Immobilization of *Pseudomonas* sp. strain ADP: a stable inoculant for the bioremediation of atrazine

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Abstract
Storage and delivery of biological products are fundamental issues determining their effectiveness. For liquid cultures of *Pseudomonas* sp. strain ADP stored at 4 and 25°C, a 1 log reduction in cfu/mL occurs after approximately 4 and 2 weeks respectively. When immobilized onto natural zeolite and stored in open containers survival at 25°C is poor. However, when the cells are immobilized with xanthan gum and stored in closed containers, survival at 25°C is superior to cells stored at 4°C. The type of growth medium, zeolite substrate and immobilization matrix excipients appear to play a role in the stabilisation of *Pseudomonas* sp. strain ADP. The bacterium remained viable and retained its ability to degrade atrazine for the complete test period of 10 weeks at 25°C.

Key Words
Immobilized cells, long-term bacterial storage, bacterial survival, bioremediation, stabilization, formulation.

Introduction

Bioremediation is broadly defined as the utilization of microbes or their enzymes to remove contaminants from soil, water and wastes. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-*s*-triazine) is a herbicide used for broad leaf control and is both persistent in soil and frequently detected in surface and groundwater at levels exceeding maximum permissible concentrations (Jablonowski *et al.* 2009; Tappe *et al.* 2002). Indigenous soil microbes commonly degrade pesticides but sometimes generate persistent degradation products in the environment through incomplete metabolism or transformation (Arbeli and Fuentes 2007; Kolpin *et al.* 2000). *Pseudomonas* sp. strain ADP was originally isolated from a site heavily contaminated with atrazine and uses atrazine as a sole nitrogen source by means of a six-step catabolic pathway (Wackett *et al.* 2002). *Pseudomonas* sp. strain ADP is the model organism for the full mineralization of this *s*-triazine herbicide (Mandelbaum *et al.* 1995). The objective of this work was to evaluate the stability of a *Pseudomonas* sp. strain ADP formulation for long-term storage at ambient temperature (25°C) and retention of degradative ability. This work is part of a larger project aimed at immobilizing the bacterium on a natural carrier (zeolite) for long-term storage and to provide a metabolically active, biological agent for bioremediation of atrazine contaminated soil.

Materials and methods

Chemicals

Technical grade atrazine (99% purity) was received from Trevor James AgResearch Ltd, Ruakura Research Centre, Hamilton, New Zealand. Flowable Atrazine™ (500 g/l atrazine and 50 g/l ethylene glycol) Nufarm NZ Limited, product number 50979-5L was purchased from PGG Wrightson. Miller's Luria-Bertani (LB) base broth and agar were purchased from Merck, Darmstadt, Germany. Xanthan gum was purchased from Danisco, China. Lupi Extra Virgin Olive Oil (Italy) was purchased from local food supply retailers.

Microbial cultures

Pseudomonas sp strain ADP (DSM 11735) was received from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) as a freeze-dried culture. The culture was plated onto atrazine agar (1000 ppm) described by Mandelbaum *et al.* (1995). A single colony was used to inoculate a 250 mL flask containing 100 mL of 100 ppm atrazine liquid medium (MB) described by Mandelbaum *et al.* (1995). After 72 h (25°C, 150 rpm) the cell density was enumerated by plating on LB and was 7.1×10^8 colony forming

units (cfu) per mL. Cells were harvested by centrifugation at 10 g for 15 minutes and then resuspended with a 40% (v/v) glycerol/LB solution. Cells were stored as 100 µL aliquots in 1 mL microcentrifuge tubes at -80°C and served as the source of culture stock for all subsequent experiments.

Pre-cultures were prepared by resuspending a culture stock microcentrifuge tube using 1 mL from a vial containing 15 mL sterile LB broth and returning the entire contents to the vial. Vials were incubated at 30°C and 200 rpm. Pre-culture vials were harvested after 18 h and 500 mL flasks containing 100 mL of LB and MB broth were inoculated with 1 mL (1% v/v) of pre-culture. Cells from flasks were enumerated after 24 h growth on a shaker (200 rpm) at 30°C.

Viable cell count enumeration was performed by duplicate sampling and serial dilutions. 0.1 M phosphate buffer solution was used as the diluent fluid. Triplicate samples of 10 µL were removed from the dilution tubes and plated onto LB agar using the tilt plate technique. Plates were incubated at 30°C for 24 h prior to counting.

Herbicide degradation
We used the clearing zone technique (Mandelbaum *et al.* 1995) modified by substituting technical grade atrazine with Flowable Atrazine in order to confirm that *Pseudomonas* sp. strain ADP retained its ability to degrade atrazine after immobilization and storage. Flowable Atrazine is more easily dispersed in aqueous media and was superior for producing atrazine agar plates of a consistent composition. Plates were prepared with an atrazine concentration of 1000 mg/l. 10 µL samples of cultures were applied to the plate and incubated at 30°C for 48 h. Cultures capable of producing a clearing zone underneath the area of sample application were considered to have degraded the atrazine and thus retained the desired metabolic activity.

Immobilization and stability assessment of Pseudomonas sp. strain ADP
A freshly grown culture of *Pseudomonas* sp. strain ADP was coated onto zeolite according to the process described in WO2008023999 (Swaminathan and Jackson 2008). Two types of natural zeolite carrier were used for the study, designated type I and type II. Additional details are commercially sensitive. Samples of culture were immobilized with and without 4% xanthan gum and olive oil, onto both types of zeolite and stored at 25°C in open and closed 70 mL HDPE screw-cap containers. Surviving cells were counted at the time of sample preparation, after 24 h and weekly thereafter. For comparison, non- immobilized cells were stored at 4 and 25°C and enumerated weekly. To assess the survival of immobilized *Pseudomonas* sp. strain ADP, a 1 g sample was added to 9 g phosphate buffer followed by serial dilution described above. Survival was calculated as the percent (%) cfu/g or cfu/mL at time T compared to the cfu/g or cfu/mL at the time of sample preparation. Results were plotted as the survival (%) against time (weeks).

Results
Short term stability
Immobilized bacteria onto type I and type II zeolite were stored at 25°C in open and closed containers. Bacteria were extracted and enumerated over a 2 week period (Figure 1). *Pseudomonas* sp. strain ADP adsorbed to type I zeolite and incubated at 25°C in closed containers survived in higher numbers compared to both type II and open samples. Poor survival of *Pseudomonas* sp. strain ADP immobilized onto type II and open samples is shown by a greater than 1 log loss in cfu/mL after 24 h (Figure 1). In type I open samples and in both type II samples the bacteria were undetected on LB plates by week 2.

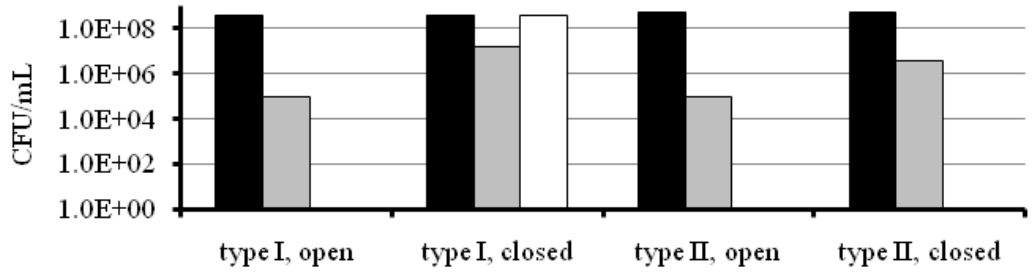

Figure 1. Short term stability of *Pseudomonas* sp. strain ADP associated with two forms of zeolite stored at 25°C in open or sealed containers. Time zero (■), 24 hour (▒), 2 weeks (□)

Stability of different zeolite types
In a longer-term experiment the type of zeolite was again found to influence the stability of *Pseudomonas* sp. strain ADP in closed containers, with at least 10 weeks survival and less than 1 log loss in population found for type I compared to less than 2 weeks for type II (Figure 2).

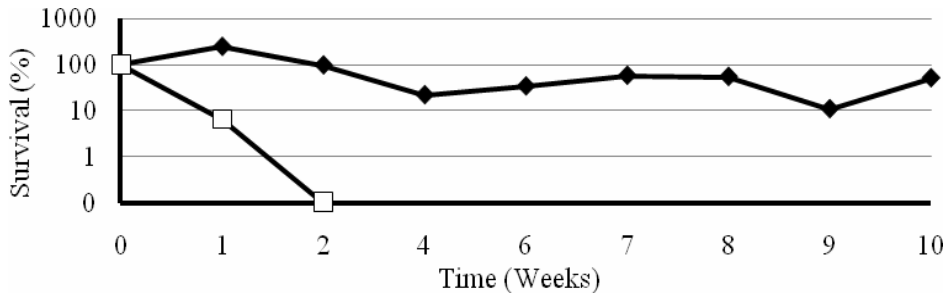

Figure 2. Survival of *Pseudomonas* sp. strain ADP immobilized with xanthan gum onto zeolite type I (♦) and type II (□) and stored in sealed containers at 25°C.

Effect of growth medium and xanthan
The type of growth medium and the excipient xanthan gum influenced the long-term stability of *Pseudomonas* sp. strain ADP cells (Figure 3). *Pseudomonas* sp. strain ADP cultured using LB and coated with xanthan gum remained within 1 log of the initial enumeration (%) for the total test period of 10 weeks. *Pseudomonas* sp. strain ADP cultured using LB and coated without xanthan or cultured using MB with and without xanthan remained within 1 log for only 7 to 8 weeks at 25°C.

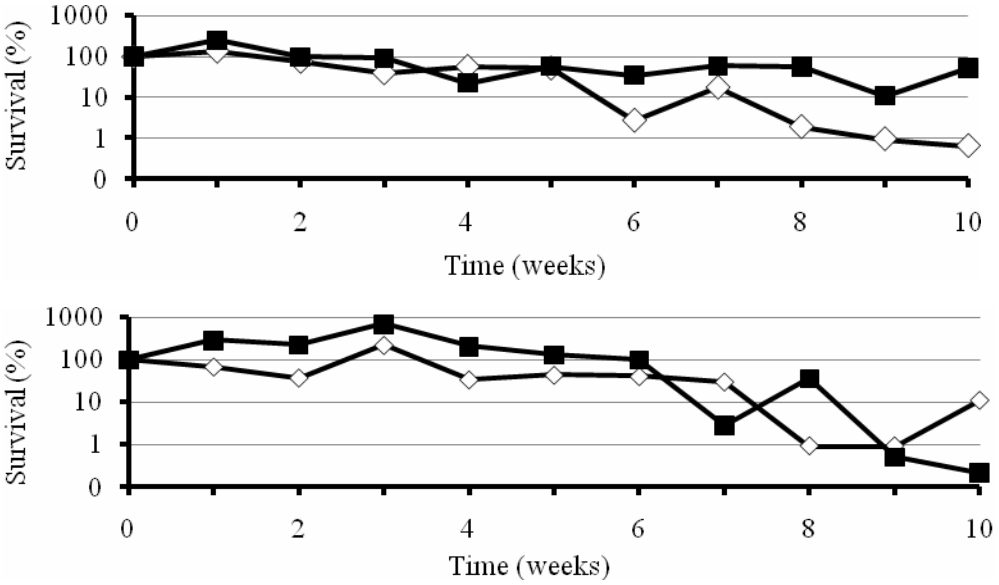

Figure 3. Average (n=2) stability for *Pseudomonas* sp. strain ADP cultured using LB (upper figure) or MB (lower figure) medium and immobilized onto type I zeolite with (■) and without (◇) xanthan gum and stored in sealed containers at 25°C.

Stability in various storage conditions
For LB cultures stored as a liquid broth at 4 and 25°C, a 1 log reduction in survival was found after approximately 4 and 2 weeks, respectively (Figure 4). When the culture was immobilised onto zeolite type I without xanthan and stored in a closed container, the survival at 25°C was the same as for culture alone stored at 4°C. For *Pseudomonas* sp. strain ADP immobilized onto zeolite type I with xanthan gum, the survival at 25°C was improved and the cells remained viable for the complete test period of 10 weeks.

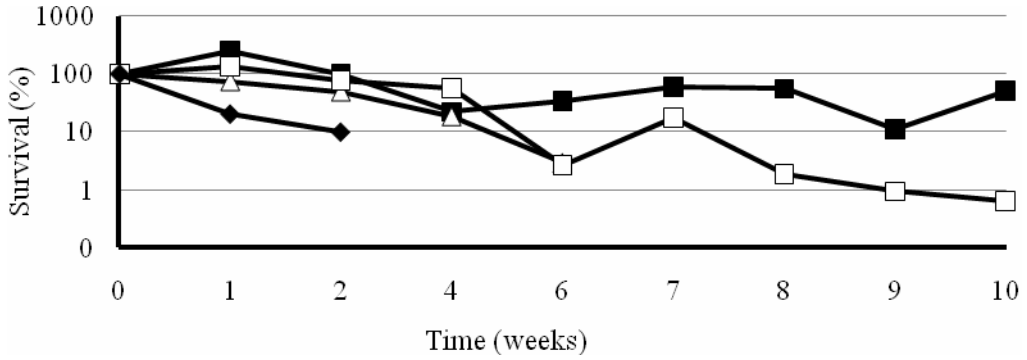

Figure 4. Survival of *Pseudomonas* sp. strain ADP cultured in LB broth stored at 4 (△) 25°C (◆), and LB broth immobilized on zeolite type I with (■) and without (□) xanthan in closed containers at 25°C.

Discussion

Pseudomonas sp. strain ADP was immobilized successfully onto a zeolite carrier. By providing appropriate formulation and packaging, *Pseudomonas* sp. strain ADP remained viable and retained its ability to degrade atrazine for the complete test period of 10 weeks at 25°C. The type of growth medium, zeolite substrate and the immobilization excipients were found to play a role in the long-term stabilisation and survival of *Pseudomonas* sp. strain ADP. Current work will evaluate the stability and atrazine degrading activity of the formulation in contaminated soils.

Acknowledgements

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