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MINI-REVIEW

Recent advances in heme biocatalysis engineering

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Abstract

Heme enzymes have the potential to be widely used as biocatalysts due to their capability to perform a vast variety of oxidation reactions. In spite of their versatility, the application of heme enzymes was long time-limited for the industry due to their low activity and stability in large scale processes. The identification of novel natural biocatalysts and recent advances in protein engineering have led to new reactions with a high application potential. The latest creation of a serine-ligated mutant of BM3 showed an efficient transfer of reactive carbenes into C=C bonds of olefins reaching total turnover numbers of more than 60,000 and product titers of up to 27 g/L^{-1}. This prominent example shows that heme enzymes are becoming competitive to chemical syntheses while being already advantageous in terms of high yield, regioselectivity, stereoselectivity and environmentally friendly reaction conditions. Advances in reactor concepts and the influencing parameters on reaction performance are also under investigation resulting in improved productivities and increased stability of the heme biocatalytic systems. In this mini review, we briefly present the latest advancements in the field of heme enzymes towards increased reaction scope and applicability.

KEYWORDS

cytochrome P450 monooxygenases (P450s), genomic data mining, heme enzymes, heme peroxidases, peroxygenases, protein engineering

1 | INTRODUCTION

The ability to oxidize a broad spectrum of substrates in a stereo- and regio-specific manner has put the focus of manifold research and also industrial applications on enzymes containing heme as a prosthetic group. Among them are cytochrome P450 monooxygenases (P450s), heme peroxidases, and peroxygenases. These heme enzymes are versatile biocatalysts with an inherent broad range of naturally catalyzed reactions, all comprising an initial formal oxene transfer. The catalyzed reactions range from C–H-activation, such as hydroxylation, dealkylation, and aromatic ring oxidation, to heteroatom oxidation, and carbon–carbon bond cleavage. In addition to the broad reaction spectrum some of them, especially P450s, also accept a vast number of different substrates, such as fatty acids and steroids, but also xenobiotic compounds like drugs and toxins. Taking these advantages together, enzymes containing heme as a prosthetic group are interesting candidates for synthetic and biotechnological applications. Important applications are the production of drug metabolites, the synthesis of fine chemicals, such as flavors, fragrances, and chemicals in cosmetics, the production of precursors for polymer synthesis or bioremediation. However, the industrial application especially of P450s has remained limited due to low activity, low organic solvent tolerance, the need of cofactor regeneration, and lack of stability. This is reflected by the fact that only a few processes meet the minimum required space-time yield for the pharmaceutical or chemical production processes, for example, the synthesis of the designer drug 4'-hydroxymethyl-α-pyrrolidinobutyrophenone or the conversion of alkenes to α,ω-dicarboxylic acids (Julsing, Cornelissen, Bühler, & Schmid, 2008, Martinez et al., 2017).

In recent years, much effort has been made to address these limitations by protein and reaction engineering. In addition, a new chemical reaction mechanism was introduced by protein engineering and new enzyme families, namely peroxygenases, have emerged as promising biocatalysts avoiding some of the intrinsic limitations of P450s. This review, therefore, reports recent advances made in the field of heme enzyme biocatalysis in the three main areas of novel biocatalysts, novel reaction chemistry, and novel reactors towards increased applicability. A special focus was placed on selected examples of 450 s and peroxygenases, which had a high impact in research during the recent years.

2 | GENOMIC DATA-MINING APPROACHES TO IDENTIFY NOVEL HEME BIOCATALYSTS

New heme proteins for productive biocatalysis can be isolated from tissue samples, natural extracts, and microbial cultures followed by a conventional in vivo activity screening. Another option is the search for novel enzymes based on sequence identity. This "from sequence to function approach" is often more time-efficient because the protein sequence of the putative new enzyme is easily obtainable and the enzyme activity and functionality can be tested.

Until the end of 2018, around 90,000 genes have been annotated as belonging to the P450 family (NCBI gene database). However, only around 2,000 P450 proteins have been described in the literature so far. Figure 1 depicts the divergence of the number of genomic sequences annotated to P450s over the last decades deposited on NCBI and the amount of verified P450s deposited on the protein database UniProt. To fill the gap between available sequences and described proteins, further data processing and sequence analysis tools are needed leading to the development of many predictive bioinformatics databases. One of the first databases available, which categorized, assembled and named P450 enzymes, was the "Cytochrome P450 Homepage" by David Nelson. According to the Nelson nomenclature, a specific cutoff in sequence identity classifies P450s in superfamilies or homologous families. Several other specialized online databases exist harboring sequence information from different species as for example the "Arabidopsis P450 database" or the "Fungal Cytochrome P450 Database". Comparison of unknown gene sequences with characterized enzyme coding sequences is a promising approach to obtain new enzymes. Using sequence alignment a new fusion enzyme, CYP505A30, from the thermophilic fungus *Myceliophthora thermophile*, sharing 57% identity with the CYP505A1 (P450foxy) from *Fusarium oxysporum*, was recently identified (Baker et al., 2017). CYP505A30 showed similar catalytic properties in fatty acid hydroxylation like CYP505A1 and CYP102A1 (BM3). The prediction of function only from protein structure similarity is however challenging as combined information about sequence, structure, and function is often still missing. This makes an activity screening inevitable. To compare proteins on a sequence-structure-function interface systematically, the BioCatNet Database System CYPED was designed (Gricman, Vogel, & Pleiss, 2015). The CYPED database combines nonredundant protein database entries, being available on protein databases such as PDB or UniProt, with the gene sequence. As experimentally obtained information about altered substrate spectra and specificities of P450 help to identify functional roles of individual residues and selectivity-determining positions, literature information was furthermore added to the database (Gricman et al., 2015). Understanding the structure-function relationship is not only important for the prediction of function but is also an integral part of the identification of selectivity and specificity determining residues to facilitate rational protein engineering strategies.

Analyzing the complete cytochrome P450 complement (CYPome) from an organism known to produce a large number of secondary metabolites is also a tool to find new P450 enzymes with unknown functions. A project determining the function of 18 CYPs from the actinomyces *Streptomyces coelicolor* uncovered a new CYP102 subfamily showing the conversion of arachidonic acid with a different regio- and stereoselectivity as reported before for the CYP102A1 (BM3) from *B. megaterium* (Lamb et al., 2010). This approach still yields great success in finding novel P450 enzymes with diverse structures and functions. In a recent study from Urlacher and coworkers the heterologous CYPome expression of the actinomyces *Streptomyces*

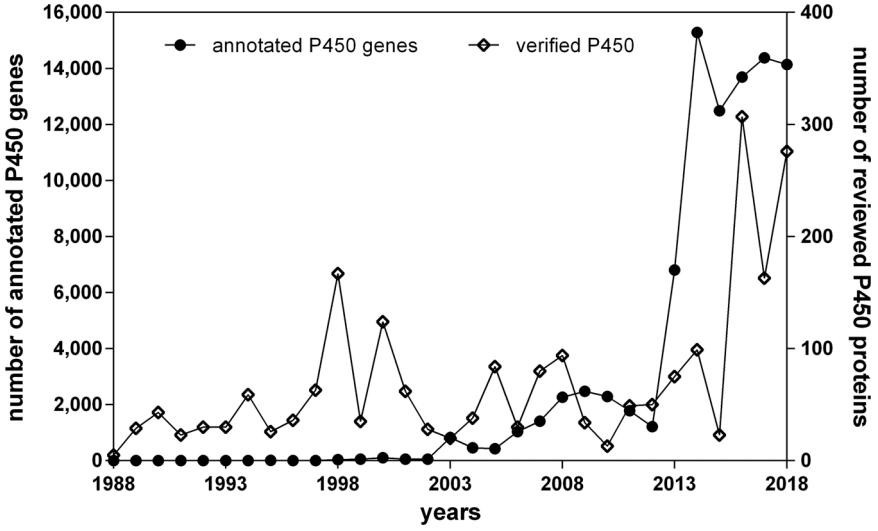

FIGURE 1 Annotated gene sequences encoding putative P450 enzymes (filled symbols, left y-axis) and a number of already published and literature described P450 enzymes (open symbols, right y-axis). Annotated genes were received from NCBI GenBank of completely sequenced genomes. The number of verified P450 comprises manually-annotated records with information extracted from literature deposited on the UniProt Knowledgebase. All values represent released records per year

platensis resulted in the identification of a novel P450 which could be assessed as belonging to the CYP107L family (Worsch et al., 2018). The P450 showed high activity for the conversion of several pharmaceutical compounds with promising application potential for preparative drug metabolite synthesis.

The discovery of a specific type of P450, that prefers H_2O_2 over NAD(P)H as an oxidant, was a breakthrough in the P450 research field. The P450s SPα from *Sphingomonas paucimobilis* and the BSβ from *Bacillus subtilis* were the first P450s showing peroxide shunt activity for the hydroxylation of fatty acids (Matsunaga, Ueda, Fujiwara, Sumimoto, & Ichihara, 1999). These P450s resemble peroxygenases in their ability to utilize both pathways making a differentiation between both families fluent. Especially, the independence on reduced nicotinamide cofactors (NAD(P)H) makes their application simple. However, H_2O_2 dependence also has the drawback that high H_2O_2 presence under process conditions significantly reduces peroxygenase activity and stability. A new family of enzymes from the kingdom of fungi, the unspecific peroxygenases (UPOs), combine the catalytic cycle from peroxidases and the P450 derived peroxide shunt (Hofrichter, Kellner, Pecyna, & Ullrich, 2015). Furthermore, they share similar reaction diversity as the P450. The first UPO described was the *Aae*UPO from *Agrocybe aegerita* and was shown to catalyze the oxygenation of aromatic, heterocyclic and aliphatic compounds (Hofrichter et al., 2015). Also, the well-established chloroperoxidase from *Caldariomyces fumago* (*Cf*CPO) was recategorized as UPO. In 2015 more than one thousand putative UPO sequences in fungal genomes were found indicating a widespread enzyme family in the whole fungal kingdom (Hofrichter et al., 2015). The currently scarce knowledge of their natural catalytic yield, compared to P450, harbors the immense potential for them to become promising biocatalysts for the synthesis of a new broad range of chemical compounds beside novel P450 enzymes.

3 | PROTEIN ENGINEERING AND DIRECTED EVOLUTION APPROACHES TO EXPAND THE RANGE OF REACTIONS

Apart from screening wild-type strains and databases, the field of protein engineering, either through rational redesign, directed evolution or a combination of both continues to provide new heme biocatalysts, for example, with broadened substrate specificity or new stereo- or regiospecificity. These protein engineering efforts mostly targeted the self-sufficient P450 BM3. Some approaches targeted the improvement of coupling efficiency, as uncoupling leads to a waste of NAD(P)H and the formation of reactive oxygen species resulting in instability of the enzyme. Furthermore, physical properties, such as solvent tolerance and thermostability were addressed.

The power of directed evolution prompted the group of Arnold not only to optimize activity, substrate selectivity and stability but to introduce new, unnatural reactions to the already existing versatile repertoire of P450 reactions (Arnold, 2015). The main approach to achieve such unphysiological synthetic reactions was to exploit the natural reactivity of the heme-cofactor and further improvement by protein engineering and directed evolution. As carbenes and nitrenes have a similar electronic structure as the above mentioned natural oxygen analog oxene, enzyme promiscuity for carbene- and nitrene-transfer reaction was likely. The major advantage of metal–carbenoid and metal-nitrenoid formation from an azide and diazo precursors compared to the natural system is that no external reductant is needed, as the iron returns directly to its resting Fe(II)-state. Transfer of carbenes through metal–carbenoid intermediates of P450 BM3 was for the first time demonstrated by Coelho, Brustad, Kannan, and Arnold, 2013 (Figure 2b). The success to generate highly active metal-carbenoids relied on the utilization of synthetic activated carbene precursors, such as ethyldiazoacetate (EDA). In a first step they could show that even free hemin could catalyze the cyclopropanation of styrenes with diazoester reagents. In a second step they replaced the conserved cysteine, serving as a ligand of the heme iron to the protein backbone, to a serine or histidine. These mutations resulted in a strong improvement in activity, showing that the enzyme backbone and the ligation state play an important role in activity. Due to this exchange cyclopropanation activity was shifted towards high turnovers (Wang et al., 2014). The engineered P450 BM3 with His-substitution (T268A-AxH) reached a total turnover number (TTN) of 7,100 in the cyclopropanation step for levomilnacipran synthesis (Wang et al., 2014). Using the serin-substituted P450 BM3 in an in vivo system of resting *Escherichia coli* BL21(DE3) cells and high substrate loading, cyclopropanation with a product titer of up to 27 g/L^{-1} and a TTN of more than 60,000 was possible (Coelho, Wang et al., 2013). Lower gram-scale synthesis of cyclopropanes in a series of aryl-substituted olefins could also be achieved with engineered myoglobin additionally showing high *trans* stereoselectivity (Bajaj, Sreenilayam, Tyagi, & Fasan, 2016). These efficient biocatalytic systems showing high TTN for nonnatural reactions opens new opportunities for a sustainable production of valuable chemicals.

In addition to an improved cyclopropanation activity via an iron–carbenoid intermediate, also the insertion of carbenoids into N-H bonds was possible when using primary amines as reaction partners (Wang, Peck, Renata, & Arnold, 2014). Another reaction that by the time was limited to synthetic transition-metal-based catalysts is the nitrene-transfer catalyzed by engineered P450 BM3 proteins (Figure 2c). By using sulfonyl azides as nitrene precursors and the subsequent formation of metal–nitrenoids C–H, amination reactions were both achieved by a cysteine–heme ligated P450 BM3 containing mutations in the active site of the enzyme (Singh, Bordeaux, & Fasan, 2014) and P450 BM3 with axial Cys–to–Ser mutation (Mcintosh et al., 2013). In both cases, similar efficiency was reported with 388 TTN and 430 TTN, respectively. Using the engineered P450 with axial Cys–to–Ser mutation also the formation of sulfimides and intermolecular aziridation could be achieved (Farwell, Zhang, Mcintosh, Hyster, & Arnold, 2015). Another highly efficient nitrene transfer reaction was the enantioselective C–H amidation with a naturally occurring hydroxamate as carbonyl nitrene precursor to construct β-, γ-, and δ-lactams (Cho, Jia, & Arnold, 2019). A P411variant (LSsp3) which was initially designed for carbene transfer was able to synthesize the desired β-lactam. Several rounds of site-directed mutagenesis and a portion-wise

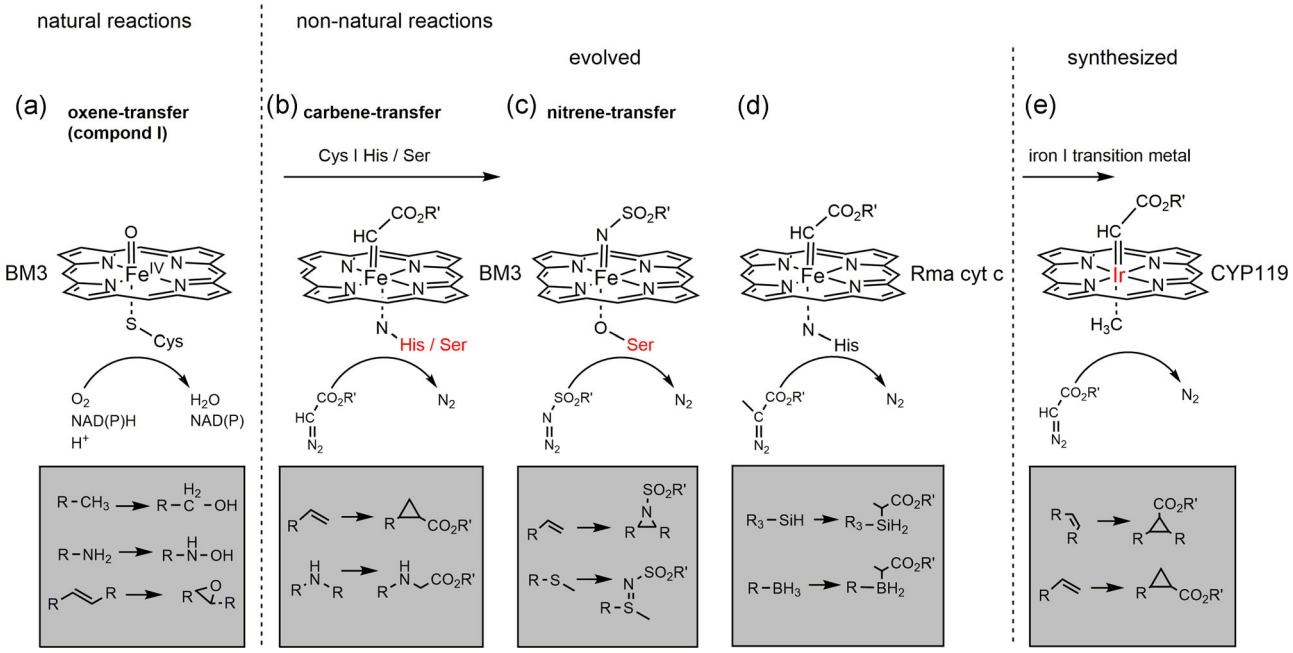

FIGURE 2 Expanding the repertoire of natural reactions of P450s towards abiological reactivity by directed evolution and design. Natural reaction via an initial formal oxene transfer (a), P450 BM3 catalyzed nonnatural reactions of carbene transfer (b), nitrene transfer (c), *Rma* cyt c catalyzed carbene transfer (d), replacement of the conserved iron to Ir(Me)-PIX (e)

feeding of LSsp3 expressing *E. coli* with the nitrene precursor yielded over 1 million TTN.

As the expansion of novel reactions within heme-proteins is limited by the inherent reactivity of the iron center, Hartwig and coworkers used a different approach of exchanging the natural iron by an abiological metal (Key et al., 2017; Figure 2e). This combination of a synthetic transition metal complex and the enzyme backbone profits both from broad reactivity, as it is typical for transition metal catalysis, and from the selectivity of the enzyme. By replacing the conserved iron in myoglobin and P450 CYP119 by Ir(Me)-porphyrin IX (Ir(Me)-PIX), the insertion of carbenes was expanded to a wide range of terminal and internal alkenes. Ir(Me)-PIX substituted variants of CYP119 could catalyze cyclopropanation of various substituted styrenes with up to 99% *ee*, 54% yield, and 300 TTN. Also the addition of carbenes to internal, unactivated alkenes, such as cyclopentene to form cyclopropane, with a 76% yield and 1,300 TTN was possible. Despite high and expanded reactivity, the specificity and substrate selectivity of the enzyme could be kept.

Another breakthrough was the first report of a heme-protein catalyzing the formation of a carbon–silicon bond in 2017, which was not known for living organisms before (Kan, Lewis, Chen, & Arnold, 2016; Figure 2d). Testing free hemin and a panel of hemoproteins for the production of organosilicon product from phenyldimethylsilane and ethyl-diazopropanoate, Kan and coworkers discovered already low TTN for the desired reaction but with low enantioselectivity. The cytochrome c from *Rhodothermus marinus* (*Rma* cyt c), however, was found to catalyze the reaction with an *ee* of 97%. *Rma* cyt c is already naturally hosting a histidine (H49) as axial coordination to the iron, which enables a high promiscuous activity for carbene-transfer via diazo precursors as it was shown in studies before. Further active site mutations were inserted and exchanges of three amino acids resulted in a 30-fold improvement compared to the wildtype reaching a 1,500 TTN and with a slight increase in optical purity to more than 99% *ee*. Furthermore, the authors could show that the synthesis of organosilicon products is also possible in vivo using whole-cell biotransformation with heterologously expressed *Rma* cyt c enzymes in *E. coli*. Their results demonstrated that enzymatic catalysis of even unnatural transformations can exceed synthetic catalysis not only in terms of selectivity but also activity for the reaction. A second reaction, the insertion of carbene into boron-hydrogen bonds, was catalyzed by the same enzyme, *Rma* cyt c (Kan, Huang, Gumulya, Chen, & Arnold, 2017; Figure 2d). To perform borylation *Rma* cyt c was expressed heterologously in *E. coli* BL21 (DE3) cells and whole-cells were incubated in presence of borane-Lewis base complexes and ethyl 2-diazopropanoate. Utilizing the wildtype *Rma* cyt c yielded in a TTN of 120 and a good selectivity (70% *ee*) that could not be achieved with other cytochrome c, cytochrome P450s and free hemin. Site saturation mutagenesis in one position of the active site that already resulted in an improvement of carbon-silicon bond formation before boosted the catalytic performance of *Rma* cyt c 16-fold with a TTN of 1,850 and an *ee* of 76%. After the insertion of other active site mutations, the turnover and selectivity could be further improved yielding a TTN of up to 15,300 and an *ee* of up to 98%. In total the in vivo borylation of 16 boron reagents in presence of a diazo ester was possible.

Apart from cytochrome c and P450s a number of reports on UPO engineering have been published during recent years (Gomez de Santos et al., 2019). Directed evolution approaches by random mutagenesis and in vivo shuffling did target expression, secretion, and stability of UPOs in yeast but also improved enzyme promiscuity

and activity. Engineered UPO variants were shown to hydroxylate their substrates to human drug metabolites (HDMs) with high efficiency. A mutant *Aae*UPO, for example, reached a TTN of 264,000 catalyzing the hydroxylation of propranolol to 5′-hydroxypropranolol when H_2O_2 was in situ generated by methanol as an electron donor (Gomez de Santos et al., 2018). In general, engineered UPOs are extremely promising biocatalysts for preparative oxyfunctionalization chemistry.

The above studies highlight the potential of heme-proteins as a starting point for unnatural reactions. It could be shown that the insertion of just a few mutations in key positions can optimize the enzyme's efficiency and selectivity for these reactions unprecedented in enzyme catalysis.

4 | NEW BIOREACTOR CONCEPTS TO INCREASE PRODUCTIVITIES

Besides the finding and generation of productive biocatalytic systems, reaction engineering strategies avoiding not only substrate or product toxicity, but also enhancing substrate solubility were investigated during the last years. This resulted in the design of novel bioreactor concepts with higher productivities of the heme-biocatalytic system (Figure 3).

When NAD(P)H is required for heme enzyme reaction, cofactor regeneration is essential for reasons of costs. Currently applied regeneration methods in industry are enzymatic methods mostly using formiate dehydrogenase and glucose dehydrogenase. However, enzymatic cofactor regeneration makes the reaction more instable and downstream processes more complex (Wang et al., 2017). Various other methods were developed for regeneration, such as chemical, homogeneous catalytic, electrochemical, photocatalytic, and heterologous catalytic routes. Alternatively, hydrogen peroxide dependent heme enzymes came into focus completely avoiding the requirement for regeneration. A versatile approach to enhance stability and minimizes enzyme deactivation of these cofactor independent enzymes such as heme peroxidases, peroxygenases and P450 monooxygenases with a high shunt pathway activity is the electrochemical supply of H_2O_2. This electro-biotechnological approach offers an elegant way to provide the cosubstrate to generate the activated heme intermediate for oxygen transfer reactions in situ at low free concentrations of H_2O_2 (Schmitz, Rosenthal, & Lütz, 2017). Because an electrochemical reaction is surface dependent, most efforts in constructing new electrobiotechnological reactors deal with the increase of the ratio of electrode surface area to reactor volume. This can be achieved by using three-dimensional electrodes such as a packed bed of electrode particles or carbon foam. In particular, as the electrochemical production of hydrogen peroxides is mostly achieved by the cathodic reduction of molecular oxygen, the use of gas diffusion electrodes (GDEs) has proven to be suitable (Figure 3b). Utilizing GDEs by direct contact of the biotransformation medium and the oxygen gas phase yielded high productivities (Krieg, Huttmann, Mangold, Schrader, & Holtmann, 2011).

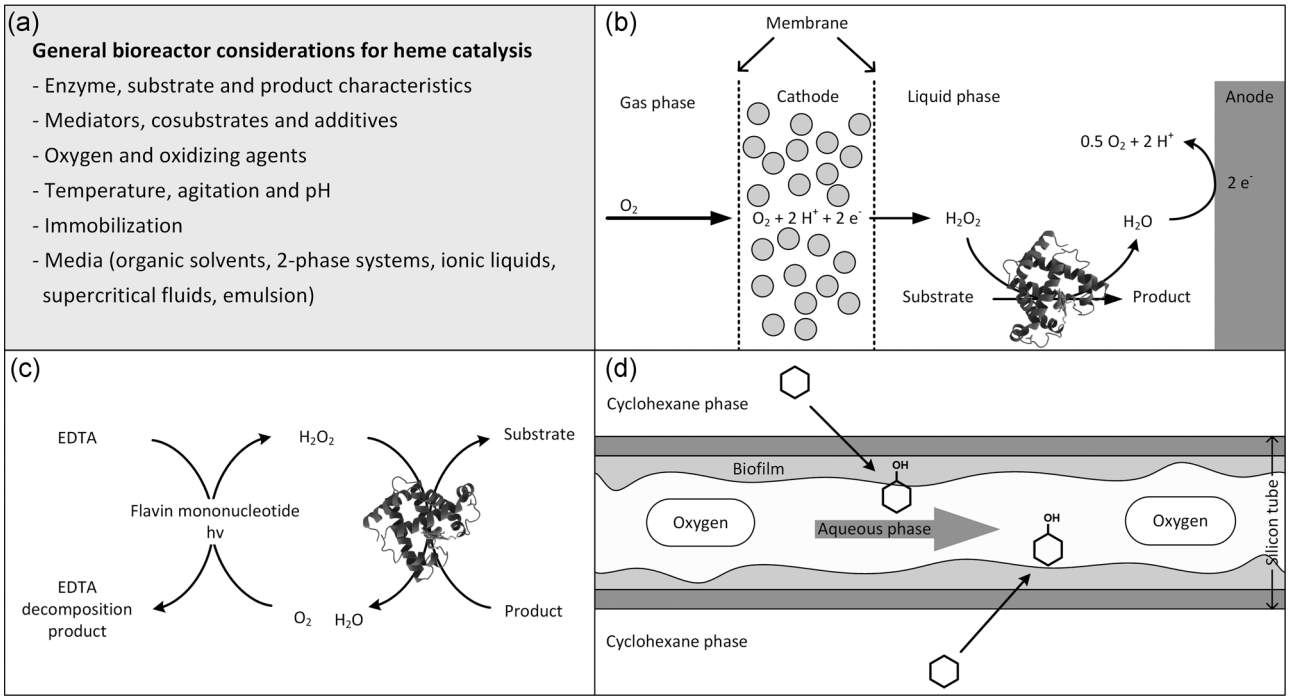

FIGURE 3 Bioreactor considerations for heme catalysis (a). Scheme of a gas diffusion electrode (GDE) (b). Oxygen is reduced to hydrogen peroxide at the cathode. Hydrogen peroxide diffuses to the enzyme and is converted into water. At the anode, water oxidizes into oxygen gas and hydrogen ions. Scheme of a light-driven in situ generations of hydrogen peroxide (c). Flavin mononucleotide is a photocatalyst reducing oxygen into hydrogen peroxide. The provided hydrogen peroxide serves as a cosubstrate for the biotransformation. Scheme of a segmented flow membrane microreactor (d). The substrate cyclohexane diffuses through a silicone membrane. The whole-cell biocatalyst grows as biofilm, attached to the inner membrane surface, converts cyclohexane into cyclohexanol. Nutrients and oxygen are provided using a segmented flow

An alternative to electrochemical H_2O_2 production is the continuous H_2O_2 supply realized by catalytic in situ generation systems based on chemoenzymatic, enzymatic or light-driven reactions (Figure 3c; Ni et al., 2016, Paul et al., 2014; Zachos et al., 2015). The H_2O_2 generation was thereby the overall rate-limiting step and thus H_2O_2 accumulation was prevented. By keeping H_2O_2 levels low, higher stabilities, enhanced productivities, and increased robustness of Heme-dependent peroxidases were achieved.

Another issue that has been raised repeatedly is substrate and product toxicity or inhibition that makes the implementation of heme enzymes for industrial processes challenging. This problem can be overcome by in situ substrate supply (ISSS) and in situ product removal (ISPR) strategies. In a study from Karande et al. (2016), three different assay formats for continuous ISSS for cyclohexane biotransformation with a P450 monooxygenase were presented (Figure 3d). Cyclohexane was supplied either via a second liquid phase, via the vapor phase, or through a membrane into a segmented flow biofilm reactor. The feeding strategy via the vapor phase reduced the presence of toxic cyclohexane in the aqueous solution and improved the biocatalyst stability over the reaction time of 1 hr. However, the substrate supply via the gas phase is challenging to implement at large scale. Therefore, the aqueous-air segmented flow biofilm reactor was used for cyclohexane biotransformation. This reactor consisted of a silicon tube in which biofilm was formed and the substrate for the biotransformation was supplied by diffusion through the silicon tube membrane. Using this bioreactor design, stable functional biocatalysis with a continuous supply of oxygen and nutrients, as well as simultaneous ISPR was achieved for several days.

The integrated development of process conditions and reactor design enables a successful application of biocatalysts which are sensitive in terms of low enzyme stability, product and substrate toxicity, and continuous electron and oxygen supply as is the case for many heme enzymes.

5 | CONCLUSION AND FUTURE PROSPECTS

Oxyfunctionalization with heme protein catalysis has proven many advantages over classical catalysis such as excellent regio- and stereoselectivity, efficiency in terms of single-step conversion and avoidance of harsh reaction conditions. However, heme protein biotransformations reported to date are mostly far away from being feasible due to limitations such as low activity, complex redox regeneration or poor coupling efficiency. Although outstanding examples were published about progression in heme enzyme reactions and processes, the mentioned limitations and especially the low stability under process conditions often hinder their application in large scale processes. Even though acceptable stability can be achieved for small scale experiments, larger scales include process conditions such as stirring and aeration, which are often not tolerated by those sensitive enzymes. Enzyme inactivation is mainly caused by ROS that is formed within the oxidative cycle (Holtmann & Hollmann, 2016). Hence, improvements can be achieved by optimizing the coupling efficiency, which in turn increases the enzyme stability and TTN (Brummund et al., 2016).

Considering the opportunities and variability of enzymatic oxidation reactions, research should focus on the improvement of heme protein applications. New genome sequences emerging on a daily basis harbor a huge potential to reveal new P450 enzymes with new substrate spectra and specificities and also new enzyme families such as the fungal UPO. To make protein engineering more time-efficient, the rational translation of sequence to function is an important step. Several residues like the heme ligation have been revealed to be crucial for activity determination in P450. A change in heme ligation, for example, could shift reactivity towards nitrene and carbene transfer showing that sequence understanding can contribute to predicting protein functions. Besides protein understanding, directed evolution utilizing random mutagenesis followed by screening was shown to result in highly efficient heme enzymes catalyzing new and even unnatural reactions such as carbon–silicon or carbon–boron bond formation. Even though the discovery and creation of productive heme dependent biocatalytic systems have been advanced, some limitations, such as toxicities of substrates and products, remain. Reaction engineering approaches contribute to overcoming these major limitations by spatial separation of toxic compounds. Furthermore, higher loading of the poorly soluble substrate is highly desirable, which can be achieved with ISSS and ISPR technologies yielding higher productivities.

Having already proven to be quite effective as individual approaches, the combination of protein engineering and reaction engineering, as well as the identification of new enzymes by sequence-based mining seems to be promising, to generate feasible processes with sufficient final product concentrations. Heme biocatalysis will continue to be a promising area leading to new processes equal or superior to conventional chemical oxygenation reactions.

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