite [18]. In a very recent study, several putative UPO genes from both long and short families were selected through careful analysis using the 3DM database to identify motifs and conserved positions in protein subsets [19]. The most promising candidates were cloned in *P. pastoris* and preliminary screened for peroxygenase and peroxidase activity. From this pool, *P. aberdarensis*, *Coprinopsis marcescibilis*, *A. novoparasiticus*, *Dendrothele bispora* and *A. brasiliensis* UPOs are highlighting in terms of activity and substrate profile, complementing the repertoire of recombinant peroxygenases reported to date.

Engineering UPOs: heterologous expression and beyond

Establishing robust heterologous functional expression platforms through which directed evolution can be performed is fundamental to UPO engineering studies. Given the problems with achieving heterologous expression of *Aae*UPO, almost one decade ago we carried out an initial directed evolution campaign on this enzyme to enhance its functional expression in yeasts

(both *S. cerevisiae* and *P. pastoris*). These studies gave rise to the PaDa-I secretion mutant, which has since formed the basis of most protein and reaction engineering carried out with UPOs to date [20]. Indeed, further directed evolution of PaDa-I (also referred to as r*Aae*UPO) led to a new portfolio of variants with strong activity and high selectivity for the production of human drug metabolites and agrochemicals [8 and references therein]. Recently, we introduced the signal leader of the PaDa-I mutant, which carries 4 secretion mutations, into two long *Pab*UPOs in order to improve their functional expression in yeasts (0.3 g/L in bioreactor) [21]. Several beneficial mutations carried by PaDa-I mature protein were also inserted into these UPOs by site-directed recombination *in vivo* (SDR), further enhancing their expression 7-fold. Therefore, inserting mutations that improve PaDa-I expression and its evolved signal peptide into other long UPOs seems to be an attractive strategy to address their poor functional expression, Figure 2.

While directed evolution is a successful approach to obtain recombinant evolved UPOs, new synthetic biology strategies are rapidly arising. A Golden Gate-based secretion system that combined 11 promoters with 17 signal peptides from different sources allowed several UPOs to be recombinantly expressed in *S. cerevisiae* and *P. pastoris*. In particular, short UPO genes from *Myceliopthora termophila* (*Mth*UPO) and *Thielavia terrestris* (*Tte*UPO) were expressed for the very first time [22]. A high-throughput episomal expression system for *P. pastoris* was also reported based on a similar modular Golden-Gate cloning strategy, whereby promoters and signal leaders were shuffled for UPO expression in yeast. This platform also proved to be useful for other enzymes, such as lipases and laccases [23]. As an alternative to these expression strategies, cell-free *Aae*UPO functional expression was achieved simply through the translational activity of lysates from filamentous fungi *Neurospora crassa* and *A. niger* [24]. While this method cannot compete with variants from directed evolution campaigns in terms of yields, it is a fast and straightforward way to get functionally expressed native UPO genes. Given the recent success in UPO engineering and expression in yeast, it would not be unreasonable to think that *S. cerevisiae* lysates could be used directly to express native UPO genes harnessing the yeast's DNA recombination machinery promoted by RAD51 recombinase and ancillary factors aimed at generating mutant libraries.

Short UPOs are easier to express functionally in common heterologous hosts like yeast or bacteria, which is why several members of this family have been subjected to site-directed mutagenesis studies without prior directed evolution to enhance expression, Figure 2. The native UPO from *Collariella virescens* expressed in *E. coli* (r*Cvi*UPO) was subjected to rational mutagenesis to increase the epoxidation of C-18 unsaturated fatty acids [25]. After exploring six substitutions in the heme access channel, a double mutant was found that improved the epoxidation of polyunsaturated fatty acids. Further experiments with hydrolyzed sunflower oil and through process optimization strikingly led to 85% epoxidation after only 1 h. While this structure—function relationship was studied using a molecular structural model, the recent crystal structure of r*Cvi*UPO obtained at a high resolution may pave the way for future engineering efforts [26].

Besides achieving reasonable protein production, the optimization of UPOs to meet industrial demands includes the engineering of enzymes that can tolerate high temperatures or the presence of inactivating organic solvents. We evolved the PaDa-I mutant to achieve high levels of activity and stability in the presence of organic solvents of different chemical nature and polarity [27]. After combining classical directed evolution with neutral genetic drift and SDR, we obtained a mutant with 23-fold enhanced activity in the presence of 30% acetonitrile, a variant that was also active and stable in aqueous acetone, methanol and dimethyl sulfoxide.

The presence of unwanted peroxidase activity is a problem that arises in many applications. Dampening such peroxidase activity while maintaining peroxygenase activity has been associated with the nature of the substrates and products, and hence, this issue must be tackled case by case. This was demonstrated in the engineering of PaDa-I for the synthesis of 1-naphthol or in the selective production of human drug metabolites [28,29]. The origin of PaDa-I mutant's peroxidase activity was recently analyzed by saturating all the radical-forming aromatic amino acids that are potentially involved in long-range electron transfer (LRET) to the heme, as also occurs in classic ligninolytic peroxidases [30]. Rather than a LRET pathway, our data indicates that the peroxidase activity mostly originates in a highly dynamic loop close to the heme access channel. However, further research must be carried out to clarify whether or not this behavior can be translated to other UPOs.

The possibility of step-wise over-oxidation by UPO is an attractive feature for use in enzyme cascade reactions, as is the case for the synthesis of furan-2,5-dicarboxylic acid (FDCA), a very important building block in bioplastics production [31]. Unfortunately, this property becomes problematic when pursuing controlled regioselective hydroxylation in the transformation of fatty acids and alkanes. With very few exceptions aside [32], the majority of P450s and UPOs described to date convert fatty acids into a mixture of hydroxy fatty acids, ranging from ω-1 to ω-9, and keto by-products. Very recently, we designed a highly regio- and enantioselective UPO for the hydroxylation of a variety of fatty acids and alkenes

while impeding overoxidation [33]. This mutant was used in the preparative regioselective synthesis of 1.4 g (ω-1)-hydroxytetradecanoic acid, becoming a variant suited to future industrial scale-up.

The short *Mth*UPO expressed in yeast was subjected to saturation mutagenesis rendering important findings in terms of chemo- and regioselectivity for the functionalization of aromatic and benzylic carbons [34]. After screening mutant libraries with a dual system that combined a split-green fluorescent protein (GFP) with a colorimetric assay based on *O*-dealkylation of a model molecule, several mutants showed regioselective for aromatic oxidations, and chemoselective for benzylic hydroxylations with 2-methylnapththalene and related molecules. In a parallel study with *Mth*UPO, a rapid GC−MS low-throughput assay was used to simultaneously test a small mutant library with 3 different substrates in order to identify different regioisomers of octane, cyclohexane and cyclohexene [35]. This multiple-substrate screening strategy seems well suited to explore substrate promiscuity and it could be translated to other UPO variants in the future. In this sense, it is of interest to develop new colorimetric methods to benchmark potential applications of UPOs. For example, a high-throughput screening assay for the *N*-dealkylation activity of UPOs was reported based on a model scaffold of the epoxy resin Hexflow® RTM6 structural motif (*N*, *N*-bis(2-hydroxypropyl)-*p*-toluidine -NNBT), a reaction that was coupled to the colorimetric reporter 4-amino-5-hydrazino-1,2,4-triazole (Purpald®) [36]. This assay was used to assess the evolution of UPO variants towards an environmental application for the first time, *i.e.* the degradation of thermoset composite-based epoxy resins.

Given that UPOs are more enantio- than regioselective biocatalysts, the generation of enantiodivergent UPOs has been a long-standing goal. Very recently, we employed the FuncLib algorithm, based on phylogenetic and Rosetta design calculations, to generate a repertoire of computationally engineered UPOs [37]. Thus, 24 functional mutants were obtained that on average contained 4−5 mutations in the active site, showing an unprecedented enantiodivergence after just a one-shot computational design. This straight computational mutagenesis approach is circumventing epistasis bottlenecks by providing a constellation of mutations with unpredictable properties that can be used as departure points for directed evolution campaigns towards new purposes.

Addressing industrial needs through reaction engineering

The PaDa-I mutant has been used extensively in a wide variety of reactions that are of synthetic interest and that may be industrially scale-up in the near future, ranging from the production of chemicals to pharma compounds. Such examples include transformations performed by UPO alone or in cascade reactions, the development of H_2O_2 *in situ* supply systems, or enzyme immobilization studies [10,11]. A biocatalytic cascade based on PaDa-I and alcohol dehydrogenase was established to obtain enantio-enriched phenylethanols from ethylbenzene derivatives as the starting material [38]. After oxidation to the corresponding ketones, the enantioselective formation of (*R*)- or (*S*)-phenylethanol was achieved by selective alcohol dehydrogenases from different sources with an *ee* above 90%. This research highlights the potential for a green chemistry approach to the synthesis of fine chemicals and active pharmaceutical ingredients (APIs). In another work, naphthalene epoxides generated by PaDa-I and related mutants were transformed into non-racemic trans-disubstituted cyclo-hexadiene derivates after nucleophilic ring opening [39]. These derivatives can be further used as building blocks to synthesize natural products and APIs. PaDa-I has been used for the selective synthesis of calcitriol from alfacalcidol -an active analog of vitamin D3-that was obtained with a high rate of conversion (80%) and a total turnover number (TTN) of 4000 [40]. Hence, there is already considerable evidence that the industrial scale-up for this process can be only met with further protein and reaction engineering. The same group reported an enzymatic cascade that combines a fatty acid photodecarboxylase with lipase and UPO (*Mro*UPO and *Cvi*UPO) to transform oleic acid and its triglyceride into the corresponding fatty epoxides [41].

Like all heme-containing enzymes, UPOs suffer inactivation by their co-substrate H_2O_2. This mechanism-based inhibition is currently being tackled by designing systems that provide a gradual supply of H_2O_2 *in situ*, including the combination of UPOs with photo-, electro- and chemo-catalysts, the use of enzyme reaction cascades and through the engineering of fusion proteins, Table 1. With nearly two dozen systems to supply H_2O_2 *in situ* having been developed in the last six years, new strategies are still likely to appear. Among some of the most creative recent studies, visible-light photocatalysis was used to supply PaDa-I with H_2O_2 in the synthesis of either (*S*)- or (*R*)-enantiomer of 1-phenylethanol from ethylbenzene [58]. In this system, heterogeneous carbon nitride material was made from urea and oxamide in molten salt (CN-OA-m), in which the choice of the color light wavelength and the buffer used was crucial to drive the selectivity of the enzymatic reaction towards the alcohol or the ketone product. Establishing such control over the outcome of a reaction with a photocatalyst that not only supplies precise dosage of the H_2O_2 to the reaction *in situ*, but also that can be scaled up, provides exciting new prospects for photo-enzymatic catalysis that can tap into difficult-to-manipulate organic chemistries.

Table 1

Systems for the *in situ* gradual supply of H_2O_2.

Strategy	Source of H_2O_2	UPO	TTN	Reaction	Reference
Enzymatic cascade (Glucose oxidase/ Formaldehyde dismutase/ Formate dehydrogenase)	Methanol as a sacrificial electron donor for the reductive activation of molecular oxygen, yielding three equivalents of H_2O_2	PaDa-I	468,500	Ethylbenzene to (*R*)-1-phenylethanol	[42]
Electrocatalysis	Electrochemical reduction of O_2 in a custom-built glass reactor equipped with a carbon-based gas diffusion electrode	PaDa-I	400,000	Ethylbenzene to (*R*)-1-phenylethanol	[43]
Photocatalysis	Methanol-driven reductive activation of ambient oxygen via Gold-titanium dioxide inorganic photocatalyst	PaDa-I	71,000	Ethylbenzene to (*R*)-1-phenylethanol	[44]
			43,900	Cyclopentane to pentanol	
			61,500	Cyclohexane to cyclohexanol	
			28,600	Cyclopentane to cyclopentanol	
			59,600	1-chloro-4-ethylbenzene to 1-(4-chlorophenyl) ethan-1-ol	
			53,500	Tetralin to 1-tetralol	
			17,500	Octane to (*R*)-octan-2-ol	
Enzymatic cascade (Glucose oxidase/Formaldehyde dismutase)	Methanol as a sacrificial electron donor for the reductive activation of molecular oxygen, yielding two equivalents of H_2O_2	SoLo mutant	264,000	Propranolol to 5′-hydroxypropanolol	[29]
Photocatalysis	Visible light-driven, photochemical water oxidation	Immobilized PaDa-I	21,000	Ethylbenzene to (*R*)-1-phenylethanol	[45]
Photocatalysis	Methanol assisted reduction of molecular oxygen with UV-illuminated titanium dioxide	PaDa-I	220,000	Ethylbenzene to (*R*)-1-phenylethanol	[46]
Chemo-enzymatic cascade	H_2O_2 generated from H_2 and O_2 via gold-palladium nanoparticles supported on TiO_2 or carbon	PaDa-I	25,300	Cyclohexane to cyclohexanol	[47]
			28,200	Ethylbenzene to 1-phenylethanol	
			61,000	Isophorone to 4-hydroxyisophorone	
			16,600	Tetralin to 1-tetralol and 1-tetralone	
			7900	Propylbenzene to (*R*)-(+)-1-Phenyl-1-propanol	
			11,700	*Cis*-β-methylstyrene to *cis*-β-methylstyrene oxide	
Enzymatic cascade	*In situ* H_2O_2 generation from formate and ambient oxygen by formate oxidase	PaDa-I	318,000	Ethylbenzene to (*R*)-1-phenylethanol	[48]
		PaDa-I	36,600	*Cis*-β-methylstyrene to (1*R*,2*S*)-*cis*-β-methylstyrene oxide	
		SoLo mutant	83,000		

				Propranolol to 5′-hydroxypropanolol	
Photocatalysis	Reductive activation of O_2 to H_2O_2 via graphitic carbon nitride (g-C_3N_4)	PaDa-I mutant	60,000	Ethylbenzene to (*R*)-1-phenylethanol	[49]
Photocatalysis	Visible light-driven activation of ambient O_2 to H_2O_2 with formate as sacrificial electron donor	PaDa-I mutant	100,000	Ethylbenzene to (*R*)-1-phenylethanol	[50]
Photocatalysis	Nitrogen-doped carbon nanodots (N-CNDs) and UV LED illumination	Immobilized PaDa-I	7806	Cyclohexane to cyclohexanol	[51]
Fusion enzymes	*In situ* H_2O_2 via aryl-alcohol oxidase (AAO)	Fusion PaDa-I – AAO	62,145	Dextromethorphan to dextrorphan	[52]
Enzymatic cascade	Reductive activation of ambient oxygen by choline oxidase	PaDa-I	150,000	Thioanisole to dimethyl (methylidyne) (phenyl)-λ^6-sulfane	[53]
Enzymatic cascade	Reduction of O_2 to H_2O_2 couple to sulfite oxidation by modified sulfite oxidases	PaDa-I	30,800	Ethylbenzene to (*R*)-1-phenylethanol	[54]
			33,400	Cyclohexane to cyclohexanol	
			28,000	*Cis*-β-methylstyrene to (1*R*,2*S*)-*cis*-β-methylstyrene oxide	
		SoLo mutant	10,540	Dextromethorphan to dextrorphan	
Enzymatic cascade	*In situ* H_2O_2 generation from formate and ambient oxygen by formate oxidase (FOx)	PaDa-I	49,000	Cyclohexane to cyclohexanol	[55]
				Cis-β-methylstyrene to (1*R*,2*S*)-*cis*-β-methylstyrene oxide	
				Ethylbenzene to (*R*)-1-phenylethanol	
Electrochemistry	*In situ* production of H_2O_2 by dielectric barrier discharge plasma	PaDa-I	13,787	Ethylbenzene to (*R*)-1-phenylethanol.	[56]
Piezobiocatalysis	Bismuth oxychloride (BiOCl) generated H_2O_2 via oxygen reduction reaction	PaDa-I	2002	Ethylbenzene to (*R*)-1-phenylethanol.	[57]
			1410	Cyclohexane to cyclohexanol	
			716	*Cis*-β-methylstyrene to (1*R*,2*S*)-*cis*-β-methylstyrene oxide	
			402	Propylbenzene to (*R*)-(+)-1-Phenyl-1-propanol	
			1489	1-chloro-4-ethylbenzene to 1-(4-chlorophenyl)ethan-1-ol	
Photocatalysis	H_2O_2 generation via carbon nitride photocatalyst (CN-OA-m)	PaDa-I	–	Ethylbenzene to (*R*)-1-phenylethanol	[58]
Enzymatic cascade	H_2O_2 generation via oxalate oxidase with oxalate as sacrificial electron donor	Pada-I	37,780	Ethylbenzene to (*R*)-1-phenylethanol	[59]
		Immobilized PaDa-I	30,699	Tolmetin to 1-Methyl-5-(4-carboxybenzoyl)-1H-pyrrole-2-acetic acid	
Photocatalysis		PaDa-I	133,000	Ethylbenzene to (*R*)-1-phenylethanol	[60]

Table 1. (*continued*)

	Reductive Activation of O_2 to H_2O_2 via graphitic carbon nitride (Pt/g-C_3N_4)		43,000	Cyclohexane to cyclohexanol	
			95,000	Tetralin to 1-tetralol	
			30,000	Cycloheptane to cycloheptanol	
			57,000	1-Ethyl-4-nitrobenzene to (*S*)-1-(4-nitrophenyl)ethan-1-ol	
			43,000	4-ethylaniline to (*S*)-1-(4-aminophenyl)ethan-1-ol	
Thermoelectro biocatalysis	H_2O_2 generation via conversion of heat by thermoelectric bismuth telluride (Bi2Te3)	PaDa-I	32,913	Propylbenzene to (*R*)-(+)-1-Phenyl-1-propanol	[61]
			6361	Tetralin to (*R*)-(−)-1,2,3,4-Tetrahydro-1-naphthol	
			10,559	Cyclohexane to cyclohexanol	
			10,770	Ethylbenzene to (*R*)-1-phenylethanol	
			18,095	*Cis*-β-methylstyrene to (1*R*,2*S*)-*cis*-β-methylstyrene oxide	
Enzymatic cascade	H_2O_2 generation via alcohol oxidase and methanol as sacrificial electron donor	Liophilized PaDa-I	–	A variety of *N*-heterocyclic compounds	[62]

Biocatalysis is often bound to circular economy approaches in regard to the application of green strategies to revalorize chemicals or in the upcycling of reaction by-products or waste. An ingenious heat-fueled thermoelectrobiocatalytic chemical conversion system has been designed to revalorize the heat in the form of H_2O_2 to drive UPO-catalyzed oxyfunctionalization reactions [61]. Based on the principle that thermoelectric bismuth telluride (Bi2Te3) directly converts low-temperature waste heat into H_2O_2, a PaDa-I mutant set-up was prepared to test the hydroxylation of characteristic peroxygenase substrates. Promising results were obtained with TTN up to 32,000, opening a window for upcycling low-quality heat waste and providing evidence of the feasibility of thermoelectrobiocatalysis.

Oxalate oxidase (OXO) has been used to feed the PaDa-I mutant with H_2O_2 from oxalate, a sacrificial electron donor that releases CO_2 as the sole by-product [59]. This new system has been validated for the oxygenation of a panel of pharma compounds in late-stage functionalization studies. As such, 33 of 64 drugs were transformed by the OXO/UPO system, with 11 of them achieving conversion rates above 70%. Given that OXO was produced recombinantly in *P. pastoris* and aggregated to PaDa-I through a CLEA (cross-linked enzyme aggregate) strategy for the oxidation of tolmetin on a semi-preparative scale, we envisage that engineering of a fusion OXO-UPO protein may work as a self-sufficient oxyfunctionalization biocatalyst.

For industrial applications, protein immobilization is a welcomed strategy that allows UPOs to be reused, increasing overall productivity by extending operational times. UPOs have been immobilized by several methods, ranging from covalent and ionic immobilization to entrapment in gels and CLEA [63]. An immobilization system based on multipoint covalent attachment of the PaDa-I mutant to an amine carrier allowed this biocatalyst to be recycled up to 7 times, albeit with a gradual drop in activity [64]. A practical application for this immobilized UPO was described through its incorporation into a packed-bed reactor for the production of (*R*)-1-phenylethanol with gradual H_2O_2 pumping [65]. In continuous operation working over more than 6 days and with a final reaction volume of 9 L, productivities of 0.25 mg (L .h)$^{-1}$ were achieved with 99.9% selectivity, without detecting any over-oxidation by-products. This proof-of-concept of a continuous UPO operation could be translated into the industry in the near future.

Conclusions

After almost two decades of research, UPOs are closer to becoming established as industrial biocatalysts. The undeniable versatility of this enzyme has become more

and more evident following its entry into the market through the appearance of UPO enzyme kits (marketed by EvoEnzyme and Aminoverse) [66,67], while pilot-scale production of recombinant UPOs is now a reality [68]. Advances in the field of protein and reaction engineering have led to the emergence of natural and synthetic UPOs designed to overcome the long-standing bottlenecks of oxyfunctionalization chemistry. We foresee a bright future for the industrial application of UPOs, whereby a combination of cutting-edge strategies in protein and reaction engineering will pave the way to implant sustainable processes that will inevitably replace their traditional counterparts. In this transition, scientists worldwide will explore the rainbow of oxyfunctionalization reactions driven by UPO, unfolding a color palette with which the current and future challenges of synthetic chemistry can be confronted.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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