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Catalytically self-sufficient cytochromes P450 for green production of fine chemicals

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ABSTRACT

This review covers our current knowledge on catalytically self-sufficient cytochromes P450.

These enzymes present all the advantages of the regio- and stereo-selectivity of typical P450s combined with the fact that, due to the natural fusion with their reductases, their application in biocatalysis does not need the addition of other proteins: only NAD(P)H and substrate are needed to trigger catalysis.

To this date only few catalytically self-sufficient P450s have been isolated from bacteria, fungi and plants. Their low number does not detract from their importance, as the reactions they catalyse and the turnover numbers of which they are capable (in some cases higher than 15,000 s^{-1}) makes them highly relevant to the

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chemical and pharmaceutical industry.

These enzymes will be covered in this review, together with their specific reactions relevant to the field of biocatalysis.

Keyword: monoxygenases; P450; heme proteins; biocatalysts;

1. INTRODUCTION.

Cytochromes P450 (P450s) are heme-thiolate enzymes representing a large superfamily of proteins widely diffused in organisms from all kingdoms of life including viruses (Table 1). They are characterized by an absorbance peak at 450 nm appearing when the heme is reduced and complexed with carbon monoxide (CO). They can perform a wide variety of regio- and stereo-selective reactions on a large range of substrates making them an attractive target as biocatalysts for green chemistry applications.

Tab. 1. Number of named P450 sequences to date (Nelson 2009).

ORIGIN	NUMBER OF NAMED P450 SEQUENCES	
Animals		10,477
Insects	6,119	
Mammals	1,666	
Other vertebrates	1,344	
Non-insect invertebrates	1,348	
Plants		13,978
Fungi		7,873
Protozoa		602
Bacteria		2,156
Archaea		52
Viruses		28
TOTAL		**35,166**

This review will concentrate on plant, fungal and bacterial catalytically self-sufficient P450s that are most relevant to chemical catalysis applications. We will also consider protein engineering approaches, by random mutagenesis on residues of the catalytic site as well as by rational design and fusion of different P450 catalytic domains with various reductase domains to obtain catalytically self-sufficient chimeric enzymes. Here the gene of P450 redox partner and of the heme domain from different P450s are fused together in a single gene producing a single polypeptide chain capable of independent catalysis, enhancing the biotechnological potential of the natural enzymes (Gilardi et al. 2002, Sadeghi et al. 2013).

2. P450 REACTIVITY.

P450s act as terminal monooxygenases performing reactions involving the transfer of one atom from molecular oxygen to X-H bonds, where X may be the –C, -N or S of a substrate, with the concomitant reduction of the remaining oxygen atom to water (Garfinkel 1958). The reaction uses the reducing power of NAD(P)H according to the scheme:

Reaction 1: $RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$

Hydroxylation is the most common reaction carried out by P450s, but a wide range of other possibilities also exist, as shown in Figure 1. These include the formation of dicarboxylic acids from alkanes, epoxidations, N-, S- and O-dealkylations, dehalogenations, peroxidations and N-oxide reductions (Guengerich and Munro 2013).

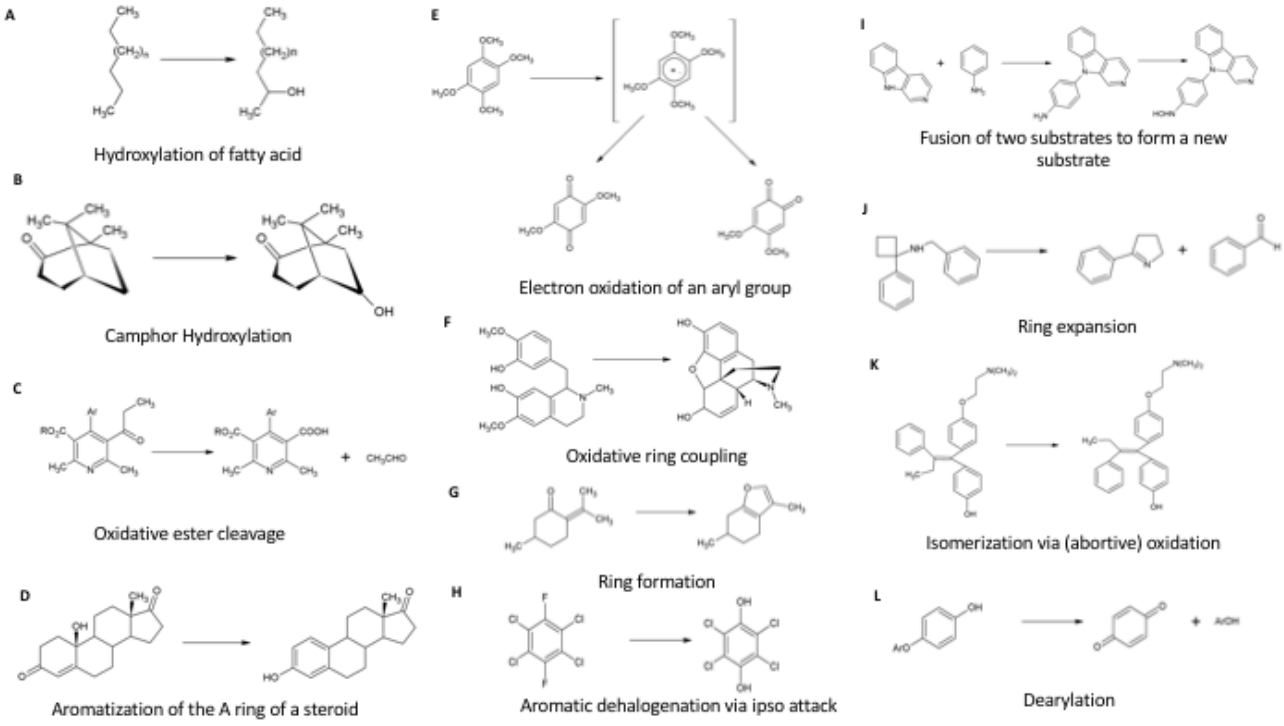

FIGURE 1. Known reactions described for P450s. (Adapted from Isin and Guengerich 2007)

Moreover, these reactions can be performed on a wide spectrum of substrates owing to the highly flexible substrate recognition sequences (SRS) present on their 3D structure (Gotoh 1992). Figure 2 shows the localisation of the SRS in the P450cam (CYP101A1).

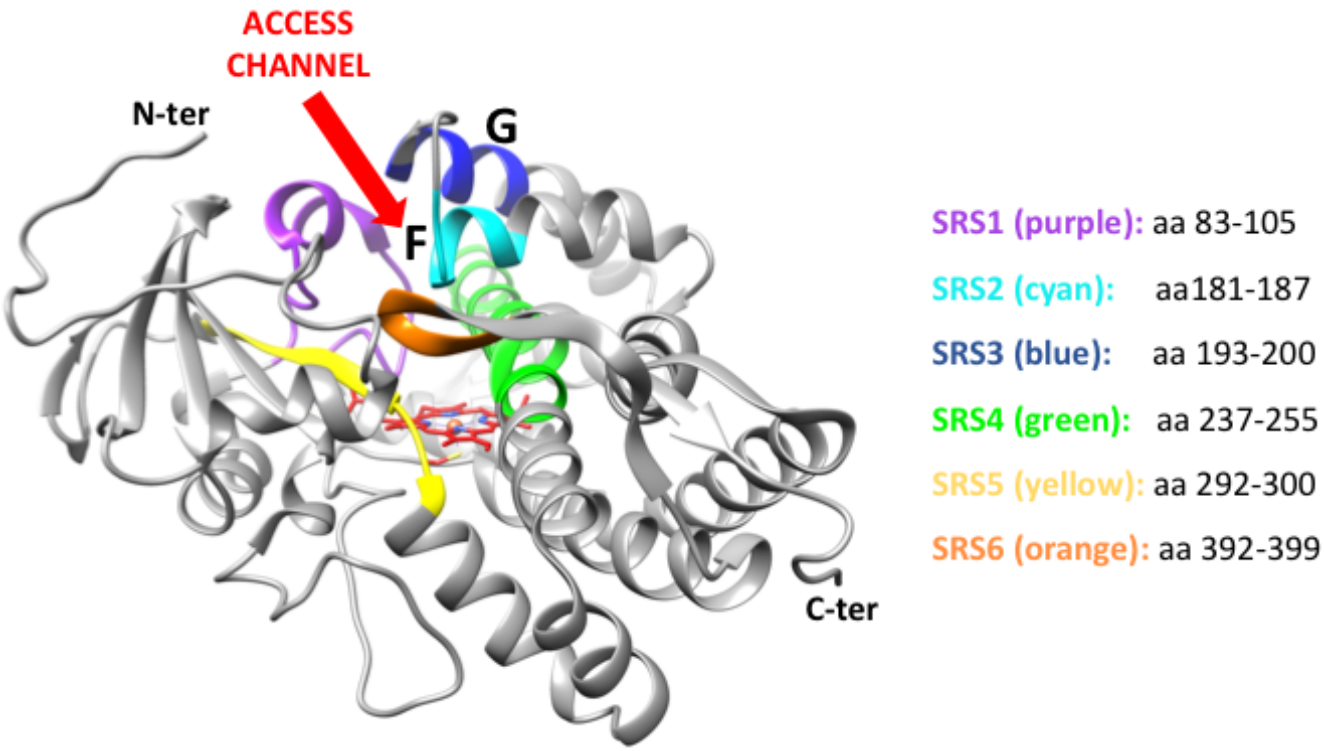

FIGURE 2. SRSs regions in the P450cam (CYP101A) X-ray structure (PDB 2CPP) (Poulos et al. 1987).

The wide range of reactions listed above, combined with the large spectrum of substrates covered by different natural and engineered P450s, makes them a good target for the production of fine chemicals by environmentally sustainable means (Valetti and Gilardi 2004, Gilardi and Di Nardo 2016).

In all cases the P450 reactivity must be sustained by an electron supply that ultimately is represented by NAD(P)H. This normally provides the electrons through a reductase partner or a domain that in turns binds and delivers two electrons to the P450 heme. The mode of electron supply forms the basis for a classification of these enzymes as illustrated in the following section.

3. P450 CLASSIFICATION BASED ON ELECTRON TRANSFER PARTNERS.

Redox protein partners or reductases are key for providing the electrons required to support the P450 catalytic cycle allowing to deliver the two required electrons one at the time to the different intermediates in the P450 catalytic cycle (Gilardi and Di Nardo 2016, Sevrioukova et al. 1999). For some P450s, its reductase is one or more separate proteins made of one or more domains. In some cases the reductase domain is fused with the P450 domain in one polypeptide chain. There, the enzyme is self-sufficient, in that only NADPH addition is needed to trigger catalysis without the need of adding separate proteins. Only very rarely P450s have been found to achieve the reductive

activation of oxygen without involving any redox partner (Toritsuka et al. 1997 and Stundl et al. 1998).

In early days, P450 had been classified in only two classes, class I and class II, but to this date, many more P450s have been discovered with a variety of electron deliver systems, leading to a new classification that comprises ten different classes (Hannemann et al. 2007), from class I to class X. Figure 3 systematically shows all classes of P450s, but here in the text we will only describe classes VII to X that correspond to the self-sufficient systems.

Class VII P450s are bacterial, soluble and cytosolic multi-domain enzymes. A single polypeptide chain comprises a heme containing P450 domain at N-terminus as well as a FMN containing reductase domain at the C-terminus. The reductase domain has been found to be closely related to the phthalate oxidase reductase (PFOR) (De Mot and Parret 2002). Class VII P450 are considered self-sufficient enzymes, as they are able to perform reactions in the presence of the only electron donor, without the necessity of a redox partner (Hannemann et al. 2007). This class is well represented by the CYP116B subfamily, that they take part in reactions involving substrates such as thiocarbamate pesticides relevant from a bioremediation point of view (Warman et al. 2012). Substrates as alkanes, aromatic compounds and terpenes were also found to be involved in different oxidation reactions involving these enzymes (Yin et al. 2014).

Class VIII encompasses cytosolic self-sufficient P450 enzymes, both prokaryotic and eukaryotic. Here a single polypeptide chain contains the heme domain at the N-terminus while a reductase domain homologous to the eukaryotic cytochrome P450 reductase (CPR), containing both FAD and FMN subdomains is present at the C-terminus.

This class is represented by CYP102A1 from *Bacillus megaterium* an important model P450 enzyme that will be described in details later.

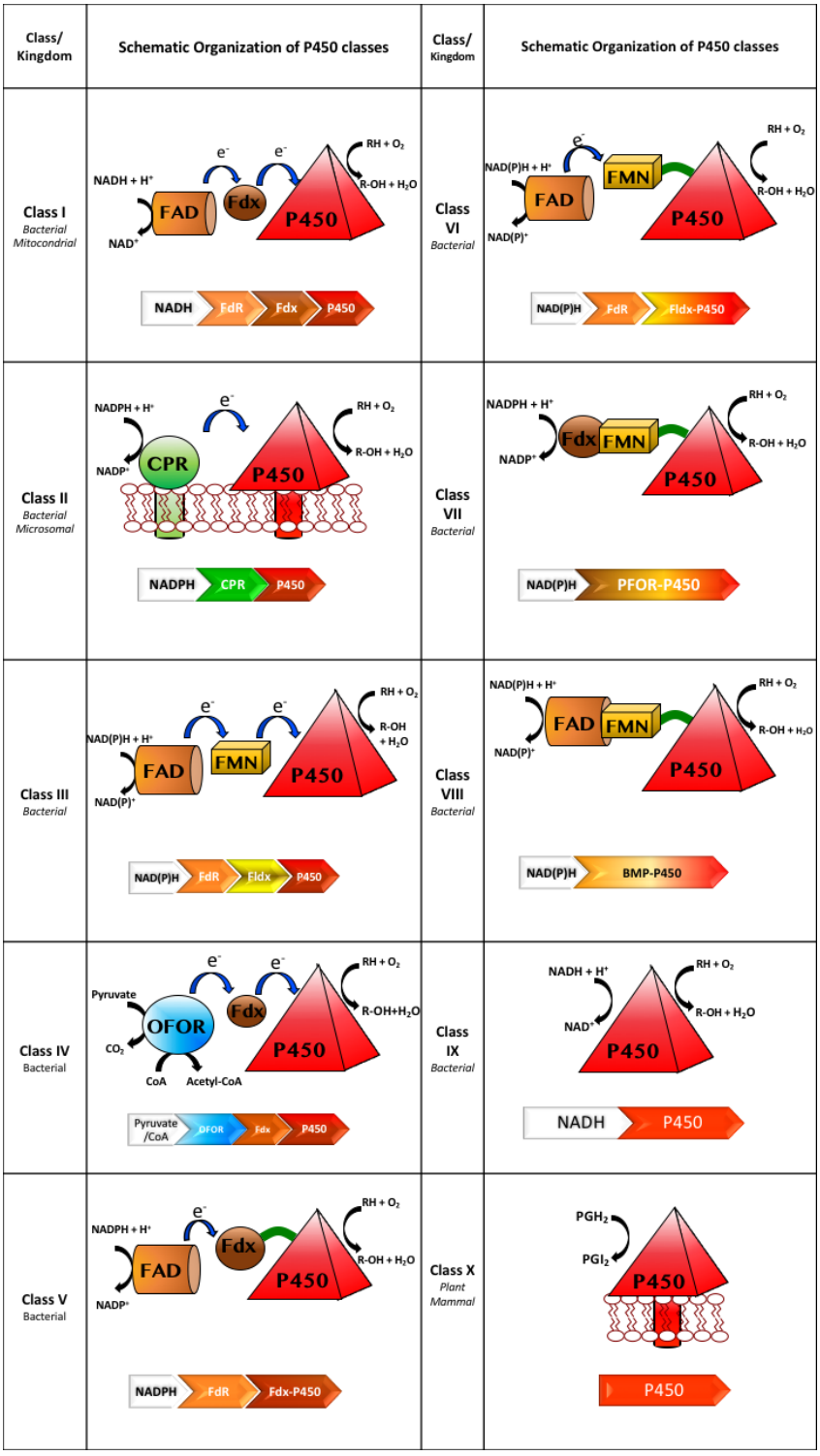

FIGURE 3. Classification of P450s on the basis of their electron transfer partners or requirements. Abbreviation: Fdx (iron–sulfur-cluster: [2Fe–2S], [3Fe–4S], [4Fe–4S], [3Fe–4S]/[4Fe–4S] type); FdR/FAD, Ferredoxin reductase (FAD); CPR, cytochrome P450 reductase (FAD, FMN); Fldx, Flavodoxin (FMN); OFOR, 2-oxoacid: ferredoxin oxidoreductase (thiamin pyrophosphate, [4Fe–4S] cluster); PFOR, phthatate-family oxygenase reductase (FMN, [2Fe–2S] cluster).

Also the self-sufficient P450 from ascomycete fungus *Fusarium oxysporum* belongs to class VIII*:* CYP505A1 (P450 foxy). It catalyses C8-C18 fatty acids hydroxylation at ω-1- ω-3 as the bacterial counterpart BM3 (Nakayama et al. 1996) as described later.

Class IX comprises nitric oxide reductase from *Fusarium oxysporum* CYP55, also known as P450nor (EC 1.7.99.7), that is a particular cytochrome involved in the denitrification process. Only a few additional members of this class have been characterized (Zhang et al. 2001 and Kudo et al. 1996). These enzymes do not have monooxygenase activity, but they show a unique electron transfer chain system. P450nor uses NADH directly as an electron donor without the need of a redox partner to reduce two molecules of nitric oxide (NO) to nitrous oxide (N_2O). This is to protect the fungus from NO inhibition, especially when dioxygen becomes limiting (Daiber et al. 2005). This is a unique reductive process in P450 reactivity. Some real technological application regarding this class of enzymes has recently been proposed (Garny et al. 2016).

Class X comprises a P450-only system similar to class IX but rather particular features. It comprises the CYP74 family. CYP74A (AOS) is an allene synthase as well as CYP74B/C that is a fatty acid hydroperoxide lyase (Shibata et al. 1995). CYP74(DES) (Itoh and Howe 2001), that is crucial in the oxidative metabolism of polyunsaturated fatty acid hydroperoxides, that are converted into different oxylipins with important roles in plant growth (Hou et al. 2016).

The catalytic features in biosynthesis and the function of these enzymes are shared in mammal P450s prostacyclin synthase and thromboxane synthase, that are involved in the arachidonic acid cascade.

The peculiar feature of these enzymes resides in the fact that they do not require O_2 or NAD(P)H for their reactions, but rather they use acyl hydroperoxide that is the oxygen donor as well as the substrate (Lau et al. 1993), forming new carbon oxygen bonds.

4. CATALYTICALLY SELF-SUFFICIENT P450s.

Catalytically self-sufficient cytochromes P450 are included in classes VII to X. Class VII and VIII P450s show a reductase naturally fused to the P450 catalytic domain, therefore for these enzymes to function and perform catalysis is only necessary to add NAD(P)H and substrate, without the need to add other electron transfer proteins. This represents a distinct advantage from the catalytic and biotechnological point of view. To date only very few self-sufficient P450s have been found, mainly deriving in bacteria, fungi and plants. Nevertheless, inspired by the protein architecture of these self-sufficient P450 systems, different kinds of artificial fusion proteins have been constructed

with protein engineering approaches (Sadeghi and Gilardi 2013). Here we report the most relevant systems for each of these groups.

4.1. Bacterial enzymes

CYP102A1 from *Bacillus megaterium*

The most intensively studied bacterial P450 enzyme is CYP102A1 (Miura and Fulco 1974), known as P450 BM3 isolated from the soil bacterium *Bacillus megaterium*. This self-sufficient P450 is soluble and is composed of a eukaryotic-like P450 reductase containing a FAD and FMN subdomains fused to a P450 domain. The solubility is a great advantage and it makes P450 BM3 a very good model system for electron transfer studies. It was one of the first bacterial P450 enzymes to be crystallized both in its open, substrate-free, and closed, substrate-bound, forms. Due to its high homology to the class II human drug-metabolizing enzymes, for many years this enzyme has been used as a paralog for the eukaryotic enzymes.

It is able to hydroxylate a range of fatty acids at ω-1, ω-2 and ω-3 positions, displaying the highest monooxygenase activity of any P450 system investigated to date, that is in the region of 17000 s^{-1} for the substrate arachidonate (Munro et al. 2002). Its catalytic activities, combined with mutagenesis studies, has produced enzymes suitable for drug metabolite production such as hydroxylation of diclofenac, ibuprofen and tolbutamide (Tsotsou et al. 2012, Sideri et al. 2013), as well as in the development of process-scale approaches (Di Nardo and Gilardi 2012), methane oxidation, and more generally has produced mutants with improved selectivity to allow the synthesis of fine chemicals (Whitehouse et al. 2012). Some of these mutants have also been characterized in details by X-ray crystallography (Di Nardo et al. 2016). Moreover, its FAD and FMN containing reductase (BMR) has been used to drive catalysis in artificial fusion systems (Sadeghi and Gilardi 2013, Astuti et al. 2004).

The 116B subfamily

CYP116B2, also referred as P450RhF, was the first enzyme in the 116B subfamily to be expressed and purified by Roberts et al. (2003). From the N- to the C-terminus, its reductase shows three distinct functional parts: a FMN-binding domain, a NADPH-binding domain and a [2Fe–2S] ferredoxin domain. Considering the general fold, the ferredoxin domain is supposed to be between the heme domain and the FMN-domain. Therefore, the electron transport is supposed to go from the primary electron donor (NAD(P)H) *via* the FMN and the [2Fe–2S] cluster of the reductase domain to the P450 domain.

In the same family is CYP116B3 that catalyses the *o*-dealkylation of 7-ethoxycoumarin producing 7- hydroxycoumarin. In the presence of NADPH, it is also capable of hydroxylation towards aromatic hydrocarbons, naphthalene, indene, acenaphthene, toluene, fluorene, *m*-xylene and ethyl benzene (Liu et al. 2006).

Recently, our laboratory has isolated a novel 116B cytochrome from *Acinetobacter radioresistens* growing on alkanes as a sole energy source. It can be used in the hydroxylation of long (C24-C36) and medium-chain (C14, C16) alkanes (Minerdi et al. 2015). At present, further analysis of P450 domain are undergoing. Early experiments on the purified P450 domain are present in this paper for the first time and indicate the presence of the typical P450 spectroscopic features (figure 4). Moreover, a crystallization screening of the heme domain is being carried out.

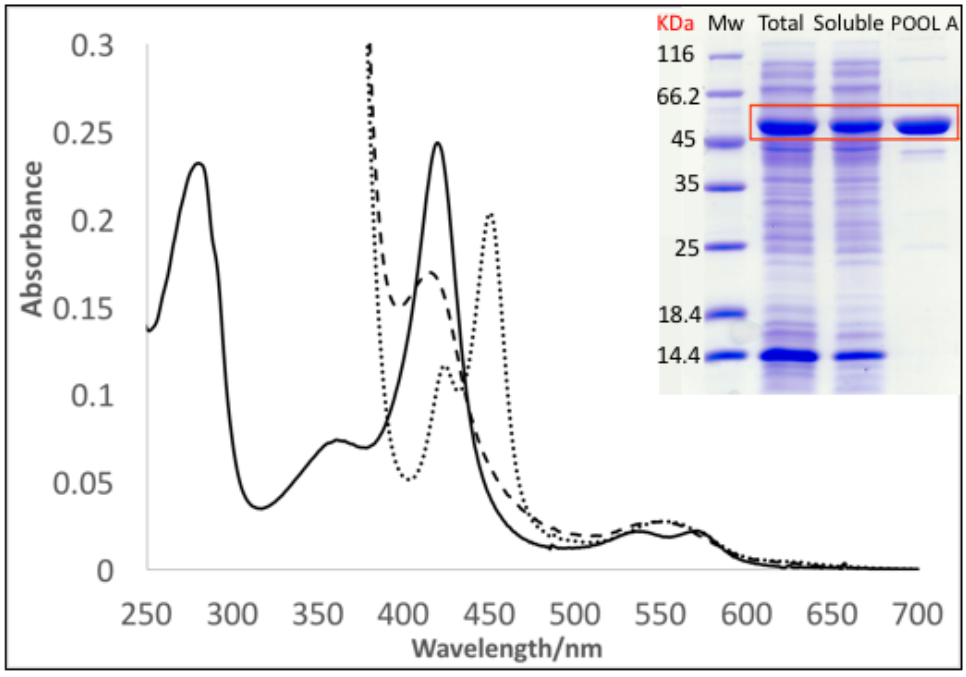

FIGURE 4. UV/Vis absorption spectra for purified 116B5 heme domain showing the purified oxidized enzyme (solid line), the dithionite-reduced enzyme (dashed line), and the reduced CO-bound complex (dotted line). The CO-bound reduced difference spectrum and the SDS gel before and after the purification are shown in the inset.

Other bacterial P450s

Very recently two novel CYP102 from the bacterium *Ktedonobacter racemifer* have been characterized as Krac0936 and Krac9955 (Munday et al. 2016). Krac0936 mainly hydroxylates long saturated fatty acids at the ω-1 and ω-2 positions and cis,cis-9,12-octadecadienoic, as well as pentadecanoic acids. Krac9955 is able to oxidize the tridecanoic, tetradecanoic and pentadecanoic

acid oxidation at the ω-4, ω-5 and ω-6 positions, together with lower activity towards unsaturated fatty acids.

Other closely related enzymes are CYP102A2 and A3 from *Bacillus subtilis*, (Gustafsson et al. 2004) A5 (Chowdhary et al. 2007) from *Bacillus Cereus* and A7 (Dietrich et al. 2008) from *Bacillus nicheliformis*.

CYP102D1 from *Streptomyces avermitilis* has been reported to be a class VIII self-sufficient enzyme. It catalyses fatty acid hydroxylation showing similar substrate preference profiles and kinetic properties with the CYP102 family (Choi et al. 2012).

4.2. Fungal enzymes

To date all fungal catalyically self-sufficient P450s have been found in the species *Fusarium*, in particular *Fusarium oxysporum* and *Fusarium verticillioides.*

4.2.1 P450s from *Fusarium oxysporum.*

CYP55: nitric oxide reductase.

Nitrogen fixation or nitrification carried out by microorganisms represents the main step in the nitrogen cycle, a fundamental process necessary to support life and key to maintaining nitrogen homeostasis on Earth. The inverse process, called denitrification, has been well characterized in bacteria where the process occurs under anaerobic conditions and it involves four reducing steps involving the following enzymes (Shoun et al. 2012):

Reaction 2, nitrate reductase (dNar): $NO_3^- + 2H^+ \rightarrow NO_2^- + H_2O$

Reaction 3, nitrite reductase (dNir): $NO_2^- + 2H^+ \rightarrow NO + H_2O$

Reaction 4, nitric oxide reductase (Nor): $2NO + 2H^+ \rightarrow N_2O + H_2O$

Reaction 5, nitrous oxide reductase (Nos): $N_2O + 2H^+ \rightarrow N_2 + H_2O$

Denitrification also occurs in fungi where the best studied system is the one of the ascomycete *Fusarium oxysporum.* Here the nitric oxide reductase activity is carried out by the cytochrome P450nor (Nakahara et al. 1993; Takaya and Shoun, 2000).

P450nor belongs to family 55 (CYP55) and it is class a IX P450 (Hanneman et al. 2007). It was isolated

from the soil fungus *Fusarium oxysporum* as a heme enzyme with a lipoxygenase activity and typical P450 spectral properties (Shoun et al. 1983). P450nor is widely diffused, mainly across the subdivision of ascomycotina (Shoun et al. 2012). It is present both in the mitochondria and in the cytoplasm of fungal cells and its molecular weight has been reported to range from 42 to 46 KDa (Nakahara et al. 1993; Usuda et al. 1995; Zhang et al. 2001). CYP55 is able to use NADH as a direct electrons donor, according to a process that is very unusual in that the two electrons from NADH are transferred here simultaneously to the heme as a hydride ion. The reaction is divided into three steps. First substrate (NO) binds to ferric (Fe^{3+}) P450nor to form a ferric-NO complex (reaction 6), then the Fe^{3+}-NO is reduced with NAD(P)H to form the specific heme intermediate (I_{heme}) that shows a Soret peak at 444 nm (reaction 7), and finally it interacts with the second NO to form N_2O (reaction 8).

Reaction 6: $Fe^{3+} + NO \rightarrow Fe^{3+}NO$

Reaction 7: $Fe^{3+}NO + NAD(P)H \rightarrow I_{heme} + NAD(P)^{+}$

Reaction 8: $I_{heme} + 2NO + 2H^{+} \rightarrow N_2O + H_2O$

The chemical nature of the I_{heme} intermediate seems to be ferric-hydroxylamine radical complex (Daiber et al. 2002). Direct electron transfer from NADH to the heme of P450nor was demonstrated by the resolution of the crystal structure of P450nor complexed with the NADH-analogue nicotinic acid adenine dinucleotide, NAAD (Oshima et al. 2004). The structure of the P450nor–NAAD complex showed a conformational change upon binding of NAAD since the entrance gate of the heme distal pocket is closed. Glu71, Arg64 and Asp88 form a salt bridge network to stabilize the protein structure (Umemura et al. 2004; Su et al. 2004). Nitric oxide reductase is employed for the quantification in solution of nitric oxide (Garny et al. 2014), biosensores development and research tools for the study of the biological roles of this important cell-signalling molecule (Mur et al. 2006).

The use of NO scavengers, toxic chemical compounds that could mask the NO function (Turpaev et al. 2004), impaired the current *in vitro* research analysis of the biological role of nitric oxide. To overcome this limit, the use of an enzymatic system of NO reduction could be a valid option. A good candidate is represented by P450nor since it is monomeric, has a single domain and accept electrons directly from its cofactor NADH/NADPH. Garny and colleagues (2016) developed a novel, dual enzyme system co-immobilizing on carboxyl-functionalized hyper-porous microspheres, the P450nor from *Aspergillus oryzae* and *Bacillus megaterium* glucose dehydrogenase with enzyme activity maintenance of 158% for P450nor and 104% for glucose dehydrogenase. The system is capable not only of continuously reduce NO but also to recycle the NADH cofactor through the action of glucose and glucose dehydrogenase. The NOR system reduces NO in a concentration-dependent manner and is able to maintain the cell viability when cytotoxic

concentration of nitric oxide are used.

CYP505: fatty acid hydroxylase.

Another self-sufficient cytochrome P450 isolated from *F. oxysporum* is represented by P450foxy belonging to CYP505 family (Nakayama et al. 1996; Kitazume et al. 2000, 2002). This is a membrane bound enzyme composed of a N-terminal heme domain naturally fused to a carboxy-terminal reductase domain in a single polypeptide. Phylogenetic analysis of the heme domain provided evidence that it is the eukaryotic counterpart of the bacterial BM3. Pfoxy catalyzes the sub-terminal ω-1 to ω-3 carbons fatty acids hydroxylation. Redox equivalents given by NADPH go to the FAD and FMN cofactors present in the reductase domain and then to the heme. Since the heme and reductase domains are naturally fused, the turnover number of Pfoxy is one hundred-fold higher than the one of conventional class II P450s, with which shares the electron transfer mechanism (Kitazume et al. 2002).

The hydroxylation on specific carbon atoms of aliphatic compound is important in industrial chemical synthesis. In fact, omega fatty acids terminally oxidized are useful for the synthesis of industrial compounds with pharmaceutical and commercial interests such as potential anticancer drugs, cosmetic intermediates, adhesives and bioplastics (Durairaj et al. 2015). Enzymatic hydroxylation of specific C-H bonds is an interesting solution since a specific carbon atoms are hydroxylated. Self-sufficient cytochromes P450 are ideal candidates for this kind of application since they do not depend upon P450 reductase, furthermore their high turn-over number allow their application in high-throughput catalytic systems development. Recombinant fungal Pfoxy expressed in *Escherichia coli* cells convert saturated fatty acids with a chain length from C7 to C16 that are present in waste lard and vegetable oils originating from recycling industry to their ω-1 to ω-3 hydroxy derivatives (Kitazume et al. 2008).

4.2.1 P450s from *Fusarium verticillioides.*

CYP505B1.

Another member of class VIII, called CYP505B1 (Fum6p), has been identified in the filamentous ascomycete *Fusarium verticillioides* showing the highest aminoacid sequence identity with P450foxy (Seo et al. 2001). *F. verticillioides* is a maize pathogen that produces the mycotoxin fumonisin that can accumulate in infected maize and cause fatal animal diseases and human esophageal cancer (Sydenham et al. 1990). CYP505 is organized in the same way as P450foxy involved in the mycotoxin fumonisin biosynthesis (Seo et al., 2001). Fumonisin is a linear 20-carbon backbone, that has a polyketide origin, substituted at various positions with an amine, one to three hydroxyl, two methyl, and two tricarboxylic acid groups. Gene

disruption analysis revealed that FUM6 gene is required for fumonisin production and Northern blot analysis revealed that its expression is correlated with fumonisin production (Proctor et al. 2003).

4.3. Plant enzymes.

CYP74 family.

Plant CYP74 is a family of self-sufficient cytochromes P450 belonging to class X with electron transfer properties very different from the typical P450s. They utilize an acyl hydroperoxide of the substrate both as the oxygen donor and as the substrate in which the new carbon-oxygen bonds are formed. Therefore, they do not require O_2 or a NADPH-dependent cytochrome P450 reductase or NAD(P)H (Itoh and Howe 2001; Lau et al. 1993; Shibata et al. 1995) and have low CO affinity. To date class X P450s have not been found to be present in mammals.

The CYP74 family comprises the allene oxide synthase (CYP74A), fatty acid hydroperoxide lyase (CYP74B, CYP74C) and divinyl ether synthase (CYP74D). CYP74A, B, C and D are present in the chloroplast membranes where they are key enzymes of the plant lipoxygenase pathway (Froehlich et al. 2001).

Fatty acid hydroperoxide lyase is an enzyme that cleaves hydroperoxides of polyunsaturated fatty acids to form short chain aldehydes and ω-oxoacid. It is a component of the oxylipin pathway and holds a central role in eliciting plant defence (Psylinakis et al. 2001). Allene oxide synthase is involved in the metabolic pathway that produces the plant growth regulator jasmonic acid from unsaturated free fatty acids (Vick and Zimmerman, 1984). Divinyl ether synthase converts hydroperoxides into the divinyl ethers (Grechkin and Hamberg, 1996).

4.4. Artificial P450-reductase fusion systems produced by protein engineering

The requirement of the addition of a redox partner protein to sustain P450 enzymatic catalysis is considered a bottle neck in biocatalysis due to the necessity of different components in solution, with a possible loss in efficiency and higher costs. This has motivated many works in which P450 enzymes of biotechnological interest that are not natural fusion with their reductase have been engineered to produce artificial multi-domain systems. In this way the enzyme and a reductase of choice are fused at genetic level to produce a single polypeptide chain. As a result, the engineered protein comprises two domains: the catalytic P450 domain and the reductase electron transfer domain requiring only addition of NAD(P)H and substrate to perform catalysis. Many such constructs have been reported to date in the literature, where their advantages in terms of the

improvement of their catalytic properties, coupling efficiency and possibly solubility are reported in comparison with their separate parent enzymes (Sadeghi and Gilardi, 2013). In some cases, human P450 fusion proteins have been shown to be useful tools for the study of drug metabolism, with consequences of drug–drug interactions and the activation of carcinogens.

The first fusion protein reported in literature in 1987 is the rat CYP1A1/rat NADPH CYP reductase system (Murakami et al. 1987) able to oxidise ethoxycoumarin. Rat reductase was successively used to build up chimeras with bovine (CYP17A1), dog (CYP2B11) and human (CYP3A4, CYP1A2) P450s (Shet et al. 1994, Fisher et al. 1992).

A general approach called the "Molecular Lego" (Sadeghi et al. 2001, Gilardi et al. 2002) was then applied to P450 enzymes by fusing class II human P450s with the soluble reductase of *Bacillus megaterium* P450 BM3 (BMR), resulting in a soluble and self-sufficient system. Examples of such fusion systems are human CYP2E1 (Fairhead et al. 2005), CYP2C9, CYP2C19, CYP3A4 (Dodhia et al. 2006), CYP2A6, CYP2C6 and CYP4F11 (Ortolani 2012, Rua 2012) and monkey CYP2C20 and dog CYP2D15 (Rua et al., 2010). The advantages reported for these chimeras are the activity, solubility and correct folding in absence of detergents, and the simple addition of NAD(P)H and substrate to trigger catalytic activity. Moreover, in a further step NAD(P)H was replaced by electrode surface that provides the electrons driving the catalysis without the use of costly cofactors (Sadeghi et al. 2011).

Another example of artificial fusion systems is the CYP116B family. Nodate and colleagues (2006) have created a fusion protein with P450cam heme domain and the reductase domain of CYP116B2 (P450RhF). P450cam-RhFRed chimera shows 100% conversion of camphor to 5-*exo*-hydroxycamphor and its specific recognition of substrates could be significantly enlarged by mutations in the different sites (Fowler et al. 2002). This chimera with Tyr96Phe mutation, combined with Tyr96Phe/Val247Ala mutations, has been studied with a high-throughput screening protocol by Robin et al. (2011), and was aimed at reaching the oxidation of useful molecules with an industrial application, such as terpenes α- and β-ionone relevant to the fragrance industry.

5. APPLICATIONS OF P450s IN INDUSTRY: PERSPECTIVES

The use of enzymes in industrial processes is a fast growing field that now is even more close to its maturity. Despite being attractive targets for catalysis, their wide application in industry still presents serious challenging due to a number of reasons. On one hand, biochemistry has made major advances in understanding their mechanisms, in controlling their proficiency and in

producing high levels thanks to DNA recombinant methods and heterologous expression technologies (Roiban and Reetz 2015).

Protein engineering both by rational mutagenesis methods and in vitro darwinian evolution strategies has offered a handle in improving their stability to temperature and pH, their resistance in organic solvent environments, their immobilisation on suitable supports. Moreover the growing legislative attention to the environment and demand for sustainable approaches to chemical synthesis has highlighted the intrinsic advantages in the use of enzymes as catalysts. In spite of their remaining limitations of possibly higher costs, more and more areas of applications have been uptaken in industry (Choi et al. 2015)

The application of P450s in industry is mainly in the area of generation of drug metabolites and synthetic intermediates as they can produce fine chemicals at high grade of purity. Heterologous expression in *Escherichia coli* or insect cells allows to obtain recombinant enzymes that can be used to produce molecules for use in the pharmaceutical industry. The cofactor regeneration and the costs associated with it, as well as narrow substrate specificity, the limited solvent tolerance are some of limitations encountered in the use of P450s on industrial processes (O'Reilly et al. 2011, Urlacher and Girhard 2012). In some case a whole-cell approach has given the best solution for specific problems. In 2003 Aventis-Pharma and the CNRS (Gif sur Yvette, France) have demonstrated the feasibility of transferring a complex biosynthetic pathway from higher eukaryotes into microorganisms producing hydrocortisone in yeast (Szczebara et al. 2003). Here complex products have been synthetized, replacing long and expensive synthetic multi-step reactions.

In 2006, P450 BM3 was implemented in the preparation of metabolites of propanolol (Otey et al. 2006). The high catalytic activity of this enzyme is very attractive, and thus the random mutagenesis approach has been largely used in order to expand the range of the wild-type (WT) protein to include different substrates and create a new product. Many different high throughput screening methods have been proposed for discovering favourable random mutations in BM3 mutants evolved to recognise specific substrates. One of such methods is represented by the alkali NAD(P)H assay that has allowed to identify mutants capable of specific reactions within a library of random mutants generated by error prone PCR (Gilardi et al. 2002, Di Nardo and Gilardi 2012).

Rational mutagenesis studies on Trp96 conserved residue, shows a primary role in the heme coordination through some key hydrogen bonds (Munro et al. 1994 and Ravichandran et al. 1993). Substitution of Phe87 makes BM3 able to hydroxylate lauric and myristic acid, mainly at the ω-position, that is not reached by the WT enzyme. Phe87Val combined with Ala82Phe mutation resulted in an increase in the active site space, allowing more substrate hydroxylation, while other

mutants (Cha et al. 2014) can make metabolites of non-steroidal anti-inflammatory drug diclofenac (mimic 4'- and 5'- P450s human metabolites).

Other interesting examples of the utility of BM3 regard the conversion of terpene to perillyl alcohol or the 1-hexane to 1,2-epoxy hexane.

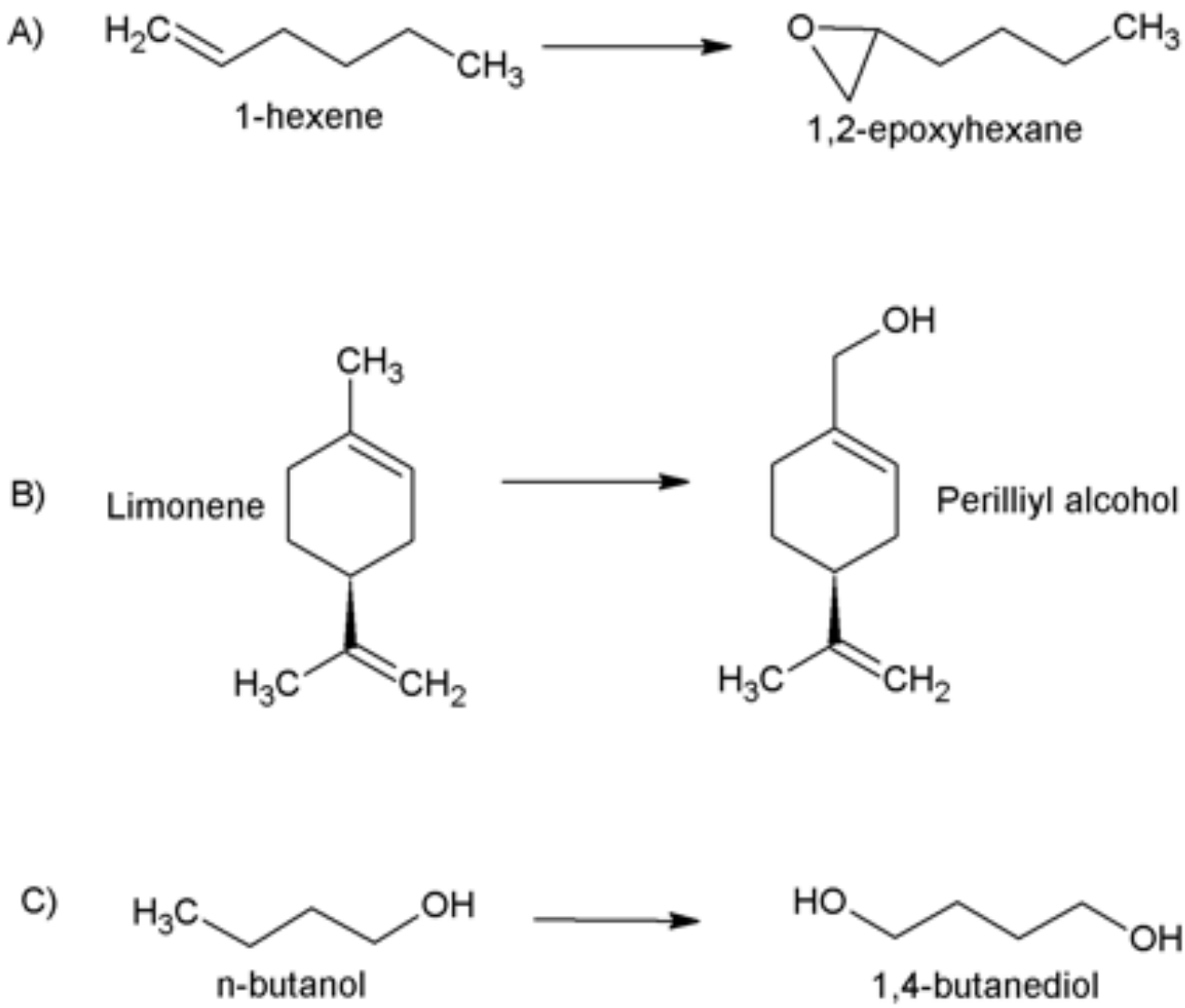

FIGURE 5. A) 1-hexene is converted to 1,2 epoxyhexane by BM3 triple mutants produced by saturation mutagenesis of the active site (Kubo 2006). B) Reaction performed by Ala264Val/Ala238Val/Lys437Phe BM3 triple mutant (Seifert 2011) C) I83M/I82T mutant of P450pyr catalysed reaction: hydroxylation of n-butanol 1 to 1,4-butanediol.

The huge protein engineering approach on BM3 is a versatile target able to perform different reactions useful in several fields.

However, using the chimera approach, interesting results have been obtained with CYP105D7, that is a class I type of CYP, that requires electrons provided through separated electron transfer proteins (ferredoxin and ferredoxin reductase) for the hydroxylation of 7-hydroxy-3-(4-hydroxyphenyl) chromen-4-one (Daidzein) an isoflavones.
Joining the reductase partners and the CYP heme domain, the fusion enzyme obtained by Choi et al. (2012) catalyses hydroxylation of daidzein more efficiently, with a k_{cat}/K_M value of 16.8 μM^{-1} min^{-1} and with very high regio-selectivity.

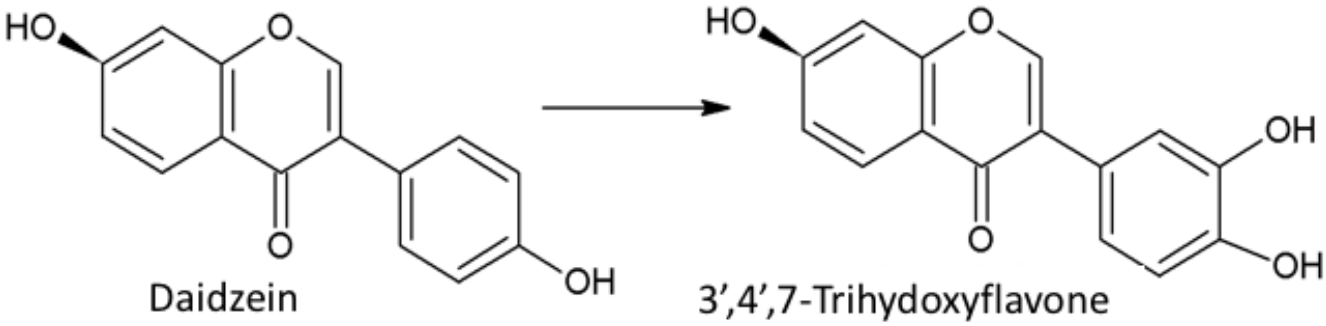

FIGURE 6. The reaction catalised by 3'-ASDH. This fusion artificial enzyme hydroxylates the 3' of the daidzein B-ring (Choi 2012).

Terminal hydroxylation of n-butanol to 1,4-butanediol, a useful reaction for industrial purposes (Barikani et al. 2009), have been reached with directed evolution, creating Iso83Met/Iso82Thr, a mutant of P450pyr monooxygenase (Yang et al. 2014) that represents a green route alternative to the synthetic traditional route for obtaining this product. This approach is fundamental in order to convert a hydrophilic compound that was not accepted by the class I wild-type enzyme.
Another field of application for P450s is the production of hydroxy-fatty acids. The enzymatic oxidation catalysed by P450s on non-activated carbon is a great advantage in different industrial processes. They are widely used in different industrial fields, such as the chemical, food, cosmetic and biofuel industries. Hydroxy-fatty acids are often used as starting materials for the synthesis of resins, polymers, biopolymers, and there is evidence that these polymers, synthesized from hydroxy fatty acids, are much more resistant and flexible than petroleum-derived ones (Burdock et al. 2006). 10- and 12-hydroxystearic acid is used in the manufacturing of lubricants, ricinoleic acid in soaps and also in the textile industry (Mutlu and Meier 2010). Due to the hydroxylation, fatty acids are more stable, more reactive and more solvent miscible, in comparison to non-hydroxylated fatty acids (Metzger and Bornscheuer 2006).
Another interesting P450 from *Bacillus megaterium*, with potential biotechnological application, is CYP106A2, a NADPH dependent protein composed of a FMN domain (megaredoxin reductase) and an iron-

sulphur protein (megaredoxin). It is able to hydroxylate various 3-oxo-D4-steroids, mainly at the 15ß – position. These hydroxylated products are very important in the pharmaceutical industry, and they are precursors in the synthesis of several steroids compounds. Moreover, Bleif and co-workers have also reported its ability to perform one-step regioselective reaction of the anti-inflammatory pentacyclic triterpene 11-keto-β-boswellic acid (KBA), and to our knowledge this is the first reported pentacyclic triterpene conversion performed by a prokaryotic P450. They have also reported a whole-cell system using *Bacillus megaterium* strain MS941 for the conversion of KBA, with a 15-fold increase in comparison with the naturally CYP106A2 expressing *Bacillus megaterium* strain (ATCC 13368).

6. CONCLUSIONS

The flexibility of P450 enzymes in the recognition of different substrates, the possibility of generating or detecting industrial relevant metabolites, as well as the use of these enzymes in environmental detoxification, are the main reasons that have made these enzymes mature as useful biocatalysts. Every year more new enzymes are discovered from different kingdoms of life, lately even from viruses (Nelson 2009). An intense study of their electron transfer chain, especially for self-sufficient enzymes that display the highest efficiency in catalysis, important in biotechnological applications. Also relevant is the design of new, artificial, self-sufficient enzymes, that overcome possible limitations of the P450 catalytic unit. Biotechnological applications, involving P450 monooxygenases, are nowadays becoming more of a reality, but further work should be focused on key aspects, such as improving the stability of the P450s and cofactor regeneration during biocatalysis. The development of efficient and self-sufficient P450s opens up new horizons in the field of the synthesis of new molecules, pro-drug activation, bioremediation and whole-cell applications.

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