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Review Article

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Cytochrome P450 Biocatalysts: A Route to Bioremediation

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ABSTRACT

The rapid increase in demand and development of industrial chemicals, to sustain and improve quality of life worldwide have resulted in the contamination and high prevalence of chemicals into environment, posing a potential threat to the biota. Hence all organisms are exposed constantly and unavoidably to these foreign chemicals, or xenobiotics, which include both manufactured and natural chemicals such as drugs, industrial chemicals, pesticides, pollutants, pyrolysis products, alkaloids, secondary plant metabolites, and toxins produced by molds, plants, and animals. The toxicity or the contamination of chemicals can be reduced by viable bioremediation solution with many intermediate or degradation products. In this remedial process, cytochromes P450 (CYPs) are key enzymes in the metabolization of all xenobiotics by catalyzing oxidations of the substrate and are polymorphic in nature. Cytochrome P450 genes constitute one of the largest gene superfamilies, with representatives in all living organisms, including bacteria, fungi, insects, plants, and animals. The catalytic versatility and substrate diversity of CYP enzymes have led to considerable interest in utilizing them as biocatalysts for many biotechnological applications (such as synthesis of drugs chemicals, metabolization of endogenous compounds, such as fatty acids and vitamins to maintain homeostasis, targeted gene therapy, cancer treatment) other thanbioremediation. In this review we primarily discuss 1) background information about CYPs including its occurrence, classification and mechanism 2) the role of CYP enzymes in bioremediation for detoxification of industrial and environmental pollutants special reference to "Morpholine" and 3) engineered CYPs enzymes and their potential role in transgenic plant-mediated phytoremediation.

Key words: Cytochrome 450, Bioremediation, Xenobiotics, Morpholine, Transgenic Plants

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INTRODUCTION

Among the various enzymatic group, cytochrome 450 (CYP) and glutathione S-transferase(GSTs) play major role in the environmental detoxification and biotransformation of drugs pesticides and xenobiotic. In addition to CYP450 oxidation, glutathione conjugation is an important mechanism for xenobiotic remediation. Glutathione S-transferase (GSTs-EC.2.5.1.18) is a family of multifunctional enzymes involved in the cellular detoxification and excretion of many physiological and endogenous substances [1]. It catalyzes the addition of glutathione to endogenous, or xenobiotic, often toxic electrophilic compounds and which are found in animals, plants and microorganisms [2]. Their role in xenobiotic metabolism is beyond the scope of present review. However CYP mechanism and occurrence are briefly explained in respective session.

The first report on the existence of a CYP enzyme or a microsomal carbon monoxide binding pigment was published in 1958 by klingenberg M. [3]. This enzyme has a unique 450 nm optical absorption peak and when its hemoprotein nature was recognized and was given the name cytochrome 450 [4], [5], [6]. Cytochrome P450 enzymes comprise a superfamily of heme proteins in which the heme iron atom is coordinated to a proximal cysteine thiolate. This thiolate ligand is responsible for the characteristic absorption maximum of the Fe^{+2}-CO complex at 450 nm and is critical for P450 catalysis [7]. It is crucial for the oxidative, peroxidative and reductive metabolism of a diverse group of compounds, both endogenous such as steroids, bile acids, fatty acids, vitamins, prostaglandins, precarcinogen and leukotrienes and exogenous including xenobiotics, most of the therapeutic drugs and environmental pollutants play an important role in homeostasis[8-11]. The large number of substrate metabolized is due to the plethora of P450 isoforms and to the broad substrate specificity of some isoforms. Over 80% of marketed drugs are converted into relatively hydrophilic compounds by CYP enzymes in the liver leading to their safe clearance from the body [12]

In almost all living organism, these enzymes are present in more than one form, thus forming one of the largest gene super families. The presence of P450 in diverse organisms, from bacteria, fungi to plants and animal simplies that the P450 superfamily is an extremely ancient enzymatic system and that all the current P450 may have descended from a common ancestral gene [13], [14], [15]. The current P450 superfamily is thought to have been formed by gene duplication and adaptive diversification [16]. The enzyme system is located in microsomes and consists of several cytochrome P450 isoforms and a nonspecific NADPH-cytochrome P450 oxidoreductase. The polymorphic xenobiotic metabolizing CYP enzymes can be mainly divided into two classesand the sites of expression and local concentrations differ for the various P450 enzymes [17].

Class I: composed of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5, which are well conserved, do not have important functional polymorphisms, and are active in the metabolism of precarcinogens xenobiotics and drugs [18]. Of these, CYP3A4 is present in the highest concentration in the human liver and is responsible for the oxidation of approximately two-thirds of all known drugs [19]. However, the other P450 enzymes listed are also important players in xenobiotic metabolism and prodrug activation.

Class II: composed of CYP2B6, CYP2C9, CYP2C19 and CYP2D6, which are highly polymorphic and active in the metabolism of drugs, but not of precarcinogens.

The notable diversity of CYP enzymes has given rise to systematic classification of individual forms into families and subfamilies. CYP450 are named primarily on the basis of the overall amino acid sequence [20]. A P450 is named CYP followed by a number, a letter and a number. P450s with > 40% of the amino acids identical are usually grouped into the same family (e.g. CYP2A6 and CYP2B6)and members with > 55% of the amino acids identical are generally grouped into the same sub family(e.g. CYP2A6 and CYP2A7), although there are exceptions to these rules. For examples, CYP6A1 and CYP6B2 are both grouped into family 6 but the amino acid identities of these two are < 40%. In this case the 40% rule was neglected because the sequences flanking the conserved cysteine were similar [21]. Given that this nomenclature system is based on the overall amino acid identity, and a single amino acid change may dramatically alter the substrate specificity of a P450[22], [23]. No information regarding the function of a P450 should be assumed from its classification within this system. However, the number of family and enzymes varies among different organisms. There are two important factors with regard to the distribution and activities of drug or xenobiotics metabolizing P450 enzymes. First, polymorphisms in the P450 genes can either decrease or elevate the activities of the individual enzymes, with higher levels of expression giving rise to higher metabolic capacity in individuals that are consequently described as ‘extensive’ or ‘hyper’ metabolizers [24], [25]. Second, most of the drug-metabolizing enzymes are subject to induction by xenobiotics or environmental factors [26], [27].

Mode of action:

Cytochrome P450s are multicomponent enzymes consisting of two separated functional classes, namely electron transfer and oxygenation. Interaction and complementation between two functional classes are necessary for the full catalytic function.

Under electron transfer, the cytochrome P450 system catalyzes the insertion of an oxygen atom into C-H and N-H bonds, the epoxidation of π bonds, and the addition of an oxygen atom to the electron pairs of nitrogen, sulfur, and

phosphorus atoms resulting in the formation of an N-Oxide or S-Oxide [28]. These basic reactions are often followed by spontaneous reactions that yield the final metabolites as described in figure1.

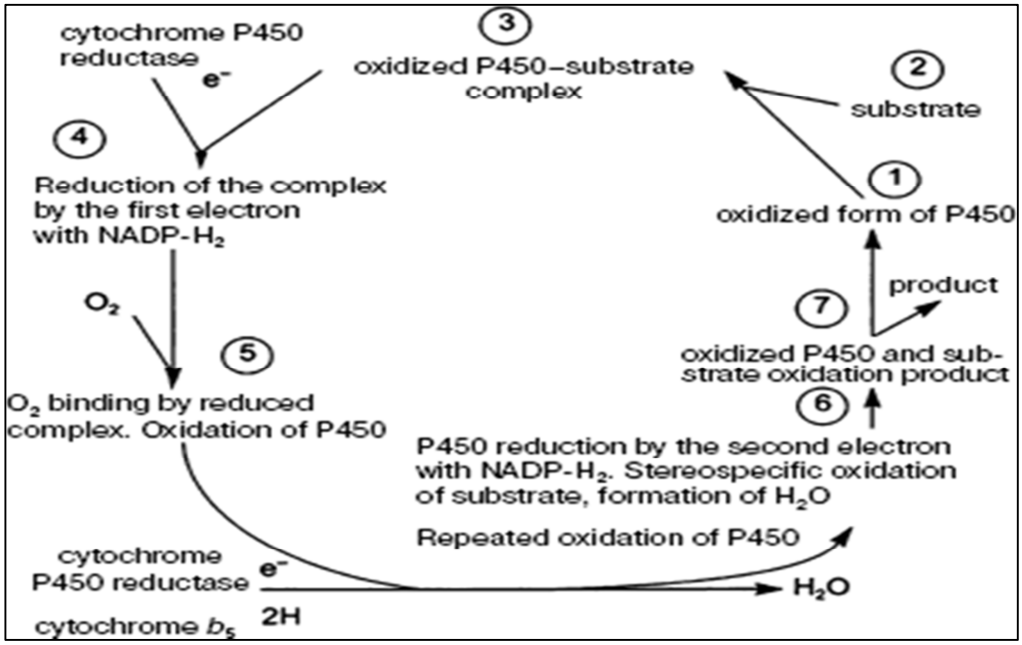


Fig. 1: The general catalytic cycle of CY450.In explanation, oxidized form of P450 (1) binds with substrate (2) and form P450-substrate complex (3). It is further reduced with NADP-H_2 first electron (4) so that oxygen molecules bind and undergoes oxidation (5). However NADP-H_2 second electron results the oxidation of substrate and formation of water molecules (6) and product (7)

In brief, the cytochrome P450 catalytic cycle is initiated by the binding of a substrate, usually with concomitant displacement of the distal water ligand. The ferric heme is then reduced to the ferrous state using electrons provided by suitable electron donor. In cytochrome P450 and many other P450 enzymes, substrate binding is widely believed to be a prerequisite for the transfer of the first electron to the iron. Reduction of the iron is followed by binding of oxygen to give the ferrous dioxy complex. Transfer of a second electron to this complex produces the ferric peroxy anion ($PorFe^{III}$-00^- where Por = porphyrin) or, after protonation, the ferric hydroperoxo complex

(Por^{III}-OOH).Heterolytic cleavage ofthe dioxygen bond in this peroxo intermediate extrudes a molecule of water and forms the putative ferryl oxidizing species (figure 2).Hydrogen bonding of the distal ferric hydroperoxooxygen, directly or via a water molecule, to a highly conserved threonine facilitates this heterolytic cleavage [29], [30], [31]. The ferryl species is thought to be responsible for most P450- catalyzed oxidations, although the ferric peroxo anion and the ferric hydroperoxo complex have been invoked as oxidizing species. Other types of cytochrome P450-catalyzed reactions also occasionally occur (the shunt pathway).It is not always, possible to circumvent the requirement for activation of molecular oxygen in a so-called "shunt pathway" by employing H_2O_2 as a co-substrate (figure 2). However, the oxidizing species thus obtained is apparently not identical to that obtained by normal oxygen activation[32]. Thus, peroxides cannot replace molecular oxygen activation in some reactions; they often give product distributions that differ significantly from those obtained by molecular oxygen activation, and they cause a more rapid degradation of the prosthetic heme group [33]. In virtually all cases, however, the resulting products are more polar, more readily conjugated, and more readily excreted. It is noted that lipophilic compounds that do not have functions suitable for conjugation and are not susceptible to CYP oxidation are difficult to eliminate.

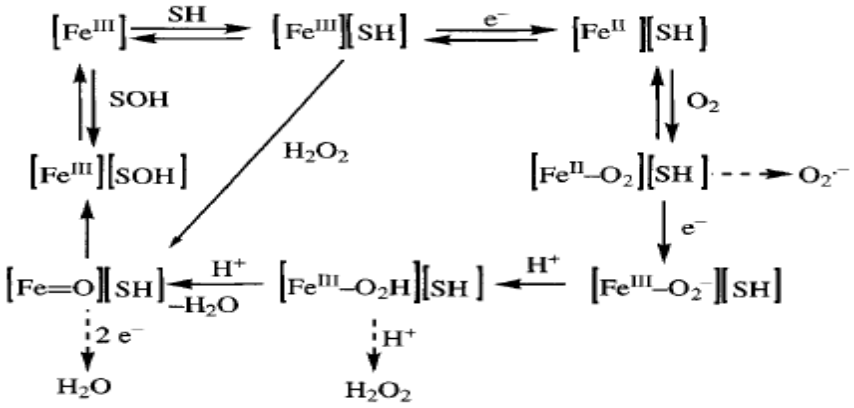
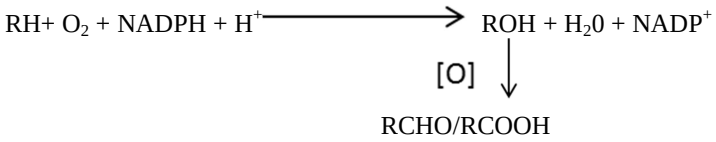


Fig. 2: The Fe^{III}stands for the resting ferric state of P450 and SH for a substrate molecule. The shunt pathway utilizing H_2O_2 is shown as are three sites for the uncoupling of the enzyme to give respectively $O_2^{\cdot-}$, H_2O_2 or H_2O

Under oxygenation, CY450 dependent monooxygenase is used which is extremely important metabolic enzymatic system involved in the metabolism of phenomenal number of endogenous and exogenous compounds [34]. In most cases bacterial P450-dependent monooxygenases are composed of three components and electrons are transferred from NADPH via an FAD-containing reductase and a small iron-sulfur protein to the cytochrome P450 where catalysis of the monooxygenase reaction takes place [35], [36]. The P450 oxidation stoichiometry requires one molecule of oxygen and two electrons from NADPH to add one oxygen atom to a substrate. The overall reaction of P450 monooxygenase mediated metabolism can be expressed as follow



Where RH is substrate. Collectively P450 monooxygenase are capable of metabolizing numerous substrates and can carry out multiple oxidative reactions [37]. Metabolism by monooxygenase generally results in detoxification of the substrate although activation is also possible in case of organophosphate insecticides and tobacco constituent's namely nitrogen-derived nitrosamines to procarcinogens, which cause lung, esophageal, and pancreatic cancers which is beyond the scope of discussion in this review [38], [39], [40].

Oxidation of Aromatic Rings: A Case study of Morpholine Biodegradation

Morpholine (C_4H_9NO) is a simple heterocyclic compound with great industrial importance. Due to its chemical nature as a cyclic ether and secondary amine, it is extensively used for various industrial purposes, e.g. as a versatile solvent in the manufacture of a range of drugs, herbicides and paints, rubber additive, anticorrosive agent, emulsifier and wetting agent in cosmetics and hygiene products [41]. Becauseof its solubility in water, significant amounts of this chemical compound could be released via industrial effluents into the environment where it undergoes chemical or microbiologically nitrosation lead to the formation of the carcinogenic compound *N*-nitrosomorpholine [42].While water is the elixir of life, people are dying out of it. The contamination in water is increasing with each passing day and detrimental to the health of human life. So removal of this pollutant from contaminated wastewater and the environment has been the subject of considerable research in recent years because its biodegradation in biological effluent treatment plants (ETP) is widely regarded as problematical [43], [44]. So occurrence of morpholine is therefore a serious potential pollutant of the environment. Despite a simple structure, it is relatively recalcitrant to biodegradation. One reason for its apparent resistance to degradation may be the fact that the only organisms reported to degrade it as a sole carbon and energy source are incapable of rapid growth and only occur in the environment in smallnumbers. The isolated organisms capable of growing on morpholine as sole source of carbon, nitrogen and energy were identified in most cases as *mycobacteria* with the exception of an SK-05 isolate [45]. The utilization of morpholine as nitrogen sources or co-metabolizing by some gram-negative bacteria has also been reported [46]. The removal of morpholine from contaminated industrial wastewaters is therefore of

environmental interest, and is possible by biological treatment since it has clearly established that morpholine is biodegradable. Degradation pathways of morpholine have been proposed by various authors [47],[48], [49], [50] It was found that when *Mycobacterium* strain MorG was grown with morpholine as sole source of carbon and nitrogen, enzymes for ethanolamine catabolism (via the ethanolamine-O-phosphate pathway) and glycollate catabolism (via the glycerate pathway) were strongly induced and 2-(2 aminoethoxy) acetate and diglycolic acid have been detected as intermediates. Other *Mycobacterium aurum* strain MO1, HE5 was also reported by different authors [51], [52].

In brief, morpholine was cleaved into two C2 units which were probably further catabolized via separate pathway branches, involving ethanolamine and acetaldehyde in one case, and glycollate in the other case. The initial ring-cleavage occurred at the C–N bond and biochemical studies suggest that a cytochromeP450 may involved in this initial step as shown in figure 3 [52], [53].

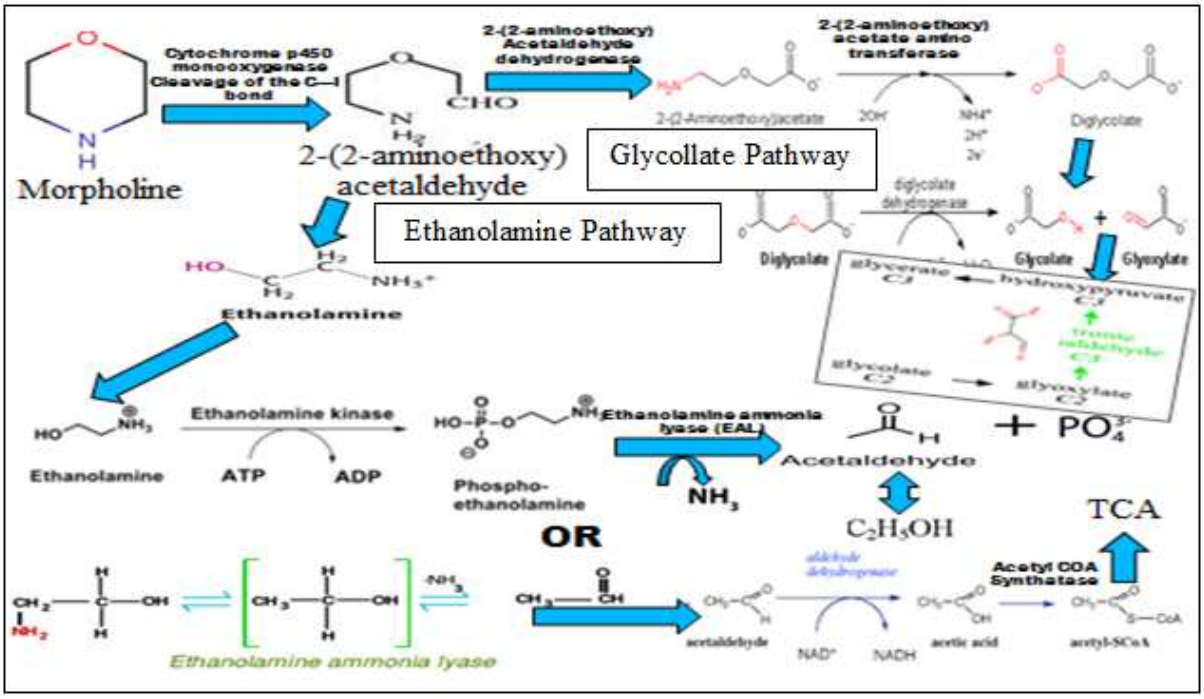


Fig. 3: Morpholine degradation pathway via the route of either ethanolamine-O-phosphate or glycollate, so called ethanolamine and glycollate pathway respectively

The oxidation of an aromatic ring bycytochrome P450 invariably involves oxidation of one of the π -bonds rather than direct insertion of the oxygen into one of the aromatic ring C-H bonds. Thus, benzene oxide has been specifically identified as a product of the oxidation of benzene by liver microsomes [54]. However, benzene oxide and the similarly unstable epoxides expected from the oxidation of other aromatic rings readily undergo heterolytic cleavage of one of the epoxide C-O bonds. This bond cleavage is followed by migration of a hydride from the carbon retainingthe oxygen to the adjacent carbocation to givea aldehyde/ketone intermediate as shown formation of 2-(2-aminoethoxy) acetaldehyde in morpholine degradation pathway by the catalytic action of CYP 450 monooxygenase(figure.3). Tautomerization of this ketone yields a phenol product. This sequence of steps is the so-called "NIH-shift" [55] as shown in figure 4.

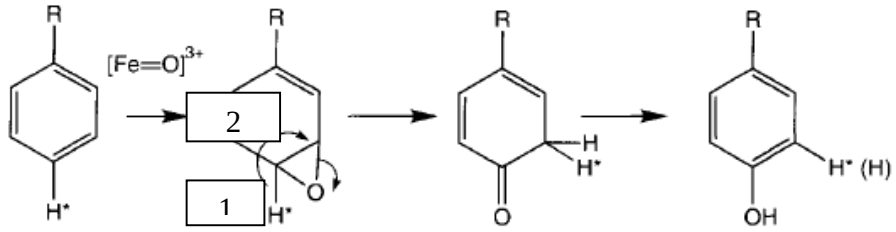


Fig. 4: The NIH shift involving initial formation of an epoxide metabolite in the oxidation of aromatic ring by cytochrome 450. The starred hydrogen shows that the hydrogen undergoes a 1,2 shift and then is partially lost in the final tautomerization step

Role to improve bioremediation (phytoremediation) of organic xenobiotics:

CYPs role for detoxification of xenobiotics in the environment through bioremediation/phytoremediation could potentially be beneficial by designing genetically modified organisms (transgenic plants). The use of transgenic plants for phytoremediation is critical because plants do not have the ability to completely catabolize toxic compounds that are common in the food chain, such as herbicides, pharmaceuticals, petrochemicals, polycyclic aromatic hydrocarbons(PAHs), and polychlorinated benzene (PCBs) [56] [57]. Since many bacterial, mammalian CYP enzymes have the capability to metabolize these compounds into relatively safe products, they can be used to create transgenic plants for such purposes. Therefore in the last decade there has been an increasing realization of the power of CYP biocatalysts for creating herbicide-resistant plants [58], [59] [60]. Although cytochrome P450 monooxygennases in higher plants pays an important role in the oxidative metabolism of endogenous and exogenous lipophilic compounds [61], [62], [63]. Molecular information on P450 species metabolizing xenobiotics in plants is quite limited; however many P450 dependent oxidations in plant microsomes have been reported [64]viz., oxidation of chlorotoluron in maize [65] and wheat [66]; linuron in wheat [67] and maize [68]; atrazine in tulip and isoproturon in yam beam [69]. It was reported 16 cytochrome P450 species responsible for the herbicide detoxification and cross tolerance in *Loliumrigidum*[70] [71].On the other hand, there are number of P450 species metabolizing xenobiotics in the microsomes of human liver because the human genome encodes 57 cytochrome P450 enzymes, roughly a third of which are involved in the biosynthesis of essential sterols, signaling molecules or regulatory factors, a third of which are largely devoted to the metabolism of xenobiotics, and a third with functions that remain unclear [72], [73]. A study of 11 human CYP450 in the CYP1, 2 and 3 families using a recombinant yeast expression system showed that they can metabolize 27 herbicides and 4 insecticides [74]. Further another study conducted by same research group found that human CYP1A1 metabolized 16 herbicides, including triazines, ureas and carbamates and CYP2B6 metabolize more than 10 herbicides including chloroacetanilides, oxyacetamides and 2,6-dinitroanilines, three insecticides and two industrial chemicals [74]. In contrast, many bacterial CYP enzymes are also used to metabolize a number of compounds; however each of mammalian or bacterial system has its own advantage and limitation (table 1). Therefore, there is a critical need to design mammalian CYPs to improve their catalytic efficiency, stability, expression, and the suitability of P450-CPR fusion enzymes, as well as to design bacterial CYPs for enhanced stability, expression, and substrate diversity [75], [76] .

Table 1: Comparative studies of mammalian and bacterial CYPs

CYPs	Advantage	Limitation	References
Mammalian CYPs	Broad substrate specificity	Require the redox partner cytochrome P450 reductase (CPR), which transfers electrons from the NADPH to the heme of CYP, to oxidize the substrate	[77]
	Expression in heterologous systems, including plants	Low turn-over number and enzyme stability	[60]
Bacterial CYPs	Do not require external redox partner for the transfer of electrons from the NADPH to CYP, rather contain reductase domain within the CYP enzymes (self-sufficient CYP). Much higher turn-over (>100) and enzyme stability	Do not show substrate diversity and metabolize limited number of compounds	[78],[79]

Due to broad substrate specificity of human and mammalian P450s, the resulted transgenic showed remarkable improvement of metabolic degradation towards single or multiple xenobiotics (table 2). Thus it is expected that the transgenic expression of both human and microbial P450 conjugation enzymes in plants will provide enhanced detoxification and therefore improved remediation of organic xenobiotics.

Table 2: Transgenic plants for enhanced phytoremediation of pesticides

Target plant	Gene(s)	Enzymes	Source	Effects	Reference
O.Sativa	CYP1A1,CYP2B6, CYP2C19	Cytochrome P450 monooxygenase	Human	Phytoremediation to atrazine and metolachor	[80]
Solanumtuberosum, O.Sativa	CYP1A1,CYP2B6, CYP2C19	Cytochrome P450 monooxygenase	Human	Resistance to sulfonyl urea and other herbicides	[81]
N.tabaccum	CYP105A1	Cytochrome P450 monooxygenase	*Streptomycesgriseolus*	Resistance to sulfonyl urea	[82]
O.Sativa	CYP2C9	Cytochrome P450 monooxygenase	Human	Tolerance to sulfonyl urea	[83]
O.Sativa	CYP2B22, CYP2C49	Cytochrome P450 monooxygenase	*Susscrofa*	Tolerance to several herbicides	[84]
N.tabaccum, A.thaliana	CYP71A10	Cytochrome P450 monooxygenase	*Glycine max*	Tolerance to phenyl urea herbicide	[85]
N.tabaccum	CYP76B1	Cytochrome P450 monooxygenase	*Helianthus tuberosus*	Tolerance to several herbicides	[86]
N.tabaccum	CYP450E1	Cytochrome P450 monooxygenase	Human	Degradation to anthracene and chloropyriphos	[87]
Transgenic plants	CYP81B2, CYP71A11	Cytochrome P450 monooxygenase	Tobacco	Degradation of chlorotoluron	[12]
	CYP1A1, CYP2B6, CYP2C19, CYP2E1	Cytochrome P450 monooxygenase	Human	Degradation of herbicides, insecticides and VOC	
	XP1A	Cytochrome P450 monooxygenase	*R.rhodochorus*	Degradation of RDX	
	CYP1A2 CYP2A6 CYP2B1 CYP2B11 CYP2Cs CYP3A4	Cytochrome P450 monooxygenase	Mammalian	Substrates for Alkoxyresorufin Indole Cyclophosphamide Ifosfamide Diclofenac Testosterone	
	CYPBM3 CYPCAM	Cytochrome P450 monooxygenase	Bacterial	Substrates for Alkanes, Benzene PCBs, PAHs	

CONCLUSION

At the global level, the use of xenobiotics has proved to assist solving of many problems facing human health and food production. However, such usage has occasionally been accompanied with hazards to man and the environment (MAE). One of biological approaches to clean up the xenobiotics is the use of microbes. Microbes are degrading organic pollutants in environment and use them for their normal metabolic processes as carbon or phosphorus source or consume the pesticides along with other source of food or energy. This bioprocess of microbes can be utilized for the development of xenobiotics decontamination and restoration of health of the environment. The use of xenobiotic degrading microbial systems requires an understanding of ecological requirements of degrading strains involved in degrading processes. There is need for further research on the biochemical and genetic aspects of degradation by microbes. For that purpose hydrolytic enzymes, responsible for degradation of xenobiotics to nontoxic products in the environment provides informed decision on which genes to engineer. The cytochromes P450 are such a diverse group of enzymes as catalytic hemoproteins for xenobiotic detoxification. The two-state model (ring cleavage to give an iminium carbon radical species followed by oxygenation) of complete catalytic action of P450 enzymes is most important mechanism for variety of xenobiotics including Morpholine. Although the precise mechanism of CYP on Morpholine removal is well understood and it may be shown that the production of negative charge on Morpholine molecule has a positive effect on biosorption and degradation of Morpholine by *Mycobacterium* and other isolates. Therefore, CYPs role must be explored for detoxification of others xenobiotics by designing genetically modified organisms for use in bioremediation. However, limited information is available with respect to the relationships among the pesticide/ insecticide type, concentration, exposure duration with CYPs and also found that CYP gene responded quite differently to different insecticides (Sun et al., 2014). Hence there is no general pattern for predicting the regulation of CYPs genes based on the pesticide classifications. No doubt, the next few years will uncover different novel aspects of P450 function and will lead to deeper and more precise understanding of the catalytic mechanisms of the amazing family of P450 enzymes for other class of xenobiotics. It has been suggested that increased understanding of the enzymatic process involved in plant tolerance and detoxification of xenobiotics will provide new directions for manipulating plant with superior remediation potential. Although more

focusing on transgenic plants has been made with conjugation of mammalian or microbial CYP450, their potential as engineered phytoremediation plants was not examined extensively in field trials as well as ecological impact and underlying economics against conventional remediation techniques. These studies are just tip of ice berg. Our knowledge continues to expand at a rapid pace, suggesting that the next decade will outpace the last in term of improving our understanding of cytochrome 450. Investigation needed for the detailed biochemical and physiological analysis of the whole process of phytoremediation – a group of innovative technological approaches should be continued; the creation of new, modified, genetically stable, environmentally safe, highly effective vegetation; the selection of microorganisms or other host system for scaling up of phytoremediation processes to handle xenobiotic harm within collaborative action plans without significant hazard to human beings.

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