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The use of live biocatalysts for pesticide detoxification

Wilfred Chen and Ashok Mulchandani

During the past decade, numerous microorganisms capable of degrading pesticides have been isolated, and detoxification processes based on these live biocatalysts have been developed. Recently, novel detoxification strategies using genetically engineered microorganisms with extended degradative capabilities have been investigated and, in some cases, shown to be more effective. One promising approach for the detoxification of organophosphate pesticides uses genetically engineered *Escherichia coli* with surface-expressed organophosphorus hydrolase. Continuous efforts in this direction are required, in conjunction with a search for microorganisms capable of degrading pesticides rapidly, to establish efficient and cost-effective large-scale processes for pesticide detoxification.

The use of pesticides has become an integral part of modern agricultural systems. However, the intensive use of pesticides has resulted in serious environmental problems because they are either recalcitrant or biodegraded very slowly[1]. Owing to their highly toxic nature, there is significant concern regarding the large quantities of pesticide wastes from, for example, excess or unused pesticide concentrates, and the dilute aqueous pesticide solution resulting from the washing of storage tanks and spraying equipment[2]. The pesticides that have received the most attention include pentachlorophenol (PCP), *s*-triazines (primarily atrazine), organophosphates and carbamates, because they are widely used and highly persistent compounds[3].

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Current methods for detoxifying pesticides rely on incineration and landfills. Although landfills function adequately in the short term, the leaching of pesticides into surrounding soil and groundwater supplies is an obvious cause for concern. Incineration, a method for the destruction of these compounds that is approved by the US Environmental Protection Agency (EPA), has met serious public opposition because of the potentially toxic emissions and is very costly, as it requires large amounts of energy to reach the high temperatures needed to destroy the pollutants. Owing to the problems associated with these methods, there is increasing interest in the use of microorganisms with unique biodegradative properties. Clearly, the challenges are to exploit existing biodegradative routes and to develop novel catabolic capabilities for a rapid, safe and economic disposal of these pesticides.

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The metabolic and genetic aspects of pesticide detoxification have been the subject of several recent reviews[3,4] and so this review will focus on the use of live biocatalysts for pesticide detoxification. Systems based on both natural isolates and genetically engineered strains have been utilized for this purpose.

Detoxification based on natural isolates

Microbial communities play a significant role in the transformation of pesticides: bacteria and fungi are the major degraders of pesticides and their breakdown products[5]. Several detoxification processes based on pure or mixed cultures have been proposed, and some of the organisms involved in these detoxification processes are listed in Table 1.

Pentachlorophenol

Pentachlorophenol (PCP) is a pesticide in widespread use throughout the world. In addition to being a major constituent of many wood-preservative formulations, it is also used as a biocide for agricultural and industrial applications. Because of the sensitivity to abrupt changes in the PCP concentration in the feed, treatments with conventional activated-sludge systems are inadequate[6,7]. Natural isolates of *Arthobacter, Mycobacterium, Flavobacterium, Pseudomonas* and *Rhodococcus* have been used as free cells in bioreactors for PCP degradation[8]. However, because of the low volumetric throughput of free-cell bioreactors caused by low growth and/or degradation rates, the use of these organisms has been investigated in a variety of immobilized-cell bioreactors. PCP-degrading cells have been immobilized by a number of methods, including entrapment in calcium alginate[9], *k*-carrageenan[10] and polyurethane foam[11], and adsorption through an extracellular polymer onto nonporous glass beads to form a biofilm[12]. Because of its low mechanical strength, incompatibility with phosphate and susceptibility to biodegradation, alginate immobilization has been found to be unsuitable for field applications[9]. Similar problems of low mechanical strength and susceptibility to biodegradation are likely to occur with *k*-carrageenan immobilization. Additionally, mass-transport resistance of reactants and products through the entrapment gels is a limitation of such immobilized-cell bioreactors; however, the magnitude of mass-transport resistance is less in polyurethane foam, because the cells are entrapped within the foam pockets and so more accessible to substrates and products[11].

Such foam-entrapped *Flavobacterium* cells were found to degrade 3.5–4.0 mg PCP (g foam)$^{-1}$ day^{-1} over a period of 25 days[11]. This high degradation rate was attributed to the reversible binding of PCP to the foam, thereby protecting the cells from high PCP concentrations. Immobilized *Arthobacter* ATCC 33790 (as a biofilm on nonporous glass beads) was equally effective at degrading over 90% of high loads of PCP for close to eight months[12]. *Arthobacter* ATCC 33790 was also coimmobilized with powdered activated carbon within the alginate gel for PCP degradation[13]. Coimmobilization with activated carbon has several advantages, for example, allowing the use of high flow rates for rapid PCP removal, lowering the toxic effect of PCP and dampening the effect of shock loading (sudden increase in PCP concentration)[13]. These advantages, however, may be better realized by immobilizing the cells on granular activated carbon, which does not require alginate-gel encapsulation.

Table 1. Examples of natural isolates able to degrade pesticides

Pesticide	Organisms	Refs
Pentachlorophenol	*Arthobacter*	12
	Mycobacterium	8
	Flavobacterium	11
	Pseudomonas	8
	Phanerochaete chrysosporium	48
s-Triazines	*Rhodococcus*	29,31
	Klebsiella	14
Carbamates	*Pseudomonas*	18
	Achromobacter	19
Organophosphates	*Flavobacterium*	38
	Pseudomonas	23,37

s-Triazines

The *s*-triazines, including atrazine and simazine, are heterocyclic nitrogen derivatives used primarily as herbicides. Attempts at degrading *s*-triazines based on either chemical or biological processes alone have met with limited success, but the use of integrated chemical–biological or physical–biological processes has been successful. The integrated chemical–biological processes have utilized pretreatment with ozone or Fenton's reagent (Fe^{2+} and H_2O_2) followed by biodegradation using *Klebsiella terragena* (strain DRS-I)[14] or a mixed culture of *Rhodococcus corallinus* and *Pseudomonas* sp. D (Ref. 15), which utilize *s*-triazine-derived products as the nitrogen source for growth. In a study combining physical and biological processes, a granular-activated-carbon column was augmented with atrazine- and simazine-degrading bacteria (*Rhodococcus rhodochorus* SL1 and *Acinetobacter junii* WT1, respectively) for the treatment of water contaminated with atrazine and simazine[16]. The triazines were first adsorbed onto the granular activated carbon and then biodegraded by the augmented bacteria. The process was successful in lowering the concentration from 10 μg l^{-1} (maximum concentration found in surface waters) to <0.003 μg l^{-1} using a residence time of 40 min[16].

Carbamates

This group of pesticides consists of esters of N-substituted carbamic acid, with four classes being used – methyl carbamates, thiocarbamates, phenyl carbamates and dithiocarbamates. Of these, the first three have been shown to be degraded by pure bacterial cultures[17]. Carbamate hydrolases and amidases are the enzymes responsible for the initial degradation steps, and they have been purified from a soil isolate of *Pseudomonas*

alcaligene[18]. Recently, methyl-carbamate hydrolase, which catalyses the hydrolysis of methyl carbamates but not phenyl carbamates and thiocarbamates, has been isolated from *Achromobacter* sp.[19] However, there have been no reports on the use of either the enzyme or the live bacteria for carbamate degradation in bioreactors.

Organophosphates

Pesticides belonging to this group, such as parathion, methyl parathion, diazinon, fenitrothion, coumaphos and malathion, are widely used agricultural pesticides. These compounds are particularly amenable to biological detoxification because they are susceptible to hydrolysis by bacterial enzymes, organophosphorus hydrolase (OPH) and organophosphorus acid anhydrase (OPA)[5]. The hydrolysis products, which are at least two orders of magnitude less toxic than the parent compounds, are prone to further rapid degradation by other biological or chemical processes[17]. OPH, isolated from *Pseudomonas diminuta* or *Flavobacterium* ATCC 27551, has been immobilized on glass and nylon beads and used in enzyme reactors for the detoxification of organophosphates[20,21].

The use of live biocatalysts for organophosphate degradation was recently demonstrated with *Pseudomonas putida*[22] and *Pseudomonas* sp. A3[23]. *P. putida* hydrolysed methyl parathion and degraded the hydrolysis product, *p*-nitrophenol, further to maleyl acetate. The immobilization of *Pseudomonas* sp. A3 in sodium alginate beads allowed 99% of 1 mM methyl parathion to be degraded in approximately 48 h in batch cultures. In another recent study, indigenous organisms present in coumaphos-contaminated soils were used in biofilter columns for the detoxification of coumaphos from cattle-dip wastes and reduced the levels from approximately 1500 mg l^{-1} to 0.1 mg l^{-1} in 7–10 days[24].

Detoxification based on genetically engineered strains

Recent advances in genetic techniques have opened up new avenues to move towards the goal of genetically engineering microorganisms to function as 'designer biocatalysts', in which certain desirable biodegradation pathways or enzymes from different organisms are brought together in a single host with the aim of performing specific detoxification(s)[25,26]. One disadvantage of culturing native isolates capable of detoxification in bioreactors is their slow specific growth rates, which make them less attractive economically. The expression of detoxification enzymes in industrially friendly strains will undoubtedly lower the overall cost of detoxification.

Pentachlorophenol

The most widely studied PCP-degrading microorganisms are *Flavobacterium* ATCC 39723 and *Rhodococcus chlorophenolicus* PCP-1. The genes coding for the enzymes responsible for initial steps of PCP degradation have been cloned into *Escherichia coli*, conferring on it the ability to degrade PCP[27,28]. Even though *E. coli* may not be suitable for field decontamination, it could be used in bioreactor systems because of its significantly higher growth rates. Alternatively, cloning these genes into indigenous strains with a high tolerance to PCP, such as *Pseudomonas* sp., would go some way towards addressing the problems of introducing genetically engineered organisms into contaminated environments.

s-Triazines

Rhodococcus strain TE1 can degrade atrazine efficiently to produce the dealkylated metabolites deisopropylatrazine and de-ethylatrazine[29], and the gene that encodes the enzyme for *N*-dealkylation, *atrA*, has been cloned[30]. An initial attempt to create a recombinant strain capable of dealkylating and dechlorinating atrazine by introducing the *atrA* gene into an isolate of *Rhodococcus corallinus* that dechlorinates and deaminates deisopropylatrazine and de-ethylatrazine[31] was not successful because of poor expression[30]. As a reverse strategy, the *trzA* gene[32] encoding the *s*-triazine hydrolase from *R. corallinus* was transferred into *Rhodococcus* TE1; the resulting recombinant *Rhodococcus* was able to dechlorinate the dealkylated metabolites[33] (Fig. 1). A plasmid carrying both the *trzA* and *atrA* gene was constructed and transformed into three *atrA*$^-$ *trzA*$^-$ *Rhodococcus* strains, rendering them able to degrade pesticides such as atrazine or simazine. Even though degradation by these recombinant strains was slow, the possibility of obtaining novel strains with hybrid pathways for enhanced degradation of atrazine was clearly demonstrated. Several attempts to express these genes at high levels in *E. coli* failed, even when they were

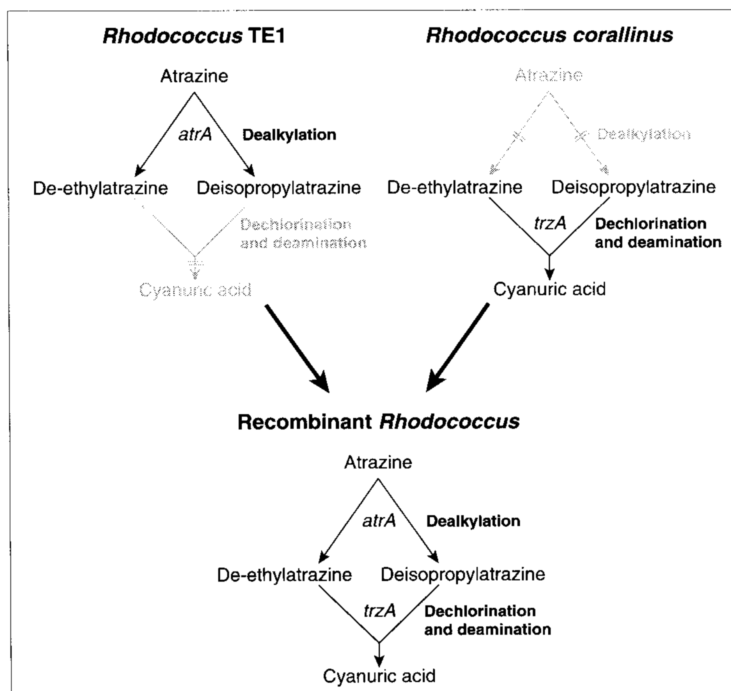

Figure 1

Construction of recombinant *Rhodococcus* for atrazine dealkylation and dechlorination. The dechlorination pathway of *Rhodococcus corallinus* was combined with the atrazine-dealkylating capabilities of *Rhodococcus* TE1, which produces the substrates required for dechlorination.

placed under the control of *E. coli* promoters[33]. Additional research into optimizing the expression of *trzA* and *atrA* in bioremedially useful organisms, such as *Pseudomonas*, should enable great progress to be made in the bioremediation of atrazine or simazine.

Carbamates

The involvement of plasmid-specified enzymes in carbamate degradation has been shown by Tomasek and Karns[34]. The structural gene *mcd*, encoding carbofuran hydrolase (MCD), was isolated from *Achromobacter* WM111 and the enzyme was shown to hydrolyse a wide range of *N*-methylcarbamates. Initial attempts to express MCD in *Pseudomonas* and *E. coli* resulted in low levels of activity[35]. It was found that the enzyme was highly active at 30°C but inactive at 37°C. Although only a limited amount of work has been done with the *mcd* gene, it is anticipated that a very attractive approach for the detoxification of carbamate pesticides could be by the introduction of the *mcd* gene into organisms that colonize the rhizospheres of plants, thereby allowing growth and degradation to occur simultaneously in the root exudates.

Organophosphates

Detoxification of organophosphate pesticides was the first to be demonstrated by genetically engineered microorganisms[36]. *P. diminuta*[37] and *Flavobacterium* ATCC 27551[38] were shown to express plasmid-encoded OPH, resulting in the hydrolysis of organophosphates, and recombinant *E. coli* expressing OPH was shown to degrade a variety of organophosphates[36,37]. However, expression was very low when under control of the native promoter, and high-level expression was only possible using the strong *E. coli lac* promoter[39]. The ability of *E. coli* to grow to much higher densities than *P. diminuta* and *Flavobacterium* in a fermenter enables the development of large-scale detoxification processes or the production of OPH in commercially feasible quantities. High-level expression of OPH has also been demonstrated in the soil fungus *Gliocladium virens*[40,41], with maximal levels of OPH activity found to correlate with biomass production. Application of these transgenic fungi provides opportunities for both *in situ* and/or *ex situ* detoxification of organophosphates because of their ability to tolerate low pH and to utilize a wide range of complex substrates.

A novel approach: cell-surface expression of degradative enzymes

For degradation to proceed, the pesticides must be transported into the cells, where the degradative enzymes reside. For recombinant strains that contain high intracellular activity of degradative enzymes, the overall detoxification rate may be limited by the transport mechanism. It has been recently demonstrated that the uptake of parathion and paraoxon is indeed the rate-limiting step in the degradation of these pesticides by recombinant *E. coli* cells expressing OPH intracellularly[42,43]. This resistance to mass transport can be reduced by treating the cells with permeabilizing agents such as EDTA, DMSO and tributyl phosphate[44]. However, not all enzymes are amenable to this treatment, and immobilized viable cells cannot be subjected to permeabilization. Although purified enzymes can be used for detoxification, in most cases the cost of the detoxification process depends heavily on the cost of

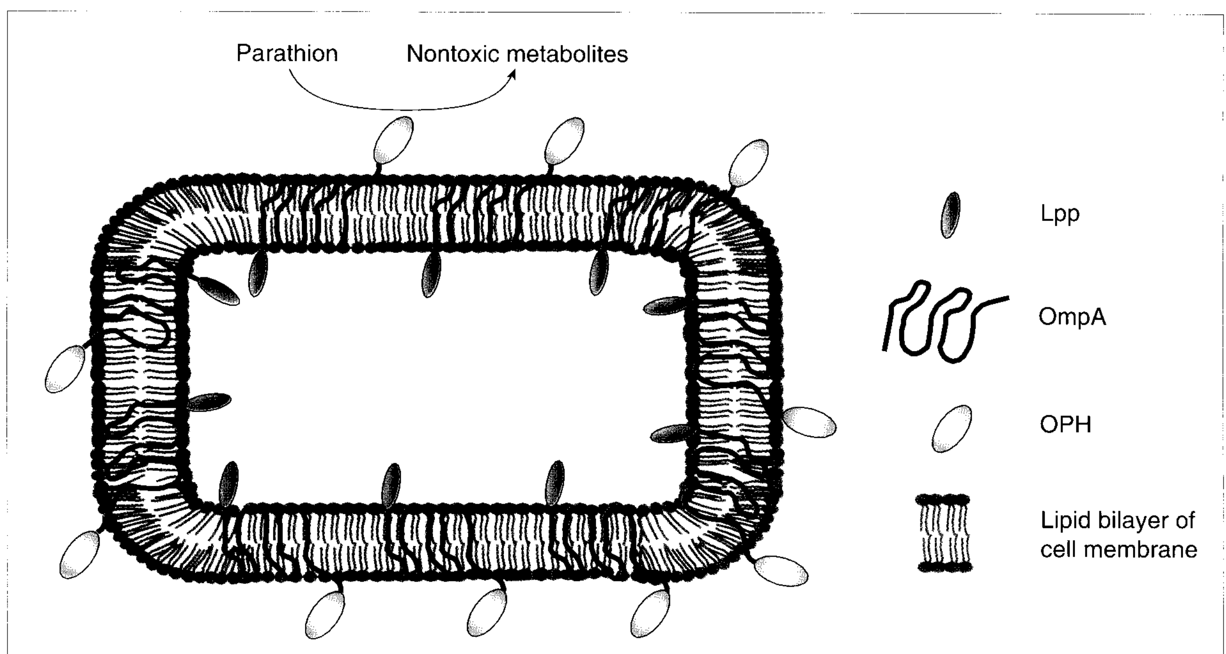

Figure 2
Schematic representation of parathion detoxification with organophosphorus hydrolase (OPH) anchored and displayed on the cell surface. The N-terminal lipoprotein (Lpp) sequence directs the Lpp–OmpA–OPH fusion to the outer membrane. The OmpA domain traverses the membrane, localizing OPH to the cell surface.

purifying the enzymes. One potential solution is to anchor and display the degradative enzymes on the cell surface (Fig. 2), thereby obviating the need for enzyme purification and eliminating the transport limitation.

The ability to display heterologous proteins on the surface of microorganisms is generating intriguing opportunities, such as recombinant bacterial vaccines, whole-cell adsorbents, and recombinant whole-cell biocatalysts[45]. For example, expression of β lactamase on the cell surface of *E. coli* eliminates diffusional limitations in the hydrolysis of β lactams by whole cells, resulting in 50-times higher rates of reaction compared with cells expressing the same level of enzyme in the periplasmic space[46]. Using the same Lpp–OmpA fusion system used for β lactamase, OPH was successfully anchored and displayed onto the surface of *E. coli*[42]. Cultures with surface-expressed OPH degraded parathion and paraoxon very effectively, without the transport limitation observed in cells expressing OPH intracellularly. Whole cells with surface-expressed OPH had seven times higher activity than whole cells expressing similar amounts of intracellular OPH, illustrating that surface-expressed OPH causes faster degradation than intracellularly expressed OPH. Therefore, an improved 'whole-cell' technology for organophosphate-pesticide biodegradation has been realized. The resulting live biocatalysts were also considerably more stable and robust than purified OPHs, retaining 100% activity over a period of one month when incubated at 37°C (Fig. 3), as well as higher activity in the presence of organic solvents. The degree of stabilization was very similar to those recently observed when OPH was immobilized within a polyurethane-foam matrix[47]. Cells displaying catalytically active OPH are in effect 'live' immobilized-enzyme particles and could be employed for organophosphate hydrolysis in a similar fashion to conventional immobilized enzymes without tedious purification. To this end, immobilization of these live biocatalysts onto a solid support could provide an attractive and economical means of pesticide detoxification in place of immobilized enzymes or immobilized whole cells expressing OPH intracellularly, affording no diffusional barrier, lower labour costs and the potential for easy regeneration.

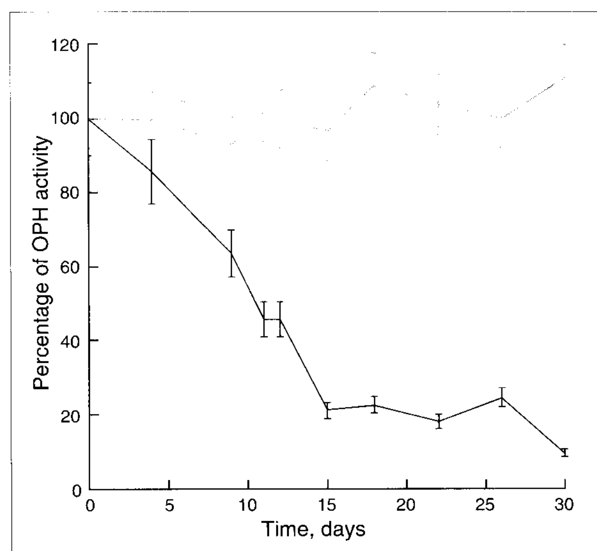

Figure 3
Organophosphorus hydrolase (OPH) activities in suspended *E. coli* cultures expressing OPH on the surface (blue) and intracellularly (red) over a 30-day period. Samples (1 ml) were taken every 2–4 days and their OPH activities determined.

Future prospects

Most of the existing technologies for pesticide detoxification have not been implemented on a large-scale basis because of the low degradation rate and high cost. There is certainly a grave need for the development of methods that are more efficient and cost effective. The application of genetic engineering and biochemical techniques to improve and evolve natural biodegradative capabilities further will ultimately create 'super biocatalysts' capable of degrading several pesticides rapidly and cost effectively. The ability to express detoxification enzymes on the cell surface of Gram-negative or Gram-positive organisms will probably allow a wide range of applications of this type. To this end, collaborations between microbiologists, biochemists and engineers will become increasingly important.

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Artificial evolution by DNA shuffling

Shigeaki Harayama

Improvement of enzymes is one of the important objectives of biotechnology. *In vitro* evolution of enzymes using DNA shuffling involves the assembly of two or more DNA segments into a full-length gene by homologous, or site-specific, recombination. Before the assembly, the segments are often subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods. Many useful enzymes and peptides have been isolated following the artificial evolution.

The use of enzymes for medical, industrial and environmental purposes is prevalent today and will continue to expand rapidly into the next century. Traditionally, the selection of useful and robust enzymes to be incorporated into commercial applications has been done by screening for enzymes or microorganisms that have become adapted to extreme environmental conditions, such as high temperature or salt concentration. In parallel, other techniques, such as enzyme immobilization and screening for improved microorganisms in chemostat cultures, have been developed[1]. However, the emergence of protein-engineering technology has resulted in completely new approaches to the discovery of novel enzymes.

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Protein-engineering technology involves creating new proteins by modifying existing ones. This approach has been made possible by progress in two techniques: site-directed mutagenesis and computer-assisted modeling of the three-dimensional structure of proteins[2]. However, this approach is only applicable to protein families in which the three-dimensional structure of at least one member protein has been resolved. Furthermore, many attempts to alter the properties of enzymes by this approach have failed because the introduced amino acid substitutions exerted unexpected

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