Cotton
RESEARCH & DEVELOPMENT

Annual, Progress and Final Reports

REPORTS

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number:		**CRC20C**
Annual Report:		Due 30-September
Progress Report:		Due 31-January
Final Report:	**X**	Due 30-September
		(or within 3 months of completion of project)

Project Title: Bioremediation enzyme for endosulfan sulphate

Project Commencement Date: 1/07/2000 **Project Completion Date:** 30/06/2003

Research Program: **Crop Protection**

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Part 3.3 – Final Reports

(The points below are to be used as a guideline when completing your final report. Postgraduates please note the instructions outlined at the end of this Section.)

1. Outline the background to the project.

Pesticide residues threaten both the production systems and the marketing of many of Australia's agricultural commodities. Commodities at risk include cotton, grain, wool, meat, fruit and vegetables, nursery plants and cut flowers. The problems will worsen because there is mounting pressure for reducing residue levels from environmental and public health groups here and overseas.

In collaboration with CSIRO Molecular Science and the project's licencee, Orica Ltd, CSIRO Entomology has a large research program that aims to develop enzymatic bioremediation technology for major pesticides used in Australian and overseas agriculture. Major applications would include the decontamination of waste waters from irrigation operations and processing plants, and the clean up of surface contaminated fruit and vegetables. CSIRO Entomology is responsible for enzyme discovery and has now isolated enzymes and the encoding genes for remediation of organophosphate, pyrethroid, carbamate and *alpha*- and *beta*-endosulfan insecticides. CSIRO Molecular Science and Orica are working on scale-up fermentation and formulation for the various enzymes.

The research project described in this report is concerned with the isolation and characterization of a gene/enzyme system for the bioremediation of endosulfan sulfate (endosulfate), the toxic metabolite of endosulfan. Endosulfan is a broad-spectrum insecticide and is one of the few remaining organochlorine insecticides in extensive use. Endosulfan differs from other organochlorine insecticides by the presence of a relatively reactive sulfite moiety. The reactivity of this group significantly reduces the persistence of this chemical in comparison to other organochloro-cyclodiene insecticides and is the reason why this compound is a potential target for enzymatic bioremediation.

Commercial endosulfan is synthesised as a mixture of two isomers, approximately 70% *alpha*-endosulfan and 30% *beta*-endosulfan. Generally, both isomers are degraded by attack at the sulfite group via either oxidation to form the toxic metabolite, endosulfate, or by hydrolysis to form the non-toxic metabolite, endodiol (endosulfan diol).

Metabolism studies have shown that endosulfate is the major residue detected in soils, and in plant and animal tissues after exposure to endosulfan. Unlike the parent compounds, which have relatively low persistence and do not bioaccumulate, endosulfate persists in soils and sediments and accumulates in animal fat. The latter is of particular concern as contamination of grazing pastures can lead to endosulfate residues in locally grown production animals. These environmental and health concerns have led to an interest in the on-farm waste-water bioremediation of this metabolite in addition to the parent compounds. The aim of this project was to isolate enzymes that degrade endosulfan sulfate and evaluate their potential for enzymatic bioremediation.

2. List the project objectives and the extent to which these have been achieved.

a) Isolation of endosulfan sulfate-degrading microorganism(s)

This objective was achieved. We have isolated a pure bacterium that degrades 20 ppm endosulfan sulfate or *alpha*-endosulfan in the time it takes for the culture to grow in our bacterial growth medium – 3 days from a 5% inoculum. This was obtained by providing endosulfan sulfate as the sole source of sulfur to microbial broth cultures derived from soil with a history of exposure to endosulfan. Successive subculturing in this broth lead to the enrichment and ultimate isolation of a bacterium capable of the required degradative activity. Degradation was determined by growth of the culture in sulfur-free media in the presence of endosulfan sulfate, concomitant with the disappearance of endosulfan sulfate and the formation of novel, chlorine-containing, organically extractable metabolites. The sequence of the 16s rDNA gene of the pure bacterium revealed it to be a member of the genus *Arthrobacter*, most similar to *Arthrobacter pascens*. We have named this bacterium *Arthrobacter* strain KW. Surprisingly, the bacterium was also able to utilise *alpha*-endosulfan as a sulfur source, producing the hydrolysis product, endosulfan monoaldehyde, as the sole chlorine-containing metabolite.

b) Characterisation of endosulfan sulfate degrading enzyme(s).

This objective was achieved. We have characterised the endosulfan sulfate degrading enzyme of *Arthrobacter* strain KW, which also degrades the *alpha* isomer of endosulfan. The endosulfan sulfate degrading activity of strain KW is similar in rate to that observed with *alpha*-endosulfan and is also regulated by the concentration of sulfur in the media. Degradation of endosulfan sulfate produces endosulfan monoaldehyde, 1,2,3,4,7,7-hexachloro-5,6-bis(methylene)bicyclo[2.2.1]-2-heptene and 1,2,3,4,7,7-hexachloro-5-hydroxy methylene -6-methylenebicyclo[2.2.1]-2-heptene. These metabolites are predicted to be non-toxic as the ring structure is disrupted and the sulfur moiety removed. The *alpha*-endosulfan degrading activity of *Arthrobacter* strain KW is much higher than that observed with strain ESD (a strain previously isolated by the Oakeshott / Russell laboratory with activity for *beta*-endosulfan, partially through CSE 77C), but similar in rate to the activity observed with strain ESD for *beta*-endosulfan. The alpha-endosulfan degrading activity of strain KW is also similar to the beta-endosulfan degrading activity of strain ESD in that endosulfan degradation is regulated by sulfur concentration and endosulfan degradation produces the novel metabolite, endosulfan monoaldehyde.

c) Cloning and expression of the gene(s) encoding the endosulfan sulfate degrading enzyme(s) and field evaluation of this enzyme.

This objective was achieved. The endosulfan sulfate degrading enzymes were isolated using activity screening of genomic libraries of DNA from the endosulfan sulfate degrading bacterium in a gram positive host that did not itself degrade endosulfan sulfate. A second approach involving screening the same library with DNA probes of previously isolated endosulfan degrading genes was also used. Both approaches identified the same gene that encoded an enzyme capable of degrading endosulfan sulfate. This enzyme was a monooxygenase enzyme that requires co-factors for activity. A provisional patent describing the endosulfan sulfate degrading enzyme systems was lodged on 7 June 2002, and the PCT patent application (PCT/AU03/00712) filed on 6 June 2003. The feasibility of conducting field trials of these enzymes in irrigation run-off was assessed in collaboration with our commercial partner, Orica Australia Limited. The requirement of co-factors for

monooxygenase activity meant that alternative, more stable co-factors would need to be found, or another (non-co-factor requiring) gene / enzyme system isolated.

3. Detail the methodology and justify the methodology used.

a) Identification of source of enzymes

Previously we have exploited the relatively reactive cyclic sulfite diester group of endosulfan as a nutrient source to enrich for endosulfan-degrading microorganisms. We used a similar strategy in this project to enrich a soil bacterial culture for organisms capable of degrading the more persistent endosulfan sulfate. This method involves incubating soil in a solution containing endosulfan sulfate as the only source of sulphur. As sulphur is an essential nutrient, this results in the gradual enrichment of soil bacteria capable of degrading endosulfan sulfate. The resultant microbial culture is then transferred to fresh solution on a regular basis as all the endosulfan sulfate is degraded. Eventually the endosulfan degrading bacteria becomes the dominant species, whereby it is isolated from the other species (usually sulphur-scavanging species that require only very low levels of sulphur for growth).

b) Cloning of enzymes

The endosulfan sulfate degrading enzymes were isolated using activity screening of genomic libraries of DNA from the endosulfan sulfate degrading bacterium in a Gram-positive host that did not itself degrade endosulfan sulfate. Previously this method had been used successfully to isolate a gene capable of degrading endosulfan. Often genes from Gram-positive bacteria are not expressed successfully in the traditional Gram-negative laboratory strains because the laboratory strains to not possess the required regulatory infrastructure. This problem is solved by expressing the genes in a bacterial strain more similar phylogenetically to the soil isolate. In this work the DNA from the *Arthrobacter* soil isolate was screened in a Mycobacterial strain – both strains being Actinomycetes Gram-postive strains. A second approach involving screening the same library with DNA probes of previously isolated endosulfan degrading genes was also used.

4. Detail and discuss the results including the statistical analysis of results.

We have isolated a pure bacterium capable of detoxifying endosulfan sulfate. 16SrDNA analysis revealed this bacterium to be in the genus *Arthrobacter* and we have named this bacterium *Arthrobacter* strain KW. Degradation of endosulfan sulfate produces endosulfan monoaldehyde, 1,2,3,4,7,7-hexachloro-5,6-bis(methylene)bicyclo[2.2.1]-2-heptene and 1,2,3,4,7,7-hexachloro-5-hydroxy methylene -6-methylenebicyclo[2.2.1]-2-heptene. These metabolites are predicted to be non-toxic as the ring structure is disrupted and the sulfur moiety is removed. The gene encoding the enzyme responsible for detoxification of endosulfan sulfate was isolated. A provisional patent describing the endosulfan sulfate degrading enzyme system was lodged on 7 June 2002, and the PCT patent application (PCT/AU03/00712) filed on 6 June 2003.

5. Provide a conclusion as to research outcomes compared with objectives. What are the "take home messages"?

We achieved our objectives in in that we isolated a gene/enzyme system that is capable of degrading the toxic metabolite of endosulfan, endosulfan sulfate. We also discovered that co-factor requiring monooxygenases are the likely sources of activity when isolating endosulfan sulfate degrading bacterial strains from soil enrichment cultures. Such gene / enzyme

systems are to be avoided if possible as cofactors are currently too unstable for applications in the clean-up of contaminated irrigation run-off. We have initiated a project to isolate such enzymes.

6. Detail how your research has addressed the Corporation's three Outputs - Economic, Environmental and Social?

The goal of cleaning up endosulfan sulfate residues is important for all three of the Corporation's outputs: 1) because it improves the quality of waste waters, 2) because it lessens non-target residue problems, which in turn helps retain registration of cheap and efficacious insecticides and 3) because it should lead to overall reduction in residues in the environment of local communities.

7. Provide a summary of the project ensuring the following areas are addressed:

a) technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.)

b) other information developed from research (eg discoveries in methodology, equipment design, etc.)

c) are changes to the Intellectual Property register required?

This project successfully identified and isolated a bacterial source of endosulfan sulfate degrading enzymes. The gene/enzyme system encoding this activity was cloned and expressed in a bacterial expression system, and characterised as a monooxygenase that requires co-factors for activity. A provisional patent describing the endosulfan sulfate degrading enzyme systems was lodged on 7 June 2002, and the PCT patent application (PCT/AU03/00712) filed on 6 June 2003. The feasibility of conducting field trials of these enzymes in irrigation run-off was assessed in collaboration with our commercial partner, Orica Australia Limited. The requirement of co-factors for monooxygenase activity meant that alternative, more stable co-factors would need to be found, or another (non-co-factor requiring) gene / enzyme system isolated.

8. Detail a plan for the activities or other steps that may be taken:

(a) to further develop or to exploit the project technology.

(b) for the future presentation and dissemination of the project outcomes.

(c) for future research.

This project was successful in achieving its objectives. Exploitation of the gene/enzyme system isolated from this study requires development of systems to deliver co-factor requirements for the enzymes. The methodology developed to achieve project outcomes is of extreme importance in the isolation of alternate gene/enzyme systems that function without co-factor requirements. Future research will centre round the isolation of such systems.

This project fits within the framework of a large bioremediation research initiative at CSIRO Entomology (see above). CSIRO Entomology, in conjunction with Orica Australia Pty Ltd. and CSIRO Molecular Science, has successfully developed enzyme-based bioremediation technologies for detoxifying pesticides in contaminated water prior to its release off-farm. A field trial of the organophosphate degrading enzyme was conducted in the summer of 2000-2001 in run-off water from a cotton field, with spectacular success. Application of the

enzyme reduced levels of insecticide in more than 80,000 litres of run-off water by more than 90% in 10 minutes. In a more recent field trial rinsate from the washdown of pesticide spray equipment was treated with the enzyme, resulting in about a 90% - 99% reduction in the concentration of insecticide in 10 - 30 minutes. In addition to remediating irrigation run-off water and rinsate, other applications that are being investigated include remediating used dip liquor from sheep dipping or post-harvest dipping of fruit, effluent from fruit and vegetable washing, as well as treating effluent from pesticide formulating plants or from factories that apply pesticides as part of manufacturing.

As well as the direct publications described below, the results of this project will be disseminated to industry in the context of the larger bioremediation project in industry and scientific publications. For example:

a) TD Sutherland, RJ Russell and M Zachariou. 2002. Using enzymes to clean up pesticide residues. Pesticide Outlook. 13:149-151

b) TD Sutherland. 2003. Using enzymes to clean up pesticide residues. Garrard's Pest review June: 4-5

9. List the publications arising from the research project and/or a publication plan.

1. Sutherland, TD, KM Weir, MJ Lacey, I Horne, RJ Russell, and JG Oakeshott. 2002. Enrichment of a bacterial culture capable of degrading endosulfate, the toxic metabolite of endosulfan. J. Appl. Micro. 92:541-548.

2. Sutherland TD, I Horne, KM Weir, CW Coppin, MR Williams, LJ Briggs, EJ Crone, SJ Dorrian, RJ Russell, ML Selleck, M Costello, H Nguyen, G Dumsday, M Zachariou, and JG. Oakeshott. 2002. Pesticide detoxification using enzymatic bioremediation. Proceedings of the 11th Australian Cotton Conference. Brisbane, Australia. pp 797-800

3. Kahli M. Weir, TD. Sutherland, I Horne, AJ Edwards, RJ Russell, JG Oakeshott. Isolation and characterisation of the gene involved in the metabolism of the organochloride insecticide endosulfan and endosulfate by an *Arthrobacter* species. In preparation for Appl Environ Microbiol.

10. Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. Where possible include a statement of the costs and potential benefits to the Australian cotton industry or the Australian community.

Pesticides provide essential protection in the production of many agricultural commodities. However, increasing pesticide use as a result of increased production has led to community concern about the social and environmental impacts of pesticide residues. Of particular concern is the contamination of irrigation run-off and drainage water, agricultural soils and horticultural products. Enzymatic bioremediation is an effective and powerful tool for the rapid degradation of pesticide residues in agricultural and rinsate water. CSIRO Entomology, in conjunction with Orica Australia Pty Ltd. and CSIRO Molecular Science, has successfully developed enzyme-based bioremediation technologies for detoxifying pesticides in contaminated water prior to its release from the farm. This technology has the potential for enormous benefits to the cotton industry because it improves the quality of waste waters, lessens non-target residue problems, which in turn helps retain registration of cheap and

efficacious insecticides and because it should lead to overall reduction in residues in the environment of local communities.

The enzyme isolated as a result of this project requires co-factors for activity so is unlikely to be released as a commercial product in the near future. However, the methodology developed during this project is invaluable in the isolation of non-cofactor requiring enzymes for bioremediation of pesticide residues.

Part 4 – Final Report Executive Summary

Pesticides provide essential protection in the production of many agricultural commodities. However, increasing pesticide use as a result of increased production has led to community concern about the social and environmental impacts of pesticide residues. Of particular concern is the contamination of irrigation run-off and drainage water, agricultural soils and horticultural products.

Pesticide residues in soil have been detoxified by introducing and/or encouraging the growth of microorganisms capable of detoxifying the residues on site – a technology known as bioremediation. This method of bioremediation is based on traditional composting techniques and relies on microbial growth to metabolise the toxicants. The detoxification process is generally slow, taking weeks to months to accomplish. Furthermore, the methodology is not suited to the generally low aeration and nutrient content of contaminated water. However, the microorganisms capable of breaking down toxicants in contaminated soil can be sources of enzymes capable of detoxifying pesticide residues in such a low aeration, low nutrient medium. The application of such enzymes is particularly suited to pesticide-contaminated water in that they can achieve rapid remediation without the addition of nutrients or aeration.

The problem of pesticide contamination of water needs to be addressed prior to its release into the waterways. CSIRO Entomology, in conjunction with Orica Australia Pty Ltd. and CSIRO Molecular Science, has successfully developed enzyme-based bioremediation technologies for detoxifying pesticides in contaminated water prior to its release off-farm. For example, an organophosphate degrading enzyme has proven to be an effective and powerful tool for the rapid degradation of pesticide residues in agricultural and rinsate water. In a recent field trial, methyl parathion levels in 80,000 L of fast flowing run-off water in cotton farm drainage channels were reduced by 90% in less than ten minutes. This is a low concentration/high volume source of pesticide-contaminated water that also contains high levels of silt and other particulate matter. In a second field trial, enzyme treatment of rinsate from the washdown of pesticide spray equipment achieved a reduction in methyl parathion concentration of 90% in 10 minutes, and 99% after 1 hour. In contrast to the run-off water in the first trial, this rinsate is a high concentration/low volume source that also contains organic solvents. The application range of the technology has been broadened further to include diazinon detoxification in spent sheep-dip liquor, and the treatment of methyl parathion residues on the surface of leafy green vegetables. In a recent laboratory trial, the concentration of diazinon was reduced from 4.7 parts per million to below 1 part per billion (99.98% reduction), within 1 hour. In the trial involving leafy green vegetables, residues on the surface of baby bok choy were reduced by up to 95%. Given the complex nature of the surface of bok choy, this trial further demonstrated the utility of the enzyme technology.

Our research currently focuses on several major insecticide classes including organophosphates, carbamates, synthetic pyrethroids and the organochlorine, endosulfan.

This project centres around the isolation of enzymes that degrade the toxic metabolite of endosulfan, endosulfan sulfate. As a result of this project we have isolated a bacterium that degrades endosulfan sulfate. This bacterial strain was isolated by providing endosulfan sulfate as the only source of sulfur to a soil microbial population. Sulfur is an essential component of living matter. Therefore only bacteria that could release the sulfur from endosulfan could survive. Removal of sulfur from endosulfan sulfate results in substantial detoxification. The enzyme responsible for this activity was cloned and characterised. The feasibility of conducting field trials of this enzyme in irrigation run-off was assessed in collaboration with our commercial partner, Orica Australia Limited. The requirement of co-factors for activity meant that alternative, more stable co-factors would need to be found, or another (non-co-factor requiring) gene / enzyme system isolated.