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Development of a simple and sensitive fluorimetric method for isolation of coumaphos-hydrolysing bacteria

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Aims: To develop a simple, rapid and sensitive fluorimetric assay to detect, isolate and characterize a soil bacterium capable of degrading the organophosphorus pesticide, coumaphos.

Methods and Results: A high throughput microtitre plate-based method was used to quantify coumaphos hydrolysis by the bacterium. The fluorescent hydrolysis product of coumaphos, chlorferon, was detected at levels as low as 10 nmol l^{-1}. Incorporation of coumaphos into agar plates allowed the rapid detection of coumaphos-hydrolysing bacteria when exposed to an excitation wavelength of approximately 340 nm. The coumaphos-hydrolysing enzyme could be visualized when bacterial cell extracts were separated on SDS-PAGE, incubated with coumaphos and exposed to an excitation source as above.

Conclusions: This method is 100-fold more sensitive than the currently used spectrophotometric method for coumaphos.

Significance and Impact of the Study: This is a unique and versatile tool to screen for bacteria possessing phosphotriesterase activity.

INTRODUCTION

Organophosphorus (OP) pesticides are potent acetylcholinesterase inhibitors with their effects on the central nervous system attributed to the accumulation of acetylcholine. The anticholinesterase activity of OPs is lost when any of the phosphoester groups in the molecule is hydrolysed by a phosphotriesterase enzyme (Serdar and Gibson 1985; McDaniel *et al*. 1988). Several bacteria have been isolated with this phosphotriesterase activity (Mulbry and Karns 1989; Sethunathan and Yoshida 1973; Munnecke and Hsieh 1974; Shelton and Somich 1988; Tchelet *et al*. 1993).

The OP coumaphos (3-chloro-4-methyl-7-coumarinyl diethyl phosphorothioate) has been used to control ectoparasites on livestock and poultry, fly larvae in poultry faecal material, as well as gastrointestinal nematodes (Krause *et al*. 1983). Coumaphos, like the common insecticides, parathion (*O,O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) and chlorpyrifos (*O,O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphorothioate), is a thion OP, containing a P = S bond (as opposed to the oxon OPs which have a P = O bond). All of these OPs have widespread use in both agriculture and domestic environments.

Previously, the detection of bacteria capable of hydrolysing coumaphos to the less toxic chlorferon (3-chloro-4-methyl-7-hydroxy-coumarin) and diethylthiophosphate (DETP) has involved cumbersome or insensitive methods such as high performance liquid chromatography (HPLC; Shelton and Somich 1988), gas chromatography (GC; Kearney *et al*. 1986) and absorbance spectrophotometry (Dumas *et al*. 1989). The HPLC method has a detection limit of 2·5 μmol l^{-1} and is quantitative, but can only be used for single point readings rather than the continuous monitoring of coumaphos hydrolysis. The absorptiometric method (using a wavelength of 348 nm) can be used to monitor coumaphos hydrolysis continuously so is well suited to kinetic studies and has a lower detection limit (1 μmol l^{-1}) than the HPLC method.

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Chlorferon is an hydroxy-coumarin derivative structurally similar to the fluorescent compound 7-hydroxy-4-methyl coumarin (β-methyl umbelliferone) which is commonly used as a fluorescent label as well as being an hydrolysis product used in several assays including lipase, phosphatase, phosphodiesterase and sulphatase assays (Roth 1969). Fluorimetric methods can be 1000 times more sensitive than spectrophotometric ones (Roth 1969) and have been used to measure coumaphos by thin layer chromatography (TLC; Surette and Mallet 1975; Caissie and Mallet 1976; Volpe and Mallet 1976). This method relies on heat treatment to hydrolyse the insecticide, releasing the fluorescent chlorferon. It has been used to detect levels of 0·01 ng g^{-1} coumaphos in lake and sewage water (Volpe and Mallet 1976).

The aim of this study was to develop a simple, sensitive and high throughput screening method based on the fluorescence of chlorferon, for the detection, isolation and characterization of soil micro-organisms capable of hydrolysing coumaphos.

MATERIALS AND METHODS

Chemicals

Technical grade coumaphos (96·2%) and chlorferon (99%) were gifts from Bayer AG (Sydney Markets, NSW, Australia). Analytical grade coumaphos (99%) was obtained from Chem Service Inc., West Chester, PA, USA.

Strains and media

A bacterium capable of hydrolysing coumaphos to chlorferon was isolated from soil obtained from a site in a domestic yard in Brisbane, QLD, Australia, in which a flea dip containing the OP insecticide diazinon had routinely been deposited at least 4 times per year for 2 years. One gram of soil was added to 50 ml of minimal medium at pH 7·0 (Tris.HCl (6·05 g l^{-1}), NH_4Cl (1 g l^{-1}), $FeCl_2{\cdot}4H_2O$ (19·9 μg l^{-1}), sodium acetate (0·68 g l^{-1}), *p*-aminobenzoate (0·9 mg l^{-1}), nicotinic acid (0·9 mg l^{-1}), to which 1% (v/v) of a trace element solution (Sutherland *et al*. 2000) and 36·3 mg l^{-1} coumaphos had been added. All cultures were grown aerobically at 28°C at 200 rev min^{-1} in an orbital shaker. When coumaphos was included in this medium it was at a final concentration of 4 μmol l^{-1}. After three subcultures over a total of 8 d, aliquots of soil culture were spread onto reduced salt (2·5% NaCl) LB agar plates (Sambrook *et al*. 1989) and incubated at 28°C for 3 d.

Analytical methods

Absorbance and fluorescence measurements All absorbance readings were measured in an LKB Biochrom Ultrospec II spectrophotometer (Pharmacia LKB, Sydney, NSW, Australia) using 1 cm quartz cuvettes. All fluorescence readings were measured in a POLARstar fluorimeter (BMG Technologies Pty. Ltd, Offenburg, Germany). The excitation wavelength was 355 nm and the emission intensity was monitored at 460 nm 96-well white microtitre plates were used (FluoroNunc plates with PolySorp surface, Nalge Nunc International, Naperville, IL, USA). A substrate blank was performed concurrently during all assays and no change in fluorescence or absorption of this blank was seen during the assay. Stock solutions of coumaphos and chlorferon were prepared in 20% methanol at 0·4 mmol l^{-1}. Standard curves were prepared in 50 mmol l^{-1} Tris.HCl (pH 7·0). The effect of pH was determined using the same buffer with the pH adjusted (range pH 7·0–9·0). The final reaction volume was 100 μl. Reactions were started by the addition of bacteria or aliquots of cell-free extracts (5 μg; see below). Hydrolysis reactions were monitored continuously at room temperature (23–25°C). For K_m determination, the concentration of coumaphos was varied while maintaining the same concentration of methanol. All measurements were performed in duplicate and average values are presented.

The fluorescence of bacterial colonies and polyacrylamide gels incubated with coumaphos was examined using a hand-held long wavelength (approx. 340 nm) u.v. light (Gelman Sciences Pty. Ltd).

Biochemical assays

High throughput screening of bacteria High throughput qualitative screening for coumaphos-hydrolysing bacterial isolates was performed in 96-well microtitre plates using a loopful of growth from bacterial plates resuspended in an assay mixture (100 μmol l^{-1} coumaphos, 50 mmol l^{-1} Tris.HCl pH 8·0) with 0·5% Triton-X-100 to lyse the cells. Assays were performed at room temperature (approx. 25°C) and monitored continuously over an 8 h period.

Once a coumaphos-hydrolysing bacterium was isolated, quantitative fluorimetric assays were performed with cell-free extracts. Triton-X-100 was excluded from the extraction and assay procedures because it quenches fluorescence (data not shown).

Preparation of cell extracts Cultures were centrifuged (8000 *g*; 15 min) during mid-log phase, and the pellet resuspended in 50 mmol l^{-1} Tris.HCl pH 8·0 and disrupted by sonication on ice (5 × 15 s bursts). Intact cells and large cell debris were removed by centrifugation at 8000 *g*. Protein concentration was determined according to Bradford (1976) using a Bio-Rad protein assay kit with bovine serum albumin as a standard. The hydrolysis of coumaphos was determined by measuring fluorescence during incubation with 100 μmol l^{-1} substrate in 50 mmol l^{-1} Tris.HCl pH 8·0.

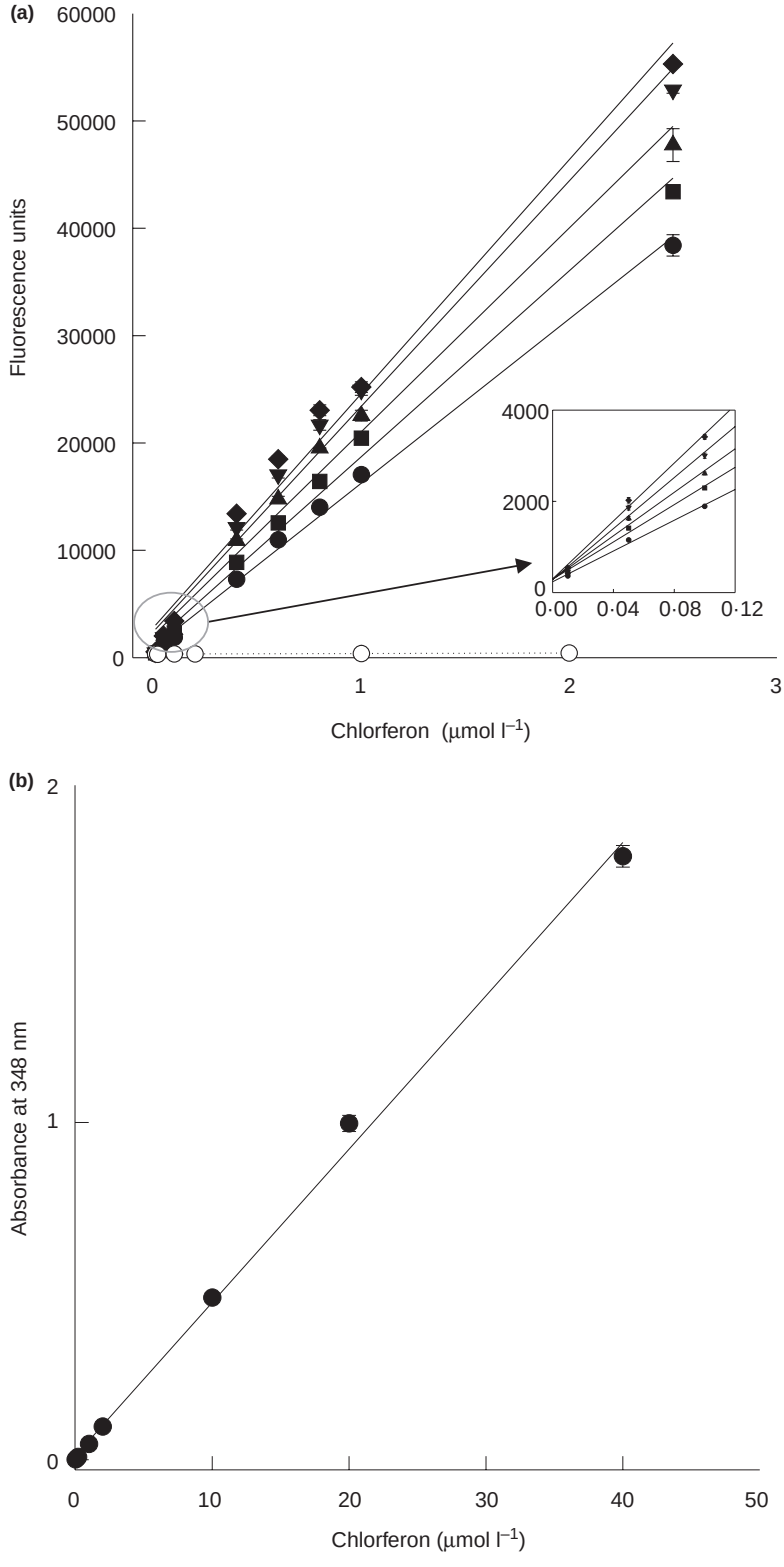

Fig. 1 A comparison of the spectrophotometric and fluorimetric properties of chlorferon. (a) The fluorescence of chlorferon in 10^{-6} mol l^{-1} at various pHs is shown. Coumaphos demonstrated no fluorescence at the wavelengths and pHs tested. (◆) pH 9·0; (▼) pH 8·5; (▲) pH 8·0; (■) pH 7·5; (●) pH 7·0. (b) The absorbance of chlorferon at 348 nm is also shown

SDS-PAGE Cell extracts (5 μg) were separated by 10% (29 : 1 acrylamide:bis) SDS-PAGE gels without the addition of β-mercaptoethanol or SDS to the sample or boiling of the sample (Laemmli 1970). After electrophoresis, gels were equilibrated for 5 min with 50 mmol l^{-1} Tris.HCl pH 8·0 and then incubated for a further 5 min in 8 μmol l^{-1} coumaphos in 50 mmol l^{-1} Tris.HCl pH 8·0. Gels were then examined under u.v. light as described above.

RESULTS AND DISCUSSION

A sensitive microtitre plate-based method for detecting the hydrolysis of the OP coumaphos has been developed based on the fluorimetric properties of the hydrolysis product chlorferon. Chlorferon has previously been detected by its absorbance at 348 nm (Dumas *et al.* 1989). Coumaphos exhibits no significant fluorescence using the wavelengths described above for excitation and emission and at the concentrations tested (Fig. 1a). The fluorescence of chlorferon is linear from 0·01 μmol l^{-1}–2·5 μmol l^{-1} (Fig. 1) at each of the pHs tested (pH 7·0, 7·5, 8·0, 8·5 and 9·0). Chlorferon emits 50% more strongly at pH 9·0 than at pH 7·0. The limit of detection of chlorferon by absorption was 1 μmol l^{-1} and was not affected by change in pH. Thus, the fluorimetric detection of chlorferon is 100-fold more sensitive than the spectrophotometric method.

The use of a microtitre plate fluorescence method allowed the high throughput analysis of many samples. We used this method to isolate a coumaphos-hydrolysing bacterium from contaminated soil. After three rounds of growth with OPs as a phosphorus source, the culture was plated out and approximately 100 colonies were picked, replicated and tested for their ability to hydrolyse coumaphos using the microtitre plate-based fluorescence assay described above. One isolate (P230) demonstrated detectable coumaphos hydrolytic activity. With 100 μmol l^{-1} coumaphos, cell-free extracts of P230 produced 0·56 ± 0·03 μmoles chlorferon min^{-1} mg^{-1} total protein.

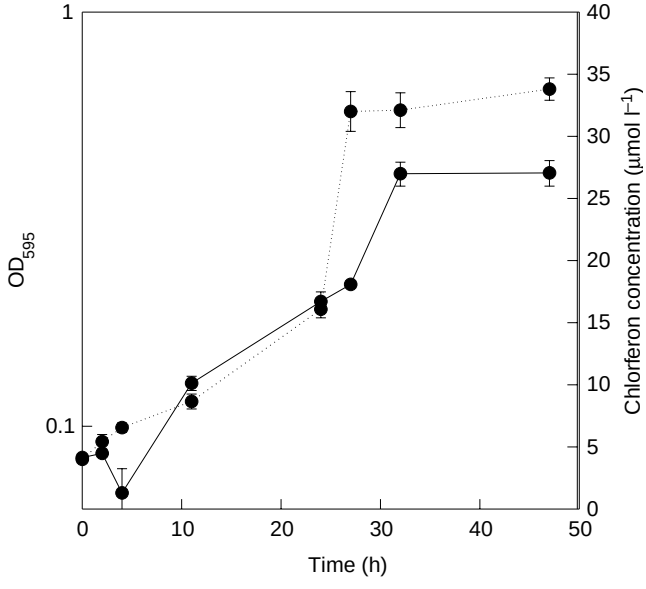

Fig. 2 Growth of isolate P230 over time (dashed line) *vs.* coumaphos hydrolytic activity as measured by the production of chlorferon (solid line)

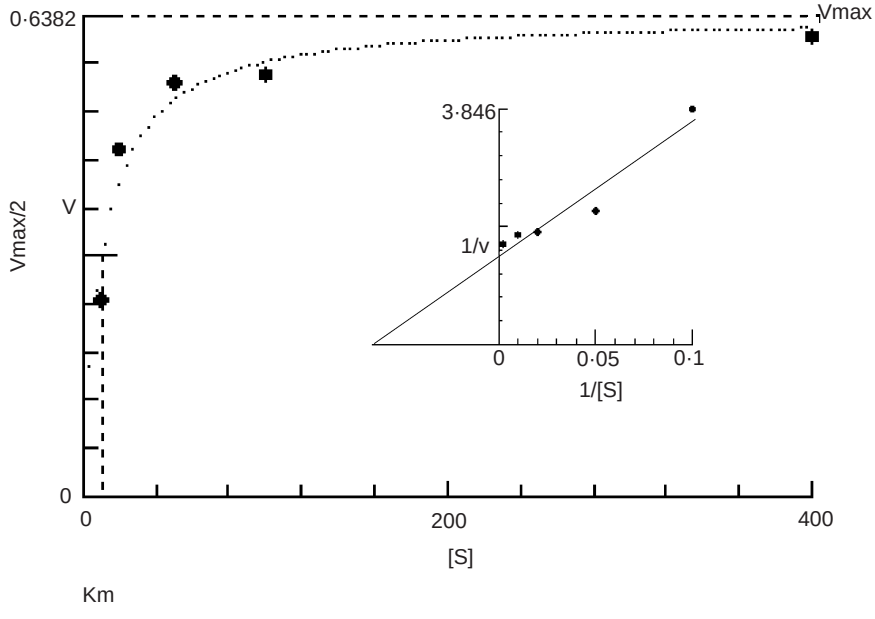

Fig. 3 The effect of varying substrate concentration (in 10^{-6} mol l^{-1}) on activity of isolate P230. The Lineweaver-Burk plot of P230 activity yielded its K_m under these conditions (see insert). ([S] refers to [Coumaphos] measured in μmol l^{-1}; v refers to the specific activity measured in μmol min^{-1} mg^{-1})

The hydrolysis of coumaphos during the growth of P230 in liquid culture was measured by quantitating the fluorescence of aliquots of the medium (Fig. 2). Most of the coumaphos in the medium was hydrolysed by mid-log phase. This demonstrates a simple and sensitive method by which the concentration of chlorferon can be monitored in a liquid medium. Previously, GC and HPLC (Kearney *et al*. 1986) were used for this purpose; however, they require derivatization and organic extraction and have problems with efficiency of recovery. The method described here has the potential to directly measure concentrations of chlorferon as low as 0·01 μmol l^{-1}, whereas the HPLC and GC methods of chlorferon quantification have a limit of detection of only 2·5 μmol l^{-1} (Kearney *et al*. 1986). In the case for which chlorferon is further degraded, an enzyme-linked assay, one that converts coumaphos to chlorferon, to quantitatively measure coumaphos remaining could be employed.

The microtitre plate/fluorescence method was used again with cell extracts for the rapid determination of phosphotriesterase kinetics. The coumaphos hydrolytic activity in cell extracts of P230 was linear over time (results not shown) and the rates changed with varying substrate concentrations, according to Michaelis–Menten kinetics (Fig. 3). The K_m of the enzyme in a crude cell extract of isolate P230 for coumaphos was estimated as $15{\cdot}8 \pm 1{\cdot}2 \times 10^{-6}$ mol l^{-1} using non-linear regression (Fig. 3).

Bacterial growth was fluorescent when isolate P230 was streaked onto an agar plate containing coumaphos when excited at approximately 340 nm (Fig. 4a). We are currently using this rapid and sensitive method to screen a genomic library of P230 genes in *E. coli* in order to isolate the gene(s) responsible for coumaphos hydrolysis.

Fluorescent detection of enzyme activities on polyacrylamide gels has previously been used for phosphatases (with 4-methyl umbelliferyl phosphate; Khan *et al*. 1995) and N-acetyl glucosaminidase (Chen *et al*. 1994). We were also able to identify the coumaphos hydrolytic enzyme using SDS-PAGE. A major fluorescent band was seen when a gel containing P230 extract was stained with coumaphos and examined under a long wavelength u.v. light (Fig. 4b). The coumaphos hydrolytic enzyme has an apparent molecular mass of 66 kDa. This fluorescence did not coincide with a major protein in the cell extracts of isolate P230 and was not observed in the absence of the substrate, coumaphos (data not shown).

Parathion and paraoxon have been used as model compounds to examine the ability of enzymes to hydrolyse OP insecticides by spectrophotometric means (Mulbry *et al*. 1987; Mulbry and Karns 1989). Fluorescent detection of chlorferon production from coumaphos hydrolysis makes this a far more sensitive assay than assays based on either parathion or paraoxon hydrolysis. The extinction coefficient (at pH 9·0) of *p*-nitrophenol (a hydrolysis product of both parathion and paraoxon), at 405 nm is 17×10^3 mol l^{-1} (Dumas *et al*. 1989). The extinction coefficient for the fluorescence of chlorferon is 22×10^9 mol l^{-1} at the same pH (determined from Fig. 1). This difference in coefficients demonstrates the potential to detect far lower levels of chlorferon than *p*-nitrophenol, and therefore this is a far more sensitive assay for the analysis of OP hydrolytic enzymes.

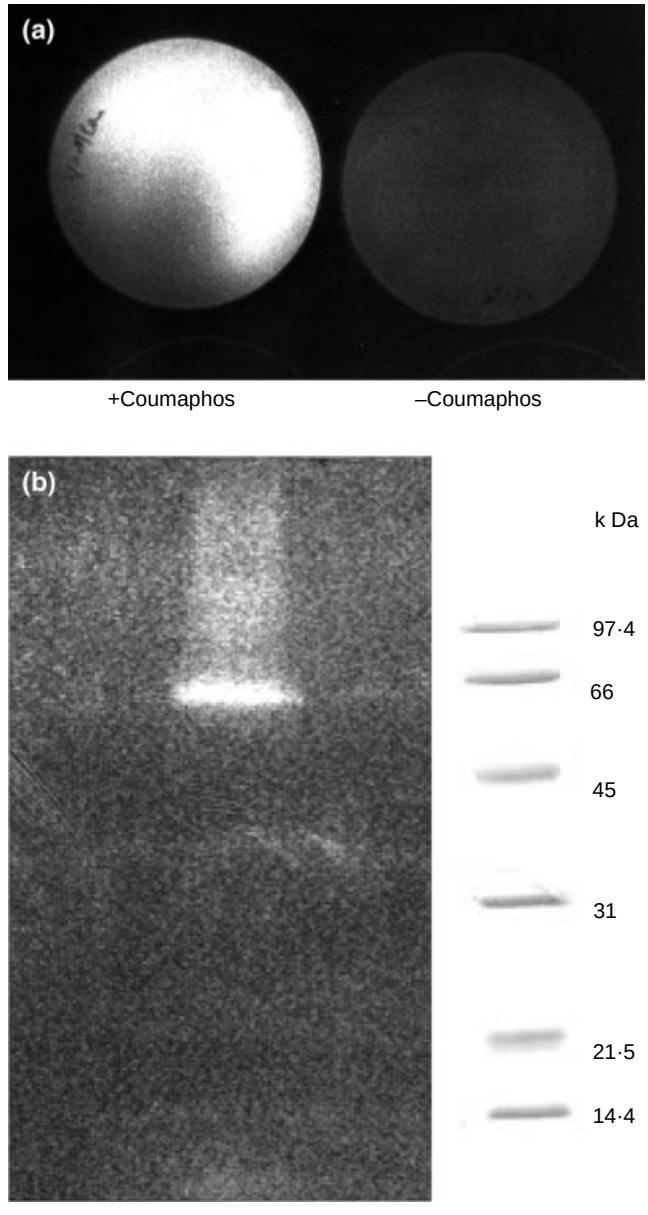

Fig. 4 (a) The detection of coumaphos hydrolysis by isolate P230 when grown on coumaphos-containing agar plates. This fluorescence was not seen in the absence of coumaphos and is only shown where P230 has grown. (b) P230 extract (5 μg) separated by weakly denaturing SDS-PAGE and the gel incubated with coumaphos. The major fluorescent band represents a protein with an apparent molecular mass of 66 kDa

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REFERENCES

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Caissie, G.E. and Mallet, V.N. (1976) The fluorimetric detection of pesticides on aluminium oxide layers. *Journal of Chromatography* **117**, 129–136.

Chen, K.S., Lee, K.K. and Chen, H.C. (1994) A rapid method for detection of N-acetyl glucosaminidase-type chitinase activity in crossed immunoelectrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels using 4-methyl umbelliferyl-N-acetyl-D-glucosaminide as substrate. *Electrophoresis* **15**, 662–665.

Dumas, D.P., Caldwell, S.R., Wild, J.R. and Rauschel, F.M. (1989) Purification and properties of the phosphotriesterase from *Pseudomonas diminuta*. *Journal of Biological Chemistry* **264**, 19659–19665.

Kearney, P.C., Karns, J.S., Muldoon, M.T. and Ruth, J.M. (1986) Coumaphos disposal by combined microbial and UV-ozonation reactions. *Journal of Agriculture and Food Chemistry* **34**, 702–706.

Khan, K.M., Brooks, S.S. and Pullarkat, R.K. (1995) Abnormal acid phosphatases in neuronal ceroid-lipofuscinases. *American Journal of Medical Genetics* **57**, 285–289.

Krause, R.T., Min, Z. and Shotkin, S.H. (1983) Determination of coumaphos and its analog in eggs and milk by using a multiresidue method with liquid chromatographic quantitation and capillary gas chromatographic/mass spectrophotometric confirmation. *Journal of the Association of Official Analytical Chemists* **66**, 1353–1357.

Laemmli, U. K. (1970) Cleavage of structural proteins of the head of bacteriophage T4. *Nature* **227**, 680–685.

McDaniel, C.S., Harper, L.L. and Wild, J.R. (1988) Cloning and sequencing of a plasmid-borne gene (*opd*) encoding a phosphotriesterase. *Journal of Bacteriology* **170**, 2306–2311.

Mulbry, W.W. and Karns, J.S. (1989) Parathion hydrolase specified by the *Flavobacterium opd* gene: relationship between the gene and the protein. *Journal of Bacteriology* **171**, 6740–6746.

Mulbry, W.W., Kearney, P.C., Nelson, J.O. and Karns, J.S. (1987) Physical comparison of parathion hydrolase plasmids from *Pseudomonas diminuta* and *Flavobacterium* sp. *Plasmid* **18**, 173–177.

Munnecke, D.M. and Hsieh, D.P.H. (1974) Microbial decontamination of parathion and *p*-nitrophenol in aqueous media. *Applied Microbiology* **28**, 212–217.

Roth, M. (1969) Fluorimetric assay of enzymes. *Methods of Biochemical Analysis* **17**, 189–285.

Sambrook, J., Fritsch, E.T. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory.

Serdar, C.M. and Gibson, D.T. (1985) Enzymatic hydrolysis of organophosphates: cloning and expression of a parathion hydrolase gene from *Pseudomonas diminuta*. *Bio/Technology* **3**, 567–571.

Sethunathan, N. and Yoshida, T. (1973) A *Flavobacterium* sp. that degrades diazinon and parathion. *Canadian Journal of Microbiology* **19**, 873–875.

Shelton, D.R. and Somich, C.J. (1988) Isolation and characterization of coumaphos-metabolizing bacteria from cattle dip. *Applied and Environmental Microbiology* **54**, 2566–2571.

Surette, D.P. and Mallet, V.N. (1975) Inorganic salts in the fluorometric detection of pesticides. *Journal of Chromatography* **107**, 141–148.

Sutherland, T.D., Horne, I., Lacey, M.J., Harcourt, R.L., Russell, R.J. and Oakeshott, J.G. (2000) Enrichment of an endosulfan-degrading mixed bacterial culture. *Applied and Environmental Microbiology* **66**, 2822–2828.

Tchelet, R., Levanon, D., Mingrelgrin, U. and Henis, Y. (1993) Parathion degradation by a *Pseudomonas* sp. and a *Xanthomonas* sp. & by their crude enzyme extracts as affected by some cations. *Soil Biology and Biochemistry* **25**, 1665–1671.

Volpe, Y. and Mallet, V.N. (1976) A mechanism for the heat-induced fluorescence of coumaphos and related compounds and the identification of their metabolites in water. *Analytica et Chimica Acta* **81**, 111–116.