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Short communication

A free-enzyme catalyst for the bioremediation of environmental atrazine contamination

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ABSTRACT

Herbicide contamination from agriculture is a major issue worldwide, and has been identified as a threat to freshwater and marine environments in the Great Barrier Reef World Heritage Area in Australia. The triazine herbicides are of particular concern because of potential adverse effects, both on photosynthetic organisms and upon vertebrate development. To date a number of bioremediation strategies have been proposed for triazine herbicides, but are unlikely to be implemented due to their reliance upon the release of genetically modified organisms. We propose an alternative strategy using a free-enzyme bioremediant, which is unconstrained by the issues surrounding the use of live organisms. Here we report an initial field trial with an enzyme-based product, demonstrating that the technology is technically capable of remediating water bodies contaminated with the most common triazine herbicide, atrazine.

1. Introduction

Herbicide runoff from agricultural lands is a major issue worldwide. It is of particular concern in the sugar cane growing region within the Great Barrier Reef catchment area and has been identified as a threat to receiving freshwater and marine environments (Lewis et al., 2009). In particular, herbicides designed to inhibit the photosystem II in plants (e.g. triazines, phenyl ureas) exceed freshwater and marine guidelines during both low flow and event/high flow conditions (Lewis et al., 2009). These ecosystems include freshwater wetlands of national or international significance and the Great Barrier Reef World Heritage Area (Lewis et al., 2009).

Atrazine, the most commonly used triazine, has been used extensively since its introduction in 1958 (Tomlin, 2006), and of particular concern as it has been linked to environmental and human health problems. Triazine herbicides are toxic to non-target photosynthetic species, including phototropic bacteria, freshwater algae, mangrove trees and corals (Bell and Duke, 2005; Jones and Kerwell, 2003; Lockert et al., 2006; Sutton et al., 1984). In addition, it has been claimed that atrazine may be carcinogenic (Huff, 2002; Huff and Sass, 2007), and may cause endocrine dysfunction in vertebrate species (Hayes et al., 2002). These issues are compounded by atrazine's relatively long environmental half-life of four to fifty seven weeks (Belluck et al., 1991) and its high level of mobility; it has been detected in both surface and ground waters in several countries (Gavrilescu, 2005; Thurman and Meyer, 1996; van der Meer, 2006), at concentrations up to 1 ppm.

Several bioremediation strategies have been suggested for decontaminating atrazine-contaminated water, including the use of transgenic plants and bacteria (Kawahigashi et al., 2006; Strong et al., 2000; Wang et al., 2005). However, technical and regulatory impediments prevent the use of live transgenic organisms in environmental settings (Watanabe, 2001). Free-enzyme bioremediation is an attractive alternative to the use live organisms, as it is not constrained by these (Alcalde et al., 2006; Scott et al., 2008; Sutherland et al., 2004). Indeed, free-enzyme systems are considered to be sufficiently safe for use in the clinical treatment of pesticide poisoning (Bird et al., 2008), and are currently used commercially for organophosphate insecticide remediation (http://www.orica-landguard.com/).

Despite the advantages of free-enzyme bioremediation, there are also constraints, and the requisite characteristics required of enzymes used in free-enzyme remediation restrict its applicability

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to a relatively narrow range of enzyme classes. The enzymes used in free-enzyme bioremediation must withstand environmental conditions for long enough to remediate their target and must be independent of diffusible cofactors, or cofactors that require active regeneration (Alcalde et al., 2006; Scott et al., 2008; Sutherland et al., 2004). Hydrolases are particularly well suited to this application, whilst other enzyme systems, such as reductive dehalogenases, monooxygenases and dioxygenases, are not (Gibson and Parales, 2000; Loffler and Edwards, 2006; Whiteley and Lee, 2006; Furukawa, 2006; Field and Sierra-Alvarez, 2008).

Suitable hydrolytic enzymes for atrazine bioremediation have been isolated: the atrazine dechlorinase (de Souza et al., 1996), AtzA, and the triazine hydrolase, TrzN (Mulbry et al., 2002). Both enzymes catalyse the irreversible hydrolytic dechlorination of atrazine to produce non-herbicidal products (Fig. 1). Generally AtzA has been explored as the most promising bioremediant for atrazine, and the generation of transgenic plants containing the *atzA* gene (Wang et al., 2005), trialling of transgenic *Escherichia coli* in field-scale bioremediation (Strong et al., 2000), and various attempts at enzyme engineering and improvement (Raillard et al., 2001; Scott et al., 2009) have all been reported. However, AtzA is limited to the remediation of chlorinated triazine herbicides (e.g. atrazine, propazine and simazine) (Seffernick et al., 2000), whilst the alternative enzyme, TrzN, acts upon a broader range of triazines including methoxytriazines (e.g. atraton) and methylthiotriazine (e.g. ametryn) (Shapir et al., 2005). TrzN is also more efficient catalytically than AtzA (de Souza et al., 1996; Scott et al., 2009; Shapir et al., 2005), making it in many ways a more attractive bioremediant than AtzA.

Here we report the first field trial of a free-enzyme bioremediant of atrazine, based on the catalytically superior enzyme TrzN.

2. Materials and methods

2.1. Preparation of TrzN-containing homogenate

The gene encoding TrzN was cloned into pET14b (Novagen) using the *Nde*I and *Bam*HI restriction sites using appropriate restriction endonucleases (New England Biolabs). The resultant plasmid was used to transform BL21 λ(DE3) (Novagen), which was then used as the expression strain. Clarified bacterial homogenate containing active TrzN was prepared from a 2 L ferment of BL21 λDE3 expressing TrzN, grown on a minimal medium (10.6 g/L KH_2PO_4, 4 g/L $(NH_4)_2HPO_4$, 1.7 g/L citric acid monohydrate, 31.3 mL/L glycerol). After autoclaving 10 mL/L of PTM4 salts (0.2 g/L D-biotin, 2.0 g/L $CuSO_4 \cdot 5H_2O$, 0.08 g/L NaI, 3.0 g/L $MnSO_4 \cdot H_2O$, 0.2 g/L Na_2MoO4, 0.02 g/L Boric acid, 0.5 g/L $CoCl_2 \cdot 6H_2O$, 7.0 g/L $ZnCl_2$, 22.0 g/L $FeSO_4 \cdot 7H_2O$, 0.5 g/L $CaSO_4$, 1 mL/L H_2SO_4) and 0.6 g/L $MgSO_4$ was added. The fermentation was fed with glycerol, supplemented with 150 mg/L ampicillin and 331 mg/L thiamine, and induced with 11.9 mg/L IPTG. The ferment yielded *ca.* 240 g wet weight of cell pellet (OD_{600} = 122). The cells were suspended in 5.2 g/L MOPS pH 6.9, then passed through a homogeniser 3 times and clarified by centrifugation. The clarified lysate was passed through a 0.22 μM filter to remove intact cells and DNaseI was used to remove intact DNA. Enzymatic activity was determined at 258 ± 19 mg of atrazine/mg of lysate/using both the UV absorbance method described by de Souza et al. (1996) and the colorimetric method described in Scott et al. (2009). The homogenate was stored at −80 °C and thawed at 4 °C when required.

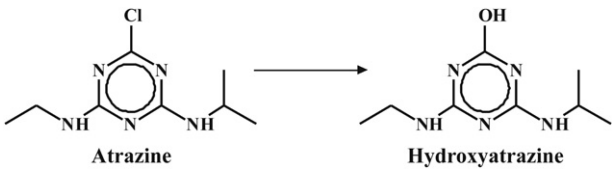

Fig. 1. Dechlorination of atrazine catalysed by the triazine hydrolase TrzN and producing non-herbicidal hydroxyatrazine.

2.2. Preparation of test dam

Holding dams are used to collect spent irrigation water so that it is withheld from entering the catchment, allowing the water to be reused in farm activities and providing time for pesticide residues to degrade by biological or photolytic routes. A *ca.* 1.5 ML holding dam at a sugar cane farm near Clare (Lat. 19:48, Long. 147:14) in the dry tropics of Queensland, Australia, was filled with headwater from irrigation of a field pre-treated with the recommended dose of atrazine (3.3 kg per hectare). 240 g of bacterial homogenate was suspended in 20 L of water, and applied by hand by spreading evenly across the surface of the holding dam. Duplicate 1 L samples were taken before the dam was filled with atrazine-contaminated runoff water, before the enzyme was added and at time intervals after the addition of the enzyme. Samples were stored immediately on ice to stop the enzymatic reaction. Samples were frozen after no more than 4 h on ice.

2.3. Determination of atrazine concentration

Atrazine concentrations were determined at two independent laboratories; Queensland Health Forensic and Scientific Services (QHFSS), by the LCMSMS method described in Lewis et al. (2009), modified to use direct injection; and by CSIRO Entomology by the following LCMS method. Briefly, 100 mL samples were acidified with HCl to pH 2.8, then the atrazine in the samples was concentrated 1000-fold by solid phase extraction using preconditioned Oasis SPE Max Cartridges (Waters, USA), and eluted in 3 mL of MeOH (with ammonia). Samples were subsequently dried and dissolved in 100 μl of MeOH. Samples were separated by HPLC and assayed for atrazine concentrations by measuring the absorbance at 265 nm, and the analyte peak area calculated using Analyst software. Replicate samples were within 10% agreement. The identity of the HPLC peak was confirmed by mass spectrum analysis, whereby atrazine ions 216 *m*/*z* were extracted on an Agilent ToF–MSD.

3. Results and discussion

The water in the holding dam contained 8–12 μg/L atrazine before the irrigation tailwater was collected (data not shown). After filling with irrigation tailwater the atrazine concentration rose to 157–170 μg/L (Fig. 2). There was a lag in the rate of atrazine depletion after addition of the enzyme, which was most likely attributable to the rate at which the enzyme mixed with the water in the holding dam. The duration of the "mixing phase" for enzyme applied in this manner is almost certainly dependent on the volume and surface area:volume ratio of the water body to be remediated; i.e. larger bodies and those with low surface area: volume ratios would require a longer mixing phase.

Notwithstanding the lag during the mixing phase, the addition of the enzyme led to >90% depletion in the concentration of atrazine in the first four hours after addition. This result suggests that a TrzN-based bioremediant for triazines is technically feasible, although the rate of remediation is slow compared with early trials of the only other enzymatic bioremediant, OpdA. OpdA reduced methyl-parathion concentrations by 10-fold reduction from 84,000 L in less than 10 min (Russell et al., 2001), 24-fold faster