Cotton Catchment Communities CRC

FINAL REPORT

(due within 3 months on completion of project)

Part 1 - Summary Details

Cotton CRC Project Number: 2.03.07

Project Title: Development of bioremediation enzymes for residues of diuron metabolites

Project Commencement Date: 1/07/2006 **Project Completion Date:** 30/06/2009

Cotton CRC Program: 2.3 On-farm storages

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Part 3 – Final Report Guide (due within 3 months on completion of project)

Background

Contamination of liquid wastes by pesticide residues presents major problems for many agricultural production and processing industries in Australia and overseas. Environmental concerns have caused governments worldwide to impose increasingly stringent maximum residue limits (MRLs). Despite the widespread uptake of best water and pesticide management practices, off-site pesticide residue issues remain a threat to the Australian cotton industry. At worst, the industry's ongoing access to irrigation water is at risk, as is the ongoing registration of some cheap and efficacious pesticides.

In response to the problems above CSIRO Entomology is developing an enzyme-based bioremediation technology for degrading key pesticides into non-toxic products. CSIRO's molecular biologists and protein chemists are responsible for the development of the enzymes, as well as limited production and formulation research and various aspects of implementation research. Until mid-2009, Orica Australia Ltd had an exclusive licence to manufacture and market CSIRO's enzyme products, but withdrew when the company's strategic focus necessitated its move out of the agricultural Watercare business. CSIRO's commercial team has now taken over responsibility for licensing and marketing while searching for alternative licencees.

CSIRO's project has been ongoing for eleven years, and enzymes that effectively degrade organophosphate (OP) Type I pyrethroid (SP) and carbamate insecticides, certain herbicides and benzimidazole fungicides have been transferred for commercialisation over the last five to six years. Several other enzymes, mainly for herbicides used in the cotton, sugar and rice industries, are also at an advanced stage of development within the CSIRO program. Orica and collaborators in California and Queensland have also carried out some remarkably successful field trials of the most advanced enzymes in various applications ranging from clean-up of irrigation tail waters (channels and dams), animal dips, spray machinery, used pesticide containers, spills and soil (after dormant sprays of tree crops), through to commodity clean-up and personal protection.

The OP enzymes are now registered and being sold for various of these uses (currently excluding commodity clean-up and personal protection, where additional formulation and pre-registration work is required) in Australia, the United Kingdom and California. Enzymes for Type I SPs and benzimidazole fungicides are now ready for registration and subsequent commercialisation. Although sales will be largely offshore initially, CSIRO is committed to early registration and use in Australia: the OP enzymes were, and the benzimidazole enzymes will be first available in Australia, albeit the Type I SP enzymes may be released essentially simultaneously in California as well.

On-going market analyses and formal Due Diligence exercises recommended that widespread utility of the technology for Australian (and overseas) horticulture depended on having enzymes to cover a broader range of pesticides, particularly insecticides and fungicides, than we had previously addressed. Accordingly, Orica and CSIRO scaled up their investment in CSIRO's R&D in 2005 in order to develop enzymes addressing a total of 15 classes of pesticide chemistry (well over a hundred registered actives). It will be a flexible technology of versatile and enduring value to Australian agricultural production and processing industries.

The goal of this project was to develop enzymes against one particular chemistry, namely the toxic metabolites of phenylurea herbicides like diuron. The reasons for this choice were that a scoping study conducted for the CCC CRC by Dr Ivan Kennedy, University of Sydney, identified diuron as the priority target for enzymatic bioremediation in the Australian cotton industry. We had already made good progress in isolating a gene/enzyme system that degrades diuron (and other phenylureas). However there are a set of aniline metabolites of phenylureas that are quite readily produced both biotically and abiotically in the environment and which have problematic levels of vertebrate toxicity (albeit they are no longer herbicidal). In the case of diuron (and some other major products like linuron) the most troublesome of these is dichloroaniline. It might be problematic to commercialise an enzyme mix to degrade phenylureas that did not also degrade these aniline metabolites.

Objectives

1. To develop sensitive biochemical assays for dichloroaniline (DCA) degradation. A high throughput, microtitre plate colorimetric assay was developed for measuring 3,4-dichloroaniline (DCA) concentration, and highly sensitive chromatographic assays were developed for metabolite analyses.

2. To obtain a bacterial culture degrading DCAs. Enrichment culturing yielded a pure strain, *Bacillus* sp. IMT21, which was able to mineralise DCA.

3. To determine the first step in the detoxification pathway for DCAs in the bacteria above. A putative metabolite was identified using gas and liquid chromatography.

4. To clone the gene/enzyme system(s) responsible for the first step in DCA detoxification in the bacteria above. A cosmid library of strain IMT21 is currently undergoing functional screening; concurrently, the entire genome of strain IMT21 is being sequenced, which will allow ready identification of the gene/enzyme systems likely to be responsible for DCA mineralisation. Additionally, a DCA-degrading gene/enzyme system (1,2,4-trichlorobenzene dioxygenase) has been sourced from another research group.

5. Carry out kinetic analysis of the DCA detoxification enzyme. Kinetic analyses were conducted on IMT21 log-phase cells as well as the out-sourced diooxygenase. Neither system has commercial levels of activity.

6. Instigate appropriate IP protection. Premature at this stage.

Methods

1. High throughput assay for DCA degradation.

This colourimetric assay relied on the formation of a coloured diazo product (Abs_{max} ~ 450 nm) with the reagent, o-dianisidine bis(diazotized) zinc double salt (Fast Blue B Salt; FBBS; Sigma). The FBBS (9 mg) was dissolved in MilliQ (MQ) water (9.75 mL) followed by addition of 5.25 mL of 10% sodium dodecyl sulphate (SDS). A 1/6 addition of the FBBS solution was made to the test solution for quantification. The assay was readily adapted to roboticised 96-well plate format, allowing high throughput screening to be achieved.

2. Chromatography and mass-spectrometry.

An Agilent series liquid chromatography (LC) system controlled by Agilent TOF Software (Version A.01.00) was used (Agilent Technologies) for quantitative analysis of diuron, DCA and other metabolites. The mobile phase consisted of acetonitrile:water (80:20 v/v, and both containing 0.1 % v/v formic acid). It was pumped at a flow rate of 1.0 ml min-1. The column employed was an Aqua® C18, 5 µm-particle size, 250 × 4.60 mm (Phenomenex), which was operated at 25ºC. The photodiode array detector (DAD) was used at a wavelength of 250 nm.

Qualitative analysis was performed using an LC/MSD TOF mass spectrometer (Agilent Technologies) with an Electrospray ionization (ESI) source. The mass spectrometer was connected to the HPLC stream after the DAD detector. Nitrogen was used at a flow rate of 12 l min^{-1} as a drying gas. The capillary temperature was 350°C and the spray voltage was 3 kv. The scanning was done in positive ion mode, and the fragmenter and skimmer were set at 120 V and 60 V, respectively, for scans in the range of 50-450 m/z.

3. Bacteria and growth conditions.

DCA degrading bacteria were isolated using standard enrichment techniques. Details of soils samples and enrichment conditions are given in Table 1. Strain IMT21 was either grown on minimal medium (described in Pandey *et. al*., 2009, *Biochem. Biophys. Res. Commun*. 380: 710-714) with DCA as a sole carbon source, or in nutrient broth as a rich growth medium. For the degradation kinetics of DCA, growth supernatant was collect at different time points and analysed by LC and LC-MS. Resting cell studies were conducted as outlined in Pandey *et al.* (2003, *FEMS Microbiol. Lett.* 229:231-236).

The trichlorobenzene dioxygenase containing plasmid pTCB144 (Werlen, *et al.,* 1996, *J. Biol. Chem.* 271:4009-4016) in *E. coli* top10 cells was maintained in Luria Bertani (LB) containing 100 mg/ml ampicillin.

Results ***(Confidential ie not for public disclosure)***

1. Biochemical assays for dichloroaniline (DCA) degradation.

A high throughput, microtitre plate colorimetric assay, the so-called "Fast-Blue assay", has been developed for measuring 3,4-dichloroaniline (DCA) concentration. The fast blue reagent (tetrazotized o-dianisidine) produces a di-azo product with DCA, which has a maximum absorbance at 450 nm. The standard curve shown in Figure 1 illustrates a linear relationship between DCA concentration and absorbance (450 nm).

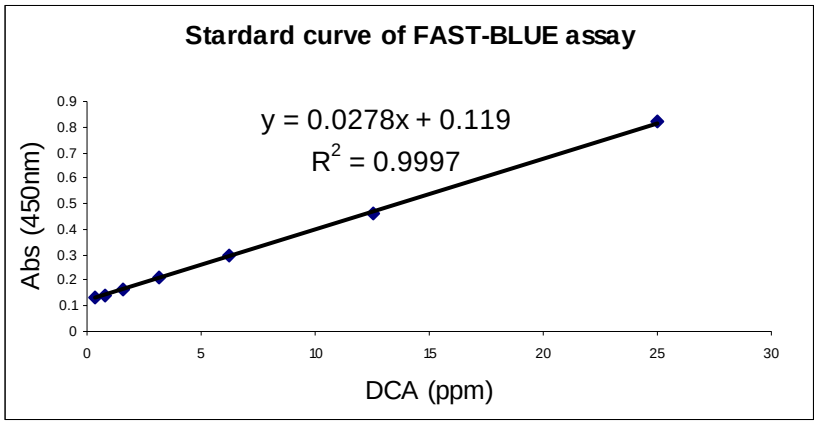

Figure 1: Standard curve for DCA detection using the "Fast-Blue" assay", illustrating a linear relationship between DCA concentration and absorbance (450nm).

Highly sensitive high-pressure liquid chromatography (HPLC) and liquid chromatography – mass spectrometry (LC-MS) assays have also been developed for the purpose of metabolite detection (Figure 2).

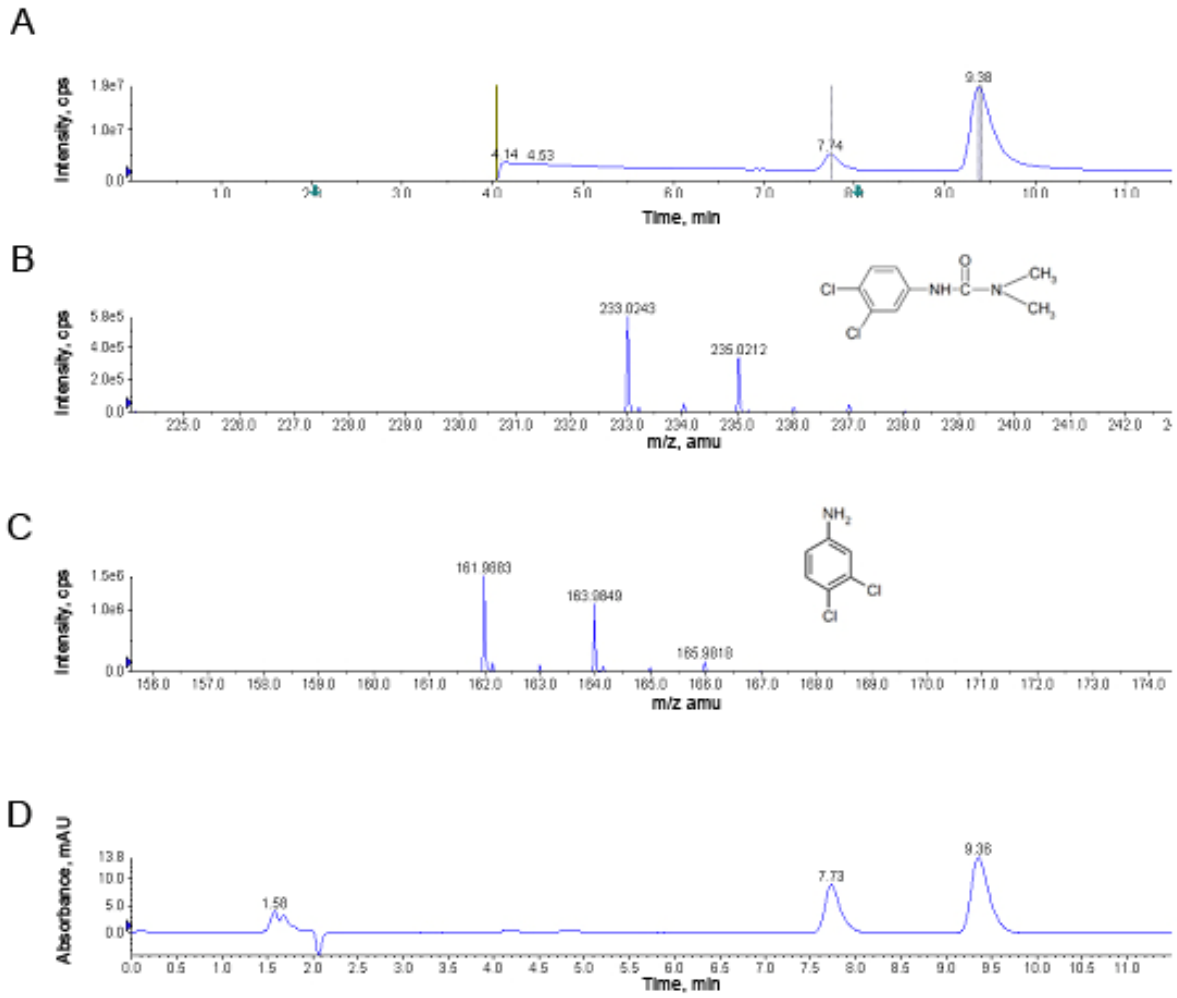

Figure 2: HPLC and LC-MS assay development for DCA: (A) Total Ion Count (TIC) in LC-MS; the two peaks at 7.74 min & 9.38 min represent DCA and diuron, respectively; (B) Mass pattern of diuron in LC-MS; (C) Mass pattern of DCA in LC-MS, and (C) HPLC assay showing resolution of DCA and diuron (7.73 min and 9.36 min).

2. Bacterial cultures degrading DCAs.

Soil samples were collected from four different locations with a history of repeated diuron exposure, and used to set up enrichment cultures in which either diuron itself, or the metabolite, DCA, were used as the sole source of carbon or nitrogen. Details of the sampling sites and the presence of pesticide degradation in the various enrichment cultures are given in Table 1.

Sampling site	Sample Description	Enrichment	Medium	Results after two rounds of sub-culturing
Site 1 (Experimental plot with 3 years diuron application)	Upper 15 cm layer of soil	Diuron	C-, N+	DU + DCA present
			C-, N-	DU mineralization
			C+ N+	No DU degradation
		DCA	C-, N+	No DCA degradation
			C-, N-	No DCA degradation
			C+ N+	No DCA degradation
	Lower 15-30 cm layer of soil	Diuron	C-, N+	DU Mineralization
			C-, N-	No DU degradation
			C+ N+	No DU degradation
		DCA	C-, N+	No DCA degradation
			C-, N-	No DCA degradation
			C+ N+	No DCA degradation
Site 2 (Regular diuron exposure over the past decade or so)	Supply channel	Diuron	C-, N+	DU mineralization
			C-, N-	DU mineralization
			C+ N+	DU + DCA present
		DCA	C-, N+	DCA mineralization
			C-, N-	DCA mineralization
			C+ N+	DCA mineralization
Site 3 (Diuron applied in 2005 and then intermittently in the past)	Paddock	Diuron	C-, N+	Diuron mineralization
			C-, N-	Diuron mineralization
			C+ N+	Diuron mineralization
		DCA	C-, N+	No DCA degradation
			C-, N-	No DCA degradation
			C+ N+	No DCA degradation
Site 4 (Contaminated with chloroanilines)	Chemical industry waste water efflux	DCA	C-, N+	DCA mineralization

Table 1: Details of soil samples and enrichment cultures for diuron / DCA degradation. C±, N± refer to the presence or absence of carbon or nitrogen in the culture media, respectively. DU = diuron; DCA = 3,4-dichloroanaline.

As shown in Table 1, diuron and/or DCA mineralizing cultures were obtained from all soil samples, and several potential 3,4-DCA mineralizing bacterial isolates were identified. The fastest 3,4-DCA degrader, strain IMT21, was chosen for further study. Based on 16S r-DNA sequencing, this strain was identified as a *Bacillus* sp. Figure 3 shows the 16S r-DNA based phylogeny of this strain. Growth of IMT21 on 3,4-DCA resulted in the production of chloride ions and ammonia in the culture supernatant (Figure 4).

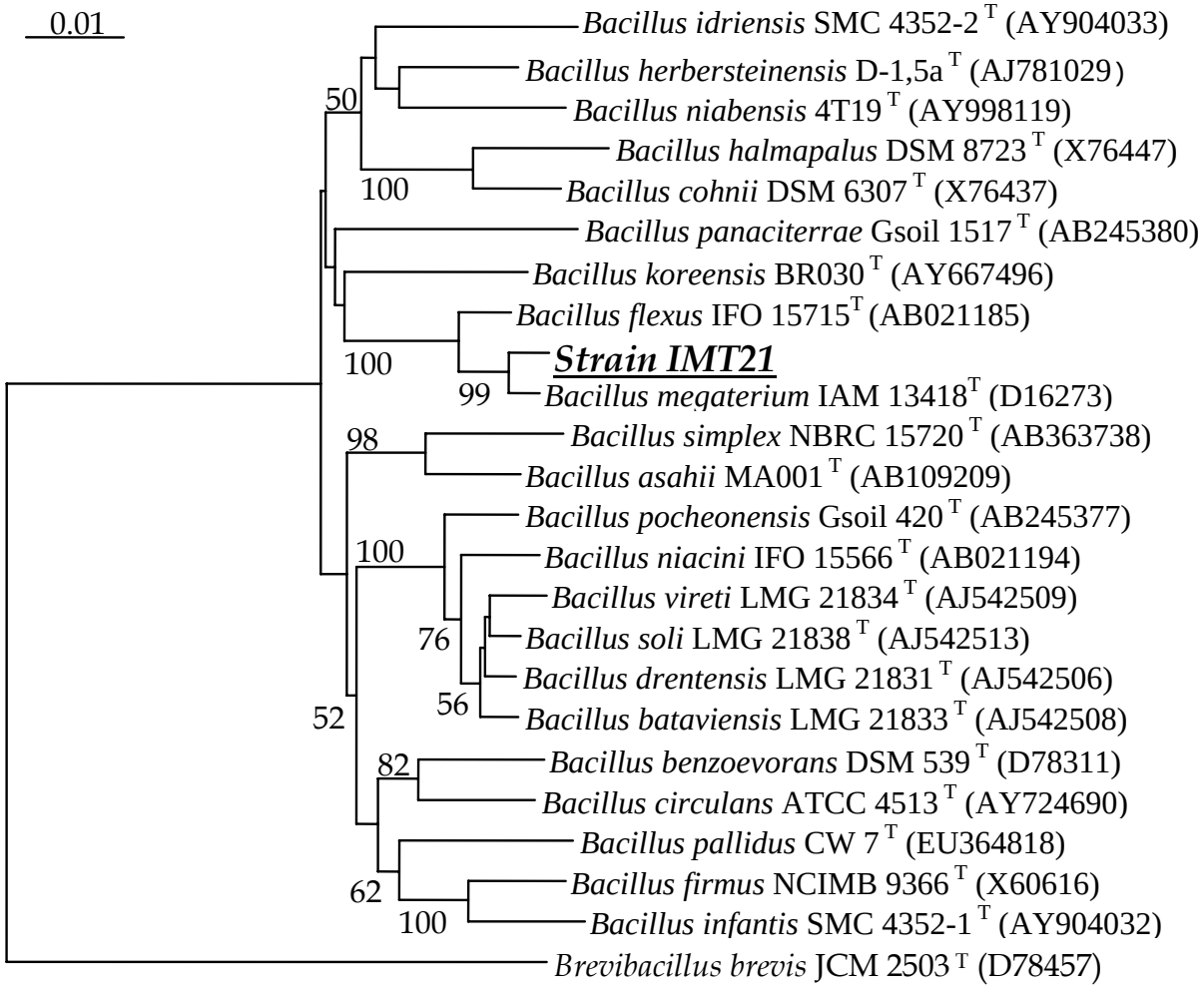

Figure 3: Neighbour-joining tree based on 16S rDNA (1483 bases) sequences, showing the phylogenetic relationship between strain IMT21 and other related members of the genus *Bacillus. Brevibacillus brevis* was used as an out-group. Bootstrap values (expressed as percentage of 1000 replications) greater than 50 % are given at the nodes. Bar represents 1 % sequence variation.

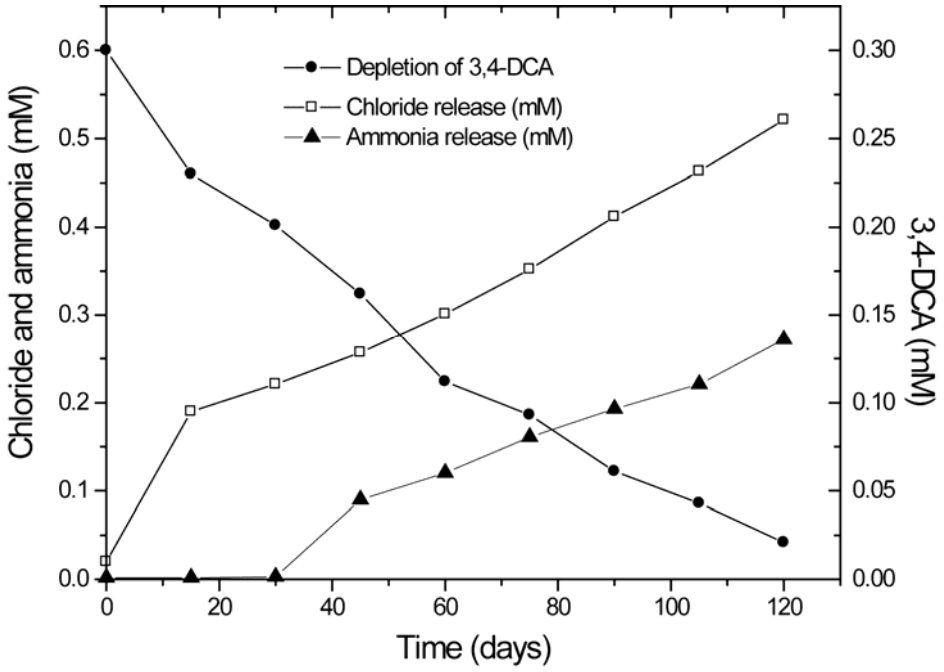

Figure 4: 3,4-DCA mineralization by *Bacillus* sp. IMT21.

3. Detoxification pathway for DCA degradation.

Gas and liquid chromatography (GC & LC) were used to identify metabolites for the initial steps in the catabolic pathway of 3,4-DCA in *Bacillus* sp. IMT21. Interestingly, two different metabolites were identified during 3,4-DCA mineralization by "resting cells" of strain IMT 21 and by log-phase cells of the same strain. Putative structures of both of these metabolites are shown in Figure 5. 3,4-Dichloroacetanilide could be a product of a non-specific enzyme occurring in the resting cells of strain IMT21. This reaction is an anabolic reaction and would not lead to the mineralization of 3,4-DCA as a carbon source by strain IMT21. However, metabolite X (as shown in Figure 5), observed during growth of the strain IMT21 on 3,4-DCA, would eventually lead to mineralization of 3,4-DCA. This oxygenated metabolite would be less toxic and less stable in the environment than 3,4-DCA. Elucidation of the complete 3,4-DCA catabolic pathway was not necessary for the current study.

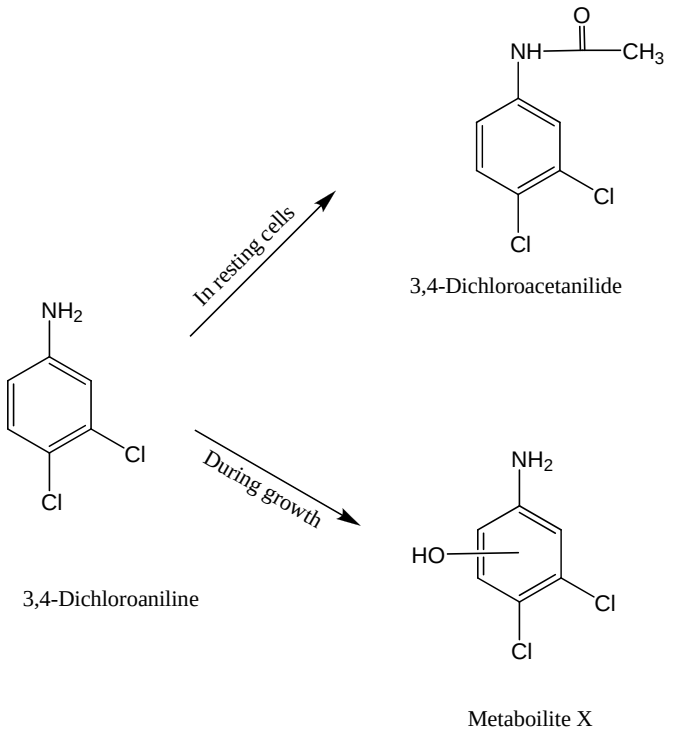

Figure 5. Putative metabolites of 3,4-DCA degradation by *Bacillus* sp. IMT21.

4. Cloning the gene/enzyme system(s) responsible for the first step in DCA detoxification in the bacteria above.

To clone the gene-enzyme system(s) responsible for converting 3,4-DCA to metabolite X (Figure 5), a cosmid library was prepared from genomic DNA of this strain and is currently being functionally screened. Functional cloning is a very time consuming approach in this case, as the wild-type IMT 21 strain itself takes 120 days to degrade 0.3 mM 3,4-DCA.

At the same time, we are therefore sequencing the complete bacterial genome of *Bacillus* sp. IMT21 using the 454 sequencing facility at The John Curtin School of Medical Research, Australian National University. Based on the structure of the metabolite X, we would clone and characterize putative oxygenases (monooxygenases/dioxygenases) from the functionally annotated genome. We are currently evaluating several pipelines for genome annotation.

In a second, independent strategy to obtain a DCA-degrading gene / enzyme system, we identified selected oxygenases acting on polychlorinated aromatic compounds from the literature, and sourced them from various colleagues for testing their DCA degrading activity. One such enzyme, 1,2,4-trichlorobenzene dioxygenase, showed activity on not only 3,4-DCA, but also on other DCA isomers.

5. Kinetic analysis of the DCA detoxification enzyme.

As mentioned above, strain IMT21 is the fastest DCA degrader among the several DCA degrading strains that we isolated. However, even this bacterium requires as long as 120 days to completely degrade 0.3 mM (~48 ppm) of DCA (Figure 4). The most parsimonious explanation for the slow DCA degradation at this stage would be that the enzyme responsible is kinetically inefficient (the first step of the aromatic catabolic pathway is considered most often as the rate limiting step) and would require protein engineering before it could meet industry standards for turnover number.

As mentioned above, the enzymatic reaction of the out-sourced 1,2,4-trichlorobenzene dioxygenase was characterized qualitatively with LC-MS and shown to transform DCA to a less stable and presumably, less toxic product. Preliminary identification of the metabolite suggests that it has a similar structure to that of metabolite X shown in Figure 5. This enzyme has now been tested on several other DCA isomers and shown to be active. Kinetic characterization of DCA degradation has been performed using resting cells as the enzyme source, rather than purified protein; while the enzyme has much better activity than the IMT21 strain (complete degradation of 60 ppm 3,4-DCA in ~8 hours by induced resting cells; Figure 6) for DCA, it would also still require improvement using protein engineering before it can meet technical performance criteria for a bioremediation enzyme.

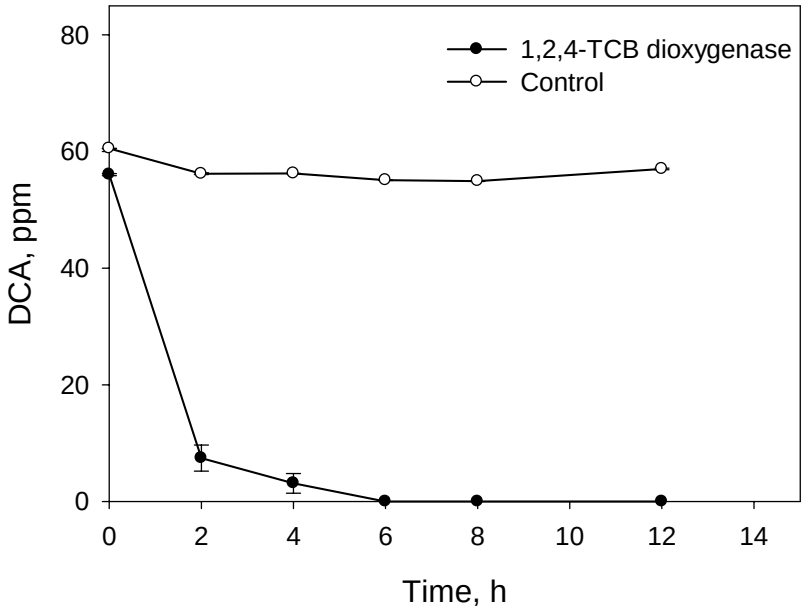

Figure 6. DCA degradation kinetics by resting cells of only *E. coli* cells (o) and *E. coli* cells expressing 1,2,4- trichlorobenzene dioxygenase (•).

The 1,2,4-trichlorobenzene dioxygenase enzyme has a requirement for cofactors, which we have previously regarded as a fatal negative for economic use in the field. However there have been several breakthroughs by other groups in the commercial use of cofactor dependent enzymes that utilize immobilization systems to boost kinetic efficiencies. We will be exploring the use of these systems for our enzymes.

6. Appropriate IP protection.

At this stage it is premature to file a provisional patent on the DCA degrading enzyme. Its activity needs to be improved and immobilization systems investigated to allow efficient cofactor utilization.

Outcomes

Although we have not succeeded in isolating an enzyme that degrades DCA as quickly as needed for commercial use, there is sufficient promise in the dioxygenase we have isolated to warrant further work to improve it. We will now seek funding from various sources to enable us to do this.

We have developed a range of high throughput and kinetic assays in the current project that will expedite the improvement work from here. The results obtained are also highly publishable. There is no value in patenting the work to date because a new patent application will be needed on the improved enzyme anyway. The diooxygenase enzyme has also been published by another group.

Conclusion

It has proven more difficult than we had hoped to isolate a dichloroanaline degrading gene / enzyme system with commercial levels of activity. Moreover the system we have isolated needs a cofactor, which makes implementation more difficult. However techniques for enzyme improvement through *in vitro* evolution are well established at CSIRO and methods for cofactor delivery are also emerging in the literature. Diuron and other phenylurea herbicides remain problematic in respect of residue issues and we have already developed an enzyme to degrade diuron to dichloroanaline, so we will seek other funds to complete the development of the enzyme for DCA degradation.

Extension Opportunities
Not applicable.

Publications

We expect following two (different) publications:

(A) Isolation and characterization of newly isolated 3,4-dichloroaniline degrading *Bacillus* sp. IMT21.

(B) Transformation of different dichloroaniline isomers by 1,2,4-trichlorobenzene dioxygenase

Part 4 – Final Report Executive Summary

Contamination of liquid wastes by pesticide residues presents major problems for many agricultural production and processing industries in Australia and overseas. Environmental concerns have caused governments worldwide to impose increasingly stringent maximum residue limits (MRLs). Despite the widespread uptake of best water and pesticide management practices, off-site pesticide residue issues remain a threat to the Australian cotton industry. At worst, the industry's ongoing access to irrigation water is at risk, as is the ongoing registration of some cheap and efficacious pesticides.

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The goal of this project was to develop enzymes against 3,4-dichloroaniline (DCA), the toxic metabolite of phenylurea herbicides like diuron. We had already isolated a gene/enzyme system that degrades diuron (and other phenylureas). However it might be problematic to commercialise an enzyme mix to degrade phenylureas that did not also degrade these aniline metabolites. We succeeded in isolating two enzymes that are able to degrade the toxic metabolites, albeit not with commercial levels of activity.

Although we have not succeeded in isolating an enzyme that degrades DCA as quickly as needed for commercial use, there is sufficient promise in the enzyme we have isolated to warrant further work to improve it. We have also developed a range of high throughput and kinetic assays in the current project that will expedite the improvement work from here. We will seek other funds to complete the development of the enzyme for DCA degradation.

For more information please contact Dr John Oakeshott (02 6246 4157; John.Oakeshott@csiro.au).