# Selective oxyfunctionalisation reactions catalysed by P450 monooxygenases and peroxygenases – A bright future for sustainable chemical synthesis

Xu, X.; Hilberath, T.; Hollmann, F.

**DOI**
10.1016/j.cogsc.2022.100745

**Publication date**
2023

**Document Version**
Final published version

**Published in**
Current Opinion in Green and Sustainable Chemistry

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in
Green and Sustainable Chemistry

# Selective oxyfunctionalisation reactions catalysed by P450 monooxygenases and peroxygenases – A bright future for sustainable chemical synthesis

X. Xu, T. Hilberath and F. Hollmann

**Abstract**

Heme-dependent oxygenases (i.e. P450 monooxygenases and peroxygenases) are highly selective catalysts for the selective oxyfunctionalisation or organic compounds. Both enzyme classes exhibit mechanistic similarities (i.e. using so-called compound I (CpdI) as active oxidation species) and differences in how CpdI is formed. From the differences also practical differences arise which may influence the scalability, economic attractiveness and environmental impact of P450 monooxygenase- or peroxygenase-catalysed reactions.
In this contribution we propose a range of performance indicators to compare the potential of both enzyme classes.

**Addresses**

Department of Biotechnology, Delft University of Technology, 2629HX, Delft, the Netherlands

Corresponding author: Hollmann, F (f.hollmann@tudelft.nl)

**Current Opinion in Green and Sustainable Chemistry** 2023, **39:**100745

This review comes from a themed issue on **Oxidative biocatalysis (2023)**

Edited by **John Woodley** and **Frank Hollman**

Available online 15 December 2022

For complete overview of the section, please refer the article collection - Oxidative biocatalysis (2023)

**https://doi.org/10.1016/j.cogsc.2022.100745**



**Keywords**

Biocatalysis, Oxyfunctionalisation reactions, Peroxygenases, P450 monooxygenases.

## Introduction

Oxyfunctionalisations comprise chemical transformations inserting oxygen atoms into (non-activated) C—H-, C—C-, C=C-bonds or onto heteroatoms. The general inertness of these bonds requires potent oxygen-transfer reagents such as high-valent metal-oxo complexes or organic peroxides. These reagents, however, are challenged by low selectivity resulting in complex product mixtures and isolation of the desired product can be tedious and time- and resource-intensive. Interestingly, many of the aforementioned oxyfunctionalising agents are 'biomimetic' i.e. they are inspired by natural catalysts such as iron- or flavin-dependent monooxygenases [1,2]. The fundamental difference between 'chemical' and enzymatic oxyfunctionalisation catalysts lies with the environment around the oxyfunctionalising catalyst. In the case of chemical catalysts this space is populated by simple ligands and solvent whereas enzymes provide a well-defined cavity (active site) that not only controls the orientation of the starting material towards the catalyst but also participates in the catalytic mechanism. Hence, it is not surprising that monooxygenases usually excel over their 'chemical' counterparts in terms of catalytic performance and selectivity.

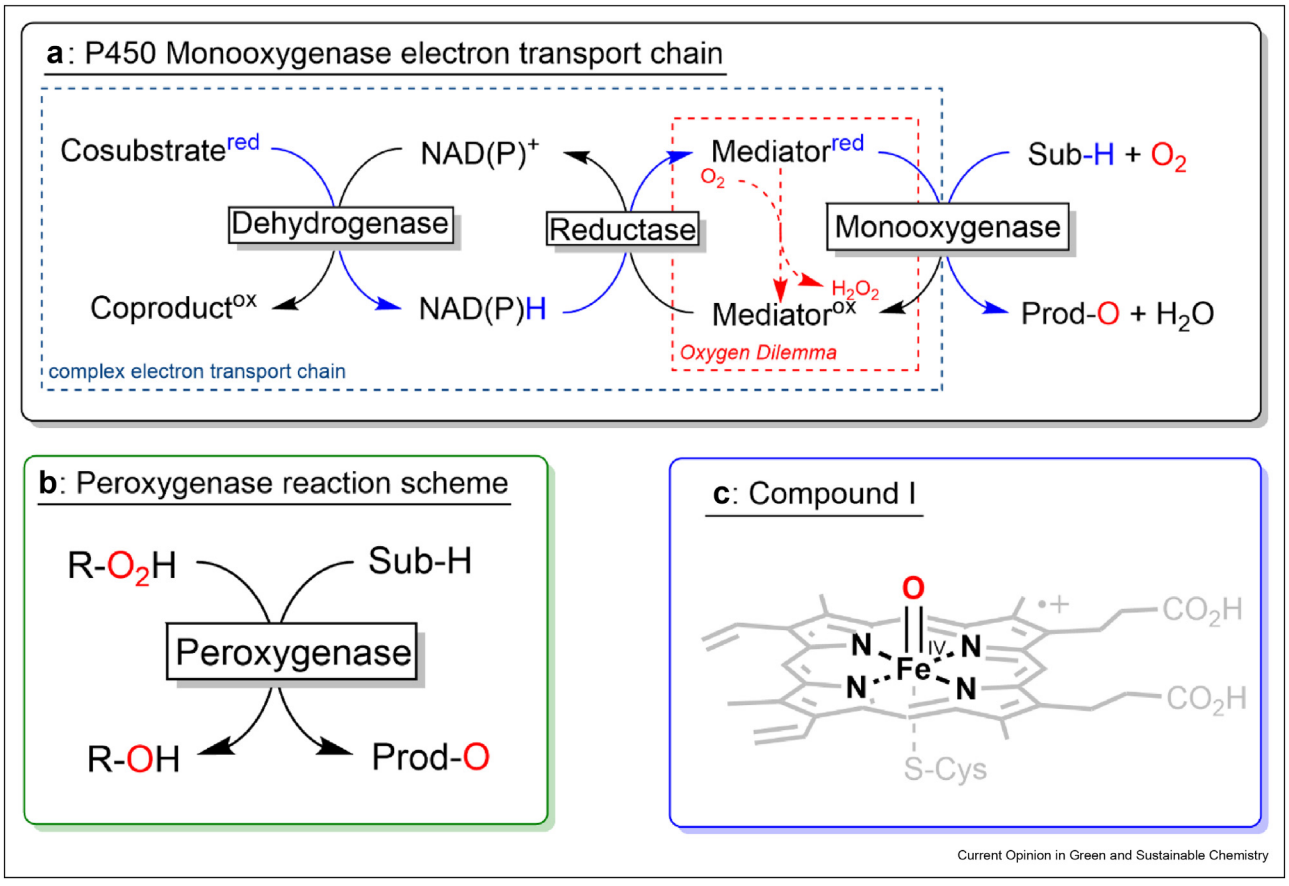
Especially cytochrome P450 monooxygenases have been the centre of attention for several decades now [3] but are increasingly challenged by so-called unspecific peroxygenases (UPOs) [4,5]. In this contribution we aim at providing a comparison between both enzyme classes with respect to their practical applicability based on recent scientific literature comparing their advantages and disadvantages.

## Mechanistic similarities and differences between P450 MOs and UPOs

Both enzyme classes make use of compound I (CpdI, Scheme 1C) as active reagent to insert an oxygen atom into their substrates. The mechanism, via which CpdI is formed, however, differs significantly between both enzyme classes. P450 monooxygenases utilise molecular oxygen as source of O and activate it reductively (Scheme 1A). The reducing equivalents required for this are obtained from NAD(P)H via more or less complex electron transport chains [6,7]. The nicotinamide cofactor itself (for economic reasons) is applied in catalytic amounts and regenerated *in situ* by the enzymatic oxidation of a stoichiometric co-substrate. It should also be mentioned that the reduced species of the electron transport chain spontaneously react with $O_2$ (needed for the catalytic reaction) and thereby partially uncouple the electron supply from the monooxygenase reaction (*Oxygen Dilemma*) [7]. As a consequence, mostly super-stoichiometric amounts of the sacrificial electron donor ($Cosubstrate^{red}$) are necessary.

Peroxygenases are much simpler as they directly utilise reduced oxygen in the form of hydrogen peroxide or organic hydroperoxides (Scheme 1B).

**Scheme 1**




Comparison of substrate oxidation via Compound I (c) formation in cytochrome P450 monooxygenases (a) and peroxygenases (b).

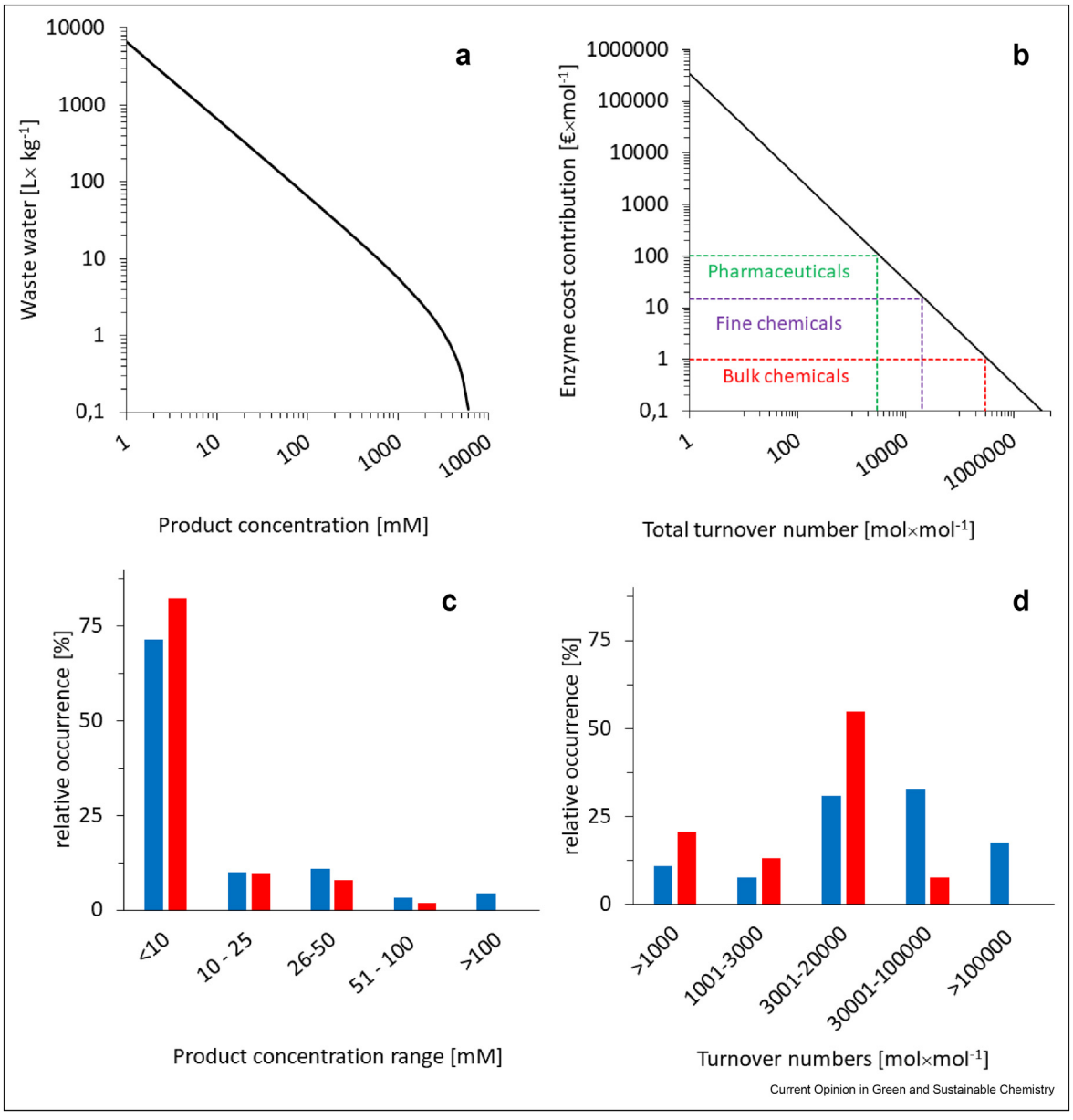
## A comparison based on performance indicators

Evaluating the environmental impact of a given (catalytic) reaction is a complex task for which many factors have to be taken into account. Nevertheless, we believe that already some simple indicators such as catalyst total turnover number (TTN) and achievable product concentration already give a reasonable indication about the economic and environmental attractiveness of a given catalytic reaction.

The importance of product concentration is rather obvious: highly diluted reaction mixtures not only use the production infrastructure insufficiently but also result in high amounts of solvent wastes and necessitate additional (time-, resource- and energy-intensive) downstream processing operations to obtain the product of interest (Figure 1a). Also, it should be taken into account that energy required for heating/cooling, mixing etc. is mostly spent on the solvent. Consequently, maximisation of the product concentration is mandatory from both an economic and environmental point-of-view. As shown in Figure 1C, the majority of recent P450- and peroxygenase-publications deal with highly diluted reaction mixtures with product concentrations beneath 10 mM corresponding to the solvent (i.e. aqueous buffer) representing more than 99% of the reaction mixture. However, in the case of P450 monooxygenases, the highest product concentrations reported so far lie around 50–60 mM [8,9]. The situation is more promising in the case of peroxygenases with more and more examples of non-aqueous applications reported, culminating in 360 mM as the, so far, highest product concentration. Particularly attractive about peroxygenases is that they can be applied under non-aqueous reaction conditions thereby circumventing the low solubility issue of many reagents of interest in aqueous media [10–14]. Nevertheless, in both cases significant improvements in final product titres are mandatory. Fortunately, the tools (such as multi-phase reactions and other non-conventional reaction media) principally exist [15,16] and are waiting to be implemented more in biocatalytic oxyfunctionalisation chemistry.

High catalyst turnover numbers (i.e. number of catalytic cycles) are desirable from an economic point-of-view to

**Figure 1**



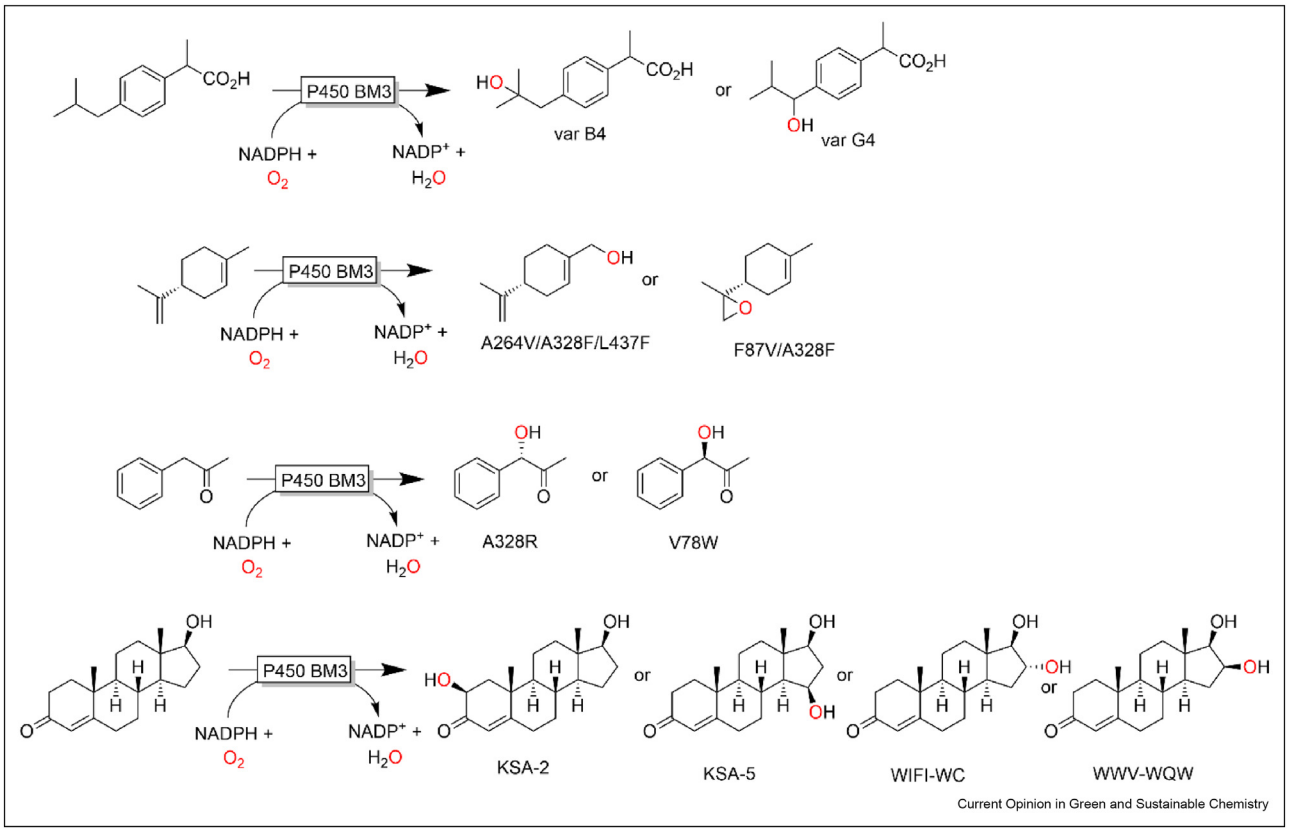

Performance indicators for biocatalytic oxyfunctionalisation reactions. a: Solvent (water) wastes generated with varying product concentrations; b: Influence of biocatalyst TN on its contribution to the final product (assumptions made: $M_W$(Enzyme) = 50 kDa, Enzyme price: 1000 € $kg^{-1}$ [17], $M_W$(Product) = 150 g $mol^{-1}$); c&d: results of a literature analysis covering 91 peroxygenase reactions and 53 P450 monooxygenase reactions.

minimise the cost contribution and environmental impact of the catalyst to the final product (Figure 1B). According to Woodley et al. [17] a minimal turnover number for an enzyme (produced on large scale) of roughly 3.000, 20.000 and 1.000.000 can be estimated to achieve acceptable cost contributions for pharmaceuticals, fine chemicals and bulk chemicals, respectively (Figure 1b). Furthermore, it should be kept in mind that the catalyst preparation causes environmental impact by consuming resources and energy [18,19]. As shown in Figure 1d the majority of both, P450 monooxygenase- and peroxygenase TNs fall more into the range of pharma- and fine-chemicals. Again, however, only with peroxygenases higher TNs approaching those required for the synthesis of bulk chemicals have been reported yet.

## Substrate scope/engineering and recombinant expression

Today, a variety of different P450s and variants thereof are known and available for organic chemists [20,21]. Currently, online databases include more than 300,000 P450 sequences from all parts of the tree of life

**Scheme 2**




Selected examples of tailored P450 (BM3) selectivity achieved via enzyme engineering [24]. hydroxylation of a) ibuprofene [25], b) limonene [26,27], c) phenylacetone [28], d) testosterone [29,30].

underlining the enormous diversity of P450 biocatalysts [22]. Furthermore, enzyme engineering has proven an efficient tool to tailor P450 monooxygenases to the substrate scope-, selectivity- or stability needs of organic chemists [20,23]. Scheme 2 gives some examples of engineered P450 BM3 to selectively hydroxylate complex starting materials at user-defined positions thereby overcoming its natural selectivity [24].

With peroxygenases we currently cannot access such a wealth of diversity. Though principally thousands of putative peroxygenase genes have been identified [31] only a handful of them have so far been functionally expressed and initially characterised [4]. Future characterisation studies will reveal whether peroxygenases are competitive in covering the broad substrate spectrum and diverse product selectivities of P450 enzymes.

Currently, the chemical space of selective oxyfunctionalisation accessible via P450 monooxygenases is enormous and approaches truly rational design. Compared to this, peroxygenase-catalysis still largely relies on wild-type enzymes, limiting their synthetic applicability.

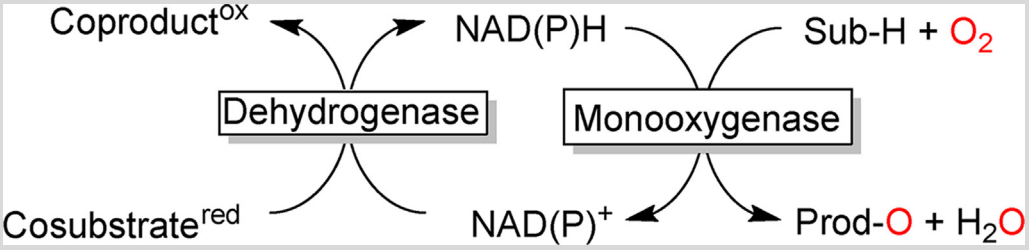
Recombinant expression systems are crucial for both, cost-efficient production of biocatalysts and their engineering. Especially prokaryotic P450 monooxygenases can nowadays be expressed in simple bacterial expression systems such as *Escherichia coli* putting the basis for mutant libraries and large-scale production [3,32]. Comparable, robust and simple expression systems for peroxygenases are still lacking today which complicates functional expression of larger libraries [4,33,34]. The enzyme titres achievable especially for eukaryotic peroxygenases are in the range of 0.5–7.5 μmol $L^{-1}$ (corresponding to approx. 25–375 mg $L^{-1}$) [33–35], which, compared to typical enzyme yield achievable with recombinant *E. coli* systems (often significantly above 5–10 g $L^{-1}$) [36] leaves room for improvement. Nevertheless, *E. coli*-based expression systems appear more favourable for enzyme production than *Pichia* simply for the dramatically reduced fermentation times (2–3 day compared to ca. 2 weeks).

## Cosubstrates/coproducts

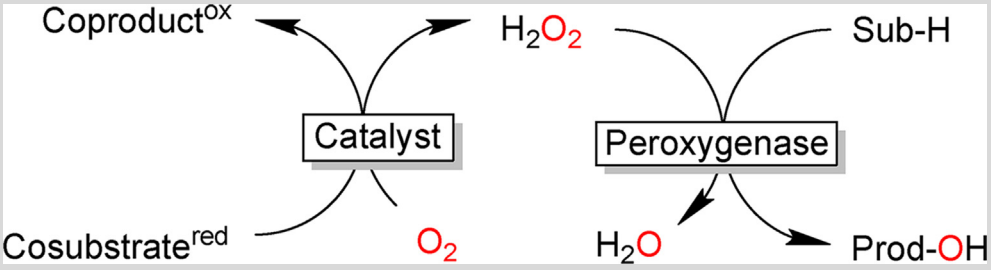
As redox reactions, oxyfunctionalisation reactions require stoichiometric cosubstrates. P450 monooxygenases require molecular oxygen and reducing equivalents for their catalytic mechanism whereas

**Table 1**

**Examples for P450 monooxygenase- and peroxygenase-in situ regeneration systems.**




| Cosubstrate/coproduct | Dehydrogenase | Waste [g mol$^{-1}_{NAD(P)H}$] | Refs |
|---|---|---|---|
| Glucose/Gluconolactone | GDH[a] | 176 | [39–42] |
| Formic acid/$CO_2$ | FDH[a] | 44 | [43,44] |
| Isopropanol/Acetone | ADH[a] | 58 | [45] |
| $H_2$/– | Hase[a] | – | [46,47] |




| Cosubstrate/coproduct | Catalyst/energy source | Waste [g mol$^{-1}_{H2O2}$] | Ref |
|---|---|---|---|
| MeOH/$CO_2$ | AlcOx, FDM, FDH, NAD, FMN/hν[a] FOx[a] | 14.7 | [48,49] |
| $H_2O/O_2$ | $TiO_2$/hν | | [50] |
| | –/plasma | | [51] |
| | –/$^{60}$Co or $^{238}$U | | [52] |
| | $Bi_2Te_3$/heat | | [53] |
| | BiOCl/ultrasound | | [54] |
| $e^-$/'none' | Cathode | 'none'[b] | [55–58] |
| $H_2$/– | Hase[a] | – | [59] |

[a] GDH: glucose dehydrogenase, FDH: formate dehydrogenase, ADH: alcohol dehydrogenase, Hase: hydrogenase, AlcOx: alcohol oxidase, FDM: formaldehyde dismutase, NAD: nicotinamide adenine dinucleotide; FMN: flavin mononucleotide.

[b] depending on the $CO_2$ footprint of the electricity used.

peroxygenases rely on hydrogen peroxide or organic hydroperoxides (Scheme 1). Envisioning larger scale applications of either enzyme class, the cosubstrate/coproduct selection has a significant impact on the economics and environmental footprint of the process.

In the case of P450 monooxygenases, glucose is a popular sacrificial electron donor because the corresponding regeneration enzyme is very active, easy to express and recycles both NAD and NADP-cofactors [37]. One issue however is the low atom efficiency of glucose as sacrificial electron donor: only one out of the 6 available carbon atoms is actually oxidised, resulting in large amounts of waste (Table 1). Moreover, the medium acidification caused by the hydrolysed by-product (gluconic acid) makes external pH control necessary. Furthermore, the viscosity of concentrated aqueous glucose solutions may pose practical difficulties for stirring and pumping such solutions. Therefore, the large-scale applicability of glucose dehydrogenase is questionable. A few other relevant cosubstrates are listed in Table 1. Overall, a broad range of practical *in situ* NAD(P)H regeneration systems are available today. They enable lab-scale applications of P450 monooxygenases and industrial applications for the synthesis of fine chemicals and active pharmaceutical intermediates but show little potential for larger scale implementation for cost- and waste reasons.

To promote peroxygenase reactions, stoichiometric supply with $H_2O_2$ or organic hydroperoxides is needed. As $H_2O_2$ also inactivates the enzymes either controlled dosage or *in situ* generation of $H_2O_2$ via $O_2$ reduction is generally applied (Table 1) [38].

As mentioned above, large-scale applications of monooxygenases or peroxygenases have to rely on readily available, easy to handle, cost-effective and low waste-

**Table 2**

**Qualitative comparison of P450 monooxygenases and peroxygenases from a synthetic application perspective.**

| | P450 monooxygenases | Peroxygenases |
|---|---|---|
| Ease of application | + a broad range of (whole cell) reaction systems are available<br>– cell-free reactions are complex | + self-sufficient catalysts |
| Substrate scope/selectivity | + very broad substrate and reaction scope | – largely limited to the selectivity of wt-enzymes |
| Cosubstrate/coproduct | – stoichiometric cosubstrates are needed generating additional wastes<br>– the *Oxygen Dilemma* still needs to be solved | + *in situ* $H_2O_2$ generation systems are at hand to minimise oxidative inactivation |
| Availability | + huge variety of engineered and wild type enzymes | – currently rather limited |
| Engineering | + well-established enzyme engineering to tailor substrate scope | + new enzymes (from natural or man-made diversity) are constantly being added |

generating energy sources to promote the biocatalytic oxyfunctionalisation reaction. Electrochemical regeneration using emission-free electrical power would represent an environmentally attractive solution to the 'regeneration issue'. First trials were reported in the late 1990s by Vilkner and coworkers [60]. Unfortunately, ever since then there has not been fundamental improvements of the productivity and robustness of direct or indirect electrochemical regeneration of P450 monooxygenases. Possibly the direct cathodic reduction of $O_2$ poses a too large technical hurdle to be solved at more than a proof-of-concept level. In contrast, this direct cathodic $O_2$ reduction to $H_2O_2$ offers, in the case of peroxygenases, a promising approach for scalable and robust reaction schemes [55,58].

Hydrogen represents another promising, waste-free cosubstrate. With the advent of $O_2$-tolerant hydrogenases [61] now also $H_2$-driven P450 monooxygenase- [46,47] and peroxygenase-driven [59] oxyfunctionalisation reactions are coming into reach. This approach, however, is still in its infancy and necessitates further development to be able to judge its practicability.

Finally, water-derived reducing equivalents would be an elegant method to drive P450 monooxygenases and peroxygenases. The thermodynamic and kinetic inertness of water oxidation, however, necessitates external energy sources and catalysts. In the case of monooxygenases, natural photosynthesis appears to be the most promising route *en route* to a water-driven oxyfunctionalisation chemistry [62–65]. The energy repertoire for *in situ* $H_2O_2$ generation from water and $O_2$ to drive peroxygenases is somewhat broader ranging from visible light [50,66,67] via γ-radiation (e.g. from nuclear waste) [52], cold plasma [51] to waste-heat [53]. Also wastes such as microplastics or lignin can serve as alternative electron donors [68]. Again, the early stage of development of the aforementioned approaches makes it difficult to predict if they will ultimately be applicable on a large-scale.

## Conclusions and outlook

P450 monooxygenases and peroxygenases bear an enormous potential for selective chemical oxyfunctionalisation chemistry. Both enzyme classes exhibit specific advantages and disadvantages over the other (Table 2).

Practical application of peroxygenases is much simpler than of P450 monooxygenases as complex and vulnerable regeneration systems can be avoided simply by adding $H_2O_2$ (or organic hydroperoxides) or utilising one of the various *in situ* $H_2O_2$ generation systems. As a consequence, turnover numbers reachable with peroxygenase-catalysts appear to exceed those of P450 monooxygenases, which gives the former an advantage in terms of cost-contribution of the enzyme catalyst to the final product. Also the application of peroxygenases in non-aqueous reaction systems appears more straight-forward (to date), which is important to reduce solvent wastes and increase the productivity of the reactions [10,12,13]. This approach, however, is still in its infancy. Particularly the low activity of the immobilised peroxygenases calls for further improvements [69].

P450 monooxygenases, on the other hand, excel in terms of recombinant expression and ease of setting-up larger mutant libraries. As a consequence, many P450 monooxygenase mutants with tailored selectivity are available whereas this number is much smaller in case of peroxygenases.

Overall, right now, it is not possible to define a 'clearly more promising' enzyme class. Future will tell if either challenge (applicability issues of P450 monooxygenases or the 'engineering gap' of peroxygenases) will be solved faster increasing the attractiveness of either enzyme class.

## Disclosure statement

Given their role as Guest Editor, Frank Hollmann had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full

responsibility for the editorial process of this article was delegated to John Woodley.

## 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

Data will be made available on request.

## Acknowledgements

Funded by the European Union (ERC, PeroxyZyme, $N^o$ 101054658). Views and opinions expressed are however those of the authors only and do not necessarily reflect those of the European Union or the European Research Council. Neither the European Union nor the granting authority can be held responsible for them.

## References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Blank LM, Ebert BE, Bühler K, Bühler B: **Redox biocatalysis and metabolism: molecular mechanisms and metabolic network analysis**. *Antioxidants Redox Signal* 2010, **13**:349–394.

2. Roduner E, Kaim W, Sarkar B, Urlacher VB, Pleiss J, Gläser R, Einicke W-D, Sprenger GA, Beifuß U, Klemm E, *et al.*: **Selective catalytic oxidation of C-H bonds with molecular oxygen**. *ChemCatChem* 2013, **5**:82–112.

3. Urlacher VB, Girhard M: **Cytochrome P450 monooxygenases: an update on perspectives for synthetic application**. *Trends Biotechnol* 2012, **30**:26–36.

4. * Beltrán-Nogal A, Sánchez-Moreno I, Méndez-Sánchez D, Gómez de Santos P, Hollmann F, Alcalde M: **Surfing the wave of oxyfunctionalization chemistry by engineering fungal unspecific peroxygenases**. *Curr Opin Struct Biol* 2022, **73**, 102342.
Concise review on peroxygenases available, their expression and engineering.

5. * Hobisch M, Holtmann D, de Santos PG, Alcalde M, Hollmann F, Kara S: **Recent developments in the use of peroxygenases – exploring their high potential in selective oxyfunctionalisations**. *Biotechnol Adv* 2021, **51**, 107615.
Concise review on peroxygenases, their scope and limitations from an organic chemistry perspective.

6. Hannemann F, Bichet A, Ewen KM, Bernhardt R: **Cytochrome P450 systems - biological variations of electron transport chains**. *Biochim Biophys Acta Gen Subj* 2007, **1770**:330–344.

7. Holtmann D, Hollmann F: **The oxygen Dilemma: a severe challenge for the application of monooxygenases?** *ChemBioChem* 2016, **17**:1391–1398.

8. ** Lu W, Ness JE, Xie W, Zhang X, Minshull J, Gross RA: **Biosynthesis of monomers for plastics from renewable oils**. *J Am Chem Soc* 2010, **132**:15451–15455.
Excellent contribution on the preparative-scale ω-hydroxylation of fatty acids. *Candida tropicalis* was engineered to optimise the hydroxylation efficiency and minimise overoxidation and metabolisation. Product titres of up to 174 g/L of 14- hydroxytetradecanoic acid have been reached, pointing towards practical applicability.

9. ** Kaluzna I, Schmitges T, Straatman H, van Tegelen D, Muller M, Schurmann M, Mink D: **Enabling selective and sustainable P450 oxygenation technology. Production of 4-Hydroxy-alpha-isophorone on kilogram scale**. *Org Process Res Dev* 2016, **20**:814–819.
Application of a recombinant *E. coli* strain for the preparative-scale, selective 4-hydroxylation of α-isophorone.

10. Nintzel FEH, Wu Y, Planchestainer M, Held M, Alcalde M, Hollmann F: **An alginate-confined peroxygenase-CLEA for styrene epoxidation**. *Chem. Commun.* 2021, **57**:5766–5769.

11. Hobisch M, van Schie MMCH, Kim J, Røjkjær Andersen K, Alcalde M, Kourist R, Park CB, Hollmann F, Kara S: **Solvent-free photobiocatalytic hydroxylation of cyclohexane**. *ChemCatChem* 2020, **12**:4009–4013.

12. Rauch MCR, Tieves F, Paul CE, Arends IW, Alcalde M, Hollmann F: **Peroxygenase-catalysed epoxidation of styrene derivatives in neat reaction media**. *ChemCatChem* 2019, **11**:4519–4523.

13. Dordick JS, Marletta MA, Klibanov AM: **Peroxidase depolymerize lignin in organic media but not in water**. *Proc. Nat. Acad. Sci. USA* 1986, **83**:6255–6257.

14. ** Zaks A, Klibanov AM: **Enzymatic catalysis in organic media at 100°C**. *Science* 1984, **224**:1249–1251.
Ground breaking early contribution on the application of enzymes in non-aqueous media.

15. * van Schie M, Spöring J-D, Bocola M, Dominguez de Maria P, Rother D: **Applied biocatalysis beyond just buffers - from aqueous to unconventional media. Options and guidelines**. *Green Chem* 2021, **23**:3191–3206.
Concise review evaluating current approaches to increase the reagent loading in biocatalysis.

16. Burek BO, Dawood AWH, Hollmann F, Liese A, Holtmann D: **Process intensification as game changer in enzyme catalysis**. *Front. Catal.* 2022, **2**, 858706.

17. * Tufvesson P, Lima-Ramos J, Nordblad M, Woodley JM: **Guidelines and cost analysis for catalyst production in biocatalytic processes**. *Org Process Res Dev* 2010, **15**:266–274.
Eye-opening contribution about factors contributing to the costs of protein production: Enzymes can be produced very economically!

18. Tieves F, Tonin F, Fernández-Fueyo E, Robbins JM, Bommarius B, Bommarius AS, Alcalde M, Hollmann F: **Energising the E-factor: the $E^+$-factor**. *Tetrahedron* 2019, **75**:1311–1314.

19. Ni Y, Holtmann D, Hollmann F: **How green is biocatalysis? To calculate is to know**. *ChemCatChem* 2014, **6**:930–943.

20. Roiban G-D, Reetz MT: **Expanding the toolbox of organic chemists: directed evolution of P450 monooxygenases as catalysts in regio- and stereoselective oxidative hydroxylation**. *Chem Commun* 2015, **51**:2208–2224.

21. Fessner ND: **P450 monooxygenases enable rapid late-stage diversification of natural products via C–H bond activation**. *ChemCatChem* 2019, **11**:2226–2242.

22. Nelson DR: **Cytochrome P450 diversity in the tree of life**. *Biochim Biophys Acta Protein Proteonomics* 2018, **1866**:141–154.

23. Charlton SN, Hayes MA: **Oxygenating biocatalysts for hydroxyl functionalisation in drug discovery and development**. *ChemMedChem* 2022, **17**, e202200115.

24. Alwaseem H, Fasan R: *Engineered cytochromes P450 for biocatalysis*. 2021.

25. Rentmeister A, Brown TR, Snow CD, Carbone MN, Arnold FH: **Engineered bacterial mimics of human drug metabolizing enzyme CYP2C9**. *ChemCatChem* 2011, **3**:1065–1071.

26. Seifert A, Antonovici M, Hauer B, Pleiss J: **An efficient route to selective bio-oxidation catalysts: an iterative approach comprising modeling, diversification, and screening, based on CYP102A1**. *ChemBioChem* 2011, **12**:1346–1351.

27. Seifert Alexander, Vomund S, Grohmann K, Kriening S, Urlacher VB, Laschat S, Pleiss J: **Rational design of a minimal and highly enriched CYP102A1 mutant library with improved regio-, stereo- and chemoselectivity**. *ChemBioChem* 2009, **10**:853–861.

28. Agudo R, Roiban GD, Lonsdale R, Ilie A, Reetz MT: **Biocatalytic route to chiral acyloins: P450-catalyzed regio- and**

**enantioselective alpha-hydroxylation of ketones**. *J Org Chem* 2015, **80**:950–956.

29. Acevedo-Rocha CG, Li A, D'Amore L, Hoebenreich S, Sanchis J, Lubrano P, Ferla MP, Garcia-Borràs M, Osuna S, Reetz MT: **Pervasive cooperative mutational effects on multiple catalytic enzyme traits emerge via long-range conformational dynamics**. *Nat Commun* 2021, **12**:1621.

30. Li AT, Acevedo-Rocha CG, D'Amore L, Chen JF, Peng YQ, Garcia-Borras M, Gao CH, Zhu JM, Rickerby H, Osuna S, *et al.*: **Regio- and stereoselective steroid hydroxylation at C7 by cytochrome P450 monooxygenase mutants**. *Angew Chem Int Ed* 2020, **59**:12499–12505.

31. Faiza M, Huang S, Lan D, Wang Y: **New insights on unspecific peroxygenases: superfamily reclassification and evolution**. *BMC Evol Biol* 2019, **19**:76.

32. Pflug S, Richter SM, Urlacher VB: **Development of a fed-batch process for the production of the cytochrome P450 monooxygenase CYP102A1 from Bacillus megaterium in E. coli**. *J Biotechnol* 2007, **129**:481–488.

33. Santos PGd, Hoang MD, Kiebist J, Kellner H, Ullrich R, Scheibner K, Hofrichter M, Liers C, Alcalde M, Master ER: **Functional expression of two unusual acidic peroxygenases from *candolleomyces aberdarensis* in yeasts by adopting evolved secretion mutations**. *Appl Environ Microbiol* 2021, **87**, e00878. -00821.

34. * Molina-Espeja P, Ma S, Mate DM, Ludwig R, Alcalde M: **Tandem-yeast expression system for engineering and producing unspecific peroxygenase**. *Enzym Microb Technol* 2015, **73–74**: 29–33.

Ground breaking contribution paving the way for the generation of peroxygenase mutant libraries.

35. Hausjell J, Halbwirth H, Spadiut O: **Recombinant production of eukaryotic cytochrome P450s in microbial cell factories**. *Biosci Rep* 2018, **38**.

36. Francis DM, Page R: **Strategies to optimize protein expression in E. coli**. *Curr Protoc Prot Sci* 2010, **61**:5.24.21–25.24.29.

37. Moore JC, Pollard DJ, Kosjek B, Devine PN: **Advances in the enzymatic reduction of ketones**. *Acc Chem Res* 2007, **40**: 1412–1419.

38. Burek BOO, Bormann S, Hollmann F, Bloh J, Holtmann D: **Hydrogen peroxide driven biocatalysis**. *Green Chem* 2019, **21**: 3232–3249.

39. Staudt S, Muller CA, Marienhagen J, Boing C, Buchholz S, Schwaneberg U, Gröger H: **Biocatalytic hydroxylation of n-butane with in situ cofactor regeneration at low temperature and under normal pressure**. *Beilstein J Org Chem* 2012, **8**: 186–191.

40. Pongtharangkul T, Chuekitkumchorn P, Suwanampa N, Payongsri P, Honda K, Panbangred W: **Kinetic properties and stability of glucose dehydrogenase from *Bacillus amyloliquefaciens* SB5 and its potential for cofactor regeneration**. *AMB Exp* 2015, **5**:68.

41. Brummund J, Müller M, Schmitges T, Kaluzna I, Mink D, Hilterhaus L, Liese A: **Process development for oxidations of hydrophobic compounds applying cytochrome P450 monooxygenases in-vitro**. *J Biotechnol* 2016, **233**:143–150.

42. Wang H, Zheng Y-C, Chen F-F, Xu J-H, Yu H-L: **Enantioselective bioamination of aromatic alkanes using ammonia: a multienzymatic cascade approach**. *ChemCatChem* 2020, **12**: 2077–2082.

43. Park H, Park G, Jeon W, Ahn J-O, Yang Y-H, Choi K-Y: **Whole-cell biocatalysis using cytochrome P450 monooxygenases for biotransformation of sustainable bioresources (fatty acids, fatty alkanes, and aromatic amino acids)**. *Biotechnol Adv* 2020, **40**, 107504.

44. Corrado ML, Knaus T, Mutti FG: **A chimeric styrene monooxygenase with increased efficiency in asymmetric biocatalytic epoxidation**. *ChemBioChem* 2018, **19**:679–686.

45. Hilberath T, Raffaele A, Windeln LM, Urlacher VB: **Evaluation of P450 monooxygenase activity in lyophilized recombinant *E. coli* cells compared to resting cells**. *AMB Exp* 2021, **11**:162.

46. Lonsdale TH, Lauterbach L, Honda Malca S, Nestl BM, Hauer B, Lenz O: **$H_2$-driven biotransformation of n-octane to 1-octanol by a recombinant *Pseudomonas putida* strain co-synthesizing an $O_2$-tolerant hydrogenase and a P450 monooxygenase**. *Chem Commun* 2015, **51**:16173–16175.

47. Preissler J, Reeve HA, Zhu T, Nicholson J, Urata K, Lauterbach L, Wong LL, Vincent KA, Lenz O: **Dihydrogen-driven NADPH recycling in imine reduction and P450-catalyzed oxidations mediated by an engineered $O_2$-tolerant hydrogenase**. *ChemCatChem* 2020, **12**:4853–4861.

48. Willot SJP, Hoang MD, Paul CE, Alcalde M, Arends I, Bommarius AS, Bommarius B, Hollmann F, FOx News: **Towards methanol-driven biocatalytic oxyfunctionalisation reactions**. *ChemCatChem* 2020, **12**:2713–2716.

49. Ni Y, Fernández-Fueyo E, Baraibar AG, Ullrich R, Hofrichter M, Yanase H, Alcalde M, van Berkel WJH, Hollmann F: **Peroxygenase-catalyzed oxyfunctionalization reactions promoted by the complete oxidation of methanol**. *Angew Chem Int Ed* 2016, **55**:798–801.

50. * Zhang W, Fernández-Fueyo E, Ni Y, van Schie M, Gacs J, Renirie R, Wever R, Mutti FG, Rother D, Alcalde M, *et al.*: **Selective aerobic oxidation reactions using a combination of photocatalytic water oxidation and enzymatic oxyfunctionalizations**. *Nat. Catal.* 2018, **1**:55–62.

First example of water-driven peroxygenase-catalysis.

51. Yayci A, Baraibar AG, Krewing M, Fueyo EF, Hollmann F, Alcalde M, Kourist R, Bandow JE: **Plasma-driven in situ production of hydrogen peroxide for biocatalysis**. *ChemSusChem* 2020, **13**:2072–2079.

52. Zhang W, Liu H, van Schie MMCH, Hagedoorn P-L, Alcalde M, Denkova AG, Djanashvili K, Hollmann F: **Nuclear waste and biocatalysis: a sustainable liaison?** *ACS Catal* 2020, **10**: 14195–14200.

53. Yoon J, Jang H, Oh M-W, Hilberath T, Hollmann F, Jung YS, Park CB: **Heat-fueled enzymatic cascade for selective oxyfunctionalization of hydrocarbons**. *Nat Commun* 2022, **13**: 3741.

54. Yoon J, Kim J, Tieves F, Zhang WY, Alcalde M, Hollmann F, Park CB: **Piezobiocatalysis: ultrasound-driven enzymatic oxyfunctionalization of C-H bonds**. *ACS Catal* 2020, **10**: 5236–5242.

55. Bormann S, Hertweck D, Schneider S, Bloh JZ, Ulber R, Spiess AC, Holtmann D: **Modeling and simulation-based design of electroenzymatic batch processes catalyzed by unspecific peroxygenase from A. aegerita**. *Biotechnol Bioeng* 2021, **118**:7–16.

56. Bormann S, van Schie M, De Almeida TP, Zhang WY, Stockl M, Ulber R, Hollmann F, Holtmann D: **$H_2O_2$ production at low overpotentials for electroenzymatic halogenation reactions**. *ChemSusChem* 2019, **12**:4759–4763.

57. Getrey L, Krieg T, Hollmann F, Schrader J, Holtmann D: **Enzymatic halogenation of the phenolic monoterpenes thymol and carvacrol with chloroperoxidase**. *Green Chem* 2014, **16**: 1104–1108.

58. Kohlmann C, Lütz S: **Electroenzymatic synthesis of chiral sulfoxides**. *Eng Life Sci* 2006, **6**:170–174.

59. Al-Shameri A, Willot SJP, Paul CE, Hollmann F, Lauterbach L: **$H_2$ as a fuel for flavin- and $H_2O_2$-dependent biocatalytic reactions**. *Chem. Commun* 2020, **56**:9667–9670.

60. Reipa V, Mayhew MP, Vilker VL: **A direct electrode-driven P450 cycle for biocatalysis**. *Proc Natl Acad Sci USA* 1997, **94**: 13554–13558.

61. Lauterbach L, Lenz O: **Catalytic production of hydrogen peroxide and water by oxygen-tolerant [NiFe]-Hydrogenase during $H_2$ cycling in the presence of $O_2$**. *J Am Chem Soc* 2013, **135**:17897–17905.

62. Jurkaš V, Weissensteiner F, De Santis P, Vrabl S, Sorgenfrei FA, Bierbaumer S, Kara S, Kourist R, Wangikar PP, Christoph CK, *et al.*: **Transmembrane shuttling of photosynthetically produced electrons to propel extracellular biocatalytic redox reactions in a modular fashion**. *Angew Chem Int Ed* 2022. https://doi.org/10.1002/anie.202207971.

63. Hoschek A, Bühler B, Schmid A: **Overcoming the gas–liquid mass transfer of oxygen by coupling photosynthetic water oxidation with biocatalytic oxyfunctionalization**. *Angew Chem Int Ed* 2017, **56**:15146–15149.

64. Erdem E, Malihan-Yap L, Assil-Companioni L, Grimm H, Barone GD, Serveau-Avesque C, Amouric A, Duquesne K, de Berardinis V, Allahverdiyeva Y, *et al.*: **Photobiocatalytic oxyfunctionalization with high reaction rate using a baeyer–villiger monooxygenase from *burkholderia xenovorans* in metabolically engineered cyanobacteria**. *ACS Catal* 2022, **12**:66–72.

65. Tüllinghoff A, Uhl MB, Nintzel FEH, Schmid A, Bühler B, Toepel J: **Maximizing photosynthesis-driven baeyer–villiger oxidation efficiency in recombinant *synechocystis* sp. PCC6803**. *Front Catal* 2022:1. https://doi.org/10.3389/fctls.2021.780474.

66. Perez DI, Mifsud Grau M, Arends IWCE, Hollmann F: **Visible light-driven and chloroperoxidase-catalyzed oxygenation reactions**. *Chem. Commun.* 2009, **44**:6848–6850.

67. Kroutil W, Reischauer S, Bierbaumer S, Winkler CK, Diaz-Rodriguez A, Edwards LJ, Kara S, Mielke T, Cartwright J, Grogan G, *et al.*: **Chromoselective photocatalysis enables stereocomplementary biocatalytic pathways**. *Angew Chem Int Ed* 2021, **60**:6965–6969.

68. Kim J, Nguyen TVT, Kim YH, Hollmann F, Park CB: **Lignin as a multifunctional photocatalyst for solar-powered biocatalytic oxyfunctionalization of C–H bonds**. *Nat Synth* 2022, **1**:217–226.

69. De Santis P, Petrovai N, Meyer L-E, Hobisch M, Kara S: **A holistic carrier-bound immobilization approach for unspecific peroxygenase**. *Front Chem* 2022:10.