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Review Article

Functional expression and regulation of eukaryotic cytochrome P450 enzymes in surrogate microbial cell factories☆

Pradeepraj Durairaj[a], Shengying Li[a,b,*]

[a] State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, Shandong, China
[b] Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, Shandong, China

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ABSTRACT

Cytochrome P450 (CYP) enzymes play crucial roles during the evolution and diversification of ancestral monocellular eukaryotes into multicellular eukaryotic organisms due to their essential functionalities including catalysis of housekeeping biochemical reactions, synthesis of diverse metabolites, detoxification of xenobiotics, and contribution to environmental adaptation. Eukaryotic CYPs with versatile functionalities are undeniably regarded as promising biocatalysts with great potential for biotechnological, pharmaceutical and chemical industry applications. Nevertheless, the modes of action and the challenges associated with these membrane-bound proteins have hampered the effective utilization of eukaryotic CYPs in a broader range. This review is focused on comprehensive and consolidated approaches to address the core challenges in heterologous expression of membrane-bound eukaryotic CYPs in different surrogate microbial cell factories, aiming to provide key insights for better studies and applications of diverse eukaryotic CYPs in the future. We also highlight the functional significance of the previously underrated cytochrome P450 reductases (CPRs) and provide a rational justification on the progression of CPR from auxiliary redox partner to function modulator in CYP catalysis.

1. Introduction

Cytochrome P450 monooxygenases (CYPs or P450s) utilize dioxygen and two units of reducing and proton equivalents to catalyze the exemplary monooxygenation reactions. This superfamily of tetrapyrrole heme-thiolate enzymes have the unparalleled ability to catalyze a vast variety of oxidative, peroxidative and reductive reactions such as C–H bond hydroxylation, C=C bond epoxidation, heteroatom oxygenation, and many uncommon transformations (Lamb and Waterman, 2013; Dubey and Shaik, 2019; Winkler et al., 2018). The multifunctional CYPs with great catalytic versatility and broad substrate scope are regarded as promising biocatalytic targets, and play essential roles in biotechnological, chemical and pharmaceutical applications, despite the challenges associated with this enzyme superfamily (Lamb and Waterman, 2013; Bernhardt and Urlacher; 2014, Wei et al., 2018). CYPs mediate pivotal steps in primary metabolic pathways and extensively participate in biosynthesis of diverse secondary metabolites (Zhang and Li, 2017). Remarkably, CYPs also act as a means of supplying higher organisms with essential molecules (*e.g.*, sex hormones, brain neurotransmitters, *etc.*) and play a vital role in protecting the biosystems (Dubey and Shaik, 2019). The promiscuous CYPs involved in the oxidation of diverse substrates display high levels of regio- and stereoselectivity (Šrejber et al., 2018; Munro et al., 2013). Interestingly, in many cases a single amino acid change can alter the CYP's reactivity, selectivity and catalytic efficiency of closely related enzymes (Sezutsu et al., 2013). Based on the global substrate specificity, CYP enzymes fall into two major groups (Urban et al., 2018): (a) the CYPs showing strong preference for a single substrate and (b) the CYPs with loose substrate specificity. Classical examples of the former group include most of the microbial and plant CYPs along with some mammalian CYPs involved in steroidogenesis and eicosanoid biosynthetic pathways, while the latter one encompasses the mammalian CYPs involved in drug/xenobiotic metabolism (Girvan and Munro, 2016; Bernhardt, 2006).

As CYPs are ubiquitous enzymes with roots in all the eukaryotic taxa, it is too abundant and intricate to elaborate all the systems in one review. Thus, herein, we comprehensively summarize the diversity, versatility and complexity of major eukaryotic CYPs with a special focus on fungal, plant and human CYP systems. This review is aimed to provide a

Abbreviations: CYP, Cytochrome P450; CPR, Cytochrome P450 reductase; Cyt B5, Cytochrome b5; CBR, Cytochrome b5 reductase; Adx, Adrenodoxin; AdR, Adrenodoxin reductase; ET, Electron transfer; TMD, Transmembrane domain.

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* Corresponding author.
E-mail address: lishengying@sdu.edu.cn (S. Li).

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rationalized and consolidated analysis of the membrane-bound eukaryotic CYPs, and the recent conceptual transformation of redox partners from auxiliary electron transfer proteins to CYP functional modulators or regulating partners. Importantly, we elaborate the critical parameters/factors involved in heterologous expression with some recent representative examples and pay special attention on the surrogate cell factories that have enabled successful expression and functional studies of eukaryotic CYPs. The focal point of this review is to address the challenges associated with the heterologous expression of eukaryotic CYPs.

2. Diversity of eukaryotic CYP systems

Evolutionarily, the CYP genes were originated several billion years ago, and are presumed to have prevailed in the last universal common ancestor (Sezutsu et al., 2013). CYPs therefore possess a conserved structural fold despite a considerable variation in their primary sequences. The ubiquitous CYPs are present in all biological kingdoms, and the explosive genome sequencing projects have identified several hundred thousands of distinct CYP genes (Nelson et al., 2013). However, there are some exceptions since a subgroup of anaerobic microorganisms and microaerophiles lack CYPs (*e.g., Escherichia coli*). The CYP numbers of eukaryotic organisms range from as low as two genes in yeast (*e.g., Schizosaccharomyces pombe*) to as many as several hundred genes in plants (*e.g.*, 455 in *Oryza sativa* and 272 in *Arabidopsis thaliana*) (Nelson et al., 2013; Urlacher and Girhard, 2012). It is evident that CYPs may not be an essential component of prokaryotes, but it is vital for the emergence of eukaryotic cells in the ancient world of prokaryotic life as sterol biosynthesis (an essential constituent of the plasma membrane) depends on the essential CYP-catalyzed reaction, namely, sterol 14α demethylation by CYP51 (Sezutsu et al., 2013; Omura, 2013). CYPs have not only played a key role in the origin of ancestral monocellular eukaryotic organisms, but also significantly contributed to diversification of eukaryotes. Adaptation of various living organisms to constant environmental changes has resulted in the divergent evolution of CYPs among different taxa, thereby causing significant differences in their catalytic activities and physiological functions (Omura, 2018). The multifunctional eukaryotic CYPs participate in primary and secondary metabolisms by playing key roles in drug/xenobiotic metabolism in humans, and natural product biosynthesis in fungi and plants (Dubey and Shaik, 2019; Bernhardt and Urlacher, 2014; Urlacher and Girhard, 2019). The major physiological functions of eukaryotic CYPs are also related to their essential roles in the synthesis and regulation of several metabolites including the phytohormones (plants), molting and juvenile hormones (insects), as well as the steroid and peptide hormones (vertebrates).

During the long course of evolution, the CYPs of prokaryotes and eukaryotes have differentiated remarkably, and as a result there are no universally conserved residues except the heme-iron coordinating cysteine across the superfamily. CYPs encompass diverse gene families with a perplexing complexity within and between species. Consequently, the numbers of CYP gene families differ a lot among different taxa – bacteria (591 CYP families), fungi (805 CYP families), plants (277 CYP families), insects (208 CYP families), mammals (18 CYP families), archaea (14 CYP families), and viruses (6 CYP families), which reflects the substantial sequence and functional diversity of CYPs (Durairaj et al., 2016; Nelson, 2018). The number of newly recognized CYP sequences has been exponentially increasing with > 300,000 candidates until 2018 (Nelson, 2018), with a possibility of reaching more than one million by 2025 through the Genome 10 K, i5k and GIGA (Global Invertebrate Genomics Alliance) sequencing projects. Though the CYP families share relatively low sequence similarities, the common CYP fold is well conserved (Šrejber et al., 2018). Hitherto, nomenclature has been assigned for > 41,000 CYPs, which includes about 16,000 plant CYPs, 8,000 fungal CYPs, 3,000 bacterial CYPs, and 2,500 mammalian CYPs (Urlacher and Girhard, 2019; Nelson, 2018).

3. Eukaryotic CYP catalytic cycle

CYPs irrespective of prokaryotic or eukaryotic origin catalyze the prototypical monooxygenation reaction with a common equation as follow: $RH + O_2 + 2\ e^- + 2\ H^+ \rightarrow ROH + H_2O$ by inserting one oxygen atom of dioxygen into the substrate, while the second oxygen atom is reduced to form a water molecule (Urlacher and Eiben, 2006; McLean et al., 2005). The general catalytic mechanism of a eukaryotic CYP-catalyzed substrate hydroxylation comprises the following sequential steps (Fig. 1): (a) Substrate binding: the substrate (RH) gains access to the active site and binds with the oxidized state of heme iron (Fe^{3+}) by displacing a water molecule over the heme iron; (b) First reduction: the major electron transfer (ET) partner, cytochrome P450 reductase (CPR) transfers the first electron to the ferric heme iron (Fe^{3+}) and forms the reducing ferrous dioxy (Fe^{2+}–O_2) complex upon binding of molecular oxygen. Herein, cytochrome b5 (Cyt B5) is not in a position to donate the first electron, as it cannot overcome the redox potential barrier (Barnaba et al., 2017); (c) Second reduction: In this rate-determining step, the second electron can be transferred either from the CPR or Cyt B5, together with the first protonation leading to a super-nucleophilic ferric hydroperoxy (Fe^{3+}–OOH, Compound 0) complex; (d) Oxygen cleavage: heterolytic O–O bond cleavage, the second protonation along with the concurrent loss of a H_2O molecule leads to the formation of the highly reactive oxyferryl porphyrin π-cation radical (Fe^{4+}=O, Compound I); (e) Product formation: The hydroxylated product (ROH) is formed upon the abstraction of a hydrogen atom by Compound I and the following OH rebound; and (f) Product release: the monooxygenated product is finally dissociated from the active site, while the enzyme restores its initial ferric state (Fe^{3+}–OH_2) and is ready to react again. An alternative route, the peroxide shunt pathway involves the direct binding of H_2O_2 to the ferric heme iron without the need of O_2, NADPH, and redox partner(s) (Zhang and Li, 2017; Munro et al., 2013; Podust and Sherman, 2012).

4. Heterogeneity of eukaryotic CYP electron transport pathways

To execute the above-described catalytic cycle (Fig. 1), CYPs recruit diversified redox partners to shuttle reducing equivalents. According to the composition of the protein components involved in electron transfer (Fig. 2), CYP systems are categorized into ten distinct classes, of which the eukaryotic CYPs fall into **Class I mitochondrial**: NADPH→[AdR]→[Adx]→[P450], **Class II microsomal A**: NADPH→[CPR]→[P450], **Class II microsomal B**: NADPH→[CPR]→[Cyt B5]→[P450], **Class II microsomal C**: NADH→[CBR]→[Cyt B5] →[P450] (CBR: NADH-dependent cytochrome b5 reductase), **Class VIII**: NADPH→[CPR-P450], **Class IX**: NADH→[P450], and **Class X**: [P450] systems (Hannemann et al., 2007).

Class I system, the most common prokaryotic (bacterial) P450 system, is also present in certain eukaryotes especially mammals, but not in plants and fungi. It is hypothesized that the microsomal CYP is the ancestor of mammalian mitochondrial CYP system, wherein the ER-targeting sequence at the amino-terminus of microsomal CYP was transformed to a mitochondria-targeting sequence possibly by accumulated point mutations during the course of evolution (Omura and Gotoh, 2017). Eukaryotic Class I mitochondrial P450 system consists of a membrane-bound CYP and a reducing system comprising two components, adrenodoxin (Adx) located in the mitochondrial matrix and NADPH-dependent adrenodoxin reductase (AdR) bound to the inner mitochondrial membrane (Fig. 2A). Most of eukaryotic CYPs (especially fungal, plants and mammals) adopt a Class II system and catalyze extremely diverse reactions in terms of both reaction types and substrate scope. Class II microsomal A system is the most common in eukaryotes, which comprises two integral membrane proteins, namely, CYP and CPR. The CPR is responsible for the sequential transfer of reducing equivalents (two electrons) from NAD(P)H to the heme-iron via the prosthetic cofactors FAD and FMN (Fig. 2B). Class II microsomal B system recruits

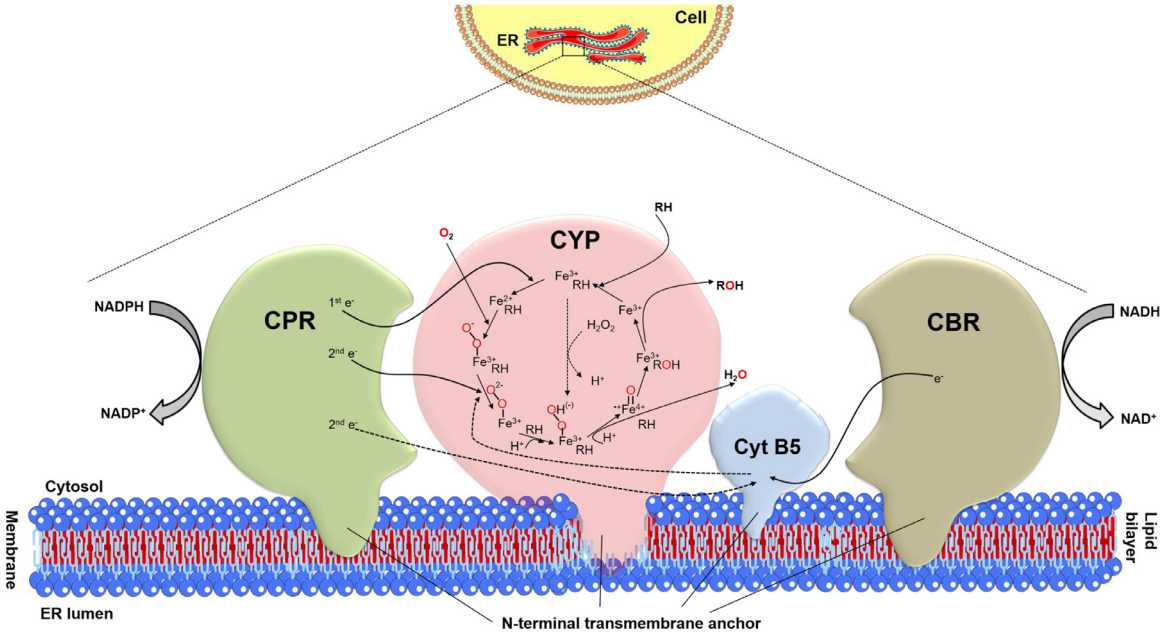

Fig. 1. Eukaryotic CYP catalytic cycle and the electron transfer mechanism of CPR/Cyt B5/CB5R redox system.

a third auxiliary protein, Cyt B5, to transfer the second electron to the oxyferrous CYP (Fig. 2C). Class II microsomal C system encompasses Cyt B5 and CBR, where the electrons are directly transferred to certain CYPs without the need of CPR (Ichinose and Wariishi, 2012; Stiborová et al., 2016) (Fig. 2D). In addition to the abundant Class II system, some fungal species also encompass Class VIII and Class IX systems. In Class VIII system, the N-terminal heme domain is naturally fused to a C-terminal CPR domain through a short peptide linker (P450foxy) (Shoun and Takaya, 2002), in which the electrons are supplied in an intra-molecular manner (Fig. 2E). Thus, this class of CYPs that are mainly originated from fungi are catalytically self-sufficient. Class IX has an exceptional single-component system (*e.g.*, P450nor), whereby CYP is able to accept electrons directly from NAD(P)H without the requirement of any additional redox partners (Shoun and Takaya, 2002). Class IX is functionally different from all other CYPs because the soluble P450nor mediates denitrification in fungi by catalyzing the reduction of two molecules of NO to form N_2O (Fig. 2F). Remarkably, while most CYP systems depend on redox partner(s) for ET, P450s belonging to Class X system neither require molecular oxygen nor any electron source for their catalysis (Fig. 2G). Class X CYPs capable of using an independent intramolecular ET system have been reported in some plant (*e.g.*, CYP74A-D) and mammalian (*e.g.*, CYP5A1 and CYP8A1) species (Hannemann et al., 2007).

5. Significance of N-terminal transmembrane domain in eukaryotic CYP systems

The eukaryotic CYPs along with its counterparts are characteristically attached on the cytoplasmic side of endoplasmic reticulum (ER) or the matrix side of the inner mitochondrial membrane. With the progress of modern biotechnology, a better understanding of eukaryotic CYPs and its association with membranes has been emerging. The eukaryotic CYPs which were once considered to be integral membrane protein with several transmembrane segments, were recently determined to be membrane-associated proteins with a short N-terminal membrane anchoring domain (Šrejber et al., 2018). To date, the knowledge about these membrane-bound CYPs has been assembled into a defined model in course of progression and attracts growing attention. The new model depicts that the eukaryotic CYPs are anchored to the ER through an N-terminal transmembrane α-helix with the N-terminus lying on the luminal side, while the catalytic domain of CYP reclines on the cytosolic side (Fig. 1) (Šrejber et al., 2018; Urban et al., 2018). The CYP catalytic domain is slightly submerged in the lipid bilayer with its proximal side facing cytosol, whereas the N-terminus and F/G loop lying on the distal side of the enzyme are deeply immersed. Apparently, the signal peptide sequence of N-terminal anchor governs the trafficking of CYPs into ER or mitochondria (Šrejber et al., 2018). The phospholipid composition in the membrane-bound CYP systems is crucial for protein folding and stability, whilst plays an essential role in the ET required for CYP monooxygenation (Barnaba et al., 2017). Of note, the composition of membrane influences the function of CYP, as the amino acid composition and configuration of N-terminal helix varies significantly in different CYP families (Šrejber et al., 2018; Gideon et al., 2012). The N-terminal transmembrane domain (TMD) is mainly composed of 20-30 hydrophobic amino acid residues which facilitates the interaction with hydrophobic ER membrane environment.

Though the primarily role of TMD is to mediate association to the lipid membrane, it also plays an essential role in the interaction between CYP and CPR, substrate binding and other downstream catalytic steps (Maroutsos et al., 2019).The major diflavin reductase, CPR carrying both FAD and FMN is also attached to the membrane similar to CYP via an N-terminal membrane-binding domain (Fig. 1) (Mukherjee et al., 2021). A typical microsomal CYP system forms a membrane-bound protein–protein complex through the electrostatic interactions between the positively charged residues on the proximal side of CYP and negatively charged amino acids of the CPR's FMN domain (Šrejber et al., 2018; Scott et al., 2016). The membrane binding domain significantly contributes to the efficient electron transfer from CPR to CYP, and the interactions correspond to the oxidation state of CPR and binding of cofactor (Mukherjee et al., 2021; Xia et al., 2019). The membrane environment thus plays a crucial role in mediating the formation of binary complex and influences the interaction/cooperation of integral CPR-CYP electron mediating system (Gideon et al., 2012). The active site of eukaryotic CYP is profoundly concealed within the structure, and connected to the protein surface and exterior by multiplex access/egress channels. As the heme is tilted towards the membrane surface, the binding and positioning of CYPs on membranes is vigorous and catalytically relevant. It has to be noted that the charge of phospholipid bilayer is likely to alter the orientation of the CYP catalytic domain relative to the membrane. The orientation of CYPs in the phospholipid bilayer could facil-

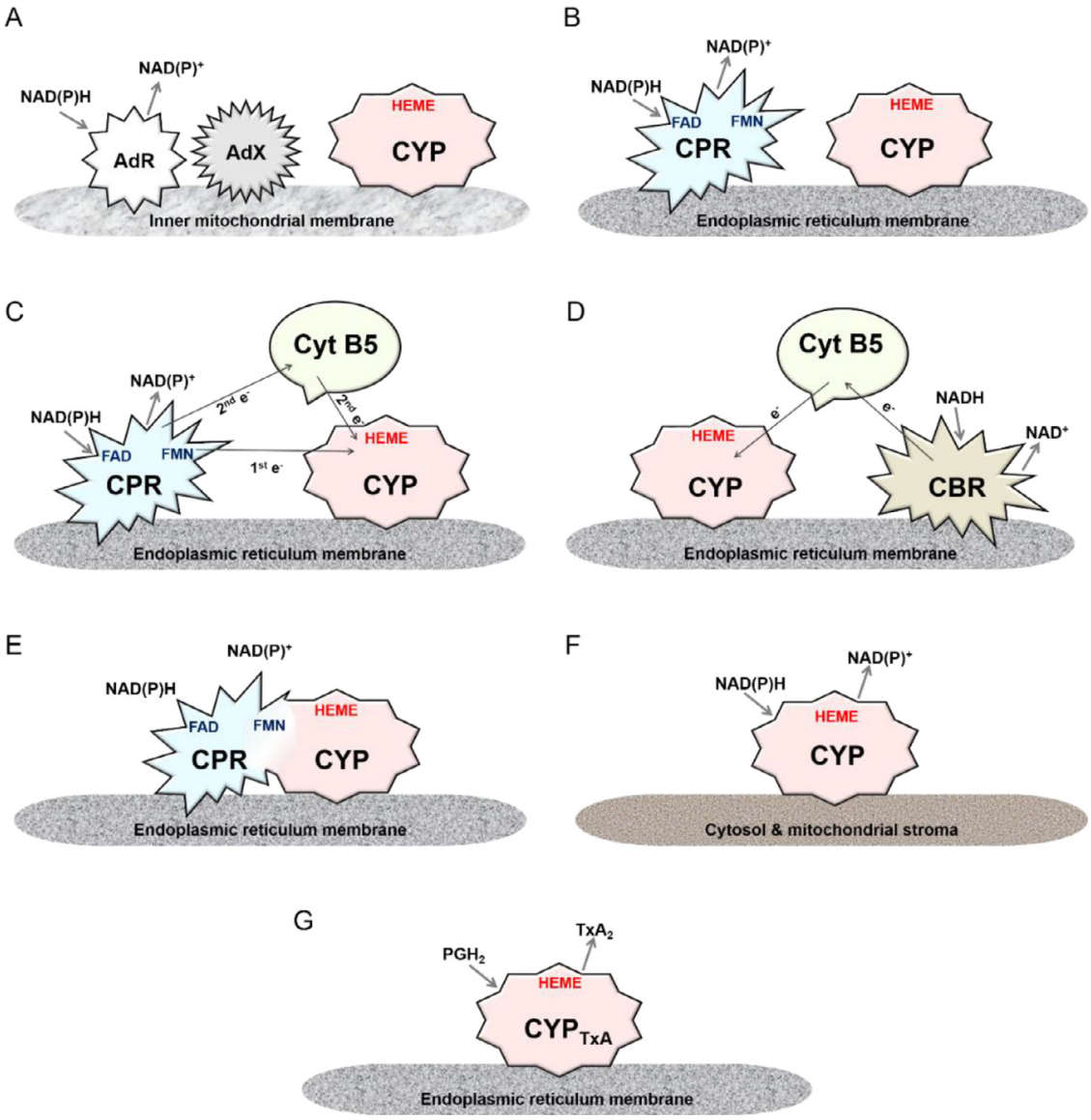

Fig. 2. Diversity of eukaryotic CYP systems based on the topology of protein components. A. Class I (mitochondrial: NADPH→[AdR]→[Adx]→[P450]); B. Class II microsomal A: NADPH→[CPR]→[P450]; C. Class II microsomal B: NADPH→[CPR]→[Cyt B5] →[P450]; D. Class II microsomal C: NADH→[CBR]→[Cyt B5]→[P450]; E. Class VIII (NADPH→[CPR-P450]); F. Class IX (NADH→[P450]); and G. Class X [P450] systems.

itate ease in substrate access and product egress channels as the B/C and F/G loops are pointed beneath the phospholipid head groups, while the solvent channel is positioned toward the membrane-water interface (Urban et al., 2018; Berka et al., 2013). In membrane-bound eukaryotic CYPs, the substrate binds to the active site of CYP (substrate binding pocket) by entering through cytosol or membrane interior resulting in possible substrate/product trafficking, whereas the substrate access and product egress are directly mediated by the same channel in bacterial CYPs.

While most of the prokaryotic CYPs are soluble, almost all the membrane-bound eukaryotic CYPs are insoluble upon bacterial expression which hinders the functional and structural studies (Denisov et al., 2012; Zelasko et al., 2013). Several eukaryotic CYPs of fungal, plant and human origins were reported to be expressed in bacterial systems mostly upon N-terminal sequence changes via deletions or modifications. Of note, the multifunctional eukaryotic CYP protein structures are still under-represented in the Protein Data Bank (https://www.rcsb.org/) owing to the difficulties associated with the membrane-bound nature. Indeed, except for the two full-length microsomal CYPs, *viz.*, CYP51 lanosterol 14-α demethylase of *Saccharomyces cerevisiae* (PDB ID: 4LXJ and 5EQB) (Monk et al., 2014) and CYP19 aromatase of *Homo sapiens* (PDB ID: 3EQM) (Ghosh et al., 2009), the TMDs are almost always cleaved in other structures to obtain complete solubilisation and subsequent crystallization. The structures obtained through N-terminal cleavage or deletion are though biochemically relevant, the reported results are devoid of knowledge relating to the structure, topology or molecular organization of these membrane-bound eukaryotic CYPs (Šrejber et al., 2018; Barnaba et al., 2017). It is worth mentioning that the truncation of TMD might not only affect the CYP's phospholipid composition leading to folding and stability issues, but could also influence the CYP-CPR interactions and impair the catalytic functionality.

6. Surrogate cell factories for eukaryotic CYP production

Recombinant expression of membrane-bound eukaryotic CYPs in surrogate (mainly microbial) cell factories provides a cost-effective avenue for large scale protein production for downstream biochemical and structural studies. Nevertheless, functional expression and production

Table 1
Holistic representation of expression of eukaryotic membrane-bound cytochrome P450s in different surrogate cell factories.

Parameters	Heterologous expression of eukaryotic membrane-bound CYPs				
	Bacterial system	Yeast system	Fungal system	Plant system	Mammalian cell-line system
Selection of vector, promoter and host strain	Required	Required	Required	Required	Required
Optimization of culture conditions	Required	Optional	Optional	Optional	Optional
Duration of culture for production	Fast (several hours to a day)	Moderate (several days)	Moderate (several days to a week)	Slow (several days to weeks)	Slow (several weeks)
Culture cost & technical demand	Low	Low	Moderate	High	High
Supplementation of heme precursor 5-aminolevulinic acid	Required	Optional	Not required	Not required	Not required
Optimization of codon usage	Required	Required	Required	Optional	Optional
Reduction of secondary mRNA structure	Required	Optional	Optional	Optional	Optional
N-terminal modification	Deletion of N-TMD or construction of chimeric CYPs is required	Not necessary	Not necessary	Not necessary	Not necessary
Post-translational modifications	Does not occur	Offers higher eukaryotic-style post-translational modifications	Offers higher eukaryotic-style post-translational modifications	Yes	Yes
Presence of endogenous CYPs	No	Very few	Several to many endogenous CYPs	Several to many endogenous CYPs	Several to many endogenous CYPs
Co-expression of redox partner	Required	Co-expression of CPR is advantageous	Optional	Optional	Optional
Co-expression of auxiliary proteins	Chaperones are required for proper folding and high protein expression	Optional	Optional	Not required	Not required
C-terminal fusion	C-terminal GFP-based platforms allow increased CYP expression and fluorescence report	Not required	Not required	Not required	Not required
Protein purification & structural studies	Highly purified CYP proteins can be achieved, allowing structural studies	Microsomes can be purified upon tedious efforts, but structural studies are limited	Hard to achieve	Hard to achieve	Hard to achieve
Biochemical characterization	Purified enzymes	Whole-cell biotransformation is preferred over purified enzymes or microsomal fractions	Whole-cell biotransformation is preferred over purified enzymes or microsomal fractions	Whole-cell biotransformation is preferred over purified enzymes	Microsomal fractions

of eukaryotic CYPs in surrogate cell factories is not simple and entails some fundamental problems (Durairaj et al., 2019). Some of the vital parameters for the heterologous recombinant expression of eukaryotic CYPs include (a) selection of host strain, (b) choice of the expression plasmid with an appropriate promoter, (c) codon optimization of exons, (d) culture conditions, (e) protein induction and so on (Table 1). It has become the foremost priority to identify an appropriate surrogate cell factory for functional expression of eukaryotic CYPs, since handling such metalloenzymes could be rather tricky owing to their membrane-bound property, low expression level, protein misfolding, ineffective substrate uptake, and need as well as tolerance for rich redox cofactors. In order to facilitate sustained expression of membrane-bound eukaryotic CYPs, a wide range of prokaryotic and eukaryotic surrogate cell factories have been successfully developed (Table 1). Herein, we will focus on the pros and cons of these microbial cell factories, as well as the established and arising engineering strategies/approaches for eukaryotic CYP production. Though the scope of this review is primarily focused on microbial cell factories, a brief overview on the higher-eukaryotic cell factories (viz., plant and mammalian hosts) are also included in order to provide a holistic understanding on eukaryotic CYP expression.

6.1. Conventional microbial cell factory systems

The most conventional and preferred surrogate cell factories for the heterologous expression of CYPs are the bacterial and yeast host systems. Several studies concerning the heterologous expression of membrane-bound eukaryotic CYPs in the traditional cell factory systems (*E. coli* and *S. cerevisiae*) have been well documented (Winkler et al., 2018; Zelasko et al., 2013; Freigassner et al., 2009; Sørensen and Mortensen, 2005; Yun et al., 2006; Ichinose and Wariishi, 2013; Emmerstorfer et al., 2014; Ichinose et al., 2015; Faiq et al., 2014; Stiborová et al., 2017; Hausjell et al., 2018; Hausjell et al., 2020; Park et al., 2020).

6.1.1. Bacterial cell factory as eukaryotic CYP production platform

Escherichia coli has always been the first-choice bacterial cell factory for heterologous CYP expression due to its unparalleled fast growth kinetics, inexpensive and rich complex media, high cell density cultures, and ease of genetic manipulation. With the extensive knowledge on genetics and physiology, many state-of-the-art molecular tools and protocols have successfully been developed for recombinant protein production and purification, facilitating functional and structural studies (Table 1). Besides, the absence of native CYP genes in *E. coli* makes it ideal for recombinant CYP production due to the advantage of lacking cross-interference. Owing to these advantages, a wide range of CYP-mediated biotransformations have been demonstrated in this bacterial cell factory leading to milligram- to gram-scale production of various compounds (Zelasko et al., 2013; Yun et al., 2006; Ichinose et al., 2015; Ajikumar et al., 2010). However, several factors concerning the prototypical elements of eukaryotic CYPs viz. the presence of N-terminal hydrophobic TMD comprising the conserved proline-rich residues prior to the CYP catalytic domain, demand for the addition of the precursor for heme prosthetic group, and dependency on ET proteins as well as NAD(P)H cofactor for electron supply complicate the heterologous

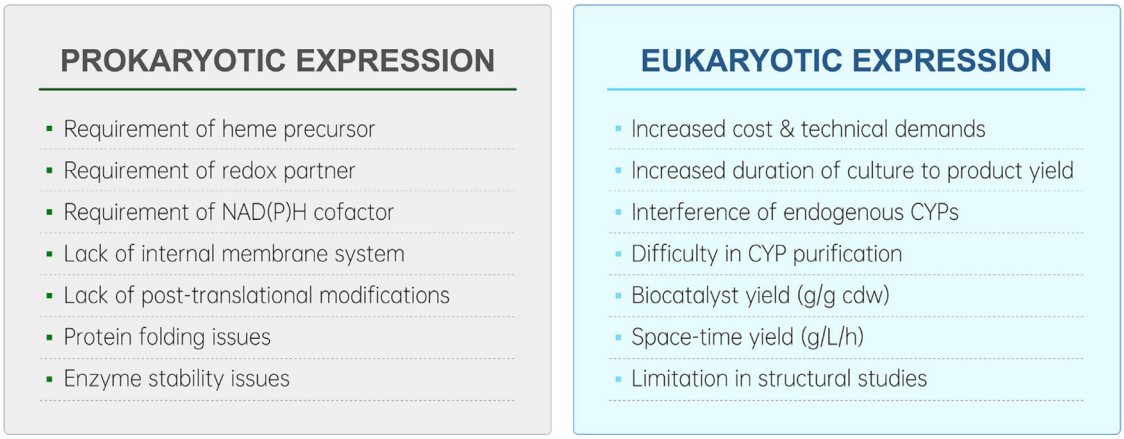

Fig. 3. Challenges associated with the prokaryotic and eukaryotic expression of membrane-bound eukaryotic CYPs.

CYP expression (Fig. 3). Remarkably, the major drawback with the bacterial cell factory is that this system often demands the membrane-bound eukaryotic CYPs to undergo significant modifications (mutagenesis/deletions) to obtain soluble fractions, possibly resulting in serious consequences. N-terminal CYP modifications though sporadically resolves the problems, the alterations could influence the native enzyme's interaction and fail the desired catalytic activity. Besides, this approach has turned out to be case-specific (not successful for all membrane-bound CYPs), since several N-terminal altered CYPs were still expressed as membrane fractions due to their indelible hydrophobic topologies. Though various strategies have been constructively developed (discussed in detail in Section 7), there is still no a universal guideline to enable successful eukaryotic CYP overexpression. The success rate varies upon a case-by-case basis, and therefore optimizations have to be performed discretely for every membrane-bound CYP protein.

6.1.2. Yeast cell factory as eukaryotic CYP production platform

Saccharomyces cerevisiae, the eukaryotic counterpart of *E. coli* serves as an ideal cell factory due to its salient features such as genetic accessibility, rapid growth, and convenient genetic and metabolic engineering approaches. The conventional yeast system is desirable for eukaryotic CYP expression as it offers rich ER environment in combination with the higher eukaryotic protein synthesis machinery, thereby enabling the recombinant expression of full-length membrane-bound CYPs without any genetic modifications/truncations (Table 1). *S. cerevisiae* offers an innate intracellular heme environment with the presence of three well characterized CYP enzymes including CYP51, CYP56 and CYP61, thereby providing an *in vivo* self-sufficient background for the heterologous CYPs ensuring complete saturation by hemin prosthetic group. The endogenous CYP environment of yeast cell factory is a significant attribute that lacks in *E. coli*, where supplementation of expensive heme precursors (5-aminolevuleic acid) is often demanded for the up-regulation of CYP activity. Besides, the added advantage of presence of native *S. cerevisiae* CPR can directly supply reducing equivalents to the heterologous CYPs, wherein an additional step of recombinant CPR co-expression is eliminated, thus avoiding ancillary stress to the host organism leading to lower CYP production. Another interesting attribute of yeast cell factory is its ability to regenerate the cofactor NADPH intracellularly since the effective coupling between NADPH and CYP is crucial for large-scale applications involving high substrate concentration (Park et al., 2020). Of note, the yeast cell factory allows biocatalytic investigations of recombinant CYPs through a one-pot biotransformation procedure achieved by either growing or resting (non-growing) cells (Durairaj et al., 2016; Lundemo and Woodley, 2015). Alternatively, the recombinant CYPs can be studied by yeast microsomes, favoring *in vitro* work concerning kinetics and reaction mechanism. Nevertheless, though yeast microsomal experiments are more precise, the factors such as sophisticated isolation process, culture-to-culture variations, and technical constraints in purification make it a complicated and cumbersome process to acquire active functional enzymes (Durairaj et al., 2016; Drăgan et al., 2011; Yan et al., 2017). As a result, yeast whole-cell biotransformation is preferred for CYP functional characterizations owing to its simplicity, straightforward analysis, and biocatalyst stability (Durairaj et al., 2016; Park et al., 2020; Lundemo and Woodley, 2015); though the kinetic and structural investigations are limited. The major drawback with the yeast biotransformation is the protein/product yield especially with the strains expressing heterologous CYPs (Fig. 3). Though gram-scale production is possible in yeast-based fermentations or whole-cell biotransformations, the range of production titer often varies and demands longer incubation (Park et al., 2020). Another limitation of yeast cell factory is that certain substrates and/or products may face some permeabilization issues, as the charged molecules are less likely to permeate multiple cell membranes (Yan et al., 2017).

6.1.3. Amalgamation of conventional bacterial and yeast cell factory platform

On the whole, our current knowledge on eukaryotic CYPs results from the composition of researches on both bacterial and yeast cell factories. On one hand, the bacterial cell factory aids higher yield of eukaryotic CYPs (14–20 nmol/mg protein), although the N-terminal modifications are inevitable. On the other hand, though the yield is relatively low (~1 nmol/mg protein), yeast cell factory enables expression of membrane-bound CYPs in its native form without any modifications (Hausjell et al., 2018). The space-time yield analysis of several CYP biotransformations in bacterial and yeast cell factories has demonstrated that though the production titer can be comparable (1-2 g/L/h), the yeast system demands longer incubation (100-200 h reaction time) compared to that of bacterial system (Park et al., 2020). Of note, the CYP activity might differ depending upon the surrogate cell factory employed in terms of not only expression rate but also functionality. For instance, the recombinant human CYP2S1 expressed in *E. coli* and *S. cerevisiae* systems gave varied results as the enzyme's catalytic specificity was altered (Wu et al., 2006, Nishida et al., 2010). Thus, direct or combinational comparison of full-length membrane-bound CYPs in both the bacterial and yeast expression systems is required to enable a better functional and mechanistic understanding. Interestingly, microbial production of plant benzylisoquinoline alkaloids were successfully established in an combinatory co-culture system (Minami et al., 2008). The transgenic *E. coli* cells expressing the branchpoint intermediate (*S*-reticuline) biosynthetic gene were co-cultured with the *S. cerevisiae* cells expressing CYP80G2 along with other required biosynthetic genes (coclaurine-*N*-methyltransferase/berberine bridge enzyme). This co-culture system ef-

ficiently produced magnoflorine and scoulerine with a final yield of 7.2 and 8.3 mg/L, respectively. Likewise, metabolic pathway distribution within microbial consortium could solve the technical difficulties and significantly enhance the production of natural products. Incorporation of robust production of taxadiene in *E. coli* and the functional expression of CYP enzyme taxadiene 5α-hydroxylase in *S. cerevisiae* resulted in the highest production (33 mg/L yield) of oxygenated taxanes (Zhou et al., 2015). Amalgamation of both bacterial and yeast based CYP expression could therefore be a novel approach to solve the limitations and exploit the advantages of each expression system.

6.2. Unconventional microbial cell factory system

Unconventional (non-*Saccharomyces*) yeasts such as fission yeast *Schizosaccharomyces pombe* (Bureik et al., 2002), methylotrophic yeast *Pichia pastoris* (Gudiminchi et al., 2013), dimorphic yeasts *Yarrowia lipolytica* (Mauersberger et al., 2013) and *Arxula adeninivorans* (Theron et al., 2014), lactose-utilizing yeast *Kluyveromyces lactis* (Engler et al., 2000) and its thermophilic sister strain *Kluyveromyces marxianus* (Engler et al., 2000) can also be employed as alternate surrogate cell factory systems for heterologous CYP production. Of which, *P. pastoris* and *Y. lipolytica* have been proven effective for the recombinant production of several membrane-bound CYP proteins with higher yields (Iwama et al., 2016; Garrigós-Martínez et al., 2021). Recently, in order to investigate whether baker yeast is a superior host to express membrane-bound CYPs, direct comparison on the expression of full-length chalcone 3-hydroxylase, a CYP essential in the flavonoid pathway was expressed in three different strains of *P. pastoris* and *S. cerevisiae* (Hausjell et al., 2020). Regarding productivity it has been reported that a highest yield of 600 pmol/mg protein was achieved in controlled bioreactor cultivations using the *P. pastoris* strain KM71H; while the yield of *S. cerevisiae* is twice as low as the lowest product yield obtained in *P. pastoris* strain SMD1168H. Interestingly, *S. pombe* which is an underestimated cell factory for CYP studies was recently demonstrated to be an ideal surrogate cell factory for expression and functional characterization of several hard-to-express and so-called orphan human CYPs including CYP4Z1, CYP2A7, CYP4A22 and CYP20A1 (Durairaj et al., 2019; Yan et al., 2017; Bureik et al., 2002; Durairaj et al., 2019; Durairaj et al., 2020). A classic example on heterologous expression and recombinant production of CYP2C9 in three different cell factories including the baculovirus transfected insect cell lines, *E. coli*, and *S. pombe* towards the hydroxylation of non-steroidal anti-inflammatory drug diclofenac resulted in the preparation of 2.2 mg, 110 mg and 2.8 g of 4'-hydroxy metabolite representing an overall yield of 28 %, 35 % and 75 %, respectively (Winkler et al., 2018; Drăgan et al., 2011; Rushmore et al., 2000; Vail et al., 2005). Besides, several human drug-metabolizing enzymes (Winkler et al., 2018) including CYP2D6, CYP2C9, CYP3A4, CYP11B1, CYP11B2 and CYP21 were successfully expressed in *S. pombe,* and the kinetic parameters were determined using the whole-cell biocatalysts. Recently, a functional library of human CYPome was constructed in *S. pombe*, where all the 57 human CYPs were co-expressed along with their natural human ET (CPR/AdR+Adx) partners (Durairaj et al., 2019). This complete library of recombinant fission yeast strains demonstrated functional expression of unmodified sequences of human PAN CYPome, and their catalytic activity were determined within a single surrogate cell factory for the first time.

6.3. Higher-eukaryotic cell factory systems

6.3.1. Fungal cell factory as eukaryotic CYP production platform

An evolutionarily closer organism, a filamentous fungus could serve as a preferred cell factory for the expression of homologous and heterologous membrane-bound eukaryotic CYPs (Durairaj et al., 2016; Zhang et al., 2021). Filamentous fungi could overcome several limitations faced in traditional expression systems (bacteria / yeast), including mRNA precursor maturation, higher splicing-rate, efficient protein secretion machinery and post-translational modifications (Tanaka et al., 2014; Nevalainen and Peterson, 2014). With the advent of modern biotechnology, several species of filamentous fungi have emerged as promising cell factories for the production of pharmaceutically relevant proteins with salient improvements. Of which, *Aspergillus* spp. (Meyer et al., 2011) dominate the scene as desirable expression hosts for eukaryotic CYP production as many studies have been reported with *Aspergillus nidulans* (Liu et al., 2019), *Aspergillus oryzae* (Cao et al., 2019), *Aspergillus niger* (Faber et al., 2001), *Aspergillus sojae* (Araki et al., 2019) and *Aspergillus aculeatus* (Thiele et al., 2020). Several fungal CYPs involved in the natural product biosynthetic pathways were functionally elucidated using the fungal cell factory with *A. oryzae* or *A. nidulans* as heterologous host (Zhang et al., 2021). For instance, the biosynthetic pathways of helvolic acid (Lv et al., 2017), fusidic acid (Cao et al., 2019), and cephalosporin P1 (Cao et al., 2019) from the ascomycetous fungi *Acremonium fumigatus, A. fusidioides,* and *A. chrysogenum* were studied using *A. oryzae* as an expression host system. Another example demonstrated the functional investigation of biosynthetic genes of fungal meroterpenoid from the filamentous fungus *Acremonium egyptiacum* in *Aspergillus* spp., wherein the genes *ascA-D* involved in the biosynthesis of ascofuranone and ascochlorin were expressed in *A. oryzae*, while AscE-G proteins were expressed in *A. sojae* high-copy expression system (Araki et al., 2019). Of which, AscE is a soluble CYP/reductase fusion protein that catalyzes stereoselective epoxidation of the terminal double bond of the prenyl group, while AscF (terpene cyclase) and AscG (CYP) are membrane-bound proteins involved in the terpene cyclization and oxidation of ilicicolin into ascochlorin. Based on the preliminary experimental evidence it is suggested that the heterologous production of fungal CYPs yields better results in fungal cell factory than CYPs of other origins. Recombinant production of the active form of mammalian CYP proteins in fungal cell factory is often limited by the differences in the mode of glycosylation of mammalian and fungal cells. Filamentous fungal system features the high-mannose type of glycosylation, but lacks the mammalian-style terminal sialylation of glycans which may affect the functionality, serum half-life and immunogenicity of recombinant proteins (Nevalainen and Peterson, 2014). In addition, factors such as DNA manipulation, incorrect processing / misfolding and secretory yields may also add up to the list of limitations associated with fungal cell factory for recombinant CYP production (Tanaka et al., 2014; Nevalainen and Peterson, 2014).

6.3.2. Plant cell factory as eukaryotic CYP production platform

Plant CYPs involved in secondary metabolism are sometimes difficult to express in a microbial cell factory, and therefore several plant-based hosts have been developed to procure sufficient protein expression and improve the yield of desired compounds. Plant-based heterologous expression offers several advantages as they permit defined mRNA and protein processing, protein subcellular localization and metabolic compartmentalization, and have essential metabolic precursors and coenzymes (Table 1). Though plant (*N*- and *O*-linked) and mammalian (terminal sialylation of glycans) cells feature different glycosylation patterns, the variances do not impair the recombinant protein production; and tremendous efforts have been made for humanization of protein *N*-glycosylation in plant cell factory (Gomord et al., 2010). A wild relative of tobacco, *Nicotiana benthamiana*, serving as an efficient production system of flu vaccines at industrial-scale (Marsian and Lomonossoff, 2016), has also acted as a competent cell factory for the heterologous expression of several plant CYPs (Reed et al., 2017). The multifunctional enzyme AsCYP51H10 involved in the modification of C and D rings of the pentacyclic triterpene scaffold into 12,13β-epoxy-3β,16β-dihydroxy-oleanane, was successfully expressed and studied using this transient plant expression system (Geisler et al., 2013). Moreover, co-expression of additional heterologous redox partner is not necessary since the *N. benthamiana* CPR provides sufficient electron equivalents to mediate CYP catalysis. Recently, the members of CYP79C family belonging to the glucosinolate (GLS) biosynthetic pathway in *Arabidop-*

sis thaliana were functionally characterized through Agrobacterium-mediated transient expression in *N. benthamiana* (Wang et al., 2020). Functional investigation of CYP79C1 and CYP79C2 in the GLS pathway engineered *N. benthamiana* facilitated simultaneous testing of substrate specificity against multiple aliphatic and aromatic amino acids. The whole-genome sequenced non-vascular plant *Physcomitrella patens* offered efficient homologous recombination, and facilitated recombinant production of several commercially important pharmaceutical proteins (Khairul Ikram et al., 2017). Furthermore, the cinnamic acid 4-hydroxylase from the aquatic plant *Anthoceros agrestis,* which could not be expressed in yeast system possibly due to high GC content and/or different codon usage, was successfully expressed in the haploid plant *Physcomitrella patens* and functionally characterized at biochemical and molecular levels (Wohl and Petersen, 2020). Specifically, *A. thaliana* serves as an excellent model for recombinant protein production and has been extensively studied for the developmental and molecular biology, as well as pharmaceutical applications (Von Schaewen et al., 2018). Remarkably, the recently developed Arabidopsis-based recombinant protein production platform (Jeong et al., 2018) is expected to serve as an efficient cell factory for the heterologous CYP production suitable for biochemical and structural studies. Interestingly, the plant cell factory system offers efficient endogenous redox partner system (*e.g., N. benthamiana* CPR) that can effectively pair up with the heterologous CYPs for direct ET. However, the rich intracellular heme environment with enormous native/endogenous CYPs may also have a downside due to the cross-link effect and/or interference with the desired recombinant CYP protein of interest. Though there are successful instances, functional studies in plant cell factories remain limited with early-stage objectives due to the restrictions including enzyme stability, relatively low turnover, yield, tedious protein extraction and purification process, and high cost of downstream processing (Schillberg et al., 2019).

6.3.3. Mammalian cell-line factory as eukaryotic CYP production platform

Development of genetically modified mammalian cell lines to functionally express membrane-bound CYPs represents another successful strategy by facilitating practical *in vitro* approaches for enzymatic characterization, drug metabolism screening and early detection of drug toxicity (Xuan et al., 2016; Satoh et al., 2017) (Table 1). The cell-line factory allows recombinant production of larger and complex proteins including membrane-bound CYPs as it offers inherent transcriptional and translational environment along with appropriate chaperonin, secretory pathway and redox assembly coupled with efficient protein folding, and excellent post-translational modifications. Though the glycosylation pattern of some of the cell lines slightly varies to that of human-type glycosylations; the cell lines can be fine-tuned by codon optimization including glycoengineering which improves the efficacy and enhances the recombinant expression (Hunter et al., 2019). Alternatively, human cell line (*e.g.*, HEK-293) systems represent an ideal source for CYP mediated drug metabolism studies, and allow post-translational modifications of membrane-bound eukaryotic proteins for functional production at high levels. Cell lines can be readily transfected or virally transduced, and the recombinant CYP proteins can be produced either transiently or by stable expression (Hunter et al., 2019). Primary human hepatocytes, hepatic cell lines, and stem cell derived hepatocytes are primarily utilized as *in vitro* models for the recombinant CYP production and functional studies. Nevertheless, high donor to donor variability, scarcity, limited lifespan, low expression and yield restrict the efficient utilization of major cell lines for enzymatic analysis involving CYP mediated drug metabolism. Currently, several alternative cell line platforms have been developed, and tremendous efforts have been put forward to determine CYP functions with the advances of synthetic biology and next-generation engineering to achieve gram-scale productivity (Hunter et al., 2019; Gutiérrez-González et al., 2019; Boon et al., 2020). For instance, human hepatoma cell lines can demonstrate increased stability, ample life-span and accessibility compared to the traditional cell-based assays using primary human hepatocytes. Transchromosomic HepG2 cell lines have facilitated expression of four major CYPs (CYP2C9, 2C19, 2D6, and 3A4) and CPR using the mammalian-derived artificial chromosome vector (Satoh et al., 2017). The expression levels were significantly higher than that of the parental HepG2 cells demonstrating a highly versatile model for the evaluation of drug-drug interactions and screening of hepatotoxicity. Another interesting study demonstrated expression of 14 human CYPs in HepG2-derived cell lines individually with the aid of lentiviral expression system (Xuan et al., 2016). In order to determine the most suitable expression platform for the *in vitro* CYP enzymatic activities, four different mammalian cell lines (COS-7, HepG2, 293T and 293FT) were functionally investigated with the typical variants of CYP2C9, CYP2C19 and CYP2D6 (Dai et al., 2015). The results indicated that the fast-growing variant of 293 cell line 293FT demonstrated higher levels of expression as well as *in vitro* activities. Recently, another interesting study successfully developed an ideal heterologous expression system with three CYP isoforms (CYP1A2, CYP2C9, and CYP3A4) using mammalian 293FT cells by optimizing high-precision conditions (Kumondai et al., 2020). Therein, the highest CYP expression which can be quantifiable by CO-difference spectroscopy was achieved in a lost-cost manner by replacing expensive transfection reagent with cost-effective and efficient substitute PEI-Max, and demonstrated significantly higher enzymatic activity by co-expressing CPR and Cyt B5. Though the mammalian cell line factory serves as an excellent system for recombinant human CYP production and *in vitro* functional studies, some issues concerning expression, reduced activity and low CYP inducibility remain to be addressed. Besides, factors such as demand for expensive culture media, complex growth requirements, technical demand, time-consuming procedures, lengthy expression phase, lot-to-lot heterogeneity, and scalability are some of the major bottlenecks to be overcome.

7. Approaches to eukaryotic CYP production in surrogate cell factories

In order to explore the functional and structural features of eukaryotic CYPs, suitable quantities of purified, well-folded and catalytically active proteins are required. The membrane-bound eukaryotic CYPs display higher complexity than most of soluble prokaryotic CYPs (Denisov et al., 2012). Recombinant production of eukaryotic CYPs remains a challenging task owing to the constraints in obtaining sufficient soluble proteins due to non-expression, protein misfolding, or aggregation into insoluble inclusion bodies (Durairaj et al., 2016; Zelasko et al., 2013). Bacterial system though serves as an ideal cell factory for prokaryotic CYPs, the membrane-bound nature of the eukaryotic CYPs often impedes their heterologous expression and recombinant production. As discussed above, membrane-bound proteins behave poorly in overexpression systems and tend to be unstable in the detergent solutions that are used in the membrane extraction and purification steps. Consequently, tremendous efforts have been undertaken in optimizing target proteins, diverse expression systems and purification strategies to yield sufficient proteins (Durairaj et al., 2016; Maroutsos et al., 2019; Zelasko et al., 2013; Yun et al., 2006; Ichinose et al., 2015; Hausjell et al., 2018). Despite great endeavors on optimizing the overexpression of membrane-bound CYPs, there still lacks a systematic approach with a solid theoretical basis. Instead, optimizations can be achieved only via a tedious trial-and-error process (Durairaj et al., 2016). Herein, we elucidate some of the key strategies that favor efficient and effective expression and production of membrane-bound eukaryotic CYPs in surrogate cell factories.

7.1. Codon optimization

With the advent of modern biotechnology, gene synthesis with adapted codon usage has become a convenient strategy to ensure sustained production of membrane-bound proteins in heterologous systems (Claassens et al., 2017). For the recombinant protein production, the

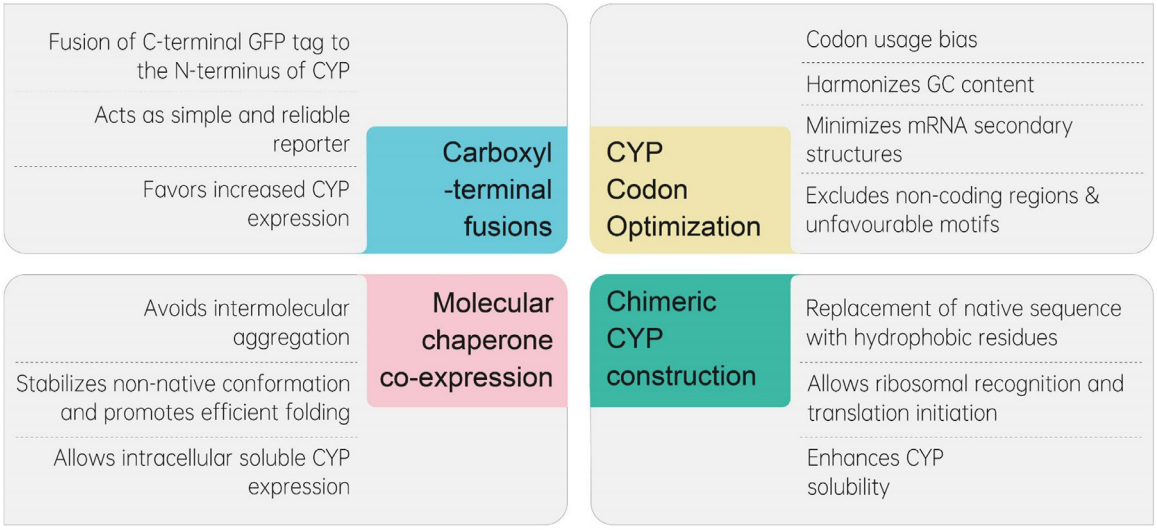

Fig. 4. Key approaches to improve eukaryotic CYP production in surrogate cell factory.

codons are optimized according to the expression host system by harmonizing the favorable GC-content, and by excluding the mRNA secondary structures at the 5' untranslated region (Fig. 4). Besides the unfavorable motifs including the repeats, Shine-Dalgarno like sequences and RNase sites also need to be excluded in order to promote heterologous protein expression. The expression levels of several mammalian and plant CYPs were significantly improved in bacterial cell factories upon codon optimization (Yamaguchi et al., 2021; Wu et al., 2009). Furthermore, transcriptional tuning and usage of rare codons has proven successful for membrane-bound proteins as it slows down the translation and allows proper co-translational folding of specific domains (α-helices and β-sheets) and insertion of membrane (Claassens et al., 2017). This not only facilitates appropriate translocation rates but also ensures membrane integration thereby avoiding accumulation of inclusion bodies.

7.2. Construction of chimeric CYPs

Construction of chimeric CYPs by exchanging the N-termini of other eukaryotic membrane-bound CYPs has proven to be an effective strategy for various mammalian and plant CYPs (Fig. 4). Especially, the modified N-terminal M-A-L-L-L-A-V-F-L residues of bovine microsomal 17α-hydroxylase (Barnes et al., 1991) significantly enhanced the solubility of several human (CYP1A2, CYP2B6, CYP2D6, CYP3A4, CYP3A43, *etc.*) and plant CYPs (CYP71E1 from *Sorghum bicolor*) (Hanna et al., 2000; Domanski et al., 2001; Pan et al., 2011). Replacement of native sequence with N-terminal hydrophobic residues L-L-L-A-V-F-L was efficiently recognized by the bacterial signal recognition particle as it allowed utmost ribosomal recognition and translation initiation. In the pursuit of exploring other effective chimeric tag insertions, modified N-terminal residues of bovine CYP17A1 and rabbit CYP2C3 such as A-L-L-A-F-V and A-K-K-T-S-S, respectively were also demonstrated to be successful for solubilisation of human CYPs (Sohl and Guengerich, 2010; Tang et al., 2010).

With respect to the fungal system, potent N-terminal domains of fungal origin were identified from various fungal CYP isoforms of *Phanerochaete chrysosporium* and *Postia placenta* (Ichinose and Wariishi, 2013; Ichinose et al., 2015). A large-scale screening of < 300 CYPs revealed 64 potential N-TMD chimeric candidates including CYP5348N1, CYP5348T3P, CYP5144C1, CYP5144C8 and CYP5348L1v2 that increased the bacterial expression of various membrane-bound fungal CYPs. Of which, the replacement with M-S-L-L-L-A-A-T-L-F-L-H-S-R-Q-K-R-Y-P-L residues from CYP5144C1 of *P. chrysosporium* drastically improved the protein expression of several fungal CYPs in *E. coli*. For instance, chimeric CYP5037E1v1 modified with the N-terminal sequence of CYP5144C1 resulted in approximately 10-time higher yield (2330 nmol P450/L) compared to the wild type. Furthermore, codon optimization for proline residues P-P-G-P in the proline-rich region encoded by the original nucleotide codons CCT-CCT-GGT-CCA also led to enhanced expression of fungal CYPs (Ichinose et al., 2015).

7.3. Coexpression of molecular chaperone

Critical factors in expressing the functionally active eukaryotic CYPs in a surrogate cell factory include proper folding, maturation and transport processes. Most of the eukaryotic membrane-bound proteins including CYPs enter the secretory pathway for folding and maturation by translocating into the ER membrane. Since eukaryotes have distinct native folding mechanisms, the prokaryotic hosts (*e.g., E. coli*) often fail to maintain the pace of transcription and translation processes for the heterologously expressed eukaryotic proteins (Francis and Page, 2010). Co-expression of ER-resident chaperones significantly improves the yield of properly folded, active and soluble proteins in bacterial systems (Fig. 4) (Schlapschy and Skerra, 2011). In particular, the GroES-GroEL bacterial molecular chaperone system is commonly co-expressed with the recombinant eukaryotic CYPs to achieve proper folding and high protein expression in *E. coli* (Sørensen and Mortensen, 2005; Ichinose and Wariishi, 2013; Ichinose et al., 2015; Wu et al., 2006; Wu et al., 2009; Hatakeyama et al., 2016). Alternatively, the Cpn60/10 chaperonin system offers an advantage of slower expression rate at low temperature (12-20°C) to ensure correct folding; however this system has yet to be explored for eukaryotic CYP expression.

7.4. Carboxyl-terminal fusions

Fusing the C-terminal GFP-based platforms to the N-terminus of membrane-bound CYPs has proven to be an effective strategy and allows direct assessment of expression levels, membrane localization and protein integrity (Fig. 4). The C-terminal GFP fusion acts as a reliable expression reporter of the eukaryotic CYP production (Christensen et al., 2017). It was reported recently that screening a library of N-terminal expression tag chimeras of CYP79A1 revealed increased expression when C-terminal GFP fusions were used (Vazquez-Albacete et al., 2017). Likewise, heterologous expression of six plant-derived CYP (CYP51G1, CYP71E1, CYP79A1, CYP51H10, CYP79B2 and CYP720B4) genes in both K- and B-type strains of *E. coli* using the workflow based on C-terminal GFP fusions along with the N-TMD truncation strategy were

proven to be successful (Christensen et al., 2017). Interestingly, the bacterial membrane anchor when fused with the eukaryotic CYPs facing the cytoplasmic and/or periplasmic site led to increased expression compared to the wild type. In addition, incorporation of bacterial signal peptide and bacterial transporters drastically improved the CYP expression when the C-terminus was fused at the cytoplasmic site. These results suggest that the host-tailored modifications of the membrane-bound CYPs could be a key to heterologous protein production.

7.5. Cleavage of transmembrane domain

Cleavage or truncation of the N-terminal TMD procures soluble expression of several membrane-bound CYP proteins of human, plant and fungal origins (Ichinose and Wariishi, 2012; Yun et al., 2006; Ichinose and Wariishi, 2013; Christensen et al., 2017). It is worth noting that the length of truncation may play a crucial role in determining the level of CYP expression. Depending upon the length of cleavage, the heterologously expressed eukaryotic CYPs can be expressed as soluble, peripherally associated, or insoluble fractions (Ichinose and Wariishi, 2012; Zelasko et al., 2013; Durairaj et al., 2015). It is often difficult to rationally propose the specific length or key residues for TMD deletion, because each CYP may require different optimal sequence modifications (Durairaj et al., 2016). As a rule of thumb, the N-terminal TMD cleavage strategy though demonstrates solubilisation of several eukaryotic CYPs, cannot be simply applied in all the cases as the results may vary (often leading to loss of CYP functionality) depending on many known and unknown factors.

7.6. Incorporation of bicistronic design

Bicistronic design (BCD) elements emerge as an alternative strategy to obtain sustained production of “difficult-to-produce” membrane proteins efficiently in bacterial systems (Claassens et al., 2019; Nieuwkoop et al., 2019). BCD system is based on a constitutive promoter, and its tuning is achieved by two ribosome binding sites (RBS) that are translationally coupled. Briefly, the first RBS initiates the strong translation of a short leader peptide, while the second RBS embedded in the leader peptide’s coding sequence drives the translation of the target protein. Though it appears that the BCD system has not been explored yet for the eukaryotic membrane-bound CYP expression, this strategy might be of potential use in the near future.

8. Eukaryotic redox partners – regulating partners in CYP monooxygenation

As discussed in the Sections 2 and 3, the redox partner CPR is crucial for CYP monooxygenation by supporting the reductive activation of inert dioxygen to drive the catalytic cycle (Fig. 1) (McLean et al., 2005). Evolutionarily, CPR is a fusion of two ancestral proteins comprising the N-terminal FMN-containing domain and the C-terminal FAD/NADPH-binding domains, which are connected by a highly flexible hinge region. NADPH supplies the reducing equivalents to FAD (reductase), which are sequentially transferred to FMN (transporter) and then to the heme-iron reactive centre. Being the imperative CYP redox partner, CPR can also supply electrons to other proteins *in vivo*, viz., Cyt B5, heme oxygenase, fatty acid elongases, and squalene epoxidase; while also transfer electrons *in vitro* to several non-physiological acceptors including cytochrome c, ferricyanide, and different drug molecules (Ebrecht et al., 2019). Interestingly, CPR exists as closed (for inter-flavin ET) or open (for electron donation) conformation depending upon the redox cycle phase (Freeman et al., 2017). The N-terminus of CPR (~6 kDa) comprises a hydrophobic membrane-binding domain and acts as a non-specific membrane anchor responsible for ER localization (Mukherjee et al., 2021; Hamdane et al., 2009). Different CYPs and CPR could form membrane-bound protein-protein complexes driven by electrostatic interactions between the negatively charged amino acids of CPR’s FMN domain and the positively charged residues of CYP at the proximal side (Nadler and Strobel, 1988). The membrane-bound CYPs and CPR are attached to the cytosolic side of ER with an estimated CYP:CPR ratio ranging from 3–15:1 (Šrejber et al., 2018). Experimental evidences also suggest that the CYP-CPR protein complex functions well at the CYP:CPR ratio of 5:1 to 20:1 in ER (Gold et al., 2018).

The interactions of CPR and CYP take place in an isoform-dependent manner involving several binding motifs of the FMN-domain (Campelo et al., 2018; Esteves et al., 2020). Interestingly, CYP-CPR interactions can not only influence the ET efficiency and substrate binding mode, but can have significant impact on the outcome of CYP monooxygenation in terms of reactivity and selectivity. Notably, the efficacy of CYP monooxygenation also relies on the abundance of CPR and its ET compatibility (Durairaj et al., 2016; Braun et al., 2012). As the functionally characterized eukaryotic CYPs are fast increasing, there emerges an important interrogation on the choice of potent redox partner. Apparently, the choice and ratio of redox partners can significantly influence the CYP’s catalytic efficiency as the second ET step is often the rate-limiting step in a CYP catalytic cycle. Based on the recent reports it is evident that in addition to the modulation of catalytic efficiency, the redox partners can also influence the substrate specificity and alter the product distribution (Durairaj et al., 2016; Durairaj et al., 2015; Zhang et al., 2018; Neunzig et al., 2013; Sagadin et al., 2019; Sagadin et al., 2018; Lah et al., 2011; Lv et al., 2019; Novak et al., 2015; Li et al., 2020; Durairaj et al., 2015). Previously, CPR was perceived as the mere power house of the CYPome, functioning solely as auxiliary electron donor partner with no much room for product profiling. With the recent breakthroughs, now it is widely accepted that the redox partners can also act as functionality-determining factor or ‘regulating partners’ for CYP monooxygenation.

8.1. Effective CYP-CPR reconstitution for efficient monooxygenation

In order to achieve optimal catalytic activity it has become a prerequisite to select an appropriate functional CPR for reconstitution of recombinant CYPs. In general, the recombinant eukaryotic CYPs are reconstituted with the redox partner belonging to three major sources of origin: endogenous CPR, homologous CPR and heterologous CPR (Fig. 5).

8.1.1. CYP reconstitution with endogenous CPRs

Eukaryotic expression host organisms encompass native or endogenous (the same source of heterologous host) redox partner(s) to supply reducing equivalents to its endogenous CYPs. Utilization of the native reductase sources to meet up the electron demand for the heterologously expressed CYPs has proven effective, and several recombinant CYPs of mammalian, plant and fungal origin were successfully studied with the endogenous CPRs (Fig. 5A). Typically, the native *S. cerevisiae* NADPH reductase naturally pairs up with the recombinant eukaryotic CYPs upon heterologous expression in yeast system (Table 2). The endogenous CPR of *S. cerevisiae* serves as a convenient redox partner as it can autonomously satisfy the recombinant CYP’s ET chain, and excludes an additional process of co-expression of exogenous CPR. Interestingly, over-expression of *S. cerevisiae* CPR in yeast expression system has also been effective to functionally study the recombinant CYPs of various origin. For instance, 425 fungal CYP isoforms from *P. chrysosporium* (Hirosue et al., 2011), *P. placenta* (Ide et al., 2012), and *A. oryzae* (Nazir et al., 2011) were successfully reconstituted with the *S. cerevisiae* CPR and functionally investigated upon heterologous expression in *S. cerevisiae*.

In addition, the endogenous reductases of other yeast expression hosts such *S. pombe* (Bureik et al., 2002), *P. pastoris* (Andersen and Møller, 2002), *Y. lipolytica* (Braun et al., 2012) can also couple and support reducing equivalents to the heterologously expressed recombinant CYPs (Table 2). Remarkably, the adrenodoxin homologue of *S. pombe* (etp1) replaces human Adx and efficiently transfer electrons to the mitochondrial P450 enzymes CYP11B1 and CYP11B2, and supports steroid

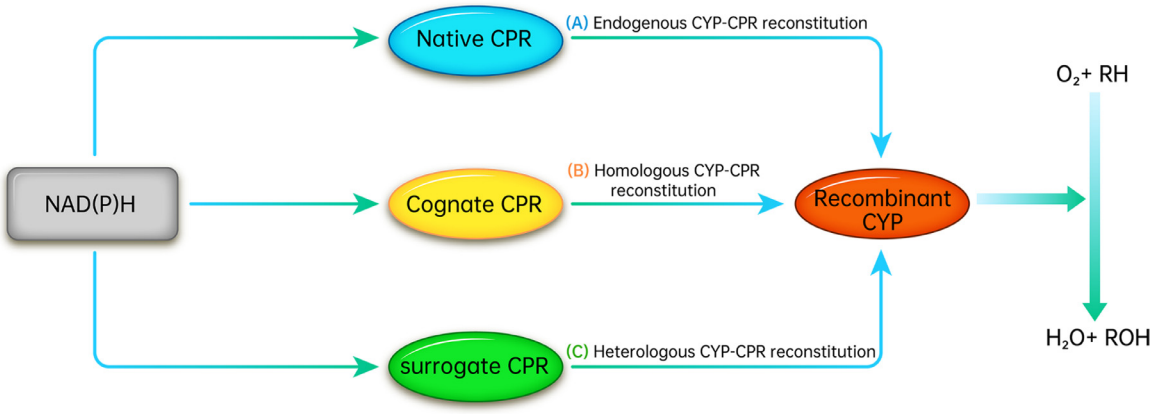

Fig. 5. Schematic representation of CYP-CPR reconstitution with different sources of origin. A. CYP reconstitution with endogenous CPR, B. CYP reconstitution with homologous CPR, and C. CYP reconstitution with heterologous CPR.

Table 2
Representative examples elucidating the impact of effective CYP-CPR reconstitution for efficient monooxygenation.

Expression host	Recombinant enzyme	CYP source	CPR source	Note	Refs.
S. cerevisiae	120 CYPs	*P. chrysosporium*	*S. cerevisiae*	Supports CYP monooxygenation, and allows rapid functional screening	Hirosue et al. (2011)
	184 CYPs	*P. placenta*	*S. cerevisiae*	Supports CYP monooxygenation, and allows rapid functional screening	Ide et al. (2012)
	121 CYPs	*A. oryzae*	*S. cerevisiae*	Supports CYP monooxygenation, and allows rapid functional screening	Nazir et al. (2011)
	CYP2U1	*H. sapiens*	*S. cerevisiae* *H. sapiens*	Only human CPR supports CYP monooxygenation	Ducassou et al. (2015)
	CYP53A19	*F. oxysporum*	*C. albicans* *S. cerevisiae* *F. oxysporum*	Homologous CYP-CPR system favored enhanced monooxygenation with broader substrate specificity	Durairaj et al. (2015)
	Astaxanthin synthase	*X. dendrorhous*	*X. dendrorhous* *S. cerevisiae*	Only the homologous CYP-CPR system supported astaxanthin synthesis	Alcaíno et al.)2008)
	CYP52M1	*S. bombicola*	*S. bombicola* *A. thaliana*	*A. thaliana* CPR favored increased production of long chain ω-hydroxy fatty acids	Liu et al. (2019)
S. pombe	50 CYPs	*H. sapiens*	*H. sapiens*	Supports CYP monooxygenation, and allows rapid functional screening	Durairaj et al. (2019)
	CYP11B1 & CYP11B2	*H. sapiens*	*S. pombe*	Supports steroid hydroxylase activity	Schiffler et al. (2004)
	CYP4Z1	*H. sapiens*	*S. pombe* *H. sapiens*	Both redox sources supports CYP monooxygenation	Yan et al. (2017) Durairaj et al. (2019)
	CYP2C9, CYP2D6, CYP3A4, CYP3A5, CYP3A7, CYP17, CYP21	*H. sapiens*	*H. sapiens* *S. pombe* *A. majus*	Mixed results. CYP3A enzymes preferred human CPR, while CYP2D6 and CYP2C9 preferred *S. pombe* and *A. majus* CPR, respectively	Neunzig et al. (2013)
P. pastoris	CYP2D6	*H. sapiens*	*P. pastoris* *H. sapiens*	Only human CPR supports CYP monooxygenation	Dietrich et al. (2005)
Y. lipolytica	CYP2D6 and CYP3A4	*H. sapiens*	*Y. lipolytica* *H. sapiens*	Only human CPR supports CYP monooxygenation	Zehentgruber et al. (2010)
	Flavonoid 3-hydroxylase	*G. hybrid*	*C. roseus* *Y. lipolytica* *S. cerevisiae*	Plant-sourced CYP-CPR pair enabled highest flavonoid production	Lv et al. (2019)
	CYP716A180	*B. platyphylla*	*A. thaliana* *C. roseus* *G. uralensis* *L. japonicas* *M. truncatula*	Among different CPR combinations, *L. japonicas* CPR and *M. truncatula* CPR yielded the highest betulinic acid titers	Jin et al. (2019)

hydroxylase activity in fission yeast (Schiffler et al., 2004). Recently, functional screening of human CYP4Z1 in *S. pombe* demonstrated that the fission yeast strains AZ1 (expressing only human CYP4Z1) and AZ3 (expressing human CYP4Z1 and human CPR) both supported the CYP activity, indicating that the endogenous fission yeast reductase can support the ET chain of recombinant CYPs (Durairaj et al., 2019; Yan et al., 2017).

Although the endogenous reductases have been proven effective for heterologous CYPs, there are several instances (discussed in next Section 8.1.2.) where the recombinant CYPs displayed lesser fraction of activity when reconstituted with the endogenous CPR (Braun et al., 2012; Dietrich et al., 2005; Jensen and Møller, 2010). For instance, a combinatorial CYP study on *de novo* production of the steviol glucosides precursor in yeast system with CYP enzymes kaurene oxidase (KO) and

kaurenoic acid hydroxylase (KAH) employed five different reductases (SrCPR1, SrCPR7, SrCPR8, ATR2 and CPR12) and the functional analysis was also compared with the endogenous *S. cerevisiae* CPR (*NCP1*) (Gold et al., 2018). Experimental results demonstrated that the endogenous yeast CPR supported the KO activity significantly in spite of other exogenous reductases. However, the endogenous yeast CPR could not efficiently pair up with the KAH resulting in the need of other CPR combinations to determine the KO-KAH activity. As the highly conserved amino acid residues of CPR are influential in determining the electrostatic interactions between CYP and CPR proteins, even slighter variations in electrostatic potentials of different homologs could affect the interaction efficacy and lower the CYP catalytic activity. In contrast to the common speculation, several experimental evidences indicate that the endogenous CPR may not be compatible for all the recombinant CYPs, and therefore reconstitution with the intrinsic (homologous) (Durairaj et al., 2015; Braun et al., 2012; Lv et al., 2019; Dietrich et al., 2005; Jensen and Møller, 2010; Ducassou et al., 2015; Alcaíno et al., 2008) or optimal (heterologous) (Ebrecht et al., 2019; Gold et al., 2018; Neunzig et al., 2013; Christ et al., 2019; Theron et al., 2019; Jin et al., 2019; Liu et al., 2019) redox partner is desirable.

8.1.2. CYP reconstitution with homologous CPRs

Several reports have established that the coupling efficiency and ET compatibility of cognate (the same source of recombinant CYP origin) or homologous redox partner is relatively higher, thus resulting in enhanced monooxygenation activities (Fig. 5B) (Durairaj et al., 2015; Braun et al., 2012; Neunzig et al., 2013; Lv et al., 2019; Durairaj et al., 2015; Dietrich et al., 2005; Jensen and Møller, 2010; Ducassou et al., 2015; Alcaíno et al., 2008; Zöllner et al., 2010; Alcaíno et al., 2012; Zehentgruber et al., 2010). For example, recombinant production of human CYP2D6 in the methylotrophic yeast demonstrated that the endogenous *P. pastoris* CPR seems to be incompatible with the heterologously expressed human CYP, therefore resulting in no catalytic activity (Dietrich et al., 2005). By contrast, co-expression of the homologous reductase human CPR favored CYP2D6 activity in *P. pastoris* and resulted in the conversion of different substrates including the 3-cyano-7-ethoxycoumarin, parathion and dextromethorphan. Similar pattern was observed when human CYP2D6 and CYP3A4 enzymes were co-expressed with the endogenous YlCPR of *Y. lipolytica* and homologous human CPR to study the steroid biotransformations in biphasic systems in *Y. lipolytica* (Zehentgruber et al., 2010). Therein, the YlCPR not only displayed stronger expression, but also showed about 40-fold higher NADPH cytochrome c reductase activity compared to human CPR. Nevertheless, this attribute of YlCPR did not reflect on the desired CYP activity, while the clones expressing human CPR demonstrated significantly higher CYP activity towards steroid biotransformations. Similarly, another example towards functional characterization of human CYP2U1 in *S. cerevisiae* showed that the strain W(R) over-expressing yeast reductase displayed stronger reductase activity than the strain W(hR) over-expressing human reductase (Ducassou et al., 2015). However, the desired CYP2U1 activity towards substrate screening was obtained only with the human CPR, while the strain encompassing yeast reductase led to an inactive CYP system. All these results clearly suggest that mammalian CYPs can effectively couple with mammalian CPRs and form desired protein-protein complexes as compared to that of endogenous CPRs (Table 2).

Remarkably, similar phenomenon was also observed in the recombinant CYPs of plant origin, where the endogenous yeast CPR seemed inefficient to ensure adequate supply of electrons to optimize CYP activity, while co-expression of plant CPR led to enhanced activity (Jensen and Møller, 2010). In a recent study on functional screening of the plant CYP enzyme flavonoid 3-hydroxylase (F3'H from *Gerbera hybrid*), one plant-sourced CPR (CrCPR derived from *Catharanthus roseus*) and two yeast-sourced CPRs (YlCPR from *Y. lipolytica* and ScCPR from *S. cerevisiae*) were examined for the biosynthesis of hydroxylated flavonoids in *Y. lipolytica* (Lv et al., 2019). Among the different combinations of F3'H-CPR pairs, the plant-sourced F3'H-CrCPR pair enabled the highest naringenin to eriodictyol and taxifolin conversion ratios (90.5% and 56.8%, respectively), and the optimal ratio of F3'H to CrCPR was determined to be 1:2. Several CYPs of fungal origin also demonstrated the preference towards homologous redox partner over the endogenous reductase. Notably, combinatorial reconstitution of *F. oxysporum* CYP53A19 with endogenous reductase ScCPR (*S. cerevisiae*), homologous reductase FoCPR (*F. oxysporum*), and heterologous reductase CaCPR (*C. albicans*) clearly elucidated the prominence of intrinsic redox partner (Durairaj et al., 2015). Therein, reconstitution of CYP53A19 with the endogenous reductase resulted in the moderate conversion of benzoic acid and 3-methoxybenzoic acid, while reconstitution with the heterologous reductase only gave poor conversion of benzoic acid alone. Interestingly, reconstitution of CYP53A19 with homologous reductase not only demonstrated increased conversion rates of benzoic acid and 3-methoxybenzoic acid, but also exhibited preference towards 3-hydroxybenzoic acid conversion. In carotenogenic yeast *Xanthophyllomyces dendrorhous*, the P450 astaxanthin synthase CrtS catalyzed the ketolization and hydroxylation of β-carotene and produced astaxanthin only when CrtS was co-expressed with homologous CPR (CrtR) in *S. cerevisiae* (Alcaíno et al., 2008). CrtS showed high specificity to CrtR over ScCPR due to the preferential electrostatic interactions, more hydrogen bonds, and ease in salt bridge formation with the FMN binding domain of homologous CPR (Alcaíno et al., 2012).

Despite the apparent prominence of homologous reductase in CYP reconstitution, several reports have exemplified the significance of underrated heterologous reductases as well (discussed in next Section 8.1.3). Besides, the cognate CPR is not yet available or identified for all the eukaryotic CYPs, and therefore reconstitution with the well-studied and optimal heterologous CPR (irrespective of expression host or recombinant CYP) is sometime plausible.

8.1.3. CYP reconstitution with heterologous CPRs

Interestingly, CPR can serve as an ideal bio-brick in synthetic biology as it can secure ET between CYP enzyme complexes of various origins based on the principle of "share your parts". In prokaryotic systems, it is a common practice to employ surrogate or heterologous redox partners (*e.g.*, spinach Fdx/FdR, Adx/AdR, Pdx/putidaredoxin reductase (PdR), and the *Sel*Fdx1499/*Sel*FdR0978) to reconstitute the activities of recombinant bacterial CYPs (Sagadin et al., 2018; Li et al., 2020). Likewise, eukaryotic CYPs can also be reconstituted with the heterologous CPRs of various origins from not only different species, but also different kingdoms (Fig. 5C) (Ebrecht et al., 2019; Gold et al., 2018; Neunzig et al., 2013; Christ et al., 2019; Theron et al., 2019; Jin et al., 2019; Liu et al., 2019; Whitelaw et al., 2015). For instance, the comparative activity study of seven microsomal CYPs (CYP2C9, CYP2D6, CYP3A4, CYP3A5, CYP3A7, CYP17, and CYP21) in fission yeast by co-expressing different CPRs viz., *Homo sapiens* (hCPR), *S. pombe* (CCR1) and bishop's weed *Ammi majus* (AmCPR) demonstrated mixed results (Neunzig et al., 2013). Therein, hCPR yielded the best results for CYP3A enzymes, while CYP2D6 displayed its highest activity when co-expressed with CCR1, and CYP2C9 preferred AmCPR. Recently, systematic improvement of betulinic acid biosynthesis was carried out in *Y. lipolytica* by using heterogenous CYP/CPR systems and engineering other functional modules (Jin et al., 2019). Specifically, 25 CYP-CPR combinations originating from 5 different P450 sources [CYP716A180 (*Betula platyphylla*), CYP716AL1 (*Catharanthus roseus*), CYP716A12 (*Medicago truncatula*), and CYP716A15 and CYP716A17 (*Vitis vinifera*)] and redox partner sources [*ATR2* (*A. thaliana*), CrCPR (*C. roseus*), GuCPR (*Glycyrrhiza uralensis*), LjCPR (*Lotus japonicus*) and MTR (*M. truncatula*)] were constructed to screen the active CYP-CPR pairing for the biosynthesis of betulinic acid from lupeol. Interestingly, the strains encompassing CYP716A180 with the heterologous CPRs (LjCPR or MTR) yielded the highest betulinic acid titers (25.62 ± 2.20 mg/L and 32.33 ± 4.34 mg/L) compared to other recombinant strains with different CYP-CPR combinations. Remarkably, an interesting study depicted the direct comparison of homologous reductase (*Starmerella bombicola*

CPR) and the heterologous reductase (*A. thaliana* CPR) with the fatty acid hydroxylase CYP52M1 from *S. bombicola* in the engineered *Saccharomyces cerevisiae* strain (Liu et al., 2019). Experimental data demonstrated that the heterologous system (CYP52M1-AtCPR1) produced 83.2 mg/L of long chain ω-hydroxy fatty acids, which was ~2.2-fold higher than that of homologous system (CYP52M1-SbCPR1). On contrary to the general proposition, all these results (Table 2) indicate that the heterologously reconstituted systems could also aid better coupling and strengthen CYP-CPR interactions leading to constructive catalysis, and therefore calls for a more in-depth investigation to elucidate the underlying mechanisms.

8.2. Significance of multiple CPRs in CYP monooxygenation

Apparently, there exists a general notion that CYP enzymes exclusively demonstrate high divergence with varied CYP counts (ranging from few to several hundreds), whereas their imperative redox partner CPR exists as a single form. Though this paradigm is true to some extent as most of the eukaryotic species from *S. cerevisiae* to *H. sapiens* possess only a single CPR that is capable of donating electrons to multiple CYPs (Durairaj et al., 2019). Remarkably, certain fungal and plant species can encompass more than one CPR, which not only reflects the diversification of CYPs, but also stipulates the need for additional redox source to satisfy the higher demand of reducing equivalents by CYPs (Urlacher and Girhard, 2019; Durairaj et al., 2016; Rana et al., 2013). Generally, the CPR count in eukaryotes can vary from one to three depending on the species, and the diversity of CYPomes. While certain plant species such as *Arabidopsis* thaliana, Gossipium hirusitum, *Withania somnifera*, Petroselinum crispum, Helianthus tuberosus, and Centaurium erythrae possess two CPR homologs (Rana et al., 2013; Koopmann and Hahlbrock, 1997; Benveniste et al., 1991; Mizutani and Ohta, 1998; Schwarz et al., 2009; Yang et al., 2010); higher plants including the Nothapodytes foetida and Hybrid poplar can encompass up to three CPR paralogs (Huang et al., 2012; Ro et al., 2002). Likewise, though several fungal and yeast species (*A. oryzae, A. nidulans, A. niger, A. fumigatus, Rhizopus oryzae* and *C. tropicalis*) are known to possess two CPRs (Ebrecht et al., 2019; Lah et al., 2008); *Fusarium graminearum* encompass three CPR paralogs, while its closer relative *F. oxysporum* contains four putative CPRs exceptionally (Durairaj et al., 2016; Durairaj et al., 2015). Though the biological significance of multiple CPRs in eukaryotes is unclear yet, recent studies have shown that the functional significance of each and every CPR paralog is different (Durairaj et al., 2016; Lah et al., 2011; Novak et al., 2015; Lah et al., 2008). In the fungal CYP system several examples depicted the prominence and versatile catalytic activities of CPR1 and CPR2 paralogs (Durairaj et al., 2016). It was hypothesized that one CPR (CPR1) might be responsible for CYPs during endogenous primary metabolism and expressed constitutively; while the other (CPR2) possibly functions in secondary metabolism and transcribed under stress, elicitors or inducers (Lah et al., 2011; Novak et al., 2015; Rana et al., 2013). Experimental evidences suggested that both the CPR paralogs could support CYP monooxygenation, but the product specificity might differ. For instance, the fungus *Cochliobolus lunatus* encompasses two CPRs, of which the CPR1-CYP53A15 system supported the conversions of benzoic acid to 4-hydroxybenzoic acid and of 3-methoxybenzoic acid to 3-hydroxybenzoic acid; while the CPR2-CYP53A15 system resulted in the formation of 3,4-dihydroxybenzoic acid for both the substrates through two-step oxidations (Lah et al., 2011). Another interesting study in the medicinal plant, *Withania somnifera* (L.) Dunal (Rana et al., 2013), propounded two divergent isoforms of CPR (WsCPR1 and WsCPR2) exist independently, of which WsCPR1 was un-inducible while the WsCPR2 transcript level increased in a time-dependent manner after elicitor treatments. Interestingly, experimental evidences suggested the possible involvement of WsCPR2 in withanolide biosynthesis (withanolide A and withaferin A) compared to WsCPR1. Recently, *de novo* production of the steviol glucosides precursor with CYP enzymes kaurene oxidase (KO) and kaurenoic acid hydroxylase (KAH) performed a combinatorial study with different redox sources including SrCPR1, SrCPR7, and SrCPR8 from *Stevia rebaudiana* (Gold et al., 2018). Among all the tested combinations, SrCPR1 demonstrated the highest turnover for KO-KAH combinations, and acted as the best coupling partner for several CYP catalysed reactions compared to other CPR paralogs from the same organism. On the whole, comprehensive analysis on multiple CPR paralogs in terms of biological and functional significance will elevate the path for pairing up the right CPR candidate to improve the efficacy of CYP monooxygenation.

8.3. Influence of alternate redox partners

In addition to the aforementioned CPR's significant role, there also exists other natural electron donor partners and several artificial strategies viz., chemical (Nazor et al., 2008), electrochemical (Krishnan and Rusling, 2016; Nerimetla et al., 2017), enzymatic (Sadeghi and Gilardi, 2013; Zuo et al., 2017), photochemical and light-activated systems (Edwards and Bren, 2020; Park et al., 2015; Kato et al., 2020) that can supply reducing equivalents to the heme domain of various specific CYPs. Though these artificial strategies are mostly explored for prokaryotic CYPs, the natural electron donor Cyt B5 has been well-studied for eukaryotic CYP systems (Fig. 1).

Cyt B5, a membrane-bound hemoprotein of ~135 amino acids (17 kDa size) with *bis*-His coordinated heme iron serves as a natural electron transporter in the CYP catalytic cycle, and can be reduced by either NADPH dependent CPR (Fig. 2C) or NADH dependent CBR (Fig. 2D). Association of CYP, CPR and Cyt B5 simulates the formation of efficient electron transport system; however, Cyt B5 is not an obligatory CYP redox partner as it can donate only the second electron (Fig. 1) due to its low redox potential (+20 mV) (Im and Waskell, 2011; Dong et al., 2013). It has been demonstrated that when the second electron is donated by Cyt B5, the catalytic activity of CYP2B4 is significantly increased by ~10- to 100-fold than in the presence of only CPR (Im and Waskell, 2011). Cyt B5 could cause a conformational change in the active site resulting in the rapid formation of active oxidizing oxyferryl species of CYP intermediate. Co-expression of Cyt B5 may stabilize the mRNA and increase the CYP expression level as it can inhibit the mRNA decay pathways during the stationary phase of bacteria (Dong et al., 2013). Two major hypotheses have revolved debating that Cyt B5 could act as an allosteric modulator or serve as a redox partner in the eukaryotic CYP mediated metabolism (Duggal et al., 2016). Binding of Cyt B5 could cause a structural change to the CYP enzyme resulting in increased product turnover by either activating the integral catalytic rate or deactivating the non-productive uncoupling channels / auto-oxidative shunt process. Cyt B5 can mediate, hinder or may not have any effect on CYP catalysis, either in a CYP-dependent or substrate-dependent manner (Bart and Scott, 2017).

Cyt B5 interacts with different drug-metabolizing CYPs (CYP2A6, 2D6, 2E1, and 3A4) with both shared and distinct surfaces, and surface interruption may reflect functionally on metabolite production and/or NADPH consumption (Bart and Scott, 2017). Cyt B5 is well known to be a key regulator for androgen synthesis, however the nature of interaction of CYP17A1 and Cyt B5 remains unclear and long debated (Akhtar et al., 2011; Sergeev et al., 2014). The bi-functional CYP17A1 acts as a branch point due to its essential role in the 17α-hydroxylation of pregnenolone and progesterone leading to glucocorticoid synthesis, or undergo a C-C bond scission in a separate lyase reaction for androgen synthesis (Duggal et al., 2016; Katagiri et al., 1995). Unlike mammals and yeasts, higher eukaryotic plants such as tobacco, Arabidopsis, soybean, *etc.* encompass multiple isoforms of Cyt B5 (Kumar et al., 2012; Smith et al., 1994; Fukuchi-Mizutani et al., 1999). In *Arabidopsis thaliana*, Cyt B5 isoform D acts as an obligate electron shuttle intermediate for biosynthesis of S-lignin as it specifically augments F5H-catalyzed reactions (Gou et al., 2019).

Interestingly, the NADH dependent CBR along with Cyt B5 can replace the canonical NADPH dependent CPR and act as the sole elec-

tron donor (Fig. 2D) (Stiborová et al., 2016). The NADH/Cyt B5/CBR system can be the exclusive donor for both the first and second reduction of human orthologue CYP1A1 during the oxidative metabolism of Benzo[a]pyrene (BaP) and formation of BaP-DNA adducts *in vitro*. Cyt B5 was shown to stimulate the CYP3A4-mediated oxidation of ellipticine in the presence of both NADPH and NADH and play dual roles: (i) sole electron donor to CYP3A4 by mediating both the first and second reduction with the aid of CBR; and (ii) allosteric modifier of CYP3A4 oxygenase (Stiborová et al., 2017). In fungi, several reports demonstrated the involvement of the Cyt B5 and NADH dependent CBR system serving as an alternative electron donor for CYP monooxygenases (Ichinose and Wariishi, 2012; Hatakeyama et al., 2016; Gutiérrez et al., 2015; Syed et al., 2011; Troncoso et al., 2008). In *Fusarium fujikuroi*, the CYP genes involved in gibberellin biosynthesis demonstrated variations not only in terms of regioselectivity, but also in reaction rate and formation of alternative products depending upon the interaction of redox partners: NADPH dependent CPR / NADH dependent CBR-Cyt B5 (Troncoso et al., 2008). The first quantitative comparison of CPR and CBR-Cyt B5 redox enzyme systems was demonstrated in *P. chrysosporium*, wherein the CBR followed a “ping-pong” mechanism (Syed et al., 2011). Moreover, CYP5150A2 exhibited substantial activity in the absence of CPR with the aid of CBR-Cyt B5, while the rate of propylbenzoic acid hydroxylation was significantly dependent on Cyt B5 concentration (Ichinose and Wariishi, 2012). Interestingly, *X. dendrorhous* contains two CBR (CBR.1 and CBR.2) genes; of which CBR.1 shows higher similarity with other microsomal CBRs, while CBR.2 is grouped with mitochondrial CBRs (Gutiérrez et al., 2015). The CBR.1-Cyt B5 served as an alternative electron donor to CYP51 and CYP61 enzymes during sterol biosynthesis, and it has been shown that the gene disruption of CPR is not lethal, as the CBR-Cyt B5 ET system enabled the CYP-mediated ergosterol biosynthesis required for cell growth.

9. Conclusion and outlook

Cytochrome P450 enzymes are an integral element towards the emergence of ancestral eukaryotes in the primeval prokaryotic era. CYPs have undoubtedly played crucial roles during the evolution and diversification of ancestral monocellular eukaryotes into multicellular eukaryotes. Eukaryotic CYPs have also been evolved into highly diverse multifunctional and versatile enzymes with their essential roles starting from organismal adaptation to diverse ecological niches, housekeeping biochemical reactions, chemical warfare in synthesizing/neutralizing metabolites and detoxification of xenobiotics. This course of evolution has brought us to a new era where CYPs on the whole has made a revolutionary progress in replacing/supplementing synthetic chemistry by synthetic biology. Nevertheless, despite the versatile and multi-functional properties, impractical attainment of sufficient recombinant proteins and product yields restricts the incorporation of eukaryotic CYPs industrially. Though some impressive examples such as the production of artemisinic acid (precursor to the antimalarial drug artemisinin) (Paddon et al., 2013) and taxadien-5α-ol (precursor to the anticancer drug taxol) (Ajikumar et al., 2010) have illustrated the industrial application of eukaryotic CYPs, there remains a serious concern in terms of cost and yield. Moreover, heterologous expression of membrane-bound eukaryotic CYPs in surrogate cell factories and exploitation of purified enzymes still remains to be a problem to be addressed. Utilization of conventional surrogate cell factories primarily of prokaryotic or unnatural CYP-producing host and eukaryotic or natural CYP-producing host are though beneficial, there exists an array of challenges (Fig. 3). Requirement of heme precursor, redox partner(s), cofactor NAD(P)H, post-translational modifications, lack of internal membrane system, proper protein folding and enzyme stability are the major drawbacks for full-length membrane-bound eukaryotic CYP expression in bacterial systems. By contrast, difficulties in extraction or purification of P450 enzymes, expression rate, crystallization studies, biocatalyst yield (g/g cdw) and space-time yield (g/L/h) are significant limitations of the yeast cell factory. Besides, heterologous expression of eukaryotic CYPs in other surrogate cell factories viz., fungal, plant, or cell-line systems is again limited by the factors such as cost, time, yield, technical demand, interference of endogenous CYPs, limitation in structural investigations and so on. To sum up the debate on membrane-bound eukaryotic CYP expression, each and every surrogate cell factories have their respective advantages, but there exists no such an ideal expression system that is devoid of any constraints or limitations (Table 1). As a rule of thumb, the choice of a surrogate cell factory for eukaryotic CYP production has to be decided based on the question on hand by limiting the downsides as low as possible. Combinational comparison or amalgamation of different cell factory systems could be a useful approach to solve the limitations and exploit the advantages of each expression system for efficient biotechnological and industrial application of eukaryotic CYPs.

With the rapid development of modern synthetic biology and protein engineering approaches, tremendous researches have been put forth to improve the efficiency and efficacy of eukaryotic CYPs as well as their redox partners, CPRs. Based on the inspiration of natural prokaryotic fusion protein P450 BM3, several studies have shown that construction of artificial eukaryotic CYP-CPR fusion proteins could facilitate intra-molecular ET in a much more precise manner favouring enhanced monooxygenation (Sadeghi and Gilardi, 2013; Hlavica, 2009). Moreover, site-directed mutagenesis of redox partners has proven to be a competent strategy in fine tuning the CYP selectivity in order to obtain the desired product patterns (Esteves et al., 2020; Sagadin et al., 2019). Remarkably, the underlying CPR-mediated CYP metabolism was recently perceived to be crucial, and the success of CYP biocatalysis also relies on the abundance, ET compatibility and coupling efficiency of its redox partner protein (Durairaj et al., 2016; Ebrecht et al., 2019; Li et al., 2020). Experimental evidences have suggested that the source and choice of redox partner can not only influence the catalytic efficiency, but also alter the substrate specificity and product distribution of eukaryotic CYPs. Apparently, the source of CPR plays a significant role in CYP mediated biotransformations as it can influence the reactivity, specificity and productivity. On that account, there emerges a new outlook affirming the functional progression or transition of CPR from ‘redox partner’ to ‘regulating partner’ in CYP catalysis. Selection of an appropriate CPR is thus imperative to regulate the CYP’s catalytic function as well as to achieve optimal activity. Especially, in order to functionally characterize an orphan CYP or to screen a P450 enzyme for a specific reaction with product selectivity as the major concern, it is profound to reconstitute the recombinant CYP with several redox partner combinations. However, it has to be noted that the decision on the choice of redox partner solely depends on the aim of study, as different redox partner combinations may yield varied results (Table 2). Nevertheless, it is not straightforward to find an ideal redox partner for effective CYP pairing; there exists a grey area on the choice of selection between the homologous and heterologous CPRs as CYP reconstitution with both the sources offering mixed results (some CYPs show preference towards homologous CPR, while some perform better with heterologous CPR). A combinatorial CYP reconstitution with different redox partners could elevate the knowledge towards preferential selection of reductases of various origins (Fig. 5). Besides, this combinatorial approach could facilitate better CYP monooxygenation by selecting the right CPR candidate in a much more consolidated way. Of note, the interest over the binary complex of CBR/Cyt B5 system is emerging, and it is appropriate to investigate whether the CPR-independent eukaryotic CYP systems could be an effective alternative redox partner system (Fig. 2D). Moreover, there arises a particular question of whether this alternative CPR-independent ET pathway can be applicable to all eukaryotic CYPs or it is CYP-specific. On the whole, an in-depth comprehensive analysis is highly expected to understand the underlying mechanism on how an auxiliary ET partner could act as functionality-determining factor in CYP monooxygenation.

Today, various challenges associated with eukaryotic CYP in terms of increased activity, selectivity, and stability have been resolved. How-

ever, efficient production of membrane-bound eukaryotic CYPs and effective construction of a compatible CYP-CPR system remain the two major unsolved problems that have been limiting the industrial breakthrough. We envision that the core bottlenecks and the solutions addressed in this review will bring a new paradigm to explore and exploit the eukaryotic CYPs with much more in-depth understandings.

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.

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Supplementary materials

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