Biotechnology for the Elimination of Agrochemical Wastes

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The disposal of aqueous pesticide wastes is a problem of growing concern for farmers and pesticide applicators. Biotechnology shows some promise for the development of safe, effective, and inexpensive methods for the disposal of waste chemicals. The Tick Eradication Program run by the Animal and Plant Health Inspection Service (APHIS) of the U.S. Dept. of Agriculture (USDA) operates a series of cattle dipping operations along the border of the United States and Mexico. These operations are designed to prevent reintroduction of the tick vectors of cattle fever [*Boophilus microplus* (Canestrini) (southern cattle tick) and *Boophilus annulatus* (Say) (cattle tick)] to the United States from Mexico. This program (operated cooperatively with the Texas Animal Health Commission) operates 42 vats with an average volume 12,500 L per vat (Kearney et al., 1986). The pesticide used in these vats is the organophosphate insecticide/acaricide coumaphos [*O,O*-diethyl *O*-(3-chloro-4-methyl-2-oxo-2*H*-1-benzopyran-7-yl) phosphorothioate]. The vats are charged to 2000 mg·L⁻¹ of coumaphos and are emptied either biannually or more frequently due to sediment accumulation or the presence of the more toxic chemical, potasan [*O,O*-diethyl *O*-(4-methyl-2-oxo-2*H*-1-benzopyran-7-yl) phosphorothioate], which is formed by the reductive dechlorination of coumaphos in the vats (Shelton and Karns, 1988). Thus, more than 532,000 L of aqueous wastes containing high concentrations of coumaphos are generated within a 2-year period.

We previously demonstrated that cultures of a parathion hydrolase producing *Flavobacterium* were effective in converting coumaphos to the hydrolysis product chlorferon (3-chloro-4-methyl-7-hydroxycoumarin), which was susceptible to oxidation by UV-ozonation or direct ozonation (Karns et al., 1987a, 1987b; Kearney et al., 1986). Potasan is also hydrolyzed to yield methylumbelliferone (4-methyl-7-hydroxycoumarin). The combination of enzyme hydrolysis and ozonation yielded products which were readily metabolized by soil microorganisms (Kearney et al., 1986). In one field trial, 2250 L of waste dipping-vat solution was successfully treated by growing the *Flavobacterium* cells in waste that was amended with the addition of carbon and nitrogen sources (Karns et al., 1987a, 1987b). A second field trial was unsuccessful due to the inability of the *Flavobacterium* to grow in the waste solution (J.S. Karns and M.T. Muldoon, unpublished results). Subsequent research demonstrated that a lack of divalent cations in the waste material prevented cell growth (J.S. Karns, unpublished results). We have investigated the use of cell-free preparations of parathion hydrolase in the disposal of waste coumaphos solutions because of the problems associated with growing pure cultures of bacteria in situ in the dip-vat wastes.

An alternative treatment we have developed involves the stimulation of natural coumaphos-degrading microbes that are present in most of the dipping vats. Previous studies indicated that most vats contained a consortium of microbes that was capable of degrading coumaphos but that were held in check by a lack of oxygen (Shelton and Karns, 1988). Upon pH adjustment and aeration, these microbial consortia could be made to mineralize coumaphos (Karns et al., 1995). A large-scale reactor based on a biofilter design was built, tested, and subsequently used to eliminate 11,000 L of waste cattle dip coumaphos within 21 d.

This paper represents a compilation of our

work on the discovery and characterization of enzymes and organisms that degrade pesticides and the development of methods for their use in the destruction of pesticides in agricultural wastes. In some cases, enzymes have been used in concert with a chemical process such as ozonation to accomplish complete degradation of a pesticide. Although none of our work specifically involves wastes that would be considered "horticultural" in origin, our hope is that the reader may see that, with a little bit of research, such techniques could be invented for the disposal of any waste material.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Flavobacterium* sp. ATCC 27551 was obtained from the American Type Culture Collection, Rockville, Md. The construction of recombinant plasmids containing the cloned and altered *opd* gene from *Flavobacterium* on pWWM44, pWWM513, or pJK33 were described previously (Mulbry et al., 1986; Mulbry and Karns, 1989). Plasmid pWWM513 contains the entire *opd* gene cloned into pUC18 as a 1.3 kb *Pst* I fragment (Mulbry and Karns, 1989). Plasmid pJK33 contains the *opd* gene with the first 33 codons of the open reading frame removed by S1 nuclease treatment inserted into plasmid pUC18 so that the parathion hydrolase gene is transcribed under the control of the *lac* promoter present on the vector (Mulbry and Karns, 1989). Plasmid pJK33 ω 2 was constructed by inserting the interposon element from plasmid pHP45 ω 2-Km (Fellay et al., 1987) as a *Bam*HI fragment into the *Bam*HI site of pJK33. All recombinant plasmids were maintained in *E. coli* strain DH5 cells.

Flavobacterium sp. ATCC 27551 was cultured on Basal Salts Medium (BSM) (Tomasek and Karns, 1989) supplemented with 0.01% yeast extract and 40 mM xylose. *E. coli* strains were grown in Lennox broth (Gibco Laboratories, Grand Island, N.Y.) supplemented with

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100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin (for strains containing pWWM44, pWWM513, or pJK33) or 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin (for strains containing pJK33- Ω 2).

Preparation of cell-free extracts. Late log phase cells from cultures of *Flavobacterium* sp. ATCC 27551 or recombinant *opd* containing *E. coli* were harvested by centrifugation (6000 g_n , 20 min, 4 °C). The cell pellets were weighed and suspended in 2 mL of ice cold 20 mM potassium phosphate buffer for each gram of cells. The cells were lysed by passing twice through a chilled French pressure cell at 110.4 MPa. The resulting extracts were centrifuged at 10000 g_n (20 min, 4 °C) and the supernatants were taken as crude, cell-free extracts. Parathion hydrolase from *Streptomyces lividans* containing the cloned *opd* gene from *Flavobacterium* (Steiert et al., 1989) was provided by Burton Pogell (Univ. of Maryland at Baltimore). This preparation was an unconcentrated culture supernatant containing parathion hydrolase that had been excreted into the culture medium by the recombinant *Streptomyces* (Steiert et al., 1989).

Enzyme purification. Parathion hydrolase was purified from *Flavobacterium* and *E. coli* cell extracts by high performance column chromatography on TSK-DEAE and TSK-phenyl columns as described previously (Mulbry and Karns, 1989). The molecular size of the polypeptides that comprised the purified enzymes were determined by SDS-polyacrylamide gel electrophoresis conducted by the method of Laemmli (1970).

Enzyme assays. Parathion hydrolase activity was measured spectrophotometrically by monitoring the increase in absorbance at 405 nm due to the appearance of the *p*-nitrophenol hydrolysis product of parathion in a Beckman DU7HS spectrophotometer. The 1-mL reaction mixture contained 50 mM Tris-HCl (pH 8.5), 0.1% Triton X-100, 0.1 mM CoSO_4 , and 0.1 mg parathion. The reaction was started by the addition of enzyme and the absorbance of a blank reaction mixture containing no enzyme was continuously subtracted from the absorbance of test reactions by the program on the spectrophotometer. Reactions were run at 37 °C. An extinction coefficient of 18,800 was used to calculate the rate of *p*-nitrophenol generation.

Hydrolysis of coumaphos was measured in waste dip-vat material provided by the USDA-APHIS-Veterinary Service Tick Task Force Headquarters, Laredo, Tex. Parathion hydrolase and any amendments (noted in Results for each experiment) were added at the start of the experiment. Reactions (5 mL in a 16 $\times$ 150-mm test tube) were incubated at 26 °C on a gyrorotatory shaker. The pH of the waste material as received may range from 4.5 to 8.5, sodium hydroxide is used to adjust the pH when necessary. At various intervals 100 μL of each reaction was removed and mixed with 900 μL of methanol to stop the reaction and solubilize the coumaphos. Samples were analyzed by high-performance liquid chromatography (HPLC) on a NovaPak C-18 radial compression cartridge column (Waters Assoc., Milford, Mass.) in a solvent system of 80%

Table 1. Molecular manipulation of the *opd* gene from *Flavobacterium*.

Construct	Activity (crude) $\mu\text{moles/min/mg protein}^z$	Cellular location ^y	Polypeptide size (Kda) ^x
<i>Flavobacterium</i>	1.526	Membrane	35
pWWM44	0.0001	---	---
pWWM513	0.1508	Soluble	37
pJK33	0.3365	Soluble	35
pJK33- Ω 2	1.3957	Soluble	35

^xRate of hydrolysis of parathion measured spectrophotometrically.

^yDetermined by fractionation of activity into 105,000 g_n pellets or supernatants.

^zDetermined by SDS-polyacrylamide gel electrophoresis of purified enzyme.

Fig. 1. Hydrolysis of coumaphos by 50 μL (panel A), or 0 μL (panel B) of a parathion hydrolase preparation (containing 28.6 I.U./mL assayed with parathion) from *Streptomyces lividans* added to 5 mL of waste cattle-dip. Samples were taken as described in the text. ($\blacktriangle$) coumaphos; ($\blacksquare$) chlorferon.

Fig. 2. Effect of Tween 80 on the hydrolysis of coumaphos by 15 μL of parathion hydrolase from *Streptomyces lividans* (same preparation as in Fig. 1). Tween 80 was added to waste cattle dip to a final concentration of 0% ($\bullet$); 0.0001% ($\circ$); 0.001% (Δ); 0.01% ($\blacktriangle$); or 0.1% ($\square$) prior to the addition of enzyme to start the reaction.

methanol–20% water brought to pH 2 with phosphoric acid. Coumaphos and chlorferon were detected by monitoring the absorbance of the effluent at 320 nm.

Large-scale enzymatic hydrolysis of coumaphos and ozonation of hydrolysis products. A limited scale-up of the combined hydrolysis and ozonation process for coumaphos disposal was performed on 76 L of authentic dip-vat waste. The waste was placed in a stainless steel cylinder [51 × 135 cm (diameter × height)] and mixed by vigorous aeration from the bottom of the tank. Sodium carbonate was added to bring the pH to between 9.5 and 10. Triton X-100 was added to 0.05% (v/v) and a sample was removed to serve as a control to assess any nonenzymatic hydrolysis. Twenty milliliters of a crude *Flavobacterium* extract containing 988 International Units (I.U.) of parathion hydrolase activity per mL was added. Ozonation was carried out in the same tank. Ozone was generated using a model GL-1B ozone generator (PCI Ozone Corp., West Caldwell, N.J.) from a dry air feed and delivered to the bottom of the tank at 850 L·min⁻¹, 75.9–103.5 KPa, and 100% ozone output (19 g·h⁻¹). Air was supplied to the generator by an air compressor which had a 40.5 L·min⁻¹ at 759 KPa maximum output (Pneumotive model LGH-210-HO2; Air Power Products, Monroe, La.). Measurements of coumaphos and chlorferon were obtained by HPLC as described above.

Biofilter degradation of coumaphos. The entire contents of a coumaphos-containing cattle dipping vat (11,000 L) was transferred to a modified 5.2 m (h) × 3.1 m (dia) fiberglass agricultural storage tank (Red Ewald, Karnes City, Tex.) that supported a 45-cm-thick polyethylene foam pad (see design presented later). The cattle dip was circulated from the bottom of the tank with a Vertiflo series 1320 pump (Odessa, Houston) at a flow rate of ≈370 L·min⁻¹. Sodium hydroxide in the solid form was added to the material as needed to maintain pH between 7.5–8.0. Samples were taken from the tank at various times and analyzed for coumaphos by HPLC as described above. Chloride levels in the tank were determined using a Buchler chloridometer.

Ozonation of pesticides on laboratory and pilot scales. The effects of ozone on numerous pesticides was tested in 200 to 500 mL batches as described previously (Somich et al., 1988, 1990).

RESULTS

Molecular manipulation of the *opd* gene.

The *opd* gene from *Flavobacterium* requires an external promoter in order to be expressed in *E. coli* (Table 1). Plasmid pWWM44 contains the entire *opd* coding region plus over 6 kb of DNA 5' to the start of the gene (thus, it contains the region that should contain the promoter of the *opd* gene in *Flavobacterium*), but *E. coli* containing this construct did not produce any measurable parathion hydrolase. Placement of a promoter known to function in *E. coli* 5' to the start of the open reading frame of the *opd* gene (pWWM513) resulted in pro-

Fig. 3. Effect of cobalt sulfate on the hydrolysis of coumaphos by parathion hydrolase from *Streptomyces lividans* (75 μ L of a preparation containing 9.3 I.U./mL). Cobalt sulfate was added to final concentrations of 0 μ M (○); 10 μ M (▲); 100 μ M (△); or 1000 μ M (●) prior to starting the reaction by the addition of enzyme.

Fig. 4. Hydrolysis of coumaphos and potasin in 76 L waste cattle-dip. (▲) coumaphos; (△) chlorferon.

duction of measurable amounts of enzyme activity. It is interesting to note that the activity is present in the soluble fraction of *E. coli*, whereas it is membrane bound in the native *Flavobacterium* strain. Manipulations of the *opd* gene such as removal of 33 of the amino terminal amino acids (pJK33) and the insertion of an interposon (pJK33omega2), which contains strong transcription and translation terminators, increased the level of expression

of the *opd* gene in *E. coli* to a level nearly equivalent to that produced in *Flavobacterium* (Table 1).

Hydrolysis of coumaphos waste by parathion hydrolase. Coumaphos in authentic dip-vat waste was hydrolyzed by parathion hydrolase to yield the hydrolysis product chlorferon (Fig. 1A). There was no hydrolysis of coumaphos in the absence of enzyme (Fig. 1B). The presence of the nonionic detergent

Tween 80 increased both the rate and extent of coumaphos hydrolysis (Fig. 2). Similar improvements were seen when Triton X-100 was substituted for Tween 80 (data not shown). The addition of cobalt sulfate also increased the rate and extent of coumaphos hydrolysis (Fig. 3). A similar stimulation of the rate of reaction by cobalt was seen when parathion was used as substrate (data not shown). Addition of CuCl_2 also stimulated the parathion hydrolase reaction, while addition of Zn^{2+} , Ca^{2+} , Ni^{2+} , Mg^{2+} , Mn^{2+} , or Fe^{2+} did not (J.S. Karns, unpublished observations).

Scale-up of enzymatic hydrolysis of coumaphos and ozonation of hydrolysis products. In a limited scale-up experiment on 76 L of authentic waste dipping-vat coumaphos suspension the enzymatic hydrolysis of coumaphos was complete within 48 h (Fig. 4). There was no hydrolysis of coumaphos in a no enzyme control (data not shown). Subsequent ozonation of the hydrolysis product chlorferon resulted in complete destruction within 10 h (Fig. 5).

Biofilter field trials. The design of the bioreactor for coumaphos degradation is shown in Fig. 6. The polyethylene filter pad provides a large surface area to which the coumaphos-degrading microbes attach. As the liquid waste passes the filter, it is spread very thin, allowing oxygen from the atmosphere to dissolve into the water. Based on previous results, this introduction of oxygen is probably the most important function of the filter. The results from the treatment of two successive batches of cattle dip in the biofilter tank are shown in Fig. 7. The coumaphos levels in each batch were reduced from $\approx 6 \text{ mM}$ ($2000 \text{ mg}\cdot\text{L}^{-1}$) to $<0.1 \text{ mM}$ ($8\text{--}10 \text{ mg}\cdot\text{L}^{-1}$) within 20 d. No further coumaphos degradation was observed after that point. Since coumaphos is relatively insoluble in water it tends to be retained on the filter pad, thus the measurements of coumaphos levels in the cattle dip liquid may not accurately reflect the degree of actual coumaphos degradation. However, because chloride ion is released late in the coumaphos degradation pathway, after more water-soluble products have formed, it can serve as a useful indicator of degradation. The release of chloride ion in amounts equal to the original coumaphos concentration shows that coumaphos is extensively degraded (Fig. 7). Determination of the amount of coumaphos remaining in the support material of laboratory-scale filters confirmed that the degradation of coumaphos was extensive, since only 6% of the added coumaphos could be recovered after extraction with organic solvents (Mulbry et al., 1996).

Treatment of pesticides with ozone. A wide variety of pesticides were shown to be susceptible to oxidation by ozone in an aqueous environment (Fig. 8). Bromacil was extremely susceptible, with the parent compound disappearing after only 15 min of treatment. Atrazine, cyanazine, metolachlor and alachlor were also substantially degraded by ozone. Paraquat, 2,4-D, metribuzin, and trifluralin required longer exposures for degradation to occur. In contrast, previous studies showed that coumaphos was not extensively affected by

Fig. 5. Destruction of chlorferon (Δ) by treatment with ozone. Reaction was performed on 76 L of waste cattle dip that was pretreated with parathion hydrolase.

Fig. 6. Design of the biofilter reactor capable of treating 15,000 L of waste cattle dip containing coumaphos.

ozonation (Kearney et al., 1986). Previous studies have shown that the products produced by ozonation of pesticides are readily degraded by soil microbes (Somich et al., 1990).

DISCUSSION

Munnecke (1976) first reported the potential for the use of parathion hydrolase for the detoxification and disposal of organophosphate pesticides. Previous work by this laboratory demonstrated that parathion hydrolase producing organisms could be used as part of

a process to accomplish the complete destruction of coumaphos in waste cattle-dips (Karns et al., 1987a, 1987b; Kearney et al., 1986). Because of difficulties in the use of live organisms for this process (delivery of fresh inocula to remote sites, differing nutrient composition between vats) we investigated the use of cell-free parathion hydrolase enzyme preparations in the treatment of waste coumaphos. This study has proven that parathion hydrolase enzyme preparations hydrolyze coumaphos when added directly to waste cattle dip. The addition of nonionic detergents and cobalt salts in-

creased the efficiency of coumaphos hydrolysis by the enzyme preparations. Parathion hydrolase is very stable at 4 °C (Munnecke, 1976; J. Karns, unpublished observations) and could be shipped to remote sites on wet ice.

One major unknown in the development of this process is the cost of parathion hydrolase. Genetic engineering may provide a means for cost effective production of microbial enzymes. The data presented here indicate that although the *Flavobacterium opd* gene is not expressed from its own promoter in *opd*-containing recombinant *E. coli* (construct pWWM44), genetic manipulations such as the insertion of exogenous promoters and terminators can markedly increase the levels of production of parathion hydrolase. In addition the enzyme is in the soluble fraction when it is produced in *E. coli*. Soluble enzyme is more likely to be amenable to efforts at purification and immobilization than is a membrane bound form. These data indicate that further manipulation of the *opd* gene in *E. coli* could result in even higher levels of expression, allowing more economical production of large amounts of enzyme. Insertion of the *opd* gene from pWWM44 into *Streptomyces lividans* by other researchers resulted in the excretion of parathion hydrolase into the culture medium in soluble form (Steiert et al., 1989). The synthesis of parathion hydrolase in an organism such as *Streptomyces*, with its extensive industrial background, also suggests that further manipulation will yield an economical source of enzyme for waste treatment efforts.

The enzymatic process for coumaphos disposal was successfully scaled-up to accomplish the complete destruction of coumaphos in 76 L of waste dip. Parathion hydrolase required 48 h to completely hydrolyze the coumaphos in this high level waste, while the ozonation process required 6 h to destroy the chlorferon product that was generated. The disappearance of chlorferon followed pseudo-first order kinetics ($k' = -6.53 \times 10^{-3} \text{ min}^{-1}$). The rate of ozonolysis of a particular compound is dependent upon the concentration of the substrate and the concentration of ozone and its degradation products ($\text{OH}^\cdot$, $\text{HO}_2^\cdot$, $\text{O}_2^\cdot$, etc.). The concentration of ozone and these various oxidants can be assumed to reach equilibrium in a short period of time, and since ozone is constantly being replenished, the rate of substrate disappearance then becomes dependent upon the concentration of substrate alone, that is, $d[S]/dt = k'[S]$, where $k' = k_1[\text{O}_3] + k_2[\text{OH}^\cdot] + k_3[\text{HO}_2^\cdot] + k_4[\text{O}_2^\cdot] + \text{others}$. Although direct ozonation of coumaphos was not effective (1), possibly due to the very low solubility of coumaphos in water, it has been shown to be useful as a first step for the destruction of a number of pesticides, some of which may be of "horticultural" relevance. Because of the wide range of compounds that ozone can attack, ozonation may be a method of choice for small-scale operations where a variety of pesticides may be in use and the volume of waste is low. With coumaphos and the other pesticides that have been tested, the application of the ozonated products to soil results in complete mineralization of the oxi-

dized products (Hapeman-Somich, 1991; Kearney et al., 1986; Somich et al., 1988, 1990), due primarily to the action of soil microbes.

The use of a consortium of microbes in a trickling filter bioreactor for the destruction of coumaphos has been enormously successful. Two full-scale units, each capable of treating 15,000 L of waste cattle dip at a time, have been placed in operation over the last year. These units have been used to treat all the wastes generated by the Cattle Fever Tick Eradication Program so that waste is no longer placed in the ground. The waste generated by this program are ideally suited to biotreatment because it is composed of one pesticide, is concentrated and is localized in one place so it can be degraded before being mixed with soil or some other matrix that can interfere with the biodegradation process. Although not covered here, we have also used our knowledge of the microbiology of cattle dipping vats and the properties of parathion hydrolase to provide APHIS with the means to prolong the life of the coumaphos in the vats, thus reducing the total volume of waste generated (Grice et al., 1996; Karns et al., 1995).

We hope that the broad range of results that we have presented here, generated over the last 12 years, convinces the reader that biotechnology can offer creative solutions to the problems associated with the use of agricultural chemicals. Although not presented here, we have also characterized organisms, enzymes (and the genes that encode them) that attack *N*-methylcarbamate insecticides such as carbofuran, carbaryl, and aldicarb (Karns and Tomasek, 1991; Tomasek and Karns, 1989), some of which may be important in cultivation of horticultural crops. We have also worked extensively on the microbial degradation of *s*-triazine compounds (Eaton and

Karns, 1991a, 1991b), which are used widely as herbicides in American agriculture. The solutions to waste management are out there waiting to be found. All that is required is the time and money to conduct research on each chemical of interest. Horticulturalists should be encouraged to bring their waste disposal concerns to the attention of their extension agents, or even their congressmen, so that agricultural experiment stations and agencies such as the USDA and the EPA are made aware that a need for the development of creative solutions exists and that resources should be made available to meet those needs.

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Fig. 7. Sequential degradation of two 14,364 L batches of waste cattle dip containing coumaphos in the biofilter reactor.

Ozonation of Organic Pesticides

Fig. 8. Destruction of several common pesticides by ozonation in aqueous solutions or suspensions.

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