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REVIEW

The enzymatic basis for pesticide bioremediation

Colin Scott • Gunjan Pandey • Carol J. Hartley • Colin J. Jackson • Matthew J. Cheesman • Matthew C. Taylor • Rinku Pandey • Jeevan L. Khurana • Mark Teese • Chris W. Coppin • Kahli M. Weir • Rakesh K. Jain • Rup Lal • Robyn J. Russell • John G. Oakeshott

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Abstract Enzymes are central to the biology of many pesticides, influencing their modes of action, environmental fates and mechanisms of target species resistance. Since the introduction of synthetic xenobiotic pesticides, enzymes responsible for pesticide turnover have evolved rapidly, in both the target organisms and incidentally exposed biota. Such enzymes are a source of significant biotechnological potential and form the basis of several bioremediation strategies intended to reduce the environmental impacts of pesticide residues. This review describes examples of enzymes possessing the major activities employed in the bioremediation of pesticide residues, and some of the strategies by which they are employed. In addition, several examples of specific achievements in enzyme engineering are considered, highlighting the growing trend in tailoring enzymatic activity to a specific biotechnologically relevant function.

C. Scott • G. Pandey • C. J. Hartley • C. J. Jackson • M. J. Cheesman • M. C. Taylor • R. Pandey • J. L. Khurana • M. Teese • C. W. Coppin • K. M. Weir • R. J. Russell • J. G. Oakeshott
CSIRO Entomology,
GPO Box 1700,
Canberra, ACT 2601, Australia

Rakesh K. Jain
Institute of Microbial Technology,
Sector 39-A,
Chandigarh 160036, India.

Rup Lal
Department of Zoology,
University of Delhi,
Delhi - 110 007, India.

C. Scott (✉)
Tel: +61 2 62464090; Fax: +61 2 62464173
e-mail: colin.scott@csiro.au

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Introduction

Enzymes are central to the mode of action of many pesticides: some pesticides are activated *in situ* by enzymatic action, and many pesticides function by targeting particular enzymes with essential physiological roles. Enzymes are also involved in the degradation of pesticide compounds, both in the target organism, through intrinsic detoxification mechanisms and evolved metabolic resistance, and in the wider environment, via biodegradation by soil and water micro-organisms.

Whether involved in their mode of action or their degradation, the enzymes involved in pesticide biology have been subjected to heavy selection pressures over the past 50 years, and some novel enzyme activities and pathways (for example, *s*-triazine and lindane catabolic pathways) [1, 2] have evolved as a consequence. Some of the degradative activities that have evolved not only constitute remarkable case studies of the influence of human activities upon natural evolutionary processes, but also form a source of significant biotechnological potential, and the basis of several strategies to reduce the environmental impacts of pesticide residues by bioremediation. It is largely, though not exclusively, the enzymes which have evolved in response to the presence of non-natural compounds that are the foundation for the pesticide bioremediation technologies that are currently available or in development.

Bioremediation of pesticide residues is becoming an important component of integrated agricultural management practices, helping to ensure that the principles of good stewardship are maintained. The subject of pesticide bioremediation has been considered in several excellent recent reviews covering bioaugmentation, biostimulation [2, 3] and enzymatic bioremediation [3, 4].

Biostimulation covers a range of technologies that encourage growth of pesticide-degrading microorganisms already resident in the contaminated soil. Not only do the appropriate organisms need to be present in the soil, but the remediated soils themselves must be suitable for the growth of the appropriate organisms in terms of physiochemical properties (e.g. pH, oxygen availability, temperature, bioavailability of the pesticide) and nutrient availability. Bioaugmentation also employs live pesticide-degrading bacteria, although these are non-indigenous inocula that can (in principle) include genetically modified bacteria. Like biostimulation, bioaugmentation requires suitable soil environment to achieve the required levels of bioremediation, and the rate of detoxification is tied to the rate of microbial proliferation.

Phytoremediation uses plants (natural or transgenic) to catabolise or accumulate contaminants. As with biostimulation and bioaugmentation, phytoremediation is entirely dependent upon the growth of the remediating plants, which can be significantly slower and dependent upon more stringent nutrient requirements than the rate of bacterial growth in biostimulation and bioaugmentation. The major advantage of phytoremediation over bioaugmentation is that GM plants can be more easily controlled and contained than GM microbes, and hence is a more freely accepted technology.

Free-enzyme remediation uses purified or partially purified enzymes to catalyse contaminant detoxification. Unlike the other technologies described here free-enzyme bioremediation is not dependent upon the growth of intact organisms, and so the rate of detoxification is directly linked to the catalytic properties of the enzyme employed and the concentration of enzyme applied. Equally, the lack of reliance on whole organisms allows the bioremediation of nutrient poor soils. However, free-enzyme bioremediation is not suitable for reactions catalysed by enzymes that are dependent upon diffusible cofactors, such as NAD, effectively limiting the enzymatic mechanisms to those free of diffusible cofactors.

Although it is not within the scope of this review to cover the details and relative merits of each of the bioremediation strategies, it is essential to acknowledge that each bioremediation strategy forms a framework in which the pesticide detoxifying enzyme must operate, and that the required biochemical and physical characteristics of those enzymes are determined by the bioremediation strategy in which they are employed. The success of any bioremediation strategy is ultimately dependent upon the presence of appropriate enzymes, and it is the enzymatic basis for pesticide bioremediation that will be covered here.

Due to the diversity of chemistries used in pesticides, the biochemistry of pesticide bioremediation requires a wide range of catalytic mechanisms, and therefore a wide range of enzyme classes. Rather than providing a comprehensive list of enzymes relevant to pesticide bioremediation (for a complete list a biodegradation pathways see the Minnesota Biocatalysis/Biodegredation Database; http://umbbd.ahc.umn.edu), this review will highlight some of the major enzymatic activities employed in the bioremediation of pesticide residues to date, using examples grouped according to their biochemical activity as defined by their Enzyme Commission (EC) classification (summarised in Table 1).

EC 1. Oxidoreductases

Oxidoreductases are a broad group of enzymes that catalyze the transfer of electrons from one molecule (the reductant or electron donor) to another (the oxidant, or electron acceptor). Many of these enzymes require additional cofactors, to act as either electron donors, electron acceptors or both. Oxidoreductases have been further subclassified into 22 subclasses (EC 1.1-1.21 and 1.97). Several of these have applications in bioremediation, albeit their need for cofactors complicates their use in some aplications (see below).

Oxidoreductases: Gox (EC 1.5.8)

Oxidases are defined as enzymes that catalyse oxidation/reduction reactions involving molecular oxygen (O_2) as the electron acceptor, whereby oxygen is reduced to water (H_2O) or hydrogen peroxide (H_2O_2). Possibly the best characterised oxidase with an involvement in pesticide bioremediation is glyphosate oxidase (GOX). The transgenic expression of GOX enzymes in plants confers upon them the ability to degrade the herbicide glyphosate, which can be considered to be a form of phytoremediation.

GOX is a flavoprotein amine oxidase from *Pseudomonas* sp LBr that catalyses the oxidation of glyphosate to form aminomethylphosphonate (AMPA) and releases the keto acid glyoxylate. Gox has a K_m of 25 mM for glyphosate and a V_{max} of 0.8U/mg, equivalent to a k_{cat} of 0.006 sec^{-1} (Fig. 1) [5, 6]. Similarly to other flavoprotein amine oxidases, such as D-amino acid oxidase (DAAO), GOX is presumed to

Table 1 Summary of example enzymes that have either the potential or proven application in the bioremediation of pesticides. Values for k_{cat} (sec^{-1}), K_m (M^{-1}) and k_{cat}/K_m ($sec^{-1}.M^{-1}$) are given for the highest known value (k_{cat} and k_{cat}/K_m) or lowest known value (K_m) for each enzyme. ND; not determined

Enzyme	E.C.	Source Organism(s)	Cofactor Requirements	Documented Target Pesticide(s)	k_{cat}	K_m	k_{cat}/K_m	Current Bioremediation Strategies Employed
Gox	1.5.8	*Pseudomonas* sp LBr; *Agrobacterium* strain T10	Flavin (FAD)	Glyphosate	5×10^{-3}	2.6×10^{-3}	1.9	*In planta*
Esd	1.13.14	*Mycobacterium* sp.	Flavin and NADH	Endosulfan and Endosulfate	ND	ND	ND	Not yet in use
Ese	1.13.14	*Arthrobacter* sp	Flavin (FMN)	Endosulfan and Endosulfate	ND	ND	ND	Not yet in use
Cyp1A1/1A2	1.14	Mammalian (Rat)	Heme and NADH	Atrazine, Norflurazon and Chlortoluron	1.5×10^{-4}	5.5×10^{-5}	4.3	*In planta*
Cyp76B1	1.14	*Helianthus tuberosus*	Heme and NADH	Linuron, Chlortoluron and Isoproturon	ND	ND	ND	*In planta*
$P450_{cam}$	1.14	*Pseudomonas putida*	Heme and NADH	Hexachlorobenzene and Pentachlorobenzene	1.4	ND	ND	transgenic *Sphingobium chlorophenolicum*
TOD	1.14.12	*Pseudomonas putida*	Fe^{2+} and NADH	Trifluralin herbicides	0.5	8×10^{-7}	6.3×10^{5}	Not yet in use against pesticides
E3	3.1.1	*Lucilia cuprina*	None	Synthetic pyrethroids and phosphotriester insecticides	1×10^{-2}	ND	ND	Not yet in use
OPH/OpdA	3.1.8	*Agrobacterium radiobacter; Pseudomonas diminuta; Flavobacterium*	Fe^{2+} and Zn^{2+}	Phosphotriester insecticides	3×10^{3}	1×10^{-3}	3×10^{7}	Free-enzyme bioremediation
LinB	3.8.1	*Sphingobium* sp.; *Sphingomonas* sp.	None	Hexachlorocyclohexane (β– and δ–isomers)	2.3×10^{-5}	ND	ND	Bioaugementation with *Sphingobium indicum*
AtzA	3.8.1	*Pseudomonas* sp. ADP	Fe^{2+}	Chloro-s-triazine herbicides	5	1.5×10^{-4}	3.4×10^{4}	*In planta* and GM bacteria
TrzN	3.8.1	*Nocardioides* sp.	Zn^{2+}	Chloro-s-triazine herbicides	2.1	2×10^{-5}	1.1×10^{5}	Not yet in use
LinA	4.5.1	*Sphingobium* sp.; *Sphingomonas* sp.	None	Hexachlorocyclohexane (γ-isomer)	ND	ND	ND	Bioaugementation with *Sphingobium indicum*
TfdA	3.8.1	*Ralstonia eutropha*	α- ketoglutarate and Fe^{2+}	2,4-Dichlorophenoxyacetic acid and pyridyloxyacetate herbicides	0.3	3.1×10^{-5}	1×10^{4}	*In planta*
DMO	1.13	*Pseudomonas maltophilia*	NADH and a Rieske Fe-S centre	Dicamba	ND	ND	ND	*In planta*

use the N(5) of FAD to abstract electrons from the targeted amine group, and then regenerate the reduced FAD via molecular oxygen. However, in contrast to DAAO and other typical amine oxidases, the GOX-catalysed reaction with glyphosate does not generate hydrogen peroxide (Fig. 1).

Comparison of the *Pseudomonas* GOX with GOX enzymes subsequently isolated from different soil bacteria (e.g. *Agrobacterium* strain T10) and variants generated by random mutagenesis allowed the design of a GOX with more efficient glyphosate degrading activity (K_m of 2.6 mM and a V_{max} of 0.6 U/mg, equivalent to a k_{cat} of 0.005 sec^{-1}). A single amino acid change (His334Arg) was responsible for the alteration in activity, and a comparison with structure-function models of other oxidoreductase flavoprotein enzymes suggests that the Arg334 may be involved in substrate alignment within the active site (unpublished data; compared to ThiO from Settembre et al.) [7].

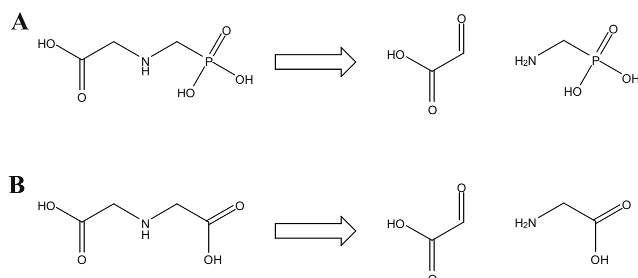

Fig. 1 Biodegradative reactions of GOX with A) glyphosate to form aminomethylphosphonate (AMPA) and glyoxylate, and B) iminodiacetic acid to form glycine and glyoxylate.

Monooxygenases: Ese and Esd (EC 1.13.14)

Monooxygenases generally catalyse the transfer of one atom of O_2 to an organic compound, with the other being reduced by electrons from cofactors to yield water [8]. Monooxygenases often play a role in the metabolism of xenobiotics by increasing either their reactivity and/or the water-solubility through the addition of an oxygen atom. Typical flavin dependent monooxygenases, unlike the novel enzymes described below, contain a tightly bound flavin

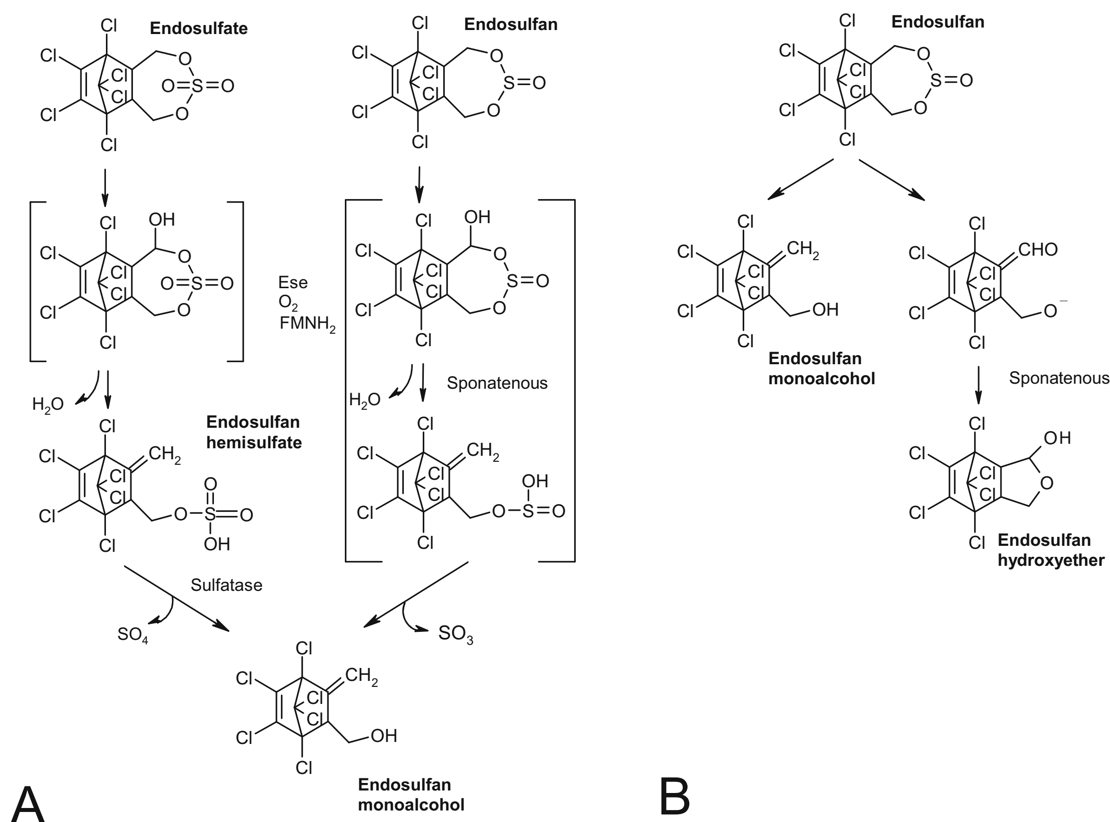

Fig. 2 Degradation of endosulfan and endosulfate by Ese and Esd. A) Proposed reaction for Ese-mediated degradation of α- and β -endosulfan and endosulfate. Parentheses indicate unconfirmed products. B) Degradation pathway for β-endosulfan by Esd.

cofactor, which can be reduced by an NAD(P)H substrate through their own catalytic activities [8].

One monooxygenase family that has a role in the fate of environmental pesticide residues is the two-component flavin diffusible monooxygenase family (TC-FDM) [9], a homologous group of enzymes unique in that the reduced flavin acts as a co-substrate not associated with the monooxygenases and is provided by a separate NAD(P)H dependent flavin reductase.

Two members of the TC-FDM family, Esd and Ese, are known to degrade the polychlorinated insecticide endosulfan and its toxic metabolite endosulfate. Endosulfate is a toxic metabolite of both isomers that is more persistent than either parent isomer (α and β-endosulfan) [10, 11]. Genes encoding the Ese and Esd enzymes were identified in bacteria isolated from endosulfan-exposed soil upon enrichment in sulfur-deficient media with endosulfan or endosulfate supplied as the sole source of sulfur, a technique that targeted the relatively reactive sulfur moiety [12, 13].

Ese performs an oxidation of one of the methylene groups of endosulfan or endosulfate (Fig. 2), producing an unstable intermediate that spontaneously dehydrates the methylene group, allowing bond cleavage and leading to the generation of a sulphur-containing intermediate. The sulphur-containing intermediate of endosulfate metabolism has been identified as endosulfan hemisulfate (Fig. 2) [14]. The equivalent metabolite for endosulfan metabolism, endosulfan hemisulfite, was not detected and likely undergoes rapid desulfurisation to form endosulfan monoalcohol. Although the degradative pathway of endosulfan via Ese has been elucidated, the enzyme has not yet been characterised kinetically.

In contrast to Ese, Esd demonstrates differential metabolism of the two isomers of endosulfan, with no detectable activity on the alpha isomer. Esd catalyses the oxidation of one or both of the methylene groups present in β-endosulfan, resulting in the formation of the endosulfan monoalcohol metabolite (Fig. 2) or endosulfan hydroxyether, respectively [15, 16]. As is the case for Ese, Esd has yet to be purified sufficiently for a detailed kinetic analysis.

Cytochrome P450 oxidoreductases (E.C. 1.14)

The cytochrome P450 family is a large, well characterised group of monooxygenase enzymes that have long been recognised for their potential in many industrial processes, particularly due to their ability to oxidise or hydroxylate substrates in an enantiospecific manner using molecular oxygen [17, 18, 19]. Many cytochrome P450 enzymes have a broad substrate range and have been shown to catalyse biochemically recalcitrant reactions such as the oxidation or hydroxylation of non-activated carbon atoms, which has led to the statement that "P450 enzymes have been compared to a blow torch" [17]. These properties are ideal for the remediation of environmentally persistent pesticide residues.

Over 200 subfamilies of P450 enzymes have been found across various prokaryotes and eukaryotes. All contain a catalytic iron-containing porphyrin group that absorbs at 450 nm upon binding of carbon monoxide (known as the Soret peak) [20]. In common with many of the other oxidoreductases described in this review, P450 enzymes require a non-covalently bound cofactor to recycle their redox centre (most frequently NAD(P)H is used), which limits their potential for pesticide bioremediation to strategies that employ live organisms (see below).

One example of a cytochrome P450 being used in bioremediation of herbicides is that of cytochrome CYP1A1 from mammalian liver (also termed aryl hydrocarbon hydroxylase), which has been shown to degrade atrazine (Fig. 3), norflurazon and chlortoluron [21, 22, 23]. The kinetics of atrazine degradation by CYP1A1 in rat liver microsomes has been examined, indicating a K_m of between 31 and 55 μM and a V_{max} of 150 pmol of atrazine per minute per mg of enzyme (equivalent to a k_{cat} of 0.009 min^{-1}, or 0.00015 sec^{-1}) [24]. The turn over of substrate by CYP1A1 would generally be too slow for free enzyme remediation technologies, but is suitable for phytoremediation and transgenic rice and potatoes expressing this enzyme have been shown to remove large quantities of atrazine, norflurazon and

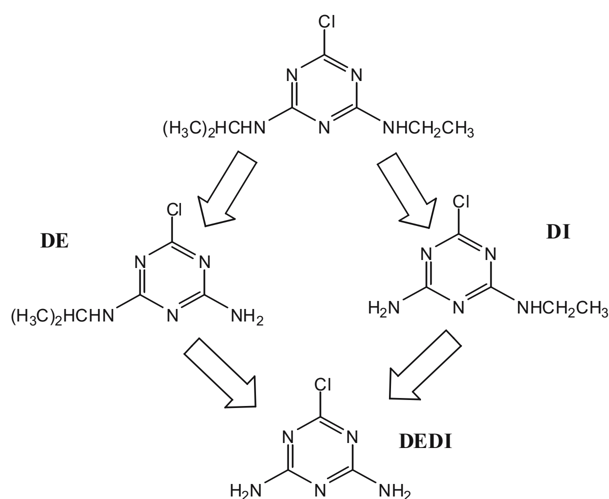

Fig. 3 Detoxification of atrazine by CYP1A1. Atrazine is sequentially oxidised by CYP1A1. In the first oxidation desethyl atrazine (DE) and desisopropyl atrazine (DI) are the products, and in the second oxidation step desethyl desisopropyl atrazine (DEDI) is the product.

chlortoluron from soil [25]. In another example, cytochrome CYP76B1 from *Helianthus tuberosus* (Jerusalem artichoke) was cloned into tobacco and Arabidopsis and shown to catalyse the oxidative dealkylation of phenylurea herbicides such as linuron, chlortoluron and isoproturon [26].

Prokaryotic P450s have great potential in bioremediation. One good example is a variant of $P450_{cam}$ (F87W\Y96F\L244A\V247L) from *Pseudomonas putida* that has been demonstrated to have significant activity against the key chlorinated pollutants pentachlorobenzene (a k_{cat} of 82.5 min^{-1}) and hexachlorobenzene (a k_{cat} of 2.5 min^{-1}) [27]. This variant of the $P450_{cam}$ enzyme has now been used to endow the capacity to completely degrade hexachlorobenzene upon a *Sphingobium chlorophenolicum* species [28]. This is especially noteworthy, as many of the most persistent pollutants (including pesticides) are polychlorinated organic compounds; several of these currently lack any satisfactory physical, chemical or biological remediation technology.

Dioxygenases: TOD (EC 1.14.12)

Toluene dioxygenase (TOD) catalyses the first reaction in the degradation of toluene by *Pseudomonas putida* F1 [29, 30]. This multi-component enzyme not only has extremely broad substrate specificity but also acts as a dioxygenase or monooxygenase. TOD acts as a dioxygenase against a range of compounds including monocyclic aromatics, fused aromatics, linked aromatics and aliphatic olefins [31, 32]. TOD also acts as a monooxygenase on monocyclic aromatics, aliphatic olefins and other miscellaneous substrates [33, 34]. By these means it converts different isomers of dimethylbenzene into dimethyl phenols and isomers of nitrotoluene into nitobenzyl alcohols and nitophenols [31, 34]. Allylic methyl group monooxygenation can be seen with different halo-propene and halo-butene isomers which are converted into butene-1-ol and propene-1-ol, respectively [35]. 2,3-Dichloro-1-propene is a substrate for monooxygenation with allylic rearrangement and gets converted into 2,3-dichloro-2-propene-1-ol [35].

TOD also has the capacity to catalyse sulfoxidation reactions, converting compounds such as ethyl phenyl sulphide, methyl phenyl sulphide, methyl p-nitophenyl sulphide and p-methoxymethyl sulphide into their respective sulfoxides [35]. TOD has been shown to work efficiently for detoxification of polychlorinated hydrocarbons, chlorotoluenes and BTEX residues (benzene, toluene, ethylbenzene and p-xylene) [35]. The broad substrate specificity of TOD makes it an ideal enzyme for bioremediation of several key pollutants, including certain pesticide residues.

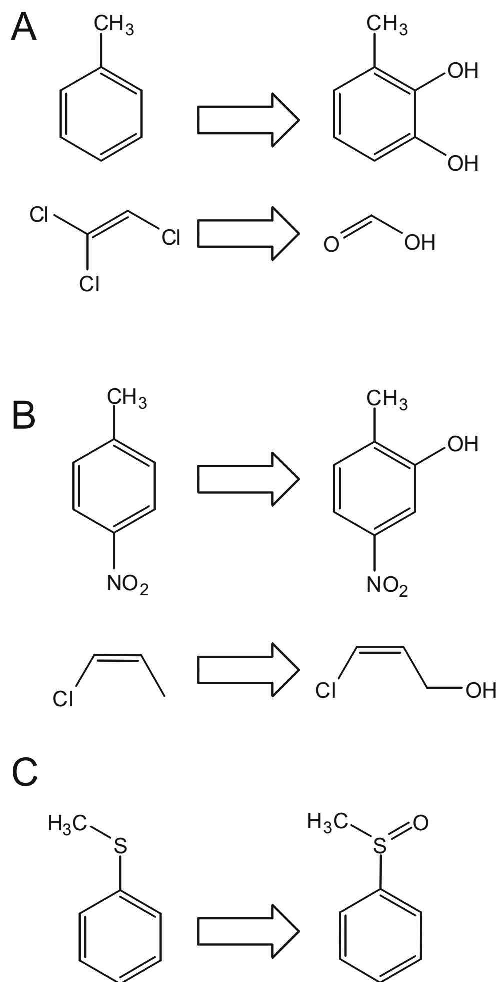

Fig. 4 Reactions representative of the major activities of toluene dioxygenase. A) Dioxygenation of toluene and trichoroethene, B) monooxygenation of 4-nitrotoluene and 1- chloro-1-propene and C), and sulfoxidation of methyl phenyl sulfide.

The TOD enzyme complex has been resolved into three components: an iron-sulphur protein (ISP_{TOL}), a flavoprotein ($reductase_{TOL}$) and an iron sulphur-dependent ferredoxin ($ferredoxin_{TOL}$) [29, 30, 36, 37, 38]. As shown in Fig. 4, the $reductase_{TOL}$ initially accepts electrons from NADH then transfers these electrons to $ferredoxin_{TOL}$. The latter reduces the terminal ISP_{TOL} that functions as the oxygenase component. Reduced ISP_{TOL} catalyses the addition of both atoms of molecular oxygen into the aromatic nucleus of toluene to form *cis*-toluene dihydrodiol, which is eventually mineralized by other enzymes encoded by the toluene dioxygenase gene cluster (*tod* operon) [39]. Although the crystal structure of the TOD enzyme complex has not been solved, the individual components have recently been crystallized by Parales and coworkers [40].

In vitro kinetics for TOD have been elucidated, despite the complex nature of the interactions between the three protein components of TOD and its various cofactors. TOD has a K_m for *o*-cresol of 0.8 μM and a V_{max} of 131 nM min^{-1} per mg enzyme (equivalent to a k_{cat} of 28 min^{-1}) [41], and a K_m for trichloroethylene of 12 μM and a V_{max} of 37 nMmin^{-1} per mg enzyme (equivalent to a k_{cat} of 8 min^{-1}) [42].

The *P. putida* TOD enzyme is a representative of a much larger family of enzymes with application in the biocatalysis of industrially/environmentally relevant reactions. Other closely related members of the toluene/biphenyl rieske non-heme oxygenase family are benzene dioxygenase, biphenyl dioxygenase and cumene dioxygenase (recently reviewed by Ferraro et al. [43]). The amino acid identities between the large subunit of the terminal dioxygenase (BphAl and TodCl), the small subunit of terminal dioxygenase (BphA2 and TodC2), ferredoxin (BphA3 and TodB), and ferredoxin reductase (BphA4 and TodA) are 65, 60, 60, and 53%, respectively. Engineering of hybrid gene clusters between the toluene metabolic *tod* operon and the biphenyl metabolic *bph* operon greatly enhanced the rate of biodegradation of the priority pollutant trichloroethylene [44, 45, 46].

EC 3. Hydrolases

Hydrolases are another broad group of enzymes commonly used in pesticide bioremediation. Hydrolases catalyse the hydrolysis of several major biochemical classes of pesticide (esters, peptide bonds, carbon-halide bonds, ureas, thioesters, etc.) and generally operate in the absence of redox cofactors, making them ideal candidates for all of the current bioremediation strategies. Several examples of hydrolases with applications in the bioremediation of pesticide residues are discussed here.

Carboxylesterases: E3 (EC 3.1.1)

Esterase 3 (E3) is an α/β hydrolase fold [47] carboxylesterase from the sheep blowfly, *Lucilia cuprina*, that operates via a catalytic triad, including a serine (S218), aspartate (E351) and histidine (H471). E3 is responsible for detoxification-mediated resistance to organophosphorous (OP) insecticides in *L. cuprina* [48]. The resistance has been attributed to both a recently occurring mutation (G137D) [49, 50, 51] associated with diazanon resistance, and the selection of mutations that were pre-existent in the natural population (W251L, S or T) associated with malathion resistance [48, 50, 51]. In addition to OPs, E3 has also been shown to be active towards pyrethroid insecticides [52] (Fig. 5).

The G137D mutation converts E3 to a phosphotriesterase, whilst abolishing its native carboxyl esterase activity. It has greatest activity towards diethyl substituted phosphotriesters (such as diazanon, Fig. 5) and a k_{cat} of up to 0.05 min^{-1} [50]. It has been proposed that the mutation alters the orientation of the water that attacks the acyl-enzyme bond, thus facilitating its attack on the phosphorylated serine [51]. In contrast, E3 with the W251L, S or T mutation retains carboxylesterase activity, whilst also gaining phosphotriesterase activity, with particular activity towards dimethyl substituted OPs such as malathion (Fig. 5) and a k_{cat} of up to 0.061 min^{-1}. It is thought that the W251L mutation creates more space in the acyl binding pocket, which in turn reduces steric hindrance to the inversion that occurs around the phosphorous when the serine-phosphorous bond is hydrolysed [50, 51]. The oxidised form of malathion (malaoxon) is also detoxified by E3 via the hydrolysis of either of its carboxyl ester bonds, a reaction that occurs more rapidly than its detoxification via the phosphotrieserase route (Fig. 5).

Commercial synthetic pyrethroid (SP) formulations are considerably more chemically complex than the OP insecticides. In general, SPs contain a central carboxylester with a gem-dimethyl group and unsaturated side chain in the acid moiety and a planar spacer bridging to a non-coplanar centre of unsaturation in the alcohol moiety. Commercial SPs are often complex isomeric mixtures; for example there are up to eight different isomers in formulations of cypermethrin. Wild-type E3 has been shown to be most active towards the *trans* isomers, whereas some mutant forms prefer the corresponding *cis* isomers [52]. Although the *cis* isomers are more toxic, the two forms are not distinguished by most regulatory bodies. This highlights the need for bioremediation strategies that incorporate enzymes with activity towards all of the components of complex isomeric mixtures.

The α/β hydrolase fold, exemplified by E3, is potentially quite versatile from a bioremediation perspective, as it is capable of tolerating large insertions that shape the substrate-binding site whilst preserving the catalytic machinery [53, 54]. The family also includes a diverse range of useful catalytic activities such as esterases, lipases, proteases, haloalkane dehalogenases, haloperoxidases, epoxide hydrolases, C-C hydrolases and even cofactor independent dioxygenases [53, 54, 55], some of which are discussed hereafter.

Phosphotriesterases: OPH, OpdA (EC 3.1.8)

The bacterial phosphotriesterases are a sub-group of the amido-hydrolase metalloenzyme family. The phosphotriesterases primarily catalyse the hydrolysis of OP triesters (Fig. 6). Two closely related (~90 % sequence identity)

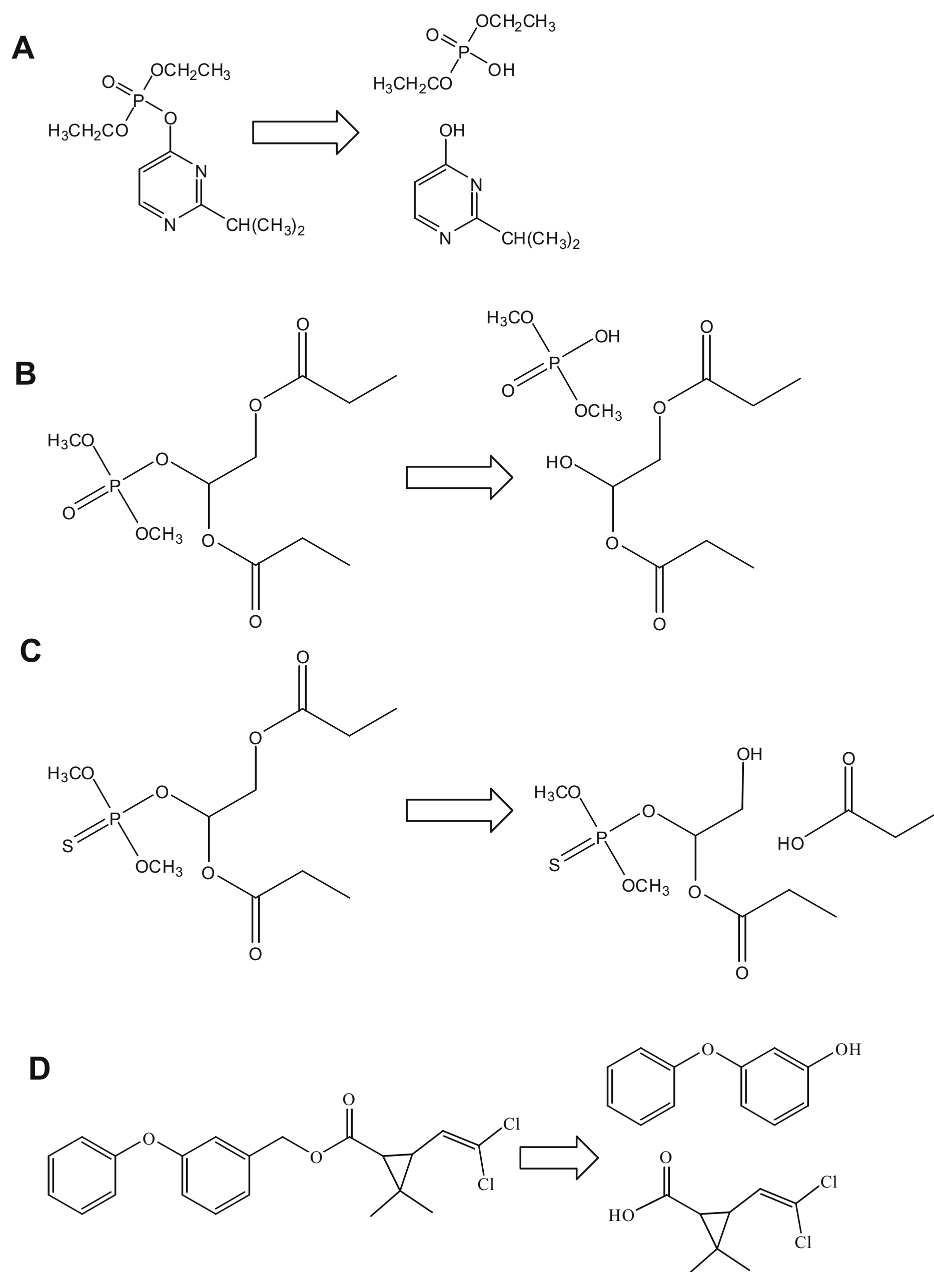

Fig. 5 Major routes of insecticide detoxification by the *Lucilia cuprina* esterase E3 and its variants. A) the phosphotriester insecticide diazinon by E3 G137D, B) the phosphotriester insecticide malaoxon by E3 W251L, C) malathion via the carboxyesterase activity of E3 (wild-type or W251L), and D) the synthetic pyrethroid permethrin via the carboxyl esterase activity of wild-type E3 or W251L E3.

bacterial phosphotriesterases have been extensively characterised: OpdA from *Agrobacterium radiobacter* [56], and OPH from *Pseudomonas diminuta* [57] and *Flavobacterium* [58]. Field trials of OpdA as a bioremediation agent have been conducted [4], and it is already in use as a commercial product to detoxify OP residues in various contaminated wastes, sold under the brand name LandGuard™ from Orica Watercare (Australia) at a cost to user considerably lower than the pesticides themselves.

Both OPH and OpdA display extraordinary catalytic efficiency for OPs, vastly superior to that of the E3 mutants described above; for instance, the k_{cat}/K_m of OpdA for the pesticide methyl parathion is in the order of 3×10^6 sec^{-1} M^{-1} [59]. The rapid turnover of OPs is made possible through the use of a binuclear metal active site, which has been characterised crystallographically [60]. Although a wide variety of divalent metal ions can be utilised, the native enzyme contains a heterobinuclear Fe^{2+}-Zn^{2+} centre [60].

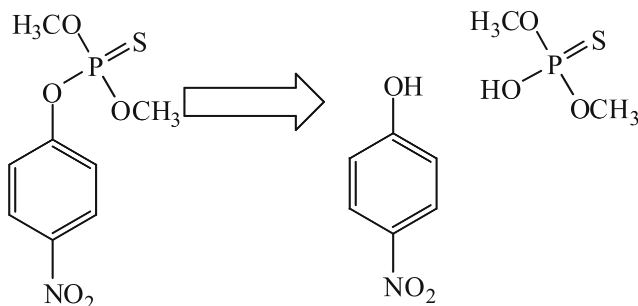

Fig. 6 Hydrolysis of the insecticidal phosphotriester parathion by the bacterial phosphotriesterase OpdA.

The catalytic mechanism is thought to proceed *via* direct in-line nucleophilic attack from a water molecule, activated through its interaction with the Fe^{2+} ion, at the electrophilic phosphorus of the substrate, which coordinates to the Zn^{2+} ion [61]. Considering that organophosphate pesticides are synthetic compounds that have only existed in the environment since the 1950s, the rapid evolution of near diffusion-limited turnover rates in these enzymes is noteworthy. Recent work has shown that their likely evolutionary progenitor is a family of bacterial lactonases that exhibit low-level promiscuous phosphotriesterase activity [62].

This latent catalytic promiscuity is an interesting hallmark of many enzymes in the amido-hydrolase family, and could explain their relatively high abundance in the field of bioremediation (other good examples are the atrazine chlorohydrolase enzymes AtzA [63] and TrzN [64], and the haloalkane dehalogenase LinB discussed below). The bacterial phosphotriesterases may be among the most promiscuous of all enzymes: in addition to their primary activity (the hydrolysis of P-O bonds in phosphotriesters) they catalyze the hydrolysis of P-S bonds [65], P-F bonds [66, 67], P-CN bonds [68], C-O bonds in esters and lactones [69, 70], and P-O bonds in phosphodiesters [71].

Haloalkane Dehalogenases: LinB, AtzA and TrzN (EC 3.8.1)

The insecticidal γ-isomer of hexachlorocyclohexane (γ-HCH, commonly known as lindane) and technical HCH (which includes α-, β-, γ-, ε- and δ- isomers) [72] have been used extensively against agricultural pests, and in malaria control programs, worldwide. The indiscriminate use and large-scale production of HCH during the past 50 years has generated dangerous stockpiles of highly persistent toxic wastes, which are illegally disposed of in the countryside, water bodies and agricultural fields in several countries.

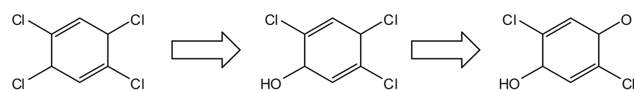

Fig. 7 Sequential hydrolytic dechlorinations of 2,4,5,6-tetrachloro-3,6-cyclohexadiene by LinB.

Genes encoding the enzymes responsible for bacterial degradation of HCH have been cloned and studied extensively. The two key enzymes are encoded by the *linA* (discussed below) and *linB* genes. LinB is a haloalkane dehalogenase of the α/β−hydrolase fold family of enzymes that shows significant similarity to three other α/β-hydrolase fold enzymes: haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10; haloacetate dehalogenase (DehH1) from *Moraxella* sp. B; and 2-hydroxymuconic semialdehyde hydrolase (DmpD) from *Pseudomonas* sp. CF600 [73]. LinB mediates the two sequential chlorohydrolase reactions converting 2,3,5,6-tetrachloro-1,4-cyclohexadiene to 3,6-dichloro-2,5-dihydoxy-1,4-cyclohexadiene [74] (Fig. 7), which are the reactions immediately following those of LinA with hexachlorocyclohexane (see below). In addition, LinB has been found to be involved in the degradation of β-HCH in *Sphingomonas paucimobilis* [75], and of β- and δ-HCH in *Sphingobium.indicum* B90A, *Sphingobium francense* SpC and *Sphingobium japonicum* UT26, although the ability to degrade β-HCH and δ-HCH differ between these strains [76]. β-HCH has been shown to be converted by LinB to pentachlorocyclohexanol with a k_{cat} of up to 4.1×10^{-3} min^{-1} [77], which is in turn converted to tetrachlorocyclohexanol with a k_{cat} of up to 1.4×10^{-2} min^{-1} [77], although these rates are highly dependent upon the source of the enzyme.

The reaction mechanism of LinB involves nucleophilic attack from the aspartic acid residue 108 at an electrophilic carbon of the substrate, followed by formation of a covalent alkyl-enzyme intermediate. The catalytic aspartic acid is then regenerated through nucleophilic attack at Asp108 upon activation of a water molecule by histidine 272 [78].

LinB not only detoxifies the product of LinA (see below), which acts directly upon γ-HCH, but also detoxifies at least two (β and δ), albeit not all of the common isomers of HCH. This highlights the fact that some bioremediation strategies require multi-step catalysis to fully detoxify the pesticide, and that multiple enzymes may be required to achieve detoxification of all of the isomers of a given pesticide.

The α/β-fold enzymes are not unique in acquiring halohydrolase activity. The amidohydrolase superfamily of enzymes also contains members that hydrolyse halide-carbon bonds, namely AtzA [63] and TrzN [64]. AtzA is the first enzyme from the atrazine catabolic pathway

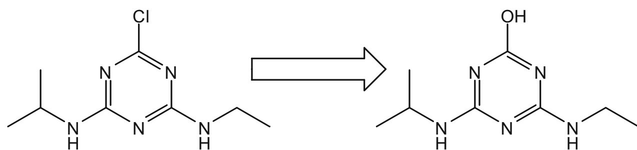

Fig. 8 Atrazine chlorohydrolysis catalysed by AtzA and TrzN.

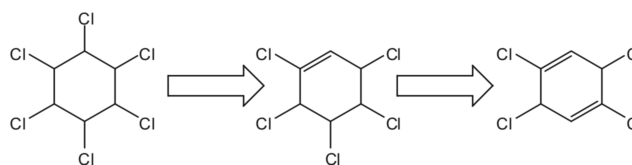

Fig. 9 Chloroelimination of γ-hexachlorocyclohexane by the hexachlorocyclohexane dehydrochlorinase LinA.

encoded by *atzA-atzF* from the transmissible pADP1 plasmid, originally isolated from *Pseudomonas* sp. ADP [63, 79]. Atrazine and related chloro-s-triazine herbicides are dechlorinated by the iron-dependent AtzA (Fig. 8), which has a k_{cat} of appoximately 5 sec^{-1} and a K_m of 149 µM for atrazine [63]. AtzA is thought to have evolved recently, and is closely related to melamine deaminse (TriA), a functional deaminase (with no dechlorinase activity despite being 98 % identical to AtzA) [80].

AtzA is functionally interchangeable with TrzN, a zinc-dependent amidohydrolase from *Nocardioides* [81], which has a far broader range of biochemical targets than AtzA, including amides, O-alkyl groups, S-alkyl groups and halides [82]. However, unlike AtzA, TrzN has proven difficult to express in heterologous organisms, requiring the use of chaperones in *E. coli* [83]. This has led to some ambiguity in the literature concerning the kinetic parameters of TrzN, with extremes of k_{cat} ranging from a value comparable to AtzA (approximately 2 sec^{-1}) [84] to approximately one tenth that value [83]. It is perhaps for this reason that AtzA, rather than TrzN, has been expressed *in planta* [85] resulting in alfalfa, *Arabidopsis thaliana* and tobacco plants capable of phytoremediating triazine herbicides.

EC 4. Lyases

The lyases are a considerably smaller group of enzymes than the oxidoreductases and hydrolases. Lyases catalyse the cleavage of bonds in the absence of redox cofactors or water, including the energetically demanding cleavage of carbon-carbon bonds (pyruvate-formate lyase, for example [86] and carbon bonds with phosphorus, oxygen, nitrogen, halides and sulfur. Here, the haloelimination reaction catalysed by lindane hydrochlorinase (active against the insecticide γ-hexachlorocyclohexane) is considered

Haloalkane dehydrochlorinases: LinA (EC 4.5.1)

The *linA*-encoded HCH dehydrochlorinase (LinA) mediates the first two steps of dehydrochlorination of the insecticide γ-HCH [87] Fig. 9), which is further catabolised by the remaining enzymes encoded by the *lin* operon (including LinB, see above). The structure of LinA has not yet been resolved, but it is predicted to belong to a novel superfamily which includes scytalone dehydratase and naphthalene dioxygenase [88]. The reaction mechanism proposed for LinA is dependent upon a catalytic dyad (Asp25 and His73) [89], where a proton is abstracted from HCH by His73 followed by release of a chloride ion and formation of a carbon-carbon double bond. This process is then repeated with the product (pentchlorocyclohexene) to ultimately yield 2,3,5,6-tetrachloro-1,4-cyclohexadiene [89] (Fig. 9).

As noted above, complete remediation of γ-HCH cannot be achieved by LinA if used in isolation, as this would lead to the accumulation of 2,3,5,6-tetrachloro-1,4-cyclohexadiene. In the presence of both LinA and LinB, however, γ-HCH is completely remediated. Therefore a strategy involving *linA* and *linB* must therefore be employed to entirely eliminate γ-HCH from contaminated sites. The simplest mechanism by which such a strategy could be realised is the use of HCH mineralising bacteria that express the enzymes encoded by the entire *lin* operon. In this regard, it is noteworthy that recent studies in which HCH contaminated soil was treated by the controlled release of a bacterium (*Sphingobium indicum*) containing the naturally occurring *lin*-operon led to significant remediation of the pesticide residue (up to 95 %) [90].

Prospects

It will be clear from the above that enzymes to degrade a wide range of chemistries can be isolated by traditional approaches such as microbial enrichments and reverse genetics. Prospects for success in enzyme discovery are now further improved by the advent of various genomic technologies and in particular metagenomics. It is arguable that enzymes could now be found that will catalyse the breakdown of all the major classes of chemical pesticide, and many other Persistent Organic Pollutants (POPs) as well. This being so, two other issues will then determine the suitability of particular enzymes for use in bioremediation,

one being their quantitative efficiencies as catalysts under the intended conditions of use and the other being their need for cofactors.

Unlike many other enzyme-based technologies, pesticide bioremediation occurs under largely physiological conditions, and in some cases occurs within cells (be they bacterial or plant). There is therefore no need to adapt the enzymes to the harsh conditions encountered in many industrial processes (high/low-pH, high temperature, non-aqueous solvent systems, etc). However, pesticide bioremediation often requires a high catalytic efficiency at very low substrate concentrations (< 1μM), and frequently requires broad substrate ranges to encompass large heterogeneous pesticide families (the phosphotriesters, for example) or stereochemistries (as is the case with the synthetic pyrethroids). These characteristic enzymatic requirements for bioremediation, together with improving solubility and expression, are particularly amenable to the use of *in vitro* enzyme evolution technologies to improve their functions.

Enzyme evolution has advanced rapidly over the last two decades, and several enabling technologies have been developed, such as low-fidelity (error-prone) PCR [91], DNA shuffling [92], ITCHY [93], SCRATCHY [94], ISOR [95], and CASTing [96], that allow the generation of random and rational gene libraries of varying diversity (between tens and >1010 different mutants per library). Various of these strategies have now been used to improve the performance of potential bioremediation enzymes and three examples of this are elaborated below.

DNA shuffling has been applied successfully to the triazine chlorohydrolase AtzA to greatly increase its substrate range. AtzA hydrolytically dechlorinates the herbicide atrazine [63], and although it is closely related to a deaminase enzyme (98 % identity with the triazine deaminase TriA [80]) it has no deaminase activity itself. Raillard and coworkers used DNA shuffling to generate sequence intermediates between AtzA and TriA [97] that were found to not only possess intermediate catalytic characteristics (both deaminase and dechlorinase activities), but also novel ones, such as hydrolytic removal of a methoxy or methylamine group.

LinA has also been improved by *in vitro* evolution (via low fidelity/error prone PCR), producing a single mutant with a 2-fold increase in specific activity and a three-fold increase in its expression compared with wild type LinA [98]. The Cys132Arg mutation responsible is most likely a solvent exposed residue, and it is thought that the mutation increases the solubility or stability of the enzyme overall. This example is particularly noteworthy because it highlights the importance of both the catalytic efficiency of the enzyme in question and its non-catalytic properties (in this case stability/solubility) as factors influencing its performance as a bioremediant.

In another example, the organophosphate-degrading bacterial phosphotriesterases (PTEs; OPH and OpdA, see above) have been modified by *in vitro* evolution. Although the bacterial PTEs can catalyse a wide variety of organophosphate pesticides, the catalytic efficiency varies considerably. For example, the k_{cat}/K_m of OpdA toward methyl paraoxon is nearly diffusion rate limited (10^7 M^{-1} s^{-1}), whereas its k_{cat}/K_m towards ethyl demeton hydrolysis is considerably lower (10^3 M^{-1} s^{-1}) [56]. For this reason, considerable effort has been made to improve its activity through *in vitro* evolution. Several mutations in these PTEs have been identified that substantially improve the catalysis of a range of pesticides [59, 99, 100]. Contrary to expectation, these mutations seemingly had little effect on the substrate binding pocket and catalytic machinery of PTEs. Instead, mutations were almost uniformly located on the surface of the enzyme, remote from the active site, and found to result in global increases in the turnover rate. Likewise, other studies have succeeded at effectively isolating variants of PTEs with increased stability and expression [59, 101].

The implementation strategy for an enzyme in bioremediation is seemingly dependent upon its requirement for cofactors. If diffusible cofactors are required, for example, the only realistic strategy at present is to use live organisms to deliver the catalytic activity of the enzyme. There are already many examples of the use of genetically modified plants in pesticide phytoremediation; glyphosate oxidase (see above), cytochrome P450 enzymes (see above), a Rieske non-heme monooxygenase (named DMO) that converts dicamba to 3,6-dichlorosalicyclic acid that has been expressed in *A. thaliana*, tomato, tobacco and soybean plants [102, 103], and aryloxyalkanoate dioxygenase enzymes (TfdA) that have been expressed in corn (patented for the degradation of 2,4-D and pyridyloxyacetate herbicides) [104]. In theory at least it would also be possible to use genetically modified bacteria containing the detoxification gene(s) as bioremediants in a bioaugmentation approach. In the absence of tight biological containment however, GM bacterial bioaugmentation appears an unlikely option in the near term.

If the enzyme is not dependent upon a diffusible cofactor, free-enzyme bioremediation can be a viable implementation strategy, and the first free-enzyme bioremediation product is now available (LandGuard™ for phosphotriester insecticides; see above). The commercial availability of LandGuard™ demonstrates that free-enzyme bioremediation can be a cost effective technology. Free-enzyme bioremediation technology avoids many of the regulatory issues

surrounding GM technologies. Herein we have described numerous detoxification enzymes using hydrolase- and lyase-based mechanisms which proceed independently of diffusible cofactors and might therefore be suitable as free enzyme bioremediants. The cost effectiveness of free-enzyme bioremediation is dependent upon the requisite enzyme dose rate, which is in turn determined by both the catalytic efficiency of the enzyme and the desired rate of remediation (i.e. relatively slow enzymes can be cost effective if given sufficient time to operate). We have also catalogued many detoxification reactions for which the only known mechanism is a redox reaction. Not all redox reactions require diffusible cofactors but many do, and many of those involve cofactor molecules that would be impractically expensive to supply at the concentrations necessary for reasonable reaction kinetics. One important challenge for the future of enzymatic bioremediation will therefore be the development of cost-effective cofactor regeneration systems.

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