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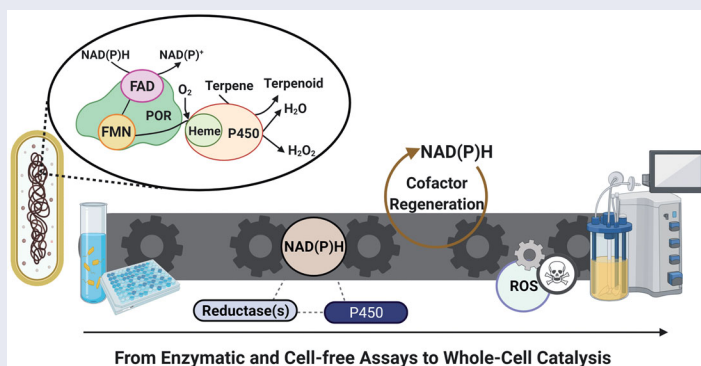
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ABSTRACT

The growing preference for producing cytochrome P450-mediated natural products in microbial systems stems from the challenging nature of the organic chemistry approaches. The P450 enzymes are redox-dependent proteins, through which they source electrons from reducing cofactors to drive their activities. Widely researched in biochemistry, most of the previous studies have extensively utilised expensive cell-free assays to reveal mechanistic insights into P450 functionalities in presence of commercial redox partners. However, in the context of microbial bio-production, the synergic activity of P450-reductase proteins in microbial systems have not been largely investigated. This is mainly due to limited knowledge about their mutual interactions in the context of complex systems. Hence, manipulating the redox potential for natural product synthesis in microbial chassis has been limited. As the potential of redox state as crucial regulator of P450 biocatalysis has been greatly underestimated by the scientific community, in this review, we re-emphasize their pivotal role in modulating the *in vivo* P450 activity through affecting the product profile and yield. Particularly, we discuss the applications of widely used *in vivo* redox engineering methodologies for natural product synthesis to provide further suggestions for patterning on P450-based terpenoids production in microbial platforms.

GRAPHICAL ABSTRACT



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Redox; biocatalysis; terpenoids; microbe; P450; reductase; microbial cell factories

Introduction

Initially, cytochrome P450 oxidoreductase superfamily (P450s or CYPs) looks an outstanding catalyst for the biosynthesis of natural products like terpenoids. They alter the biological properties of the terpenoids through regio- and enantioselective hydroxylation, rendering them the largest structurally diverse group of plant natural products with distinct characteristics

[1–3]. However, while more than 300,000 P450 sequences have been reported from all trees of life up to 2018 [4], these have not all been exploited for industrial production in versatile microbial systems. It is undeniable that our limited knowledge of the function of terpene synthesis networks in non-native environments has hindered this goal. Therefore, the majority of review papers have mainly focused on P450 enzyme and

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related metabolic engineering [5–7]. However, a thorough discussion on the redox topic, particularly for terpenoid synthesis in microbial cells, is missing. Here, we highlight the importance of reductase partner engineering as a promising approach for feasible P450-based biocatalysis in microbial cells. Our main points include: assessing the P450-reductase fusion protein construction and reducing equivalents regeneration with regards to P450 activity and product profile. Conversely, P450 enzymes have also been recognised for generating redox signaling molecules, which exert a physiological impact on the cell [8]. Consequently, as a precautionary approach, we highlight the drawbacks to excess cofactor and reductase expression, and we propose potential strategies to minimise the cause of oxidative stress for more efficient P450-based biocatalysis.

Classification of P450s by reductase partners

While widely known for their hydroxylation activity, the cytochrome P450 enzymes can catalyse many other natural reactions such as: N- and S-oxidation, dealkylation, hydroxylation, peroxidation, epoxidation, aromatic hydroxylation, oxidative and reductive dehalogenation, and deamination. Some of these have been extensively covered in other excellent reviews [9–11]. These flexibilities make P450 enzymes superior to lengthy, low-yield and not eco-friendly organic chemical syntheses. There are several patents associated with P450-catalysed microbial biosynthesis of terpenoids such as: carotenes [12], sesquiterpenes such as: isovalencene, spirovetiva-1(10), 7(11)-diene and valencene [13], flavored nootkatone, vanillin, and valencene-containing alcoholic beverages [14], and diterpenes [15], with commercially well-known examples being: artemisinin and nootkatone by Amyris [16] and Oxford Biotrans and Isobionics companies, respectively. Despite these success stories, the general complex activity of P450s and, potentially, the species-specific dependency on electron transfer proteins hamper their usage in wider areas. Based on these interacting proteins which shuttle the electron, the cytochrome P450 enzymes, including P450s, are categorised into ten different classes, among which the most widely studied are classes I, II, III, VII, and VIII as shown in Figure 1. There are several past review papers about the classification of P450 enzymes [22–27]. However, the important point and the common scenario is that, through redox partners such as: FAD-containing ferredoxin reductase (FdR), ferredoxin (Fdx), FAD- and flavin mononucleotide (FMN)-binding cytochrome P450 reductase (POR, formerly known as CPR), or FMN-dependent small redox protein (flavodoxin;

Fld), the electron is shuttled from the reducing equivalent (NADPH) to the heme-iron of P450 enzyme, where the oxygen is activated to convert the terpenes to terpenoids.

The regulatory effect of reductase partner on P450 function

In bacterial systems, the electrostatic interactions between the ferredoxin (Fdx), P450 heme and the reductase domains, as well as the negatively and positively charged amino acids on the Fdx iron-sulfur cluster and P450 proximal site, mediate the conformational changes of the Fdx for electron transfer to P450s [28,29]. In addition, the substrate binding to P450 induces P450 conformational change to increase its preference for Fdx through electrostatic and steric complementarity [30].

However, the plants as one of the main abundant sources of terpenoids [31,32], employ a more complex, endoplasmic membrane-bound class II P450 enzymes. These systems are composed of a P450 and FAD- and flavin mononucleotide (FMN)-binding cytochrome P450 reductase (POR) [22]. Similar to bacterial P450s, the interaction efficiency between POR and P450 is known to be modulated by the electrostatic potential of each protein and docking of acidic POR residues from the negatively charged FMN to the basic, positive charge region at P450 prosthetic heme site [33–35], although it cannot be generalised to all P450s as reported with human microsomal P450s [36]. Despite having a lower electron transfer rate to soluble bacterial P450s, POR binding can reorient the P450 catalytic domain, and vice versa, to decrease the interaction of P450 with the membrane, while the interaction of POR FMN and NADP domains with P450 can enable the electron transfer [37]. Furthermore, the presence of several hydrophobic residues at the P450 might play a role in mediating P450-POR interaction and in POR recognition by P450 [38,39]. It has also been proposed that both POR upward orientation outward the membrane and FMN-binding domain rotation might co-occur to promote the POR and P450 binding and electron transfer to P450 heme iron [40], and the latest studies have shown that the closed conformation of POR is also important for intramolecular electron transfer for heme oxygenases [41]. Here, NADP⁺ binding and dissociation regulate the specific induced-fit-like changes in POR with highly interspecies-conserved FMN-domains to have an open, electron-donating conformation, while also influencing the interflavin and POR-P450 electron transfers [36,42–44]. This enhances P450 competing

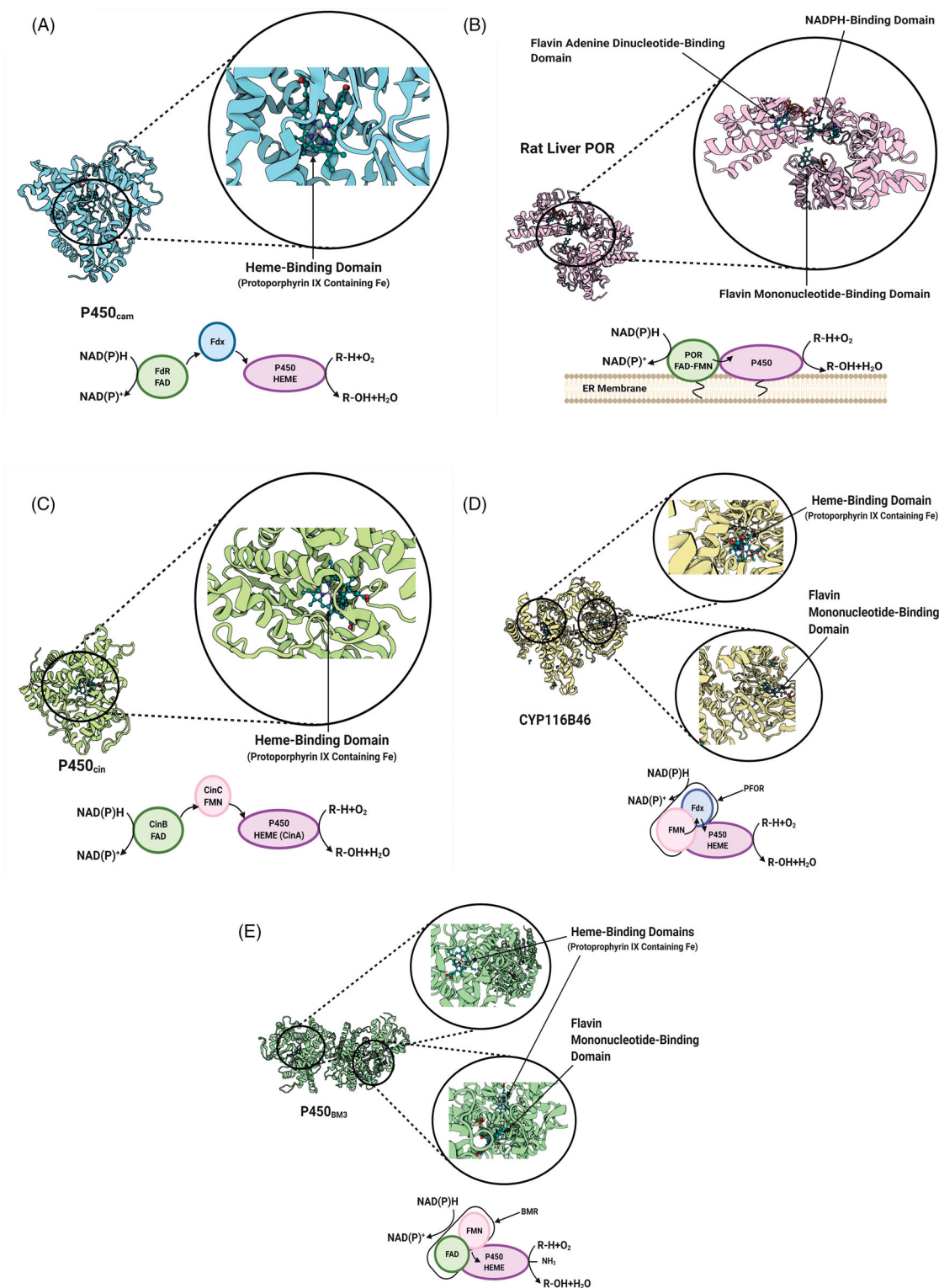


Figure 1. Examples of several widely investigated P450 classes with examples. (A) Class I-P450_{cam} [2GR6]; (B) Class II and Rat Liver POR [1AMO [17]]; (C) Class III-P450_{cin} [1T2B [18]]; (D) Class VII-CYP116B46 [6LAA [19]]; (E) Class VIII-P450_{BM3} [1BVY [20]]. Fdx: ferredoxin; FMN: flavin mononucleotide; PFOR: phthalate family oxygenase reductase; POR: cytochrome P450 reductase; FAD: flavin adenine dinucleotide; CinA: P450_{cin} heme active site; CinB: P450_{cin} FAD containing protein cindoxin reductase; CinC: P450_{cin} FMN containing flavodoxin [21]. Figure created with [BioRender.com](https://www.biorender.com).

capability to interact with a specific POR among many other structurally diverse P450s [45]. Various studies have highlighted the importance of POR in P450-

mediated terpenoid biosynthesis. For example, the early attempt to build the paclitaxel biosynthetic pathway in *Saccharomyces cerevisiae* yielded a very low

hydroxylated product titer of taxa-4(20),11-dien-5 α -ol, being about 25 μ g/L when only *CYP725A4* was expressed [46]. One claim was that the absence of a POR partner did not promote hydroxylation [46]. This assumption was later confirmed in our recent study, where tandem, heterologous expression of both *Taxus* sp. *CYP725A4* and *POR* in *S. cerevisiae* resulted in approximately 78 mg/L oxygenated taxanes produced at 1 L bioreactor scale [47].

Some P450 enzymes require other interacting partners such as cytochrome b5 (Cb5), a small membrane-anchored haemoprotein, that forms a heterodimeric complex with P450 to provide additional redox support as an electron buffer for P450 and POR interaction [16,33,48–51]. Cb5 reductases also exist in all human cell types, facilitating some P450-catalysed steroidogenesis [52] and the functional deficiency of this enzyme has been associated with congenital methemoglobinemia [53,54]. Due to either being more thermodynamically favorable [55] or because of its higher redox potential (by 320 mV), being 20 mV [50], Cb5 can transfer the second electron to P450 ferric-superoxo heme species (Figure 2). However, it does not need to necessarily act in electron transfer, but it can modify the P450 tertiary structure and its active site as an allosteric effector, which can increase the substrate affinity, coupling efficiency and breakdown from products [55,58–62]. Cb5 can also increase P450-peroxide anions stability and faster second electron transfer for more rapid product formation [51,58,63]. For instance, Bart and Scott [64] reported a 60% less NADPH consumption using wild type Cb5 and *CYP2A6* for catalysing *in vitro* coumarin 7-hydroxylation, despite no increase in product yield.

Estrada et al. [65] reported that Cb5 promoted the androgen-forming, 17 α -hydroxypregnenolone lyase activity of *CYP17A1*. Hence, they are likely to be useful

for terpenoid biosynthesis applications. A recent study by Wang et al. [66] showed that triterpenoid acid titer was increased by eight-fold upon integration of *Glycyrrhiza uralensis* *Cb5* in *S. cerevisiae* strain with integrated *CYP88D6*, *CYP72A154*, B-amyryn synthase, and *Arabidopsis thaliana* *POR1* genes and overexpressed key mevalonate pathway genes (*ERG20-ERG9*, *ERG8*, *ERG10*, *ERG13*, *ERG1*, and a truncated *HMG1*). Similarly, Paddon et al. [16] reported up to 40% improvement in artemisinic aldehyde intermediate in artemisinic acid production by co-expressing *A. annua* *Cb5* in *S. cerevisiae* expressing *Artemisia annua* *CYP71AV1* and *POR*. However, in Sagwan-Barkdoll and Anterola [67] study, co-expressing the *Taxus x media* *Cb5* gene with *CYP7254*, *POR*, and other paclitaxel biosynthesis genes resulted in the complete absence of main product (taxa-4(20),11-dien-5 α -ol), while decreasing the concentration of undesired oxygenated taxanes. These results denote that the Cb5s are P450-specific or the P450s have different numbers of positive Cb5 binding sites [68]. Also, the competition of Cb5 and POR in binding to P450 proximal surface due to overlapping binding sites on P450 [36,64] should not be ignored. Despite this, it has been proposed that, at a highly coupled substrate metabolism rate, the reaction is not sensitive to Cb5 [62] and the POR-P450 ionic strength can also modulate the Cb5 competing capability [36].

Engineering the expression of the P450 and interacting reductase genes

The first rational step for increasing terpenoid productivity is to improve the substrate availability. However, this approach is at the risk of an irreversible mechanism-based inactivation of the enzyme [69]. Instead, more studies have attempted to control the P450 expression and improve the product formation in terms

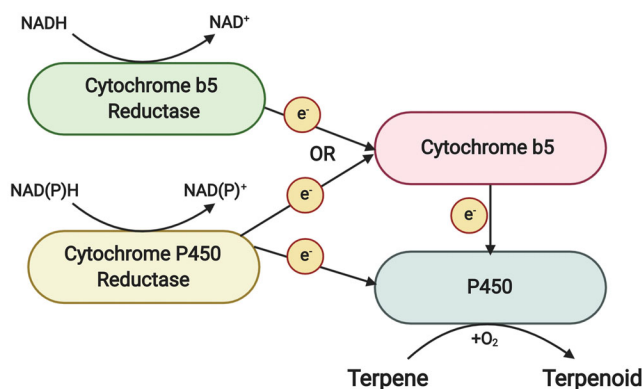


Figure 2. Cytochrome b5 mechanism of action. Cytochrome b5 (Cb5) acts as an electron buffer for P450 enzyme and can transfer the second electron from cytochrome b5 reductase or cytochrome P450 reductase to P450 enzyme. Adapted with modifications from [56,57]. Figure created with BioRender.com.

of the reductase power. An example of such work is depicted in Emmerstorfer et al. [70] study where overexpression of *ICE2* led to POR (AtCPR1) stabilisation and enhanced (+)-valencene conversion by 40% due to improved P450 enzyme and mRNA stability and increased *S. cerevisiae* endoplasmic reticulum membrane. In Chen et al. [71] study, the hydrocortisone production was also improved by approximately 3.4-fold through: screening a variety of PORs, deleting the enzymes associated with C20-hydroxylation by-products formation NADPH-dependent aldo-keto reductase (*YPR1*) and glycerol dehydrogenase (*GCY1*) [72], careful promoter selection, increasing the CYP5311B2 (R126D/Y398F) genomic copy numbers to two and genomic expression of *Absidia orchidis* *Cb5*. Although modulating the P450 and reductase gene dosages seems a common practice in P450 microbial research, here, increasing the gene copies of *A. orchidis* POR did not make any significant improvement to hydrocortisone production. This is beneficial considering the possibility of oxidative stress and reactive oxygen species (ROS) generation. This phenomenon can happen with overexpressing reductases or P450s, leading to toxicity and reduced growth rate as occurred with overexpressing *S. cerevisiae* endogenous POR, *NCP1*, for abscisic acid production [73]. Similarly, according to Biggs et al. [74], the optimum activity and highest oxygenated diterpene concentration by N-terminally truncated CYP725A4 was achieved using a low-copy number plasmid (five-copy) and a weak promoter (Trc). In contrast, in the Sarrade-Loucheur et al. [75] study, the genomic overexpression of human and yeast POR genes did not impair the *S. cerevisiae* growth, and Zhao et al. [76] obtained up to 96% efficiency in triterpene protopanaxadiol production in yeast using three copies of P450-POR fusion protein without affecting the cell growth. Modulation of P450 and POR copy numbers in the Gold et al. [70] study also showed an increase in steviol production upon increasing the rate-limiting P450, KO, expression at the ratio of (KO[P450]: KAH[P450]: POR = 2:1:1). Therefore, the outcome is thought to be dependent on the final product and the P450 enzyme. As the risk of metabolic load through high expression of recombinant genes is undeniable [77], a balance of heterologous productivity with cellular fitness needs to be achieved. We therefore suggest the readers refer to [78] for an excellent review on advances in terpene production through microbial host engineering. Notwithstanding, we envisage the future of microbial biocatalysis will rely on microbial consortia to distribute the metabolic tasks, while using more sophisticated exoelectrogenic and riboflavin-secreting microorganisms like *Shewanella* sp.

and *Geobacter* sp. to facilitate the P450 biocatalysis [79,80]. However, a few relevant studies have been conducted in P450 area. An example is the production of up to 33 and 30 mg/L of oxygenated taxanes or nootkatol, respectively, using microbial consortia of *Escherichia coli* and *S. cerevisiae* [81].

Synthetic biology tools for P450 expression optimisation

Back to our main discussion, in a single microbial chassis, the P450 expression can be more effectively controlled *via* standard tools offered by synthetic biology such as pre-evaluated promoter libraries. Generally, the inducible promoter systems are preferred in P450 bio-transformation, however strong constitutive promoters can favor higher terpenoid titers. For instance, a 3.1-fold increase in β -nootkatol and (+)-nootkatone titers in *S. cerevisiae* were achieved using HXT7 and CYC1 promoters instead of GAL1 for *Hyoscyamus muticus* pre-naspirodiene oxygenase (*HPO*) and *A. thaliana* AtCPR, respectively [82]. However, given a small set of characterised promoters and the lack of such promoter-terminator toolkits for P450 research, other regulatory elements can also be utilised to further modulate the expression of these genes in parallel. For instance, the carotenoids titers were increased by four-fold in *E. coli* using a small library of relatively short ribosome binding sites (RBS) [83] or a 51% increased β -carotene production was achieved using degenerate RBS for tuning the genomic expression of highly-transcribing mevalonate pathway *mvas* and *Hmg1* genes in *E. coli* [84].

The transmembrane linkers of the membrane-bound eukaryotic P450s and reductases can regulate their possible interactions with adjacent macromolecules on endoplasmic reticulum, while the P450 linker assists in its folding to obtain its functional state at the cytoplasmic interphase [85]. However, these insoluble P450s and PORs promote the inclusion body formation upon expression in bacterial cells like *E. coli*. Although P450 overexpression in *E. coli* have been shown to decrease the translation of upstream enzymes in methylerythritol 4-phosphate (MEP) pathway [74], much research has been undertaken to circumvent their aggregation problem through N-terminus modification and truncation [86], addition of small peptide tags like polyhistidine, 28-codon leader and small ubiquitin-related modifier and using bovine microsomal P450 17 α -hydroxylase membrane anchorage sequence (MALLLAVF), which however, do not guarantee the optimal enzymatic activity [87–92]. In this regard, a list of N-terminal sequence modifications including bacterial membrane

anchors and signal peptides for improving the P450 expression in *E. coli* cell factories have been suggested as P450-expression enhancing toolbox [93] and have been thoroughly covered in other studies [94,95].

Immobilisation techniques for improved P450 activity

The replacing technologies such as immobilisation have been instrumental for supporting redox activity for P450s while rendering these endoplasmic reticulum-localised enzymes homogenous and soluble [96]. On this point, nanodiscs, which are stable, self-assembled, nanoscale lipid bilayers of plants that are solubilised by amphipathic scaffold proteins, have been successfully used to immobilise P450s and reductases [97,98] (Figure 3(A)). For example, Rouck et al. [100] used nanodiscs to incorporate (N-terminally modified) CYP725A4-(N-terminally truncated) POR chimera constructs to produce the hydroxylated product taxa-4(20),11-dien-5 α -ol. In another interesting immobilisation study, Lee et al. [99] harnessed *E. coli* specific inclusion bodies called “intracellular poly(3-hydroxybutyrate) (P(3HB)) granules” for P450 catalysis. Here, by attaching phasin (P(3HB)-associated protein)-P450 enzyme to the granules (Figure 3(B)), the stability and activity of the P450_{BM3} was improved regardless of changes in reaction conditions. The recovery and purification of the enzymes from bacteria was also easy and the enzyme preserved its activity upon repetitive use. Therefore, investigating this approach for long-term terpenoid biosynthesis would be promising. The optimisation of P450 enzymes can also be achieved through many other approaches such as: codon optimisation, expression of homologous P450s, protein engineering and bioprocess optimisation. However, since the focus of this review paper is on redox state and reductases

rather than P450 enzymes, we invite the readers to refer to some very good P450-focused reviews [48,101,102] to complement the knowledge.

The effect of surrogate reductases on P450 activity

There remain many unknowns regarding the P450s in natural product biosynthesis pathways. In fact, neither the specific biological activities nor the cognate electron transfer partners of all P450 enzymes have been elucidated [103], given that the effect of evolution for selective interactions of P450s with particular reductases cannot be ignored [25]. However, this cannot be generalised to more versatile P450s with detoxification activities in both bacteria and eukaryotes. Besides the traditional *in vitro* assays, there has been extensive research using omics tools to characterise more putative PORs in eukaryotic systems for improved natural products biosynthesis [104]. Despite this, the research outcomes can sometimes show a better P450 activity using surrogate redox partners, particularly because the endogenous microbial redox state might not always adequately promote the P450 activity. Examples include *E. coli* flavodoxin (Fld) and flavodoxin reductase (Fpr) that weakly supported production of 15-hydroxyabietic by CYP105A1 [105], or the overexpression or basal expression of *S. cerevisiae* NCP1 that did not favor sesquiterpenoid abscisic acid and 4-hydroxy-diclofenac production [73,75].

Utilising bacterial surrogate redox partners can also alter or control the site-selectivity and product profile of P450 enzymes. For example, Zhang et al. [106] found that monooxygenase MycG activity was altered with standalone P450_{RhF} reductase domain (RhFRED) which affected the substrate binding availability and rendered the enzyme capable of unnatural N-demethylation of

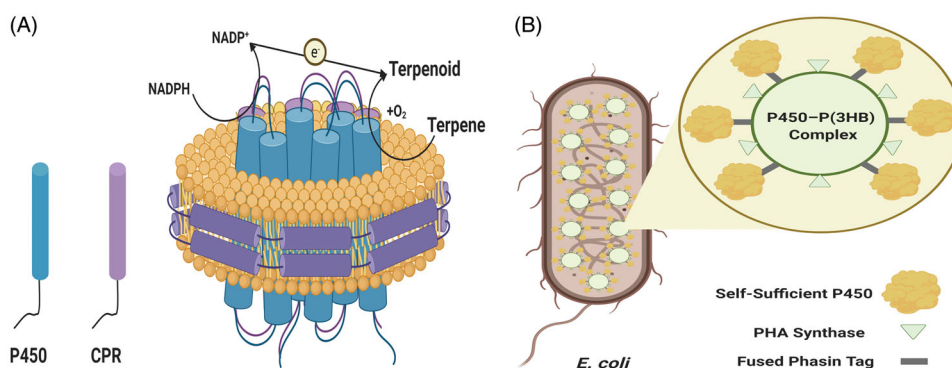


Figure 3. Immobilisation techniques with potential for P450-mediated biocatalysis. (A) Nanodiscs resemble the plant lipid bilayer membranes for P450 and reductase immobilisation and can improve P450 solubility (B) Immobilisation of self-sufficient (reductase-free) P450s on *E. coli* poly(3-hydroxybutyrate) granules increases the P450 enzyme solubility and activity under harsh reaction conditions. Adapted with modifications from [99]. Figure created with [BioRender.com](https://www.biorender.com)

mycinamicin substrates. Among the surrogate reductases, *Pseudomonas putida* putidaredoxin reductase (PdR; CamA) and putidaredoxin (Pdx; CamB) have been utilised for characterisation of the orphan P450s [103]. This was exemplified by Lim et al. [107] who characterised lauric acid regioselectivity using *Sulfolobus acidocaldarius* CYP119 with these redox partners. Likewise, using *P. putida* CamA/CamB for a mutant variant of myxobacterium *Chondromyces apiculatus* DSM436 versatile P450, CYP109Q5 (A280V/T229L), enabled the hydroxylation of β -ionone, valencene and fatty acids in *E. coli*. However, little or no activity was observed using *E. coli* JM109 (FAD-containing ferredoxin reductase) FdR/Fdx and *Bos taurus* FdR/AdX constructs [108]. Apart from the more widely-used and smaller bacterial redox genes, several other studies have relied on eukaryotic PORs to control the P450 chemistry. For instance, Sun et al. [109] used two PORs from *G. uralensis* and one POR from *A. thaliana* and *Medicago truncatula* along with a mutated CYP726A3 (T338S) and achieved higher aldehyde production in triterpenoid licorice biosynthesis pathway in *S. cerevisiae*.

Self-sufficient P450 systems: P450-reductase fusions

Fusing P450s to reductase genes can improve the biocatalysis

Although protein-protein interactions and gene expression levels are crucial for efficient P450-catalysed reactions, there is still a necessity to improve these enzymes in terms of the turnover rate, stability, electron supply source, substrate specificity and promiscuity in a non-native host [110–112]. Fusing the genes in multi-gene biosynthetic pathways have been shown promising for increasing the terpene and oxidised terpenoid products [113,114] without necessarily impacting the protein production level. Following them, the P450s can be fused to reductase. However, it is argued that fusing P450 and redox enzymes might have little or no influence on: enzyme performance, electron coupling and product formation [48,76,115]. For instance, Zhang et al. [116] showed that fusing *Zingiber zerumbet* CYP71BA1 (α -humulene 8-hydroxylase) to *A. thaliana* POR did not result in sesquiterpenoid 8-hydroxy- α -humulene formation, in contrast to low-yield separate expressions. In the same way, Sagwan-Barkdoll and Anterola [67] did not obtain an improved product formation when fusing CYP725A4 to POR gene in *E. coli*. In contrast, Albertsen et al. [113] suggested that enzyme trimming can be used to optimise the active site positioning of enzymes in a fusion construct. A recent study

by Haslinger and Prather [117] showed that linking the P450 and reductase partners outperformed their separate expression for caffeic acid production in *E. coli*. Indeed, the nature has favored mankind with plenty of naturally evolved fusion proteins where the P450 heme domain is covalently bound to a reductive domain [118] and currently, new electron transfer pathways in these self-sufficient P450s are being discovered [119]. Examples include the widely-studied *Bacillus megaterium* self-sufficient P450 (P450_{BM3}) [120], which has an excellent electron transfer system and the fastest substrate oxidation rate among the P450s owing to the proximity and specific positioning of its domains [9,103], *Rhodococcus* sp. ECU0066 P450_{S_{MO}} [121], and *Rhodococcus* sp. NCIMB 9784 CYP116B2 (P450_{R_HF}) [122]. These natural systems can be effectively mimicked for constructing the artificial P450-reductase fusion proteins. For instance, to systematically generate the self-sufficient P450s, Neil Bruce's group [103,123] proposed the ligase- and restriction enzyme-free LICRED cloning method using P450_{R_HF} reductase domain (RhFRED).

The first successful, heterologous self-sufficient P450 system was reported by Sibbesen et al. [124], which was composed of a triple fusion of *P. putida* putidaredoxin reductase (PdR), putidaredoxin (Pdx) and P450_{cam} domains to produce 5-exo-hydroxycamphor and 5-oxo-camphor from camphor in *E. coli*. In another non-terpenoid example, fusion of RhFRED to *S. venezuelae* P450 *PikC* with a 16-aa linker improved the enzyme catalytic activity by four-fold towards both 12-membered ring macrolactone YC-17 and 14-membered ring macrolactone narbomycin [125]. In terpenoid research, a self-sufficient P450 system, consisting of *Thermus thermophilus* CYP175A1, ferredoxin (Fdx), and ferredoxin-NADP⁺ oxidoreductase (FNR) improved the beta-carotene hydroxylation up to 25-fold through improved electron transfer [126]. Artificial fusion of the P450_{cam} variant (Y96F/V247L)-P450_{S_{MO}} reductase domain using a GS linker also enabled the hydroxylation of the terpenes limonene, α -pinene and camphor in Luan et al. [127] study.

Enhancing the electrostatic interactions between P450 and reductase proteins can improve protein-protein interactions and electron transfer [128], resulting in higher P450 turnover number [129,130]. In fusion proteins, this can potentially occur through perfecting the repositioning of redox center and the reduction potential. Indeed, the shorter distance between the Fdx iron-sulfur cluster and P450 heme-iron center as well as between the FAD components and the Fdx iron-sulfur cluster like in plastidic-type (FAD-containing ferredoxin reductase) FdRs (6–8 Å), can enable more optimal

electron transfer as can be seen in plastidic-type FdRs [28,30]. Other studies have also suggested a distance of 6.2 Å based on *Pseudomonas aeruginosa* NAD(P)H:rubredoxin reductase (RdxR)-rubredoxins (Rdxs) electron transfer system [131] or a distance of 14 Å or less between redox center edges for maximised and robust electron transfer rate [132]. However, current limited knowledge and the lack of protein structure of many P450s with terpenoid biosynthesis activity confine advising on proper distancing between the Fdxs. Despite this, previous studies have emphasised on the key role of Fdx aromatic amino acid (F65) and FNR hydrophobic amino acids (L76, L78, and V136) for Fdx as well as flavodoxin (Fld) binding and orientation [128,133]. Therefore, a proper design must be considered not to interfere with biocatalysis which stress the need for auxiliary structures to facilitate this process.

Linkers influence the P450 productivity

Linkers can properly distance the P450 and its active site from redox gene partners and improve their internal spatial and steric orientations [134]. These artificial amino acid chains aim to mimic the natural linkers found in self-sufficient P450 systems or in NADPH-FAD binding domains that closely connect the FAD and FMN domains for improved electron transfer [135]. However, the scientific community has been skeptical about applying these artificial constructs for creating the P450-POR fusions. Contrary to these disagreements, there has been some success in using linkers as previous works suggest. For instance, Li et al. [125], using a 16-aa linker (covalent linkage) stabilised P450-redox partner interaction in PikC-RhFRED system and

positively influenced the electron transfer. Zao et al. [76], using flexible linkers to fuse the truncated cytochrome P450-type protopanaxadiol synthase (PPDS) to a truncated *A. thaliana* reductase (ATR1), improved the yield by 71% and terpenoid substrate (dammarenediol-II) conversion by 4.5-fold in *S. cerevisiae*.

The previous studies have stated a few key parameters for constructing successful P450-redox partner fusions, particularly when using linkers. Beside their lengths and amino acid contents, their position relative to the P450 and the redox gene are important. In this regard, two interesting studies by Vlada B. Urlacher's group have focused thoroughly on the effect of linkers for constructing P450 fusions. In the first study, DualLinX method is proposed as a single-cloning fusion methodology which introduces two distinct linkers simultaneously. Here, *E. coli* flavodoxin (FldA) was fused to its flavodoxin reductase (Fpr) with either a flexible or rigid linker [136] (Figure 4). Using *Bacillus subtilis* CYP109B1 and FldA, and Fpr as reductase partners, the best performance was achieved with a linker length of more than 15 residues as well as with rigid linkers. Similar results using a free mixture of the redox partners at 4:4:1 (Fpr:FldA:CYP109B1) ratio were achieved upon using the ([E/L]PPPP)₄ linker, both reaching around 62% myristic acid conversion and 2.75-fold cytochrome c reductase activity *in vitro*. Also, a later study by this research group [137] demonstrated the best electron transfer for *B. megaterium* CYP106A2 and bovine CYP21A2 using rigid, proline-rich linkers. In the same study, fusing the *B. subtilis* flavodoxin (YkuN) to N-terminus of *E. coli* Fpr with ([E/L]PPPP)₅ linker increased the myristic acid conversion rate and coupling efficiency of CYP109B1 to 94% and by 1.7-fold,

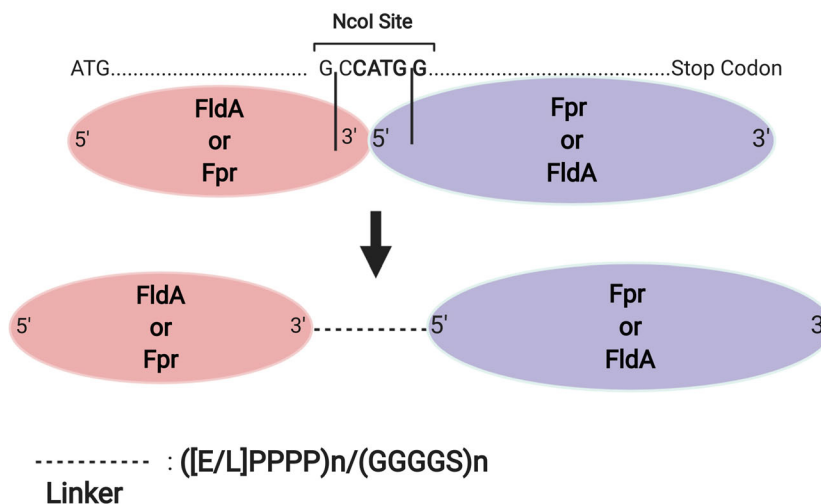


Figure 4. DualLinX method. In this system, *E. coli* flavodoxin (FldA) and flavodoxin reductase (Fpr) are fused with a proline- or glycine-rich linker in any of N- or C-termini attachment orientations, through insertion of an NcoI restriction enzyme site between these two genes. Figure created with [BioRender.com](https://www.biorender.com).

respectively, compared to separate expressions. In addition, the proline-rich linkers were the best in coupling the electron transfer with *B. megaterium* CYP106A2 and bovine CYP21A2.

The proline-rich, rigid linkers have been shown to enable more dynamic conformational changes due to their disordered loop shape [138,139]. Moreover, they promote the electron transfer due to better control of enzyme spatial arrangements and structural isolation of fusion domains from the surrounding amino acids and redox partners [136,140,141]. Despite this, the electron transfer improvement using glycine-rich, flexible linkers has also been reported [142,143]. Bakkes et al. [137] reported that glycine rich-linker showed the highest coupling efficiency of 81.2% with up to 8.1-fold decrease in NADPH oxidation rate. Some good applications of flexible linkers in terpenoid biosynthesis are described in [143,144] studies in *Yarrowia lipolytica*. Li et al. [144], using a GSTSSG linker for fusion of *M. truncatula* CYP716A12 to an N-terminally truncated *A. thaliana* POR (*ATR1*), improved the β -amyrin to triterpenoid oleanolic acid conversion to 100% compared to 72.7% with no linker. The same authors had previously engineered *Y. lipolytica* for production of another triterpenoid, compound K [143]. Here, using the same linker for P450 *PPDS* and *A. thaliana* truncated *ATR1* reductase fusion improved the substrate, dammarenediol II, conversion and final protopanaxadiol titer by 98% and 1.33-fold, respectively. Interestingly, this linker, however, resulted in around 16-fold lower (+)-nootkatone titer using fusion of nootkatone synthase CYP706M1 to truncated *A. thaliana* reductase (*46AtPOR1*) in *Y. lipolytica*. This denotes the importance of other key parameters in tuning the effect of linker, like linker length. Indeed, the linker length can affect the orientation, folding, correct conformation and flexibility of the

enzymes [21,145]. Supportive of this statement, the recent study by Moon et al. [142] showed that the fusion of steviol biosynthesis gene CYP714A2 to *A. thaliana* POR by a series of flexible (GGGG) $n=1-3$ linkers in *E. coli* was only improved by 1.7-fold using a linker length of 3 (15 amino acids) in comparison to length of 1. As evident, a more standardised approach for determining suitable linker seems essential. In this regard, a recent study by Gräwe et al. [146] has proposed a new type IIS restriction enzymes- and T4 DNA ligase-assisted iterative functional linker cloning (iFlinkC) strategy, with a variety of rigid, flexible, short and semi-flexible linkers for fusion protein construction.

Linker-mediated P450-reductase self-assembly systems

The protein self-assembly methods and scaffolds are other flexible tools for co-localising the P450 and redox genes and bringing the redox centers to close proximity for an enhanced electron transfer [111,130]. In this regard, Truyuki Nagamune group, introduced a self-assembling system using *Sulfolobus solfataricus* heterotrimeric proliferating cell nuclear antigen (PCNA) to form a complex known as PUPPET (PCNA-utilized protein complex of P450 and its two-electron transfer-related proteins). Here, *P. putida* P450_{cam} components were localised through fusing the N-termini of *P. putida* P450 components (P450_{cam}, putidaredoxin: Pdx, and putidaredoxin reductase: PdR) to C-termini of three PCNAs, which enabled up to two-fold *in vitro* catalytic activity while achieving 25% of native P450_{cam} activity [147,148] (Figure 5). Later, the same group, Haga et al. [140], showed that improving the Pdx positioning through optimising the linker between Pdx and PCNA, particularly with a rigid linker (G4SP20G4S), enhanced

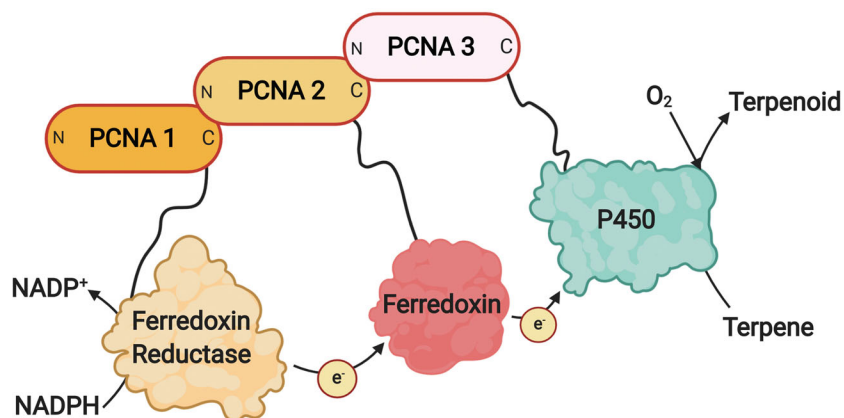


Figure 5. PUPPET system. In this system, the N-termini of *Pseudomonas putida* P450 components are attached to C-termini of *Sulfolobus solfataricus* heterotrimeric proliferating cell nuclear antigen (PCNA), which enables the colocalisation of the redox and P450 enzymes. Adapted with modifications from [147]. Figure created with [BioRender.com](https://www.biorender.com).

the P450_{cam} activity by 1.9-fold, despite not achieving a similar enzyme turnover number as in P450_{cam}. With using glycine-rich linkers ((G4S)*n*, *n* = 1–6), no improvement was achieved, and this was justified by the short end-to-end distance of the linker that potentially resulted in unfavorable interaction of Pdx with P450_{cam}. In their more recent study, Haga et al. [148] successfully recovered 92% of P450_{cam} activity by assembling it with more reductase moieties; three PdRs and two Pdxs moieties. Haslinger and Pratha [117] were the first to deploy the PUPPET system for whole-cell catalysis to produce caffeic acid polyphenol in *E. coli*. In this study, up to six- and eight-fold increase in caffeic acid titer was obtained using palustrisredoxin (Pux)/ putidaredoxin reductase (PdR) and putidaredoxin (Pdx)/PdR, where they fused these ferredoxin reductase (FdR), Fdx and *Rhodospseudomonas palustris* CYP199A2 F185L Δ 7 counterparts to PCNAs 1, 2 and 3, respectively.

Are linkers capable of altering P450 kinetics and product profiles?

Variable study outcomes have made it very difficult to reach a consensus on the type of effect that linkers have on P450 enzyme kinetics. For example, Haslinger and Prather [117] reported that the fusion construct developed using proliferating cell nuclear antigen (PCNA) technology accelerated the P450_{cam} enzymatic activity compared to the free enzymes system. However, the fusion construct (PCNA1–PdR:PCNA2–PdX:PCNA3–P450_{cam}) did not alter its kinetics [147]. Hence, the intermolecular interactions between the redox partner and P450 genes need to be properly investigated to evaluate the efficacy of the linkers. In line with it, Belsare et al. [21] suggested that the heme and FMN sites in P450_{cin} CinA (heme active site)-10aa linker-CinC (FMN-containing flavodoxin) fusion were not fully interacting. This resulted in around 1.7-fold less product formation rate compared to free components mixture. From another viewpoint, the linker effect might be determined by its position in relation to the reductase enzyme. In Jin et al. [149] study, the total triterpenoids concentration decreased by around two-fold or no catalysis occurred when the linker was fused to the N-terminus and C-terminus of POR, respectively. This was regardless of the rigid or flexible linker type for fusing *Betula platyphylla* CYP716A180 to *Lotus japonicus* POR or *M. truncatula* POR for sequential oxidations of lupeol to triterpenoid, betulinic acid. Also, the linkers might only have minimal effect on the activity and stability of the properly-folded P450 as was shown for the endogenous linker of P450 2C2 [150]. It is noteworthy

that the linker structure has proved to be a more important parameter than flexibility for determining the distance and orientation of enzyme partners in a fusion construct [151]. This would potentially play a part in tuning the P450-reductase interactions and their overall activity. Therefore, overall, caution should be exercised to carefully design them to eliminate the possible.

Different viewpoints also exist regarding the potential role of linkers in determining the product spectra. For instance, Biggs et al. [152] showed that fusing the N-terminally modified and truncated CYP725A4 and *Taxus* POR proteins with a GSTGS linker shifted the diterpenoid product: by-product ratio to 1:1, still favoring the by-product (5(12)-oxa-3(11)-cyclotaxane) formation in *E. coli*. This was similar to an older study where fusing the truncated *Taxus* POR to N-terminally modified and truncated CYP725A4 with the same linker resulted in almost equal ratio of main product (taxa-4(20),11-dien-5 α -ol) and by-product (5(12)-oxa-3(11)-cyclotaxane) formation in *E. coli*. However, these results do not seem sufficient to provide a sound base for claiming that the linkers are the sole players in novel P450 product synthesis. Indeed, the role of evolution for the superior kinetic properties of self-sufficient enzymes shall not be ignored. For instance, the linker-containing, self-sufficient enzyme like P450_{BM3} with remarkable kinetic properties, has long served as a model for engineering the other P450s, while also being used to catalyse the sesquiterpene valencene oxidation in *E. coli*, albeit with low selectivity [153]. However, looking at this issue differently, an interesting discovery by Winzer et al. [154] revealed that the presence of a natural fusion protein called "STORR: [(S)- to (R)-reticuline]" which links P450 (CYP82Y2) to aldo-keto reductase (oxidoreductase in high-reticuline (an alkaloid)-producing mutants of poppy lines), prevented the accumulation of highly reactive or unstable intermediates. Therefore, it remains a question whether the by-products are actually the result of inefficient electron coupling due to inefficient linker design or P450-reductase interactions, and they are the actual transient intermediates (metabolons) in the metabolic pathway [155] that rapidly form and are replaced with more stable compounds, or they are simply formed as a result of underground metabolism in the heterologous host [156], or are formed due to low P450 enzymatic activity [73,157]. Clearly, the eventual decision over fusing or separating P450 and reductases and the linker type depends on the context and the individual enzymes. On this matter, the complementary computational protein modeling tools like I-TASSER have proven to be useful for fusion protein design [158,159] to reduce the

possibility of the inefficient electron transfer and formation of by-products.

Regeneration of reducing equivalents for efficient P450 activity

Bioelectrocatalysis and photochemical catalysis: developing cell-free systems for cofactor regeneration

In addition to whole-cell systems, a variety of cell-free techniques such as bioelectrocatalysis have been used in P450 research to provide the required reducing environment. The direct NAD(P)H supplementation in the reaction mixture or P450 immobilisation along with a reductant and electron mediator such as zinc dust and cobalt(II) sepulchrate trichloride have been shown to overcome the self-sufficient P450 enzymes instability and increase the electron transfer rate [110,130,160,161] (Figure 6). For instance, in a recent groundbreaking study by Frank et al. [162], a three-electrode setup in conjunction with a 96-multipotentiostat and vertical-divided cell architecture and indium tin oxide as the electrode material, preserved up to 80% bioelectrocatalytic activity of the immobilised flavin-deficient variant of P450_{BM3}. In another study, by Ulrich Schwaneberg's group [163], the 2- β -hydroxy-1,8-cineole formation rate by *Citrobacter braakii* P450 CinA-10aa-CinC fusion protein [164] was increased by 1.5-fold using either zinc/cobalt (III) sepulchrate or platinum/

cobalt (III) sepulchrate, in comparison to equimolar of CinA, CinC and Fpr mixture in presence of NADPH. However, several studies have shown that bioelectrocatalysis is also at the risk of direct O₂ reduction by the cathode and reactive oxygen species (ROS) generation, unfavorable enzyme orientation, infeasible electrochemical reaction [165,166], and P450 conformation changes [161]. For instance, in Mie et al. [161] study, using lower molecular weight redox partners like ferredoxin (Fdx) compared to ferredoxin reductase (FdR), optimising the concentration of the purified enzyme and using larger and surface-modified electrode (ITO-AUPA) outperformed the conventional NADPH and catalase supplementation methods in reducing the electron uncoupling and side reactions, while increasing the 25-hydroxyvitamin D₃ concentration. However, the P450 activity was decreased by 17% with time in presence of sufficient substrate, which was attributed to some of the most common drawbacks for applying these bioelectrochemical systems. These include the electrode low stability and its deterioration and/or enzyme deactivation and ROS generation. Also, similar to Frank et al. [162], using whole-cell, unpurified *E. coli* crude extract decreased the product titer by 70%, potentially due to electron loss from mediator or fouling of high molecular weight or competing biopolymers that masked the electron transfer [162].

Self-assembly of enzymes with nanomaterials is also another bioelectrocatalysis method which can aid in

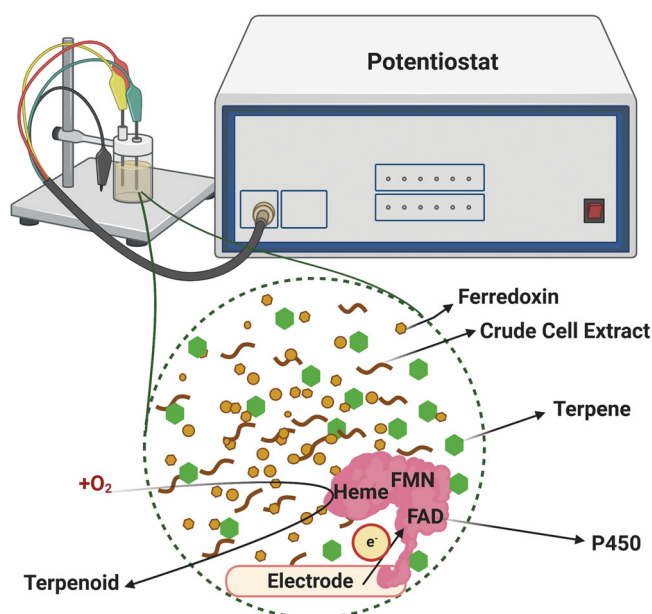


Figure 6. A hypothetical representation of bioelectrocatalytic, cell-free system for self-sufficient P450-mediated production of terpenoids. Either the self-sufficient P450-expressing crude microbial cell extract with ferredoxin or electrode-bound purified P450 enzyme can be used to potentially hydroxylate the terpenes. The redox potential is simultaneously monitored with a potentiostat device. Figure created with [BioRender.com](https://www.biorender.com).

electron delivery and improve the enzyme catalytic activity [130]. For instance, Darimont et al. [167] used carbon nanotube for anchoring the P450_{BM3}, which, together with low pH buffer and high catalase concentration, improved the coupling efficiency and NADPH-dependent activity by 32- and 13-fold, respectively. However, inadequate reduction equivalents reduced the hydroxylation activity, re-emphasising the key role of electron supply pool. Furthermore, apart from being costly for large scale applications, the accumulated NAD(P) can be inhibitive to NAD(P)H utilising enzymes and can lead to formation of enzymatically inactive NAD(P)H isomers (1,2-NAD(P)H and 1,6-NAD(P)H) [88,168]. New studies have shown that it is possible to build more efficient and stable photoelectrochemical systems like nanostructured electrodes from inexpensive materials that are devoid of inactivate NAD(P)H dimers formation [169]. However, it is questionable if they can sustain production of P450-catalysed natural products like terpenoids in long term.

In contrast to bioelectrochemical systems, the light-driven, photochemical reduction systems rely on exogenously supplementing and covalently attaching light-harvesting units (photosensitisers) such as fluorescent eosin Y dye as electron shuttles for rapid electron

injection and transfer to P450 heme active site. However, most of the photochemical-based P450 catalysis have so far relied on P450_{BM3}. In Park et al. [170], novel cofactor-free and reductase-independent platform from bacterial P450_{BM3} and human P450s (CYP1A1, 1B1, 1A2, 2A6, 2E1 and 3A4) were developed using photosensitiser eosin Y which enabled 7-ethoxycoumarin o-dealkylation and drug bioconversions, respectively. However, eosin Y dye suffers from operating under certain light intensities for providing photons that facilitate P450 turnover and cofactor regeneration and there is a risk of photobleaching at higher light irradiation [171]. However, there are reports using other good photosensitisers such as: riboflavin, FAD and FMN [172], Cadmium telluride (CdTe) quantum dots [173] as well as deazaflavin [174] (Figure 7). For example, Spradlin et al. [175] studied the attachment of P450_{BM3} heme domain to ruthenium(II)-diimine photosensitiser *via* a non-native single cysteine residue and a single point mutation enabled up to 60 and 90% more photocatalytic activity and product conversion, respectively. Overall, better electron coupling and lower electron transfer rate have been reported in photochemical systems [171,175] compared to convenient NADPH-based assays. Therefore, using them in combination with

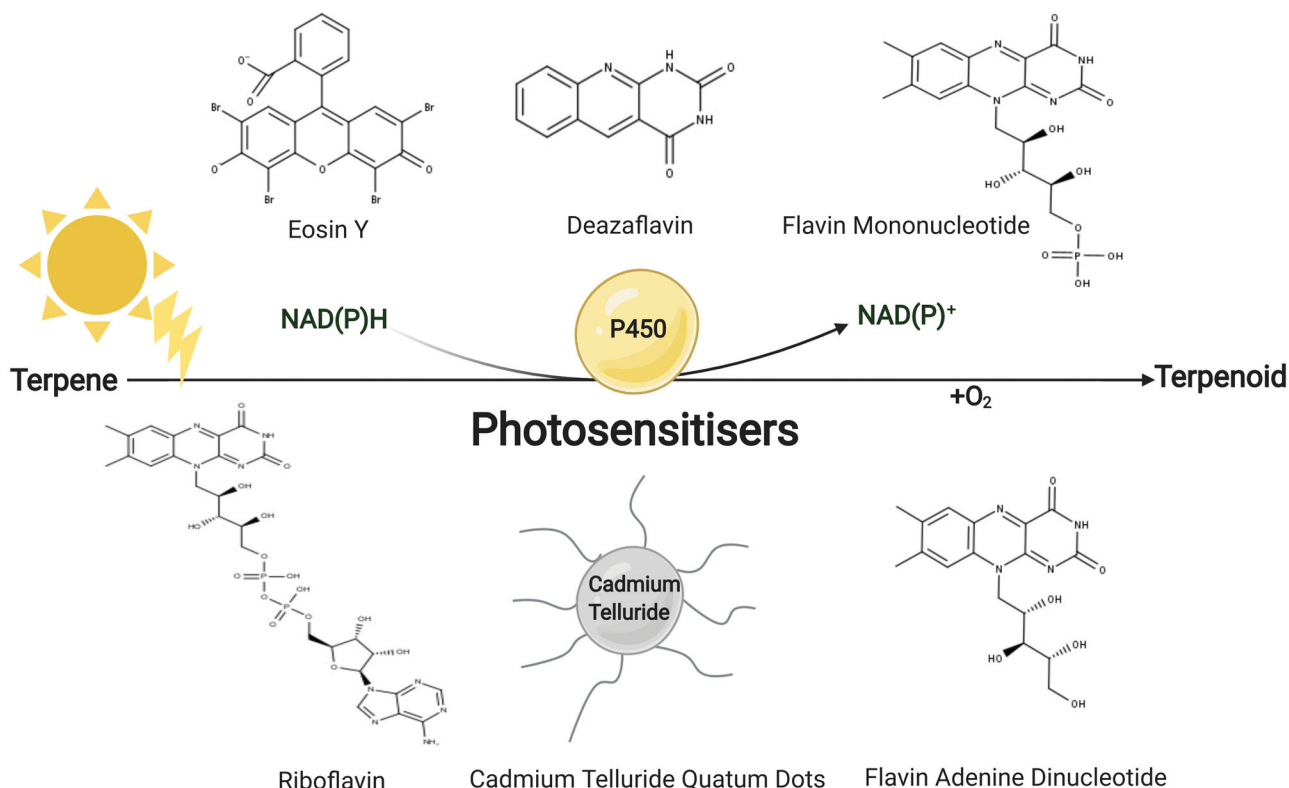


Figure 7. Schematic of photochemical-mediated P450 catalysis in *in vitro* systems for terpenoids synthesis. Photosensitisers are light-harvesting units that provide electrons to P450 enzymes for biosynthesis. MarvinSketch 20.17.0 was used for displaying the chemical structures, ChemAxon (<https://www.chemaxon.com>). Figure created with BioRender.com.

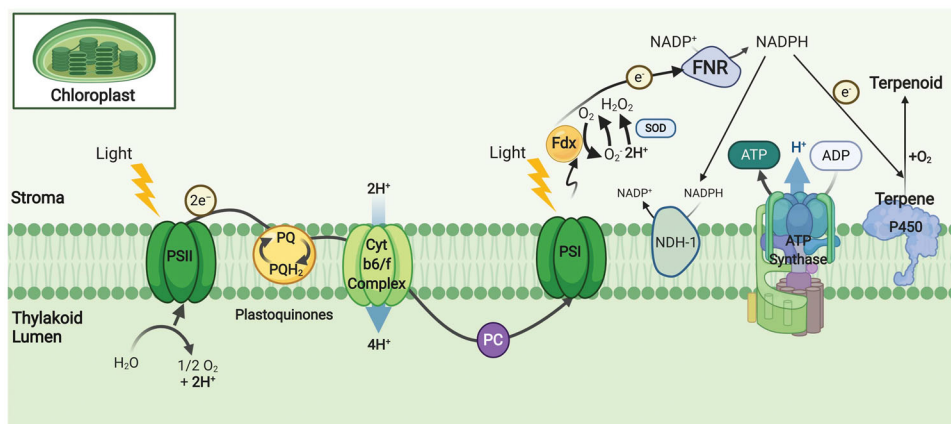


Figure 8. Photosynthetic electron transport chain reduces NAD(P) to fuel terpenoid biosynthesis by P450 enzymes. PSI: Photosystem I; PSII: Photosystem II; PQ: Plastoquinone; FNR: Ferredoxin-NADP⁺ oxidoreductase; Fdx: Ferredoxin; NDH-1: NADPH dehydrogenase; SOD: Superoxide dismutase; Cyt b6/f Complex: Cytochrome b6f complex. Adapted from “Light Dependent Reactions of Photosynthesis”, by BioRender.com (2021). Retrieved from <https://app.biorender.com/biorender-templates>

microbial cells can be a new avenue for terpenoid biosynthesis research. However, similar to any other process upscaling, their economic feasibility must be initially assessed.

Not going so far, the photosynthetic organisms are natural, *in vivo* photochemical systems. Thanks to the reducing environment of chloroplast stroma, the P450 stabilisation and activity is promoted together with a great pool of ferredoxin (Fdx) and oxygen [160,176]. On this matter, an emerging technology, although still in its infancy stage, is to couple the P450 to chloroplast or cyanobacterial thylakoids. Here, the photosynthesis is mediated through electron carriers found in thylakoid lumen and stroma and this favors P450 activity with a continuous supply of reducing factors [160] (Figure 8). Upon water oxidation at photosystem II (PSII) and electron transfer to the photosystem I (PSI), the electrons will reduce the Fdx isomers and they will be majorly spent for NADP reduction through sequential binding of two Fdx molecules to ferredoxin-NADP⁺ oxidoreductase (FNR) [177,178]. The generated NADPH can potentially be synchronised for P450-mediated terpenoid biosynthesis and the competing electron sinks can also be deleted to further optimise the desired P450 activity [179]. For instance, Berepiki et al. [180] utilised cyanobacterium *Synechococcus* PCC 7002 photosynthetic electron transport chain to drive heterologous expression of *CYP1A1* by using electrons from water splitting and knocking out *NDH-1* (a subunit of type I NADH dehydrogenase complex in cyclic photosynthetic electron flow) which doubled the P450 activity.

Despite the advantages the natural, light-driven P450 biosynthesis offers, it requires sufficient electron allocations in competition with endogenous processes that rely on Fdx. These include: FNR, lipid and

chlorophyll biosynthesis, nitrate and sulfur assimilation, thioredoxin-mediated redox regulation of chloroplast metabolism and cyclic electron flow [160,181,182]. NADP⁺ and P450 also both compete for reduced Fdx produced from PSI. This is consistent with Nielsen et al. [176] findings, who reported that electron flux is directed toward FNR in the excess of exogenous NADP⁺, resulting in 64% reduction in *CYP79A1 in vitro* activity present in isolated thylakoids. In this scenario, a possible way to maximise the heterologous P450 activity would be to either mutate the P450 interacting phase of Fdx or to fuse the P450 to Fdx to manage electron partitioning as also indicated previously [160]. Applying the later strategy, Goñi et al. [183] presented a hybrid electron transfer chain in which *B. megaterium* ATCC 13368 *CYP106A2* was reduced by cyanobacterium (*Anabaena* sp. PCC 7119) photosynthetic electron transfer flavodoxin (Fld) and Fdx *in vivo*. However, from another viewpoint, although the photosynthetic organisms can accumulate NADPH and ATP under macronutrient depletion [184,185], the reaction of oxygen with these high potential electrons can lead to reactive oxygen species (ROS) generation. This phenomenon that can even occur under mild light irradiation, is unlikely to be compensated with alternate electron transfer pathways that do not yield NADPH [180].

Enzyme-mediated cofactor recycling

There is a growing interest in *in vitro* systems and chemo-enzymatic cascade reactions for the synthesis of useful chemicals [186–188]. Here, the reaction depends on exogenously supplementing the NADP(H) cofactor for purified enzyme or crude cell extract. However, this cofactor has proven to be the most expensive

component for the generally costly cell-free synthesis [189]. Therefore, unless an NADPH-rich, lyophilised cell is utilised [190], adapting *in vitro* systems instead of whole-cell biocatalysis must be re-considered economically from the aspect of P450 enzyme overall contribution. It must be noted that interesting cofactor analog such as carba-NADP(H) has also been recently proposed [191]. But, its weaker activity compared to the commonly used NAD(P)H justifies the superiority of whole-cell biocatalysis. There might be also some concerns over the lack of sufficient microbial research infrastructures in chemistry labs [129]. However, the valuable open-source microcontroller-based toolkits and 3D printing tools can effectively assist in constructing relatively low-cost miniature bioreactors from scratch for effective control of the microbial systems [192,193].

Going forward, to promote the whole-cell biocatalysis, the enzymatic methods have been extensively used for NAD(P)H cofactor regeneration (Figure 9), among which hydrogenases, dehydrogenases and oxidases being the most applied. Hydrogenases have 100% atom efficiency and are by-product free compared to commonly used dehydrogenases [166]. As they can be deactivated in the presence of oxygen, aerobic-tolerant hydrogenases like *Ralstonia eutropha* [NiFe] hydrogenase, with inhibition tolerance of 20% at 60% oxygen of total gas (0.7 mM of O₂) can be used for P450 reaction [194], although this has not been reported for terpenoid biosynthesis so far. However, perhaps, a good

example is a study by Lonsdale et al. [195], where it was reported that using *R. eutropha* soluble NAD⁺-reducing hydrogenase for *Polaromonas* sp. JS666 alkane-oxidising CYP153A in *P. putida*, increased the 1-octanol yield by three-fold. The first use of hydrogenases for oxygen-dependent enzymes was recently shown by Preissler et al. [196] using a mutant variant of *R. eutropha* O₂-tolerant NAD⁺-reducing hydrogenase (E341A/S342R), which supported the activity of P450_{BM3} for octane oxidation.

From dehydrogenases, aldehyde, glucose and formate dehydrogenases, have been used for optimising the P450 product yield [197,198]. However, formate dehydrogenase is probably the least optimal due to its low specific activity and stability, preference for NAD⁺, as well as product loss in the form of CO₂ (197). However, formate dehydrogenases from *Burkholderia cepasia* and acidobacterium *Granulicella mallensis* with both NAD⁺ and NADP⁺ specificity, have been identified [198,199]. While being recognised for *in vitro* P450 catalysis using bacterial crude cell extracts [200] or for co-immobilisation [201], glucose dehydrogenases are used in microbial terpenoid biosynthesis, a notable example being Luan et al. [127]. Here, the glucose dehydrogenase coexpression improved (–)-limonene conversion to (–)-isopiperitenol by 10-fold in *E. coli* expressing *Rhodococcus* sp. ECU0066 P450_{S_{MO}} reductase-GS linker- *P. putida* PpGI P450_{cam} (Y96F/V247L). Brummund et al. [202] also used a crude, cell-free

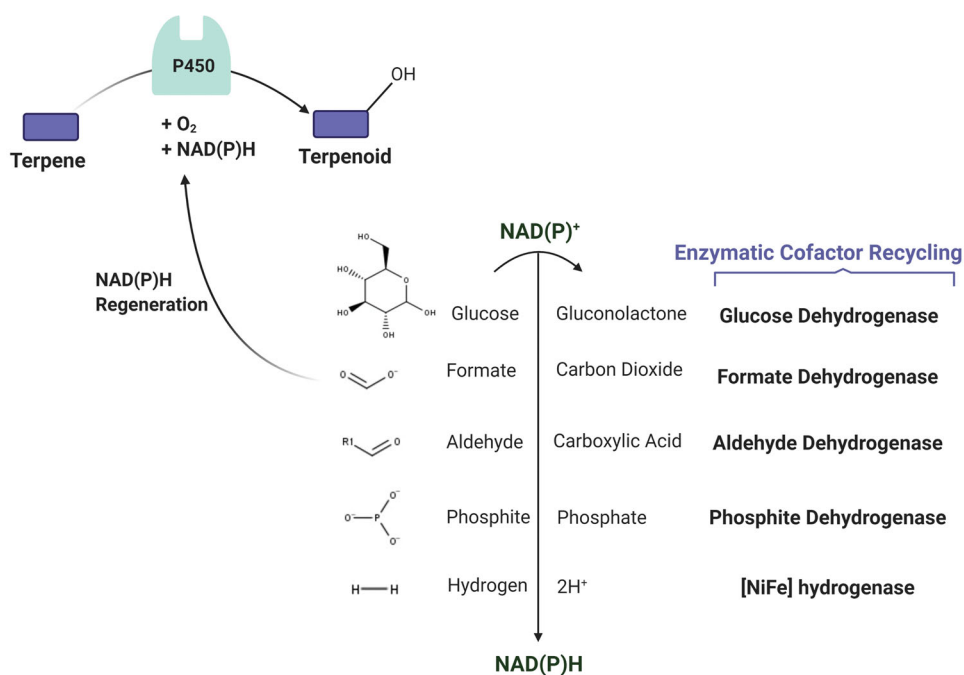


Figure 9. Selection of enzymatic routes to NAD(P)H regeneration for P450-mediated terpenoid synthesis. Figure created with BioRender.com.

extract of *E. coli* K12 derivative to convert sesquiterpene α -ionone to its oxidised form using a mutant variant of P450_{BM3} (R47L/F87V/L188Q), which relied on commercial glucose dehydrogenase system for NADPH regeneration. Chen et al. [203] also reported up to 14-fold increased artemisinic acid production when coexpressing *A. annua* aldehyde dehydrogenase with N-terminus truncated *A. annua* CYP71AV1-POR fusion in *S. cerevisiae*. Likewise, using *B. megaterium* glucose dehydrogenase and *E. coli* transhydrogenase improved the titer up to two- and four-folds, respectively. Thermostable phosphite dehydrogenases have also been used in fusion with self-sufficient Baeyer–Villiger monooxygenases (CRE2 (coenzyme regenerating enzyme)/BVMO) for NADPH regeneration for ketone conversion using *E. coli* crude cells [129,204]. Although these studies did not use P450s, they lend the opportunity to deploy phosphite dehydrogenases for P450 biotransformation through using phosphite-oxidising microbes in co-culture systems [205,206]. However, using glucose dehydrogenase involves the co-production of acidifying gluconate/gluconic acid in the medium, which although they might be overcome with biphasic culture systems, increased glucose feeding and pH control, they would increase the costs and complicate the identification and purification of substrates from the products [197,200,207].

The dehydrogenases might act distinctly when partnered with P450s for terpenoid biosynthesis as illustrated in Meng et al. study [208]. Here, while using short-chain dehydrogenase/reductase (SDR) superfamily dehydrogenases (*Z. zerumbet* ZSD1 and *Citrus sinensis* ABA2 or *P. pastoris* dehydrogenase (AHD-C3)) yielded between 49 and 60 mg/L of (+)-nootkatone in *S. cerevisiae*, the endogenous dehydrogenases (ADH2 and AHD6) did not significantly change the product concentration. This was attributed to their interference with P450 and reductase enzymes (*H. muticus* premnaspirodien oxygenase (HPO) variant (V482I/A484I) and *A. thaliana* cytochrome P450 reductase (ATR1)). The other general disadvantage of dehydrogenases is that they can get deactivated by NADPH-mediated product inhibition, lending the opportunity to NAD(P)H oxidases (NOXs), despite not have been reported for P450-catalysed terpenoid biosynthesis so far. NOXs show more specificity towards NADH, even in species with dual (NADH and NADPH) specificity [197]. However, a water-forming *Lactobacillus reuteri* NOX with equal efficiency for both NADPH and NADH and higher activity with NADPH, has been reported to improve the *Gluconobacter oxydans* sorbitol dehydrogenase total turnover by around 53-fold [209]. Moreover, dual-

specific NOXs can be artificially constructed as was shown with P450_{BM3} for vanillin production [210].

Excess cofactor regeneration: good or bad?

Despite the great potential of cofactor regeneration systems, the cofactor supply might not be always the rate-limiting step for terpenoid formation. Li et al. [211] demonstrated that increasing the exogenous supplementation of mutated 2,3-butanediol dehydrogenase (mBDH1) substrate, acetoin, although initially improved the betulinic acid concentration by 1.5-fold at 20 mM concentration at the price of decreased growth rate, it did not further increase the product concentration at 100 mM. Similarly, recently, Otto et al. [73] reported that the titer of sesquiterpenoid, abscisic acid (ABA) produced by an engineered *S. cerevisiae* strain expressing *Botrytis cinerea* four-step ABA biosynthesis pathway genes (*bcaba1234*), did not depend on the NADPH supply. Instead, the rate-limiting enzymes were the P450s *BcABA1* and *BcABA2*, which improved the ABA titer by 4.1-fold upon overexpression. Janocha and Bernhardt [105] had also shown that the co-expression of *Lactobacillus brevis* alcohol dehydrogenase and dissolving the substrate, abietic acid, in 2-propanol as co-substrate for NADPH regeneration only enhanced the 15-hydroxyabietic acid formation rate by 10% from whole-cell *E. coli* JM109 system, transiently expressing resin acid diterpenoid hydroxylase (*Streptomyces griseolus* CYP105A1), *Schizosaccharomyces pombe* adrenodoxin reductase homolog 1 (*Arh1*) and electron transfer protein 1 and ferredoxin domain (*Etp1^{fd}*). However, the limited concentration of the reducing cofactor might also not be favorable to P450 catalysis. For instance, Ensari et al. [212] could only recover the efficiency of hydroxy- and keto-fatty acid methyl esters production by using ethanol as a co-substrate for producing NADH cofactor in *E. coli* expressing *Candida parapsilosis* alcohol dehydrogenase 5 (cpADH5 (W286A)) and P450_{BM3} (R47S/Y51W/T235S/N239R/I401 M). Therefore, although excess NAD(P)H might inactivate P450s, the efficient P450 biocatalysis strongly depends on proper redox balance. Such shortcomings in previously explained cell-free or whole-cell enzymatic methodologies can be systematically addressed through systems metabolic engineering and mathematical modeling approaches. As a common practice, the NADPH-consuming central carbon metabolism reactions are downregulated or knocked out and NADPH generating pathways are upregulated, albeit at the risk of reduced strain fitness. Instead, the genome-scale models are becoming more popular to help scientists modulate the microbial

growth and productivity. However, not all microbial horse powers are favored to have a good model yet and the models for more widely used microorganisms like *S. cerevisiae* are still evolving to enhance their prediction power [213]. In recent years, the metabolic and regulatory target predicting algorithms [214–218] have been integrated into programming work benches to address these issues [219–223]. Among relevant applications to the P450 biocatalysis are the cofactor swapping methodology for redox balance [224] and cofactor modification analysis [225]. Also, there has been an emerging interest in improving the microbial biocatalysis through computational approaches in addition to flux balance analysis and multi-omics data integration [226–229]. Hence, it is not ambitious to envisage that mathematical modeling tools will reach a level to be the next players in optimising the microbial terpenoid biosynthesis with the least effect on cell activities as compared to rather blind enzyme selections described above.

Practical considerations for redox manipulation: reductase-associated toxicity

The natural, inevitable uncoupling in P450s is possibly negligible compared to higher reactive oxygen species (ROS) and proton leak caused by mitochondria and oxidative phosphorylation, a price that cells pay for aerobic energy transformation [230,231]. As discussed, the excess reductase expression can lead to electron transfer uncoupling, ROS production and reduced terpenoid titer. This might explain the higher ratio of P450s to PORs in plants, reaching up to 15:1 [33,232]. Despite this, the ROS problem is commonly tackled using scavenging systems and antioxidants like superoxide dismutase and catalase. However, these methods are inefficient for removing the lower hydrogen peroxide levels [233] or are associated with energy-intensive uncoupling [127,231]. Although hydrogen peroxide is blamed for causing cellular death, it has been shown to improve antioxidant carotenoids production by three-fold [234] and it can be effectively controlled to power P450-based catalysis. For instance, Jiang et al. [235] used *Streptomyces coelicolor* A3(2) alditol oxidase (AldO) and commercial lipase (CRL) for hydrogen peroxide formation for *in situ* free fatty acids decarboxylation by an artificial three-enzyme cascade of OleTJE P450 peroxygenase and fatty acid decarboxylase. This approach was also found to be FMN- and NADPH-saving and preserved the enzyme from hydrogen peroxide-mediated deactivation and instability, despite loss of activity at

higher hydrogen peroxide concentrations. Zhang et al. [106] also observed the partial contribution of superoxide to novel N-demethylation activity of P450 MycG enzyme. An old study by Cirino and Arnold [236] also showed the applicability of hydrogen peroxide for improving the hydroxylase activity of a P450_{BM3} variant in *E. coli*. For this to occur, other than developing more stable P450s, the laboratory microbial strains need to be equipped with better oxidative stress resistance through adaptive evolution at adverse conditions [237] to facilitate the large-scale P450-based terpenoid biosynthesis.

Although not tested, it would be interesting to explore microbial cells with superior endogenous hydrocarbon degrading mechanisms such as n-alkane utilising *Alcanivorax dieselolei* strain B-5 [238] and alkylguaiaicols-utilising *Rhodococcus rhodochrous* EP4 [239], which might better handle the ROS-caused toxicities for improved P450-based terpenoid production. Despite their potential benefits, the tools and knowledge for genetic manipulation and cultivation of these microorganisms are limited. There are, however, more widely used host microbes like *P. putida* which has remarkable oxidation capacity for terpenoid production [240]. It also has unique and elaborate oxidative stress response owing to its Entner–Doudoroff pathway [241], adaptive membrane fluidity, chaperone-assisted protein refolding and efflux pumps in some of its strains [242]. The other stress-resistant microorganisms are *Bacillus* spp. with a network of metal- and thiol-based oxidative stress response mechanisms [243]. The diatom *Phaeodactylum tricornutum* has also been reported to well-tolerate the P450 and POR enzymes without affecting the cell growth, suggesting its potential for wider applications in industrial P450 biotransformation [244]. Alternatively, increasing the ergosterol and triacylglycerol biosynthesis rate or exogenous ergosterol supplementation seem promising particularly for non-oleaginous microbial chassis [237,245]. This is because the microorganisms with more oxidative stress resistance like two non-conventional yeast strains, *Torulaspota delbrueckii* and *Metschnikowia pulcherrima*, have been reported to have higher ergosterol/squalene and phosphatidylcholine/phosphatidylethanolamine content in their plasma membrane, which are postulated to increase their hydrogen peroxide permeability as reported before [246]. Contrary to these findings, studies like Brennan et al. [247] have not reported ergosterol synthesis upregulation in limonene-producing yeast with known toxicity. Hence, this approach might involve preliminary trial and error experiments.

Conclusions and prospects

Cytochrome P450s can be undoubtedly one of those most versatile and popular choices for industrial biotechnology, either for producing natural products or to synthesise new-to-nature chemicals. Aiming to hopefully replace the sophisticated organic chemistry approaches as a cheap and eco-friendly alternative, there is a need to finely tune them to meet industrial requirements for high productivity. Despite the scientific advances and exploratory attempts to reach a comprehensive understanding of redox state and its functional dimensions for P450-catalysed terpenoid biosynthesis, there are moderate industrial achievements.

Among many decisive parameters for their optimal functions, the current paper focuses on the important regulatory role of NAD(P)H electron-deriving reductases in microbial hosts for heterologous production of terpenoids. The optimal construction of P450-reductase fusion proteins and selection of proper linkers can both impact the P450 functionality and product profile through affecting the P450-reductase interaction. However, the P450 research community still needs user-friendly standardised methodologies, synthetic biology and protein modeling toolkits to accelerate the protein fusion design for the biosynthesis of P450-catalysed terpenoids and other natural products.

Beside the fusion proteins, several non-enzymatic and enzymatic NAD(P)H regeneration approaches also exist that could exert strong impact on the activity of whole P450-redox partner systems. Although the course of this paper was based on the whole microbial cell biosynthesis, a number of the latest cutting-edge discoveries in cell-free P450-redox tuning were discussed as potential avenues to be adapted and combined in whole-cell systems. However, as these methodologies increase the risk of redox imbalance and oxidative stress, using more stress-resistant microbial hosts or developing microbial consortia are suggested. While non-model microorganisms from extreme environments could possess superior catalysis functionalities relative to commonly used laboratory strains, their common usage is still far from reality. Instead, it is anticipated that evolving new microbial strains, while not being superior to extremophiles, can remarkably enhance the microorganism characteristics for P450-catalysed terpenoid biosynthesis.

With these limitations, there is still room for improvement with regards to cofactor regeneration and modulation of P450 and reductase expressions at whole-cell. Indeed, P450 research can instead benefit from systems biology-guided metabolic engineering to decrease the associated costs and risks, and

increase the flux towards terpenoid synthesis, through engineering the cell factory and/or modifying the bio-processing parameters like growth medium composition.

Having broad selectivity and being favored with available cofactor pool, microorganisms provide ample opportunities to extend our reach to a diverse range of terpenoids with roots in P450-dominated biosynthetic pathways. We therefore close by noting that we have just scratched the surface of whole-cell biocatalysis and to our belief, microorganism-wise modulation of reductase partner and cofactors can systematically shape the next generation of P450 whole-cell catalysis research.

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