

Expert Opinion

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Engineering cytochrome P450 biocatalysts for biotechnology, medicine and bioremediation

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Importance of the field: Cytochrome P450 enzymes comprise a superfamily of heme monooxygenases that are of considerable interest for the: i) synthesis of novel drugs and drug metabolites; ii) targeted cancer gene therapy; iii) biosensor design; and iv) bioremediation. However, their applications are limited because cytochrome P450, especially mammalian P450 enzymes, show a low turnover rate and stability, and require a complex source of electrons through cytochrome P450 reductase and NADPH.

Areas covered in this review: In this review, we discuss the recent progress towards the use of P450 enzymes in a variety of the above-mentioned applications. We also present alternate and cost-effective ways to perform P450-mediated reaction, especially using peroxides. Furthermore, we expand upon the current progress in P450 engineering approaches describing several recent examples that are utilized to enhance heterologous expression, stability, catalytic efficiency and utilization of alternate oxidants.

What the reader will gain: The review provides a comprehensive knowledge in the design of P450 biocatalysts for potentially practical purposes. Finally, we provide a prospective on the future aspects of P450 engineering and its applications in biotechnology, medicine and bioremediation.

Take home message: Because of its wide applications, academic and pharmaceutical researchers, environmental scientists and healthcare providers are expected to gain current knowledge and future prospects of the practical use of P450 biocatalysts.

Keywords: biocatalyst, cytochrome P450, directed evolution, drug metabolism, drug toxicity, protein engineering

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1. Introduction

Cytochrome P450 monooxygenases (CYP) are a superfamily of ubiquitous heme proteins that perform a number of difficult oxidative reactions, such as C-H bond hydroxylation, *N*-dealkylation, *N*-hydroxylation, *O*-dealkylation, *S*-oxidation and epoxidation of numerous endogenous and exogenous compounds (reviewed in [1]). They exhibit strict as well as overlapping substrate specificity, in addition to substrate regio- and stereoselectivity and non-Michaelis-Menten kinetics (reviewed in [2]). CYP enzymes are central to the study of toxicology because they are involved in the clearance of a majority of marketed drugs and abused substances, activation of prodrugs, drug-drug and drug-food interactions, and metabolism of carcinogens and other pollutants. Currently, there are > 7000 CYP sequences known, of which 57 are human [3]. Their catalytic versatility, substrate diversity and atypical kinetics have led to considerable interest in utilizing CYP as biocatalysts in biotechnology, medicine and bioremediation.

While bacterial CYP are soluble and metabolize only a limited number of natural substrates, such as fatty acids, vitamins, styrene, erythromycin and terpenes, mammalian CYP are membrane-bound and metabolize an array of substrates, such as steroids, fatty acids, drugs, prodrugs, carcinogens, pesticides and herbicides (reviewed in [4]). Bacterial P450_{BM3} (CYP102) is catalytically self-sufficient, that is, the electron transfer flavin mononucleotide/flavin adenine dinucleotide reductase domain and the P450 monooxygenase domain are in a single peptide [5]. In contrast, mammalian CYP require additional redox partners, such as cytochrome P450 reductase (CPR) and often cytochrome *b*₅, an expensive cofactor NADPH (nicotinamide adenine dinucleotide phosphate-oxidase), and lipids [1,2]. Furthermore, bacterial CYP show a much higher turnover, expression in *Escherichia coli* and coupling efficiency (utilization of NADPH in substrate metabolism versus formation of superoxide, peroxide and water molecules) compared to that of their mammalian counterparts [1,2,6]. These contrasting features of bacterial and mammalian CYP clearly suggest that bacterial CYP are easy to use as biocatalysts, whereas mammalian CYP have much broader applications in pharmaceuticals/biotechnological industries and in green chemistry.

Over the past 3 decades, CYP have been reviewed numerous times with an emphasis on CYP's regulation, atypical kinetics, drug metabolism, drug interactions and structural-function studies [1,2,4,7-11]. In 2002, Guengerich wrote the first review on the proposed application of CYP biocatalysts, especially in the synthesis of drugs and other fine chemicals [12]. Thereafter, several reviews reported on proposed applications of bacterial and mammalian CYP [13-18]; for example, Gillam recently highlighted a need for engineered CYP in such applications [15]. Here, we review the recent advancements and future prospects for the use of CYP enzymes as biocatalysts in biotechnology, medicine and bioremediation, as well as recent success in engineering techniques and examples of engineered CYP for the proposed applications (Figure 1, Tables 1 and 2). To the best of the author's knowledge, this is the first review that combines the potential applications of natural and engineered CYP enzymes.

2. Applications of cytochrome P450 biocatalysts

In recent years, there has been an increasing realization of the power of CYP biocatalysts for the industrial synthesis of bulk chemicals, pharmaceuticals, agrochemicals and food ingredients, especially when high regio- and stereoselective hydroxylation is required [12-14,18]. In addition, there is an increasing demand for CYP biocatalysts in the detoxification of environmental contaminants [19-21] and gene-directed enzyme prodrug therapy (GDEPT) for cancer treatment [22-24].

2.1 Biotechnology

CYP enzymes have a tremendous potential in the synthesis and discovery of drugs, as well as in drug development. Although several microorganisms have been used historically for the synthesis of drugs using a hydroxylation reaction, the well-established commercial application of CYP is in the biotransformation of steroids, such as the 11 β -hydroxylation of reichstein S to hydrocortisone by *Curvularia* sp. [25,26] at a scale of ~ 100 tons per year. In addition, *E. coli*-expressed CYP is used to make provastatin from compactin [27], and CYP102 is utilized in the production of epoxyeicosatrienoic acid, leukotoxin B and eicosanoid epoxides [28]. Many synthetic steps of antibiotics involve bacterial P450s, for example, synthesis of erythromycin and tetracenomycin requires P450_{eryF} (CYP107) [29]. Similarly, several plant CYPs are involved in the biosynthesis of the most widely used chemotherapeutic drug taxol [30]. Achieving a complete taxol synthetic pathway in microorganisms is still challenging. Therefore, through CYP a semi-synthetic approach could be used to produce new taxol analogues with enhanced drug efficacy and potency. In another example, the production of the anticancer drug perillyl alcohol from limonene was achieved by CYP153 expressed in *Pseudomonas putida*, resulting in a 6.8 g/l yield [31]. Thus, the true potential of CYP in the production of drugs or drug intermediates can be further exploited.

The CYP-derived drug metabolites in the liver and other organs/tissues are often biologically active, and understanding their effects is crucial in evaluating a drug's efficacy, toxicity and pharmacokinetics. Such studies, however, require large quantities of the pure metabolites, which may be difficult to synthesize by chemical methods, especially when regio- and stereoselective hydroxylation is required. An alternate route for the synthesis of drug metabolites is the use of human CYP. When this approach was used in a 1-L reaction, CYP2C9 co-expressed with CPR produced 110 mg 4'-hydroxydiclofenac with a conversion rate of 93% [32]. However, the limitations in these approaches include poor activity, stability and expression in *E. coli*. Recently, an enzyme immobilization approach has been developed with several CYP enzymes to synthesize metabolites [33-35]. The immobilized enzymes exhibited an improved half-life. For example, the immobilized CYP102 had a half-life of 29 days at 25°C compared with 2 days for free enzymes [35]. More recently, CYP2C9 was attached to gold electrodes such that the resulting constructs maintained the ability to bind and metabolize substrates in the presence of CPR and NADPH [36]. Similarly, Mie *et al.* showed that CYP3A4, which was immobilized on gold electrodes, can be used for electrochemically driven drug metabolic reactions [37]. They also found that the CYP3A4-mediated reactions are facilitated in the presence of CPR. The activity obtained with the immobilized CYP2C9 and CYP3A4 on gold was similar to that obtained using a standard reaction in addition to exhibiting an increased stability. This opens up the possibility of the construction of bioreactors using biochip technology to synthesize drug metabolites.

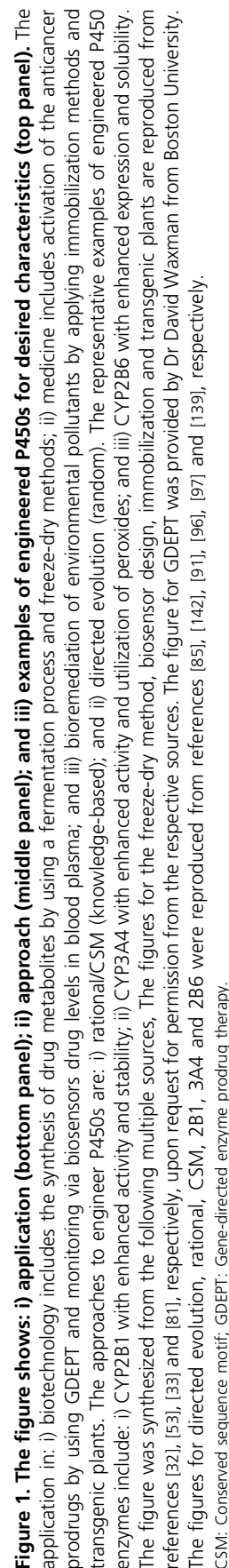


Table 1. Multiple ways to perform cytochrome P450-mediated reactions for practical purposes.

Reaction requirements	Enzyme source	Activity	Cost	Possible applications	CYP
CYP + CPR + NADPH	Purified	High	High	Synthesis, bioremediation	1A2, 2C9, 3A4
CYP + CPR + NADP ⁺ + G6P + G6PDH	Purified	High	Medium	Synthesis, bioremediation	1A2, 2C9, 3A4
CYP–CPR + NADPH or NADP ⁺ + G6P + G6PDH	Purified/whole cell	High	Medium	Synthesis, bioremediation, GDEPT	1A1, 1A2, 2A6, 2B6, 2B11, 2C18, 3A4, 71B1
CYP + peroxides	Purified/whole cell	Low	Low	Synthesis, bioremediation	101, 102, 2B1, 2C8, 2C9, 2C18, 2C19, 3A4
CYP + metal powders	Purified	Low	Low	Synthesis, bioremediation	102
CYP + metal electrode	Purified	Medium	Medium	Synthesis, biosensor	101, 102, 1A2, 2B4, 2C9, 2D6, 2E1, 3A4

GDEPT: Gene-directed enzyme prodrug therapy.

Table 2. List of important cytochrome P450 enzymes, screening strategies and possible practical applications.

CYP	Screening strategies	Possible applications in biotechnology, medicine and bioremediation
102	Fatty acids, alkanes, drugs; H ₂ O ₂	Synthesis of alcohol, indigo and indirubin, and metabolites of propranolol and buspirone; degradation of pollutants, such as PAHs and petroleum components, such as alkanes
101	Alkanese; H ₂ O ₂	Synthesis of alcohols; degradation of petrochemicals, such as alkanes
1A2	7-MeQ, 7-MR; NADPH	Synthesis of metabolites of propranolol and warfarin; detoxification of PCBs
1A1/1A2	Luciferin	Synthesis of metabolites of CYP1A1/1A2s as well as novel drug substrates
2A6	Indole, 7-MR; NADPH	Syntheiss of indigo compounds
2B1	7-EFC; H ₂ O ₂	To re-engineer 2B6 or 2B11 for the activation of CPA and IFA
2C8*	C152; CuOOH	Synthesis of metabolites of taxol, chloroquine and amodiaquine; biomarkers for cancer
2C9*	C152; CuOOH	Synthesis of metabolites of diclofenac, warfarin and flurbiprofen
2C18*	C152; CuOOH	Activation of CPA and IFA
2C19*	C152; CuOOH	Synthesis of metabolites of diclofenac and warfarin; activation of CPA and IFA
2Cs	Luciferin; NADPH	Synthesis of metabolites of CYP2Cs as well as novel drug substrates
3A4	7-BQ; CuOOH, NADPH	Synthesis of metabolites of several drugs; activation of IFA

*Directed evolution of these P450s is underway: 2Cs: 2C8, 2C9, 2C18 and 2C19.

CPA: Cyclophosphamide; IFA: Ifosfamide; PAH: Polycyclic aromatic hydrocarbon; PCB: Polychlorinated biphenyl.

In addition to drugs and drug metabolites, several other commercial products, such as dyes and pesticides, could be synthesized by using CYP. Dioxygenases have been used commercially to produce dyes; for example, in the production of indigo from tryptophan [38]. Recently, investigators have shown that CYP can readily form indigo and indirubin in bacterial cultures and transgenic tobacco plants, which points to its important role in the dye industry [39,40]. Another application of dye production by CYP involves horticulture. For example, the production of flowers with unusual colors, such as, blue roses, is performed by transferring CYP coloration genes from other plants [41]. Accordingly, the introduction of CYP, which oxidizes indole into a variety of colored compounds, into flowering plants is an alternative method that is proposed in the horticultural industry.

2.2 Medicine

GDEPT that increases the chemosensitivity of tumor cells by stimulating tumor cell-catalyzed activation of anticancer prodrugs offers a unique opportunity to improve the efficacy of cancer chemotherapeutic agents. In addition, GDEPT may potentially decrease or eliminate the adverse effects from: i) toxic metabolites arising from side reactions, such as N-dechloroethylation; and ii) toxicity of a prodrug at a high dose, if taken orally or intravenously [22,24]. Over the past decade, Waxman and colleagues have performed elegant studies involving the development of the CYP-based activation of cyclophosphamide (CPA) and ifosfamide (IFA) through GDEPT for cancer treatment (reviewed in [22,24]). To study the activation of CPA and IFA in tumor cells, they constructed a CYP-internal ribosomal entry site CPR plasmid. Human CYP2B6 showed a strong cytotoxicity in

9L-glioma cells with both the prodrugs, CPA and IFA [42-47]. Human CYP2C18 and CYP3A4 showed a strong cytotoxicity with CPA and IFA, respectively, despite their several-fold lower levels of expressed proteins than CYP2B6 in 9L-glioma cells [44,45]. In addition, low- K_m human CYP2C9 and CYP2C19 enzymes are capable of activating both CPA and IFA [48].

Preclinical GDEPT studies with CYP2B6 using CPA demonstrate a substantial improvement in antitumor activity, with a concomitant decrease in systemic toxicity when compared to that found with conventional chemotherapy [49]. Subsequently, results of Phase II clinical trials using human CYP2B6 showed a targeted activation of CPA and IFA, leading to the selective death of the tumor cells with a minimal effect on normal cells [50]. Furthermore, canine CYP2B11, a low K_m CPA 4-hydroxylase ($K_m \sim 100 \mu\text{M}$) when compared with a high K_m human CYP2B6 ($K_m \sim 1 \text{ mM}$) showed enhanced activation of CPA and IFA [51,52]. Furthermore, the activation of CPA *in vivo* solid tumors that express CYP2B11 was investigated; the findings demonstrated that when compared with human CYP2B6, canine CYP2B11 showed an increased intratumoral concentration of 4-OH-CPA [53]. These findings provide the proof-of-principle for the use of a low K_m CYP to augment intratumoral prodrug activation at pharmacologically relevant dosages. The potency of CYP-mediated GDEPT can be further improved by: i) enhancing the stability and expression of CYP enzymes in tumor cells; ii) increasing the bioactivation (4-hydroxylation) and decreasing the detoxification (N-dechloroethylation) pathways; and iii) finding genetic variants of drug-metabolizing enzymes and drug transporters [22,24].

Another application of CYP enzymes in medicine is to use them as biosensors to monitor drug levels in blood plasma [54]. Monitoring drug level consistently is critical, because the presence of genetic variants or drug-drug/drug-food interactions lead to altered drug response and drug toxicity [55]. For example, CYP2C9 and CYP2D6 have several variants with low, high or no activity, which arise from genetic, environmental, physiological or pathophysiological factors [55]. Similarly, numerous drugs have been shown to inhibit or activate human CYP enzymes, which alter the metabolism of other drugs, resulting in toxicity [10]. In addition, CYP biosensors can be used to detect food contaminants, such as hazardous carbamate and organophosphate pesticides, in order to improve the food safety [54]. Thus, an immobilized CYP enzyme on an electrode, which either binds to drugs or converts them into oxidized products, can act as a biosensor. An electrochemical potential generated upon binding or metabolism can be used to monitor the drug/food contaminant levels.

CYP as a biosensor ranks third after cytochrome c and glucose oxidase [54]. Currently, P450_{CAM} (CYP101), CYP102, CYP1A1, CYP1A2, CYP2B4, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP4A1, CYP11A1 and CYP17A have been used for sensor constructions to monitor drug levels in blood plasma [54,56-64]. For example, an amperometric drug metabolism biosensor consisting of CYP3A4 encapsulated in a

didodecyltrimethylammonium bromide vesicular system on a platinum disk electrode was developed for the determination of indinavir, a protease inhibitor antiretroviral drug [57]. Some of the common electrodes used for enzyme immobilization are gold, platinum, tin oxide, glassy carbon, pyrolytic graphite, edge-plane graphite and carbon cloth. However, the use of polymer or a polyelectrolyte enhances the preservation of the native structure, thereby, increasing electron transfer between the enzyme and the electrode. Recently, a molecular Lego approach (assembling non-physiological redox modules and generating artificial redox chains, which lead to a new multi-domain construct) has been developed to improve the electrochemical and catalytic properties of CYP102 [65].

2.3 Bioremediation

There are three major classes of ubiquitous global industrial pollutants present in air, water and soil, namely, polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated biphenyls (PCBs) [66,67]. PAHs are ubiquitous compounds originating from the natural and anthropogenic pyrolysis of organic matter, such as forest fires, fossil fuel consumption and processes in the oil industry. CYP101, CYP102, CYP1A1, CYP1A2 and CYP1B1 are known to metabolize PAHs [1,2,4]. CYP1A1 shows high activity toward dibenzo-*p*-dioxin (DD), and mono-, di- and tri-chloro-DDs, while the CYP1A1 mutant, F240A, exhibits activity toward 2,3,7,8-tetra-chloro-DD [68,69]. Furthermore, recombinant cells (*Saccharomyces cerevisiae*, *basidiomycete*, *Dehalococcoides* and *Rhodococcus* species), expressing CYP1A1, successfully biodegrade PCDDs [68,70]. PCBs are metabolized by several CYP enzymes, and the major factors that determine the degree of metabolism are the extent of chlorination and position of chlorine atoms on the biphenyl nucleus [71]. For example, a marked species variation exists in the metabolism of 2,2',3,3',6,6'-hexachlorobiphenyl, and it has been shown that only dog CYP2B11 is capable of metabolizing PCBs [72]. In contrast, CYP1A1 is also known to bioactivate these pollutants into genotoxic and carcinogens leading to high risk for lung cancer [73].

Herbicides provide labor-saving ways of improving crop yield and quality, because weed infestation reduces crop yield and decreases market prices. In addition to chemical methods, many herbicides are removed from the environment by bacterial and plant CYP enzymes, which convert herbicides to less lipophilic and toxic metabolites [74]. Many CYP-dependent oxidations have been reported, including those of chlorotoluron and linuron in maize and wheat. However, molecular information is limited regarding plant CYP related to xenobiotic metabolism. Only some herbicide-metabolizing CYP genes have been cloned and characterized, including CYP73A1 and CYP76B1 from Jerusalem artichoke, CYP71A11 from tobacco and CYP71A10 from soybean [74].

Detoxification of the contaminants can be performed by immobilizing CYP enzymes on a surface. Recently, plant CYP71B1-reductase fusion protein was immobilized to

metabolize chlortoluron [33]. The immobilized CYP71B1 enzyme shows 10-fold higher activity than does the free enzyme for the demethylation of erythromycin, and retained activity for > 24 h. In addition, creating CYP-expressed transgenic plants has potential for developing herbicide-resistant plants and for reducing the environmental impacts of agrochemicals [75-77]. Recently, several CYP enzymes, such as CYP1A1, CYP2B6, CYP2C9 and CYP2C19, have been introduced in various plants for effective phytoremediation of herbicides [78-83]. For example, a rice plant in which the CYP2C9-57R2 gene was introduced showed resistance to sulfonylureas, while the transgenic rice plant CYP2C19-12R1 showed cross-resistance to several herbicides, such as atrazine, norflurazon, metolachlor and sulfonylureas [78]. *Arabidopsis thaliana* expressing xplA cyclotrimethylenetrinitramine (RDX)-degrading CYP was grown in RDX-contaminated soil and found to be resistant to RDX phytotoxicity, producing shoot and root biomasses greater than those of wild-type plants [79].

3. Cost-effective source of electrons

CYP enzymes require a complicated source of electrons via redox partners and NADPH, and, therefore, it is critical to find alternate strategies to bypass these requirements for practical applications. Table 1 shows various ways to perform CYP-mediated reactions, their cost-effectiveness, practical uses and examples. The most difficult and expensive strategy is to use NADPH and purified CYP, CPR and cytochrome *b*₅. The cost of NADPH can be reduced by using an NADPH regenerating system (glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP⁺). This strategy can mainly be used for synthesis of specific compounds and bioremediation. The requirement of redox partners can be overcome by expressing CYP and CPR in either the same plasmid (bicistronic expression) or a different one (co-expression) (reviewed in [84]). Several bicistronic CYP-CPR plasmids, such as those from CYP2B6, CYP2B11, CYP2C9, CYP2D6, CYP2C18 and CYP3A4, have been constructed and expressed in *E. coli*, yeast, insects and mammalian cells ([43,44,85-88], unpublished observations). More recently, Purnapatre *et al.* have reviewed methods of production of recombinant CYP enzymes in various expression systems, including *E. coli*, yeast, insects and mammalian [87], which offer simple and economical modes of production of target-based anticancer drugs. We do not discuss various CYP or CYP-CPR expression systems here as they are beyond the scope of this review. Bicistronic construct is an absolute requirement in the case of GDEPT [24] and is cost effective in the case of industrial synthesis of chemicals and bioremediation (Table 1).

The most cost-effective and simple method, however, would be to bypass the requirements of CPR, cytochrome *b*₅ and NADPH by using alternate oxidants, such as peroxides (hydrogen peroxide (H₂O₂), cumene hydroperoxide (CuOOH)), metal powders (Zn, Pt) or metal electrodes (gold, glassy carbon) (reviewed in [20,54,89]). These strategies

can be used for synthesis, bioremediation and/or biosensor applications. The use of peroxides and metal powders is the most cost-effective way to synthesize drugs, drug metabolites or other chemicals, whereas the use of a metal electrode is economical for biosensor design (Table 1). However, not all CYP enzymes show measurable activity with these alternate oxidants, and if they do, the activities are usually very low (> 10-fold lower than the NADPH-dependent activity) [20,89]. The general mechanism by which peroxide supports CYP-mediated substrate oxidation remains unclear, because significant activity requires an appropriate CYP, substrate and peroxide. Thus, there is an opportunity to engineer CYP enzymes for enhanced utilization of alternate oxidants. Recently, several CYP enzymes were engineered by rational and directed evolutionary approaches for such a purpose (see section 5).

4. Approaches to engineer cytochrome P450

A detailed approach for CYP engineering has been recently reviewed [89-91]. In this section, we briefly describe these approaches, followed by some promising approaches that should be undertaken in the future (Figure 1, Table 2).

4.1 Rational approach

A rational approach requires previous knowledge of CYP structure-function relationships through chimeragenesis, site-directed mutagenesis, X-ray crystal structure, protein modeling and solution thermodynamics [11]. Information obtained from the above different sources provides an insight into the functional role of amino-acid residue(s), which can be replaced by another residue(s) for enhanced solubility, stability, expression and activity, as well as for altered substrate specificity and regioselectivity [11]. This method has been used to engineer several CYP enzymes, which are described in section 5.

4.2 Semi-rational approach

In this approach, the relative activity, stability or expression of CYP enzymes within the same subfamilies, such as CYP2B enzymes (2B1, 2B4, 2B6 and 2B11), is determined. Then, the amino-acid sequences between the set of enzymes, which show high or low activity, stability or expression, are compared to determine the residues that might be responsible for their functional differences [11]. Furthermore, the extent of solvent accessibility of these amino-acid residues is determined by using computational analysis based on available X-ray crystal structures or structural models generated through crystal structures. The selected residues that are thought to be responsible for decreased activity, stability or expression are then replaced by their counterpart residues by site-directed mutagenesis. By using this approach, Kumar and colleagues have recently engineered CYP2B6 and CYP2B11, which is described in section 5.

4.3 Conserved sequence motif

Conserved sequence motif (CSM) analysis is performed by multiple sequence alignment by using a linear combination of

237 dimensional physicochemical properties of 20 natural amino acids [92]. Braun's group has developed and applied this approach to extensively identify and investigate CSM in several proteins [93,94]. Recently, CSM approach has been applied to the CYP2 family and identified 20 motifs [95]. A significant correlation was found between the motifs/residues within the motifs and their known functions from genetic variants and site-directed mutants. This suggests that CSM analysis is a critical approach to identify the functional role of the residues within motifs. Furthermore, functional analysis of CSM 8 in CYP2B4 showed that Arg¹⁸⁷, which is extremely conserved within and across the family, is critical for protein stability and binding/metabolizing small ligands. However, in rare examples, CYP1A1 and CYP2E1 contain His at that position, suggesting that these enzymes can be engineered for enhanced stability and ligand selectivity by replacing His by Arg. Thus, this approach could be important to engineer CYP for enhanced expression, stability and activity as well as altered substrate specificity.

4.4 Directed evolution

Directed evolution does not require previous knowledge of protein structure and function. Rather it is performed by creating variants using error-prone PCR, saturation mutagenesis or recombination followed by screening and selection of variants for desired characteristics, such as enhanced activity, stability or expression, or for altered substrate specificity [89-91]. Directed evolution has been successfully applied to the design of industrial biocatalysts for enhanced catalytic efficiency, novel activity and increased stability [96,97]. The main requirement of directed evolution is to develop a high-throughput activity screening (HTS) system to screen/select mutants with desired properties (Table 2).

Development of an HTS requires finding a simple, cost-effective and efficient method to measure enzyme activity directly on a multi-well microplate. Therefore, the use of alternate oxidants, such as peroxides, which bypass the requirements for redox partners, lipids and NADPH, is highly desirable. Recently, a sensitive and cost-effective activity screening system has been developed for CYP101, CYP102, CYP1A2, CYP2A6, CYP2B1, CYP2C8, CYP2C9, CYP2C18, CYP2C19 and CYP3A4 by screening a number of enzymes, fluorescence substrates, peroxides and buffer systems (Table 2; unpublished observations) [89,98-104]. The activity screening was further expanded to screen/select CYP mutants for enhanced catalytic tolerance to temperature and organic solvents. This was performed by incubating mutants at T_{50} and $DMSO_{50}$ (temperature and DMSO concentration, respectively, at which the enzyme retains 50% activity), and selecting the mutants with higher activity than the wild type [105-107]. CYP mutants with increased stability can further be screened by monitoring protein unfolding transitions (induced by guanidium-HCl or temperature) by tryptophan fluorescence as described in the literature [108].

Flow cytometry has been recently applied to HTS applications in directed evolution for the analysis of single cells carrying mutant genes/proteins with improved activity [109-111]. The approach requires a sensitive and relatively rapid reaction that uses a fluorogenic substrate in whole-cell suspensions. The individual clones with improved activity are then sorted by fluorescence activated cell sorting techniques, which can sort ~ 2000 – 3000 cells/s. The author performed preliminary experiments with CYP2C9 using 7-dimethylamino-4-trifluoromethylcoumarin (C152) as a substrate and peroxides as an oxidant to validate the feasibility of this approach in *E. coli*. The results, in brief, showed that the cells that produced 7-amino-4-trifluoromethylcoumarin, the product of C152, showed approximately twofold higher fluorescence intensity (unpublished observations). Although the results are promising, this technique requires further optimization. This *in vivo* approach will potentially replace the conventional *in vitro* approach and significantly improve the cost effectiveness of directed evolution of CYP enzymes.

5. Examples of engineered cytochrome P450

5.1 Bacterial cytochrome P450

Because of very high turnover, solubility and expression in *E. coli*, bacterial CYP are relatively easy to design as biocatalysts. Among them CYP102 is the most exploited enzyme for this purpose because it contains both CYP and reductase domains in a single polypeptide, is characterized extensively and has a turnover rate of several thousands. CYP102 was successfully engineered by directed evolution for new and efficient hydroxylation pathways and for enabling the utilization of alternate oxidants, such as H_2O_2 [98]. CYP102 F87A mutant was created by site-directed mutagenesis, which is capable of hydroxylating lauric acid and myristic acid exclusively at ω -4 rather than at ω -1, ω -2 and ω -3 positions as done by wild type [112,113]. Subsequently, by using directed evolution, Arnold and other research groups have created several CYP102 mutants that displayed the following properties [112-124]: i) a more than twofold higher activity towards shorter chain length fatty acids; ii) higher activity for indole hydroxylation to indigo and indirubin; iii) hydroxylation of several other substrates, such as octanoic acid, *n*-octane, α - and β -ionone, naphthalene and anthracene; iv) up to a 100-fold increased activity with unnatural alkane substrates, such as benzene, styrene, 1-hexane and propane; v) enhanced activity towards smaller alkanes, especially direct conversion of ethane to ethanol; vi) strict substrate specificity and enantioselectivity; for example, the 77-9H mutant showed a 52% selectivity for the ω -position of *n*-octane and other mutants exhibited a range of oxidation of terminal alkanes to either (*R*-) or (*S*-) epoxides; vii) increased total activities by using air as oxidant in whole-cell bioconversions of propane to propanol under mild conditions; viii) increased activity towards indole hydroxylation; ix) generation of reactive metabolites from the drugs

clozapine, diclofenac and acetaminophen; and x) activity towards many non-natural substrates.

In addition, CYP102 was engineered for enhanced utilization of H_2O_2 , thermal stability and catalytic tolerance to temperature and organic solvents [105,106]. The evolved enzymes enhanced T_{50} from 43 to 61°C and displayed enhanced catalytic tolerance up to 10-fold in 2% tetrahydrofuran and 6-fold in 25% DMSO. Recently, a novel approach of recombining segments of the CYP102 gene from three different bacterial sources has given an unprecedented opportunity to design CYP102 with diverse characteristics. Upon screening and selection of several recombinant variants, > 73% of the chimeric CYP proteins were found to be catalytically active peroxygenases, and some were more thermostable than the parent proteins [90]. One CYP102 variant showed a > 10°C enhanced T_m compared with the wild type. Accordingly, these engineered CYP enzymes with increased thermostability can be used for industrial biocatalysis.

Achieving CYP-catalyzed oxidation of ethane is a key step in the pathway to CYP-catalyzed methane oxidation and opens new opportunities for bioconversion of natural gas to fuels and chemicals. Although the rate, total turnover number and coupling efficiency of ethane hydroxylation are low for practical purposes, it has been shown that continual improvement of CYP102 by further directed evolution will generate a biocatalyst with similar productivity to that obtained with propane (6000 total turnover) [117,121]. The CYP102 random mutant has been shown to be proficient in the oxidation of indole to indigo, and the catalytic efficiency is similar to that of the human CYP2A6 enzyme [122]. This suggests its potential application in the dye and horticulture industries. More recently, CYP102 was designed to metabolize several important drugs, such as buspirone, dextromethorphan, 3,4-methylenedioxymethylamphetamine (MDMA) and propranolol [118,119,123,125]. For example: i) the evolved CYP102 enzyme showed a 180 turnover in the H_2O_2 system and could produce > 70 mg of propranolol metabolites in a 1-L *E. coli* culture; ii) the CYP102 mutant 9-10A-F87A (50 nM) converted buspirone to (*R*)-6-hydroxybuspirone with a 3800 total turnover, 8.9% conversion; and iii) random clones of CYP102 enhanced the activity with dextromethorphan and MDMA by ~100-fold. Currently, the CYP102 mutants produce ~100 mg of metabolites per liter of bacterial cultures, which is close to an optimal requirement for industrial production (150 – 200 mg).

CYP101 primarily catalyzes the hydroxylation of camphor to 5-exo-hydroxycamphor. Using site-directed mutagenesis, CYP101 was engineered to create mutants for efficient oxidation of alkanes, halogenated hexanes, PCBs, PAHs and unnatural substrates [126-130]. While some variants showed enhanced activity by ≥ 19 -fold compared with the wild type towards linear alkanes such as pentane, hexane and heptanes, the F87W/Y96H/T101L/V247L mutant oxidized *n*-butane to secondary alcohol with a turnover of 750 min^{-1} [126,127]. The V247L and F87W/Y96F/V247L

mutants showed an increased turnover with the highly insoluble pentachlorobenzene without the need for surfactants or organic co-solvents [128]. The F87W and Y96H mutants greatly showed an enhanced activity with PAHs: phenanthrene, fluoranthene, pyrene and benzo[a]pyrene [129]. Furthermore, CYP101 was engineered with increased activity for the oxidation of diphenylmethane (by Y96F/I395G mutant) and styrene, and ethylbenzene (by Y96F/V247L mutant) [130]. In particular, the Y96F/V247L mutant shows a coupling efficiency of ~ 60% for styrene and ethylbenzene oxidation, with substrate oxidation rates of ~ 100 min^{-1} . More recently, CYP101 was engineered by directed evolution via a step-by-step adaptation to smaller alkanes, from hexane to butane to propane, and finally to ethane [131]. The turnover of ethanol synthesis was 78 min^{-1} .

5.2 Mammalian cytochrome P450

The design of new mammalian CYP biocatalysts is of high priority, because of their extreme substrate diversity and ability to metabolize numerous xenobiotics. Guengerich and colleagues successfully performed directed evolution of CYP1A2 and CYP2A6 initially by using random mutagenesis of substrate recognition sites and colony-based colorimetric and genotoxicity activity assays, respectively, followed by random mutagenesis of the whole cDNA and *in vitro* fluorescence-based enzyme assay systems [38-40,102,103,132-136]. The engineered CYP1A2 enzymes showed more than fivefold enhanced activity with 7-methoxyresorufin, 7-ethoxyresorufin, phenacetin and 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline [102]. Similarly, several CYP2A6 mutants exhibited higher activities towards several indole derivatives [103]. Furthermore, Guengerich's group generated a transgenic tobacco plant harboring engineered CYP2A6, together with an indole synthase (BX1) from maize, which accumulated an indole precursor, indican; a step forward towards development of indigo dyes [40].

Kumar, Halpert and colleagues have engineered several mammalian CYP2B enzymes for enhanced expression, stability, activity, substrate specificity and regio-selectivity by using rational and directed evolution approaches (reviewed in [11] and [89]). CYP2B1, CYP2B4, CYP2B6 and CYP2B11 were engineered for enhanced expression and solubility by using a rational approach [137]. CYP2B enzymes were engineered based on pioneering work by Cosme and Johnson, who engineered N-terminal (membrane-bound domain) of CYP2C5 by deleting 21 residues and replacing 5 hydrophobic-to-basic residues [138]. The engineered CYP enzymes (termed dH; N-terminal deleted and C-terminal His-tagged) were soluble and exhibited a 5- to 10-fold higher expression than the wild type. N-terminal engineering led to a breakthrough in the crystallization of the first mammalian CYP enzyme CYP2C5 [139], followed by several other mammalian CYPs, such as CYP2B4, CYP2C5, CYP2C8, CYP2C9 and CYP3A4 (reviewed in [11,140,141]).

Based on known chimeragenesis, protein modeling and X-ray crystal structures, several CYP2B enzymes were engineered for enhanced activity, stability and expression as well as for altered regio- and stereoselectivity by site-directed mutagenesis of active-site, substrate access channel and CSM residues (reviewed in [11,89,91,142-147]; unpublished observations). Some examples are: i) I480V of CYP2B1 and L363V of CYP2B11, which exhibited higher, 2',3,3',6,6'-hexachlorobiphenyl hydroxylase activity; ii) residue 209 of F-helix when replaced to Ala in CYP2B1dH, CYP2B4dH and CYP2B11dH showed a 5-, 50- and 4-fold enhanced catalytic efficiency, respectively, with testosterone; iii) replacement of seven simultaneous active-site residues of CYP2B1dH to the corresponding CYP2C5dH residues converted progesterone 16 α - to 21-hydroxylase activity; and iv) active-site substitution Ile³⁶³→Ala in 2B4 showed a 150-fold enhanced catalytic efficiency for testosterone hydroxylation.

More recently, Kumar, Halpert and colleagues have engineered human CYP2B6dH for enhanced expression and stability by using a semi-rational approach (Figure 1) [142]. The mutants L198M, L264F and L390P showed an approximately threefold higher expression than did CYP2B6dH. In addition, L264F exhibited an enhanced stability against thermal and chemical denaturation compared to that with CYP2B6dH. Furthermore, CYP2B6dH and CYP2B11dH were engineered by replacing their residues, which are identical in both, to the corresponding residues that are identical in relatively more stable CYP2B1dH and CYP2B4dH. The CYP2B6dH and CYP2B11dH mutants showed increased stability compared to their respective wild-type enzymes (unpublished observations). The engineering of CYP2B6dH led to its structure-function studies, which used biochemical, biophysical and X-ray crystallography ([142]; unpublished observations). The engineered CYP2B6dH enzymes could be better candidates for GDEPT, provided these show enhanced expression and stability in tumor cells.

Directed evolution of CYP2B1dH L209A created V183L/F202L/L209A/S334P mutant, which enhanced the utilization of H₂O₂ for 7-ethoxy-4-(trifluoromethyl) coumarin (7-EFC) *O*-deethylation by sixfold (Figure 1) [100]. Subsequently, the F202L/L209A/S334P mutant was constructed in CYP2B1dH and CYP2B1 full-length backgrounds, which are among the most active P450s for the metabolism of several substrates, such as 7-EFC, 7-benzoyloxyresorufin (7-BR), testosterone and benzphetamine in the standard NADPH system. Furthermore, L209A/S334P and L209A/V183L mutants showed a >2.5-fold enhanced catalytic efficiency with the anticancer prodrugs CPA and IFA for 4-hydroxylation compared with the wild type [100]. These mutants also showed more than twofold decreased catalytic efficiency for N-dechloroethylation (toxic reaction). The V183L/F202L/L209A/S334P mutant (QM) was further subjected to directed evolution to enhance catalytic tolerance to temperature and DMSO [107]. While the CYP2B1dH QM/L295H mutant exhibited a significantly higher activity

than did QM at a broad range of temperatures (35 – 55°C), the QM/K236I/D257N/L295H mutant displayed a more than twofold higher activity than did QM at nearly the entire range of DMSO concentrations (Figure 1). Furthermore, based on the mutations found in the CYP2B1dH QM, corresponding substitutions were made in CYP2B11dH (the most active enzyme for the activation of CPA and IFA) [143]. Among the mutants created, V183L showed a: i) 4-fold increased k_{cat} for 7-BR debenzoylation; ii) 4.7-fold increased k_{cat}/K_m for testosterone 16 α -hydroxylation; and iii) 1.7-fold higher k_{cat}/K_m for the activation of CPA and IFA, compared with CYP2B11dH. Especially, the CYP2B11dH V183L mutant showed an approximately fourfold decreased K_m (0.060 and 0.030 mM for CPA and IFA, respectively) compared to that with CYP2B11dH [143].

Gillam and colleagues applied a restriction enzyme-mediated DNA shuffling approach within the subfamily to design CYP1A and CYP2C enzymes [20,148-151]. These engineered CYP enzymes showed distinct and novel activity profiles, including those with drugs. For example, a random sample of 26 CYP2C clones (shuffled from 2C8, 2C9, 2C18 and 2C19) revealed two clones with activity towards luciferin 6'-methyl ether, one towards 6'-deoxyluciferin and five towards diclofenac 4'-hydroxylation [149]. Of 96 clones screened on solid media, one showed elevated indigo production compared to the parental forms. Similarly, from the clones obtained upon CYP1A (shuffled from CYP1A1 and CYP1A2) different activity profiles were seen with higher specific activity on individual compounds (e.g., clone 22; nine times the CYP1A1 specific activity toward luciferin 6'-chloroethyl ether); novel activities (e.g., clone 35; activity toward 6'-deoxyluciferin and *p*-nitrophenol) and broadening of substrate range observed in particular clones (e.g., clone 9; activity toward both selective substrates luciferin 6'-methyl ether and luciferin 6'-chloroethyl ether as well as toward 6'-deoxyluciferin and *p*-nitrophenol) [150].

CYP2D6 was recently engineered (T309V) by site-directed mutagenesis of the active site residues, Thr-309, which showed a 2- to 4-fold enhanced activity for CuOOH-mediated N-demethylation of 7-methoxy-4-(aminomethyl)-coumarin, MDMA and dextromethorphan [152]. CuOOH-supported activity of T309A, however, was enhanced by 70-fold for the oxidation of bufuralol compared with the findings with the wild type, suggesting its application in the synthesis of bufuralol metabolites. Subsequently, a T309A mutant was created in CYP3A4, which showed the two and fourfold enhanced utilization of CuOOH ($k_{cat}/K_{m,CuOOH}$) for the oxidation of 7-benzoyloxy-4-(trifluoromethyl)coumarin and 7-benzoyloxyquinoline (7-BQ), respectively, compared with the wild type [101]. Directed evolution of CYP3A4 yielded several mutants (L216W, F228I, F242V and T433S), which showed a 2- to 4-fold enhanced utilization of CuOOH with 7-BQ compared with the wild type [101]. More importantly, T433S in the presence of cytochrome *b*₅ in a CuOOH-supported reaction showed an 80% conversion of

testosterone to 6 β -OH testosterone with a total turnover of 180 min⁻¹. An effort was made to engineer CYP3A4-CPR for altered enzyme cooperativity for 7-BQ. 7-BQ yields a sigmoidal curve with CYP3A4, which become hyperbolic in the presence of 25 μ M α -naphthoflavone [101,153]. Upon directed evolution, we created two CYP3A4 mutants, which exhibited altered enzyme cooperativity with 7-BQ (unpublished observations).

6. Conclusion

In the past decade, a need for CYP biocatalysts has emerged because of their use (Figure 1) for: i) synthesis of drugs and drug metabolites in the pharmaceutical industry; ii) synthesis of indigo-based colored compounds in the dye and horticulture industries; iii) synthesis of agrochemicals and other fine chemicals in the food and chemical industries; iv) P450-based GDEPT for the activation of the anticancer prodrugs CPA and IFA; v) biosensor design to monitor drug levels on blood plasma in clinics and hazardous components in the food industry; and vi) bioremediation using transgenic plants and immobilized systems. Because the pharmaceutical/biotechnological industries and scientific community have recognized the need for CYP biocatalysts, they are now engaged in designing CYP by rational, semi-rational, CSM and directed evolution approaches to improve catalytic efficiency, stability, expression, cofactor requirements and the suitability of CYP-CPR fusion enzymes, as well as regio- and stereoselective hydroxylation of specific substrates (Table 2). In addition, researchers are actively searching for alternate oxidants, such as peroxides, metal powders and metal electrodes (Table 1), to acquire simple, cost-effective and efficient ways of performing CYP-mediated reactions. Engineering of CYP enzymes by rational, semi-rational and CSM approaches is limited because of an incomplete understanding of the structure-function relationships of these enzymes. Therefore, directed evolution involving random mutagenesis and DNA shuffling, followed by simple and effective HTS methods, can potentially engineer CYP for novel and enhanced properties (Table 2). In addition, developing HTS by using flow cytometry is an extremely powerful approach for designing CYP. Rational and directed evolution approaches have been successfully applied to engineer bacterial and mammalian CYP enzymes for enhanced activity, stability and expression in *E. coli*, as well as for altered substrate specificity and regio- and stereoselectivity and redox partner requirements. Thus, the future of engineered CYP enzymes in industrial, medical and environmental applications appears bright.

7. Expert opinion

7.1 Applications of cytochrome P450 biocatalysts

Approximately 30% of drug candidates are terminated at clinical trials due to drug and metabolite toxicities in animals and humans [154]. Therefore, 'drug metabolites in safety testing' has become an integral part of drug development

in the 21st century [155]. This has led to an increased demand for drug metabolites to facilitate research for studying their possible adverse effects in animals and humans. Thus, freeze-dried and immobilized CYP are expected to be used for bulk synthesis of drug metabolites. In addition, a CYP nanobiochip has the potential as a bioreactor to generate drug metabolites. Due to differential drug responses as a result of CYP polymorphism and drug interactions, we expect to see a rapid growth in the next 5 – 10 years in the design of CYP biosensors. Application of CYP biosensors can further be expanded into nanotechnology, which would require coupling of CYP enzymes with electrical circuits in an electrode in nonphysiological environments. We predict rapid developments in the practical use of targeted CYP-based GDEPT for cancer treatment because it has a tremendous potential for reducing adverse effects of chemotherapy. However, although the clinical trials using CYP2B6 are promising, delivery of these genes and prodrugs to targeted cancer cells without affecting the non-malignant cells still poses a serious concern. CYP-cloned transgenic plants that have ability to detoxify soil contaminants, such as atrazine, norflurazon, metolachlor and sulfonylureas, will not only improve agricultural production, but will also increase unused lands that are polluted by industrial toxic wastes. In addition, the researchers have proposed the use of recombinant CYP in bacterial systems for wastewater treatment. An increasing concern about environmental pollution and global warming reflects a need for green chemistry that uses CYP biocatalysts in the 21st century. However, the use of transgenic plants may be of general concern in public regarding their possible long-term health issues, especially in the European countries.

7.2 Cost-effective approaches to utilize cytochrome P450 biocatalysts

There are significant opportunities for optimizing artificial recombinant fusion proteins by improving the coupling between CYP and reductase domains and exploiting alternative electron transport partners. In most cases, the catalytic rates with fusion enzymes are not optimal, which suggest to us that there is a potential for further improvements in creating more effective fusion enzymes. Because fusion enzymes still require the expensive cofactor NADPH, alternative means have been proposed: i) light-driven NADPH synthesis in chloroplast in the presence of CYP-CPR fusion enzymes [156]; and ii) engineering CYP system for increased specificity towards relatively inexpensive NADH [157]. Although *in situ* NAD(P)H (re)generation may be the most practical source of electrons, recent studies have provided evidence that peroxides and metal powders could be a cheap and effective alternative for *in vitro* applications, especially in industrial synthesis (Table 1). In the case of biosensor design, metal electrodes immobilized with CYP enzyme are the only source of electrons. Bistronic expression of CYP and CPR in a retroviral vector has proven to be the most efficient way to activate CPA and IFA in cancer cells. A co-expression system

also appears to be the most effective in generating transgenic plants for phytoremediation. Alternatively, CYP enzymes can be engineered to interact with the host cell reductase(s) as well as for their subcellular and tissue distributions.

7.3 Mammalian versus bacterial cytochrome P450 biocatalysts

It is generally believed that bacterial CYP enzymes, which possess higher turnover, stability and expression than their mammalian counterparts, are the most suitable candidates for practical purposes, even for the synthesis of non-natural substrates [14]. Furthermore, bacterial CYP biocatalysts can function or can be tailored to function under extreme conditions of temperature, pH, buffer system or solvent. Indeed, bacterial CYP enzymes have been recently engineered for the metabolism of several non-natural substrates, such as smaller alkanes and drugs. However, it remains to be seen whether bacterial enzymes can be tailored for the regio- or stereoselective synthesis of drugs and drug metabolites of variable size, shape and geometry. Recent discoveries revealed that, in contrast to the bacterial, mammalian CYP enzymes have large and flexible active sites, which are capable of adapting their conformation based on the size, shape and geometry of substrates [140,141]. Thus, for regio- and stereoselective chemistry, the engineering of mammalian CYP enzymes for enhanced activity for their own array of substrates is easier than engineering bacterial CYP to accommodate the vast majority of substrates of mammalian CYP. In addition, the engineering of mammalian CYP enzymes may be the only way to improve cancer gene therapy using GDEPT and phytoremediation using transgenic plants. Recently, several mammalian CYP enzymes have been engineered, which are expected to find application(s) in drug and chemical synthesis, dye production, horticulture, biosensor, cancer gene therapy and/or bioremediation.

- Cytochrome P450 (CYP) enzymes are central to the study of toxicology because they are involved in the clearance of a majority of marketed drugs and abused substances, activation of prodrugs, drug–drug and drug–food interactions, and metabolism of carcinogens and other pollutants.
- In recent years, there has been an increasing realization of the power of CYP biocatalysts for the industrial synthesis of

bulk chemicals, pharmaceuticals, agrochemicals and food ingredients, especially when high regio- and stereoselective hydroxylation is required. In addition, there is an increasing demand for CYP biocatalysts in the detoxification of environmental contaminants and gene-directed enzyme prodrug therapy for cancer treatment.

- CYP enzymes require a complicated source of electrons via redox partners and NADPH, and, therefore, it is critical to find alternate strategies, such as CYP-reductase bicistronic expression system, peroxides and metals to bypass these requirements for practical applications.
- We briefly describe CYP engineering approaches, such as rational, semi-rational, conserved sequence motif and directed evolution.
- Because of very high turnover, solubility and expression in *Escherichia coli*, bacterial CYP are relatively easy to design as biocatalysts. Among them, CYP102 is the most exploited enzyme for this purpose because it contains both CYP and reductase domains in a single polypeptide, is characterized extensively, and has a turnover rate of several thousands. The design of new mammalian CYP biocatalysts is of high priority because of their extreme substrate diversity and ability to metabolize numerous xenobiotics.
- Rational and directed evolution approaches have been successfully applied to engineer bacterial and mammalian CYP enzymes for enhanced activity, stability and expression in *E. coli*, as well as for altered substrate specificity and regio- and stereoselectivity, and redox partner requirements.
- The future of engineered CYP enzymes in industrial, medical and environmental applications appears bright. In fact, several bacterial and mammalian CYP enzymes have been engineered, which are expected to find application(s) in drug and chemical synthesis, dye production, horticulture, biosensor, cancer gene therapy and/or bioremediation.

Declaration of interest

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