INOCULANT PRODUCTION AND FORMULATION OF *Pseudomonas* sp. strain ADP

A thesis
submitted in the partial fulfillment
of the requirements for the Degree
of
Doctor of Philosophy in Chemical and Process Engineering
in the
University of Canterbury
by
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University of Canterbury
2011

Abstract

In this work, a model microbial agent for bioremediation was improved using fermentation and formulation methods. The outcomes of the fermentation work include the development of a new culture medium which increased the cell productivity greater than one order of magnitude. A robust functionality to degrade the herbicide atrazine was expressed. The new medium was scaled-up to a 2L bioreactor.

Liquid bacterial culture was not inherently stable and lost viability at both 4°C and 25°C storage. When liquid bacterial culture was formulated by encapsulation in a biopolymer gel and applied to zeolite the transfer of cells from bacterial culture to formulated carrier was highly efficient. No loss of viability was measured from the immobilization process, and the functionality of the agent was retained. The formulated agent expressed an extended shelf life of at least 10 weeks when stored in ambient (25°C) temperature.

When the formulation granules were inoculated into sterile soil, viability of the granules was stable and also retained the maximum level of functionality for the full test period of 10 weeks. The soil surrounding the formulation granules was also enumerated. The number of cells in the soil increased after a single inoculation of the formulation and the maximum level of functionality was conveyed from the formulation to the surrounding soil.

The formulated inoculant constitutes an improvement for a bioremediation strain to stabilize the agent, produce an extended shelf life at ambient temperatures, and maintain the functionality of a microbe to utilize atrazine. In this thesis we have used a biopolymer formulation in which an inoculum is simply mixed into a gel and applied directly to the surface of the zeolite with no special equipment, drying, temperatures, or secondary re-growth steps required. It is a simple model system consisting of a carrier, and a artificial biofilm. As a technique to produce stable functional inoculants for bioremediation, the work presented here demonstrates an approach that is simple, practical, effective, and robust.

Dedication

This work is dedicated to my wife, daughter and son; Rachel, Mackenna, and Owen. When there are a multitude of reasons to quit, there are just a few which keep us going forward.

Acknowledgement

Previous to beginning this mid-life challenge I worked on the fishing fleet in the Bering Sea, the oilfields of Texas and Oklahoma, as a bioremediation contractor for contaminated soils and groundwater, managed large employee numbers, started 3 businesses, and served as the Operations Manager of two Hazardous Waste Treatment Facilities in Oklahoma and Ohio. More importantly, I also served as a coach, captain and mentor for 20 years. The memory of the people from these endeavors, along with family and friends, inspired me to persevere through this PhD process. Notably it was also the inspiration of the thousands of young athletes I once coached and often instructed to "never quit".

My deepest appreciation goes to Grandfather Bill Swanda (April 9, 1922 to Feb. 14, 2010) who gave his blessing for me to leave the Country, move to New Zealand, and attempt this degree. 'So long Cowboy'. Now you can ride as far as you wish in a place with no fences.

Without measure I am thankful for the contribution my parents have made. Raymond and Diana were raised in the remnants of the Dust Bowl in southwestern Oklahoma. It exemplifies the worst of Native American Reservations and remains a place of poverty and sadness. By escaping they gave their children a chance. They instilled in me the virtues of perseverance, giving my all, and making something out of nothing.

I owe Dr. Anwar Sunna a large debt of gratitude for his kindness, and clear observations. A well timed compliment and a few words of empathy can make all the difference. Similarly I thank Dr. Richard Burns for observing that the project was organized backward and his advice to "start in the soil first, and ferment last". Both Anwar and Richard provided much needed, and appreciated, mentoring and feedback during the preparation of our publications.

There are a number of people at AgResearch who I owe many thanks, Jayanthi Swaminathan for her knowledge of formulations, David Wright for his fermentation expertise, Sandra Young for genetic typing, Sandra Jones for always knowing the

right answers, Celine Blond for her kind donation of soil and conversation, and Rebecca “Bex” Kenny for keeping things stirred up in a good way.

With great appreciation I would like to thank Dr. Craig Robert Bunt for his optimism, unreserved support, and positive spirit of collaboration. The progress and success of this project would simply not have been possible without his involvement. Craig had ample reason and opportunity to leave me early-on in the PhD process. Yet he remained alongside through the morass, like a Virgil to my Dante as we performed a farcical rendition of *The Inferno*. He was the ideal companion for my journey. For his endless sacrifices I have thanked him, and apologized to him, for the last three years. It was my exceptional privilege to share space with a person of consistent kindness, principle, and courage.

Lastly, I acknowledge my gratitude to the elements which made my PhD experience especially abnormal. Whether necessary or not, each requirement tasked me to go the hardest way possible and in the end I profited greatly. In the fullness of time I came to appreciate that these elements had required my most positive and strongest aspects. It forced this old captain to be uncommon again. For that I am grateful.

Table of Contents

List of Figures

List of Tables

List of publications

Publications arising from this thesis:

Refereed papers

Stelting, S., Burns, R., Sunna, A., and Bunt, C., Survival in sterile soil and atrazine degradation of *Pseudomonas* sp. strain ADP immobilized on zeolite, Submitted (2013)

Stelting, S., Burns, R., Sunna, A., Visnovsky, G., and Bunt, C., "Immobilization of Pseudomonas sp. strain ADP: a stable inoculant for the bioremediation of atrazine", *Applied Clay Science*, Special Issue: 19 WCSS (2010)

Stelting, S., Burns, R., Sunna, A., Visnovsky, G., and Bunt, C., "Immobilisation of Pseudomonas sp. strain ADP: an approach for bioremediation of herbicides", *Proceedings of the symposium on MICROBIAL PRODUCTS Exploiting Microbial Diversity for Sustainable Plant Production,* (2010) 97-102.

Refereed full conference papers

Stelting, S., Burns, R.G., Sunna, A., Visnovsky, G., and Bunt, C.R., "Characterization of stabilized formulations of the atrazine degrading bacterium Pseudomonas sp. strain APD", *Proceedings of the International Symposium on Controlled Release of Bioactive Materials*, 37 (2010) 2pp.

Stelting, S., Burns, R., Sunna, A., Visnovsky, G., and Bunt, C., "Immobilization of Pseudomonas sp. strain ADP: a stable inoculant for the bioremediation of atrazine", *World Congress of Soil Science*, 19 (2010) 4pp.

Refereed conference abstracts

Bunt, C., **Stelting, S.,** Wright, D., and Swaminathan, J., "Preformulation characterisation of zeolite core materials in biocontrol products", *New Zealand Plant Protection* 63, (2010), 284.

Stelting, S., Visnovsky, G., and Bunt, C., "A room temperature stable Pseudomonas ADP formulation for bioremediation", *Australasian Pharmaceutical Sciences Association*, (2009), Hobart, Australia

Stelting, S., Visnovsky, G., and Bunt, C., "Bioremediation of herbicides", Microbial Products: Exploiting Microbial Diversity for Sustainable Plant Production, *New Zealand Plant Protection Society Pre-conference Symposium*, (2009), Dunedin, New Zealand.

1 Introduction

1.1 Bioremediation using microbes

There are a variety of substantial problems which can be approached with microbial solutions. Examples include insect pest control, fungal pathogen control, seedling inoculation and plant growth enhancement. Additional microbial utilizations include the increased availability of nutrients such as nitrogen and phosphorous, and the removal of contaminants from soils and waters (Bashan, 1998; Paau, 1988; van Veen *et al.*, 1997). The contaminations in our soils and waters are of mostly human origin and there remains a responsibility to address and repair our damage. One approach to this obligation is bioremediation. Bioremediation can be broadly described as the utilization of microbes to remove contaminants from soil, water, and wastes. A recent review of the considerable breadth of bioremediation has been published (Juwarkar *et al.*, 2010). A wide range of target contaminants can be metabolised (removed) by many microbial species, Table1.

Table 1. Microbial species and target contaminants they have been reported to be able to metabolise.

Microbial species	Target contaminant	Reference
Alcaligenes spp	Halogenated hydrocarbons, linear alkylbenzene sulfonates, polycyclic aromatics, polychlorinated biphenyl	(Lal & Khanna, 1996)
Arthrobacter spp	phenol, organophosphorus, nitriles, cyanide, benzene, hydrocarbons, pentachlorophenol, phenoxyacetate, polycyclic aromatics	(Alexander, 1999; Jogdand, 1995)
Azotobacter Spp	Aromatics, branched hydrocarbons, benzene, cycloparaffins, hydrocarbons	(Dean-Ross *et al.*, 2002; Jogdand, 1995)
Bacillus spp	Aromatics, long chain alkanes, phenol, cresol, halogenated hydrocarbons, phenoxyacetates	(Cybulski *et al.*, 2003)
Corynebacterium spp	Aromatics	(Jogdand, 1995)
Flavobacterium spp	Naphthalene, biphenyl, aromatics, branched hydrocarbons	(Jogdand, 1995)
Mycobacterium Spp	Hydrocarbons, polycyclic hydrocarbons	(Jogdand, 1995; Park *et al.*, 1998)
Nocardia spp	Phenoxyacetate, halogenated hydrocarbon diazinon	(Jogdand, 1995)
Phanerochaete spp	Chlorophenols	(Singh, 2006)
Pseudomonas spp	Atrazine, pentachlorophenol, benzene, anthracene, hydrocarbons, polychlorinated biphenyl	(Biglione *et al.*, 2008; Clausen *et al.*, 2002; Cybulski *et al.*, 2003; Desouza *et al.*, 1995; Garcia-Gonzalez *et al.*, 2005; Garcia-Gonzalez *et al.*, 2003; Klein *et al.*, 2009; Lima *et al.*, 2009; Mandelbaum *et al.*, 1995; Newcombe &

		Crowley, 1999; Rietti-Shati *et al.*, 1996; Sadowsky & Wackett, 1999; Zhao *et al.*, 2003) (Cassidy *et al.*, 1997)
Rhodococcus spp	Atrazine, aromatics	(Vancov *et al.*, 2005; Vancov *et al.*, 2007)
Trametes spp	Chlorophenols	(Singh, 2006)
Variovorax spp	Linuron	(Owsianiak *et al.*, 2010)
Xanthomonas spp	Polychlorinated biphenyl, polycyclic aromatics, biphenyls	(Jogdand, 1995)

Agricultural chemical like herbicides are one source of contamination in soil, surface water and groundwater. Atrazine ($C_8H_{14}ClN_5$, 215.68 g/mol) (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). A high incidence of global contamination has prompted researchers to consider its long term effects and solutions (Govantes *et al.*, 2010). The bacterium *Pseudomonas* sp. ADP (P.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. ADP has become a reference strain for the bioremediation of atrazine and has been extensively studied (Ralebitso 2002).

A large number of bacteria and fungi and their catabolic enzymes have been screened for their potential to degrade environmental contaminants. The white rot fungi such as *Trametes versicolor* and *Phanerochaete chryosporium* (Singh, 2006) are active against chlorophenols and have been the subject of much research. Bacterial strains (e.g. *Arthrobacter citreus*) also have been heavily researched (Alexander, 1999) and have capabilities for utilizing phenol, organophosphorus, nitriles and cyanide.

These bioremediation studies have been primarily conducted in laboratory settings using a single species or type of organism. In these controlled settings the ability of bacteria and fungi to degrade many environmental contaminants is well proven. Thus the potential of microorganisms to degrade organic pollutants is well accepted.

Although the capability of microbes to degrade contaminants at lab scale is well accepted the utilization of the microbes in field scale settings remains controversial. Indeed there is significant controversy "between academic scientists and engineering practitioners"(Prince, 1998), especially with regard to the area of bioaugmentation. This issue is further discussed and is summarized by (Singer *et al.*, 2005) as, " inoculum survival remains the 'Achilles heel' for bioaugmentation of contaminated land". Without academic proof of inoculum proliferation in the field soil ecosystem, the controversy continues. In addition to the controversy of bioaugmentation, bioremediation as a method of contaminant removal is limited in some ways by scientific merit, but more so by its application at a larger scale. Many of the challenges of bioremediation, and bioaugmentation, are thus problems of delivering a science from the lab to the field.

1.1.1 Enhanced or Accelerated biodegradation

While a lack of academic acceptance for bioremediation has limited its acceptance at the field scale level, the actual research of bioremediation is seldom conducted at the field scale. Fortunately agricultural scientists have described a phenomenon that is functionally identical to bioremediation. The effect is known as Enhanced or Accelerated biodegradation and is a good example, at field scale, of microbial ability to remove pesticides from soil. The phenomenon was first noted in the 1940's and is well known to both agricultural soil scientists and pesticide manufacturers (Felsot, 1989).

Enhanced or Accelerated biodegradation is a natural acclimation of soil bacteria to repeated applications of a type, or family, of pesticides. It is known that modern pesticides are degraded by indigenous soil microbes. When soil microbes are exposed to repeated applications of pesticides they respond by increasing, or accelerating, their removal of pesticide in the soil (Shaner *et al.*, 2007). For agriculturists, the accelerated removal of the herbicide used for weed protection is viewed negatively and the increased removal rate kinetics can render the application of pesticides uneconomic (Arbeli & Fuentes, 2007). Felsot (1989) describes microbial degradation of pesticides as a double edged sword, on one hand reducing the environmental hazards and on the other hand producing ineffective pest control.

Enhanced biodegradation is a microbial adaptation response and an unintended consequence of a chemical application. Microbes are widely known for the ability to adapt and respond to

new circumstances. Another well-known example includes microbial adaptation to antibiotics and the appearance of antibiotic resistant strains (Neu, 1992). These unintended responses illustrate that microbial abilities are innately effective. For those who would develop microbial technology like bioaugmentation, enhanced degradation is a field-scale demonstration that microbes are naturally capable of prosperous activity and contaminant removal outside of the laboratory.

1.1.2 Bioaugmentation

Bioaugmentation is a bioremediation method that inoculates microbes into the remediation site. The inoculants for bioaugmentation are produced in controlled conditions where consistent outputs of quality and density are possible. As introduced in 1.1, it remains a controversial practice. The justification for augmenting degrader organisms to a contaminated property is to create a more immediate response to the contaminant and to produce an overall more rapid rate of mineralization (Singer *et al.*, 2005). Reviews of bioaugmentation have been produced (Gentry *et al.*, 2004; vanVeen *et al.*, 1997; Vogel, 1996).

The principle weakness of bioaugmentation is inoculum survival in live soil (Singer *et al.*, 2005). Live soil is a competitive, inhibitory, and predatory ecology to microorganisms, and inoculants frequently disappear from soil soon after their introduction (vanVeen *et al.*, 1997) (Gentry *et al.*, 2004). The lack of reliability for inoculated organisms to survive, proliferate, and remain active in live soils is also described by Alexander (1999).

The practice of bioaugmentation has uses other than bioremediation and there are a variety of potential applications for augmentation of microbial inoculants. Traditional inoculant products include *Rhizobium* for legumes, probiotics, silage, and inoculants for food and drink fermentation. Agricultural uses include plant protection by pest and pathogen control, plant growth stimulation by both seedling inoculation and hormone production, and the improvement of both soil structure and the increased availability of nutrients such as nitrogen and phosphorous (Bashan, 1998; Paau, 1988; van Veen *et al.*, 1997). The use of microbial agents for plant protection is driven by the emergence of new or expanded restrictions placed upon current chemical control agents (Bashan, 1998; Gerhardson, 2002; Lewis & Papavizas, 1991). The potential role of microorganisms, for these and other beneficial processes, is limited by their survival and proliferation in soil (Bashan, 1998; van Veen *et al.*, 1997).

There are a variety of mechanisms considered to be the causes for decline in inoculant activity when applied to soil. Chemical and physical factors which can induce stress on the inoculant include pH, temperature and moisture availability. In general, the chemical and physical factors can be identified and minimized where needed at lab and field scale. A lesser understood, and more difficult to address mechanism of inoculant limitation is the biotic stress from predation, competition and inhibition due to the indigenous soil biology.

The importance of biotic factors is clearly observed when inoculants introduced into sterile soil do not exhibit a decline in population magnitude(van Veen *et al.*, 1997). In a sterile soil environment population size and activity can increase. This effect highlights the importance of biotic forces as the prime limitation to the successful introduction of microbial agents. To overcome these biotic limitations, soil inoculants are in need of improvement

The approach of protecting inoculated microbes from the biotic influences of soil has been investigated previously. Alginate has been commonly utilized for encapsulation, yet due to material and scalability issues has yielded little, if any, commercial application(Bashan, 1998). From a methodological standpoint, the survival of alginate encapsulated cells in soil was reported to be higher than unencapsulated cells for 9 weeks (Trevors *et al.*, 1993). Cells encapsulated by this method were considered less sensitive to moisture fluctuations in soil.

Conventionally, the bioremediation approach to bioaugmentation has utilized a once-only, single application of the inoculant as a liquid bacterial culture or irrigation additive. Commonly this approach produces poor survival of inoculant, the rapid loss of inoculants activity, and an unreliable reputation of bioaugmentation. An alternative approach is to apply the liquid which contains the inoculant more than once. The logic of using a repeated application approach is in providing "excess, active, degrader organisms" (Singer *et al.*, 2005).

The utilization of repeated applications of degrader organisms rather than the conventional single application was described as a successful improvement for a liquid inoculant type (Newcombe & Crowley, 1999). *Pseudomonas* sp. strain ADP and an atrazine degrading consortia were utilized at microcosm and field scale. When *Pseudomonas* sp. strain ADP was applied every 3 days in soil microcosms containing 100 ppm atrazine, 72% and 90% of the atrazine was removed in 18 and 35 days respectively. A single application of *Pseudomonas*

sp. strain ADP removed 36% over the same time period. Similar results were reported at field scale with 72% atrazine removal in 11 weeks after 8 applications of an atrazine degrading consortia. The author's results suggest *degradation activity rapidly declines when soils are treated with only a single inoculation of atrazine degraders*. The repeated application of an inoculant has achieved the greatest results for bioaugmentation (Singer *et al.*, 2005).

Soil survival can be enhanced by repeated delivery of liquid inoculants (Newcombe & Crowley, 1999; Silva *et al.*, 2004) and also formulation (Bashan, 1998; Lewis & Papavizas, 1991). In order to produce either liquid inoculants or formulation requires the fermentation of the microbial strain to a useful cell concentration. Fermentation technologies are available to "maximize the volumetric productivity" ($g\ L^{-1}\ h^{-1}$) for products such as single-cell protein (Riesenberg & Guthke, 1999). Fermentation can produce cell numbers in the order of $>10^{10}$ colony forming units (cfu)/ml.

To be identified as a candidate agent (inoculant) a microbe must express a suitably specific functionality for a beneficial task. In general, these microbial inoculants display stability and delivery challenges, with perhaps the only predictably stable bacteria being Gram-positive spore formers (Emmert & Handelsman, 1999). A challenge to naturally instable microbial products is to retain functionality, and an effective dose titer, while being delivered into soil, water, or other inhospitable targets. Such challenges can be overcome or partially addressed by formulation (Bashan, 1998; Lewis & Papavizas, 1991).

Formulation methods to overcome performance constraints include protecting the cells by immobilization and encapsulation (Cassidy *et al.*, 1997; Gentry *et al.*, 2004; Vancov *et al.*, 2007). The methods of immobilization and encapsulation have had little application in bioremediation or bioaugmentation (for contamination). These formulation style methods have the potential to increase the survival and activity of the inoculant by protecting the cells from the competitive, inhibitory and predatory soil environment. Dispersal alternatives to the liquid delivery of microbial based bioremediation products are needed but have received little published attention.

1.1.3 Bioremediation failure

In reviewing decades of bioremediation research the conspicuous question that remains is the reason for bioremediation inconsistency and failure. While provocative, this author suggests

that the foundations of the science are currently incapable of answering such a question. To highlight the point a discussion of bioremediation standards will be discussed.

The design of an appropriate bioaugmentation experiment in a field soil environment requires an understanding of the parameters that control the bioaugmentation process. Unfortunately, the critical parameters for the design of a bioaugmentation process are, as yet, not defined. As is the case with other sub-visible microbial processes (i.e. fermentation and formulation), microbial application work at the field scale is largely an empirical process. To address the difficulties of characterizing, quantifying and evaluating bioaugmentation research, Vogel (1996) suggests using standardized parameters for evaluating a bioaugmentation inoculum in soil. They are:

1. Pollutant characteristics
   Bioavailability, concentration, and microbial toxicity.
2. Soil physico-chemical characteristics
   Humidity, water content, organic matter content, clay matter content, and pH.
3. Microbial ecology
   Presence of predators, interspecies competition.
4. Microbiology
   Presence of co-substrates, genetics of relevant organisms, enzyme stability and activity.
5. Methodology
   Inoculation concentration, method of inoculation, the presence/absence of indigenous activity, and inoculum heterogeneity.

To initiate a hypothesis about the reason for bioremediation failure, the Methodology section (number 5 above) is recommended. The first four standards are supported in the literature from the fields of chemistry, agriculture, and microbiology. However, the fifth item, methodology, is the frontier of the undeveloped. For example, a standard inoculum type does not exist in the literature. Importantly, a *stable* control inoculum type does not exist in the literature. More importantly, a *stable* control inoculum type has not been defined, or applied, or quantified in either lab or field settings. Herein is a vital limitation. The foundations of delivering the science of bioaugmentation, the methodology and application in the field, are currently not developed. To that end this thesis is aimed.

In further consideration of bioremediation failure, it may be illuminative to observe that bioremediation has had success in any aspect. As a process, bioremediation lacks a standard inoculum type, a standard inoculum rate, standardized application methods (tillage, watering, moisture levels, nutrients, soil contact and depth). Yet there are successes. Furthermore, the conventional liquid inoculum type is inherently unstable and the conventional academic treatment regime is to apply the inoculum only once. Yet there are successes. Fortunately, it is now academically accepted that soil survival can be enhanced by repeated delivery of liquid inoculants (Newcombe & Crowley, 1999; Silva *et al.*, 2004). A case can be easily made that bioremediation, especially bioaugmentation, is designed to fail without a coherent, and scientific, methodology.

1.1.4 Bioremediation Hypothesis

Aside from methodology weaknesses, the author's hypothesis for bioremediation failure is directed primarily at the applicant…the human element, the person delivering the treatment. From the author's perspective, the applicant is the least accountable of the bioremediation approach and has the greatest impact. Except for the microbial culture, the other factors in a bioremediation approach are conditions. Conditions are elements addressed in the treatment design. Conditions are aspects that can be controlled. The physical conditions are dealt with first, then the chemical conditions, followed by the biological conditions. Lastly, is a stimulation or introduction of a microbial culture. This final step is a development or inoculation of microbial products. A well designed bioremediation follows that order. However, there is no accounting for the applicant in the design. This hypothesis is important for why this project was conducted.

Placing bioremediation failure as the responsibility of the product applicants is not a criticism. It is unscalable to expect that every technician in the field would hold a PhD in bioremediation, and that is a weakness of biological technology in general. Biological technology requires a high degree of technical information and skill to achieve consistent results. Liquid inoculants are not stable and need a high degree of proficiency in application. For a highly skilled applicant, liquid inoculums have produced good outcomes. However, in the hands of a less well trained applicant the inherent weaknesses of a liquid microbial culture are a clear limitation to success.

The person in the field needs a more robust technology to be successful. A manager in the office requires a person in the field which has an inoculant technology that is easy to apply, remains viable, retains its functionality to do a beneficial service, carries an effective payload of material which is stable, remains storable without loss of functionality, and can be quickly scaled up to large batches in the downstream production area. Therefore, to contribute to the methodology of the bioremediation science, *this thesis is about inoculant production and formulation*. This work seeks to make the microbial agent more robust and stable and therefore less reliant on the applicant.

In an attempt to frame the authors hypothesis, this research seeks to answer the questions the author had from 15 plus years of working in bioremediation (personal communication). Namely, how to improve liquid inoculants? They are not stable. In Industry, the author's company was forced to pour our inoculants into the sewer after 30 days in refrigerated storage due to loses in viability. To be obvious, that is not an ideal business model. That experience has brought the author to this thesis research. Inoculant stability needs improvement for storage and field application. It is this author's opinion that the applicant needs the most help, and an improved inoculant is one way to provide that help. Many times the author has witnessed a technician with liquid inoculant in their truck left over from the previous week. Unfortunately the applicant did not understand the nature of a living liquid and how it differs from a container of chemicals. The inoculant baked in the sun with no oxygen and no refrigeration for the full weekend. The manager in the office expected that that inoculum would work. Unsurprisingly, it would not. When the inoculum is not handled, conditioned, or presented to be successful, the bioremediation project has little chance. The reputation of bioremediation is that it is inconsistent. That inconsistency is driven by a lack of robustness in the biological component…the microbial agent. This work seeks to make the microbial agent more robust and stable.

In a final attempt to develop this hypothesis the author will make an overtly simplistic and necessary example. Bioremediation has a multitude of similarities with agriculture, turf production, and gardening, but there is one obvious difference. Bioremediation does not have a stable seed. For agriculture, turf production, and gardening, a stable seed is the fundamental element. The seed is the cornerstone for describing the methodology and application in the field. Without a stable seed as a starting material each of these developed sciences would likely be analogous to bioremediation; fraught with inconsistency and failure. This primary

essential building block is absent in bioremediation. There is no stable seed from which to develop a methodology.

1.1.5 Novelty

The reader is correct to conclude that the basis for Section 1.1.3 and 1.1.4 is largely conceived on hypothesis and personal experience. The author agrees. It is an inherent challenge to reference work that has not been done previously. To that end the author concedes that the results of this work must speak for themselves. In further sections the thesis will resume a traditional referencing format as the technical aspects of fermentation and formulation are discussed.

1.1.6 A Model system

To facilitate an approach for producing a "stable seed" material, a model system will be utilized. A model system allows the work conducted here to be set within a well-developed framework of previous investigators.

The model system for the current work consists of the herbicide atrazine and the bacterium *Pseudomonas* sp. ADP (P.ADP). Atrazine ($C_8H_{14}ClN_5$, 215.68 g/mol) (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). A high incidence of global contamination has prompted researchers to consider its long term effects and solutions (Govantes *et al.*, 2010). The bacterium *Pseudomonas* sp. ADP (P.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. ADP has become a reference strain for the bioremediation of atrazine and has been extensively studied (Ralebitso 2002).

A further benefit of using a model organism and a widespread herbicide to test the approach of this research was to avoid the intellectual property issues which restrict publication in this field.

1.2 Fermentation

1.2.1 Medium development

A prime factor in any fermentation process is the fermentation medium. The medium is vital to research and critical at the industrial scale (Zhang & Greasham, 1999). During the fermentation, the medium supplies the nutritional needs of the microbes and is utilized for the production of biomass, and metabolite biosynthesis. The components that comprise the fermentation medium therefore directly affect the process, productivity, and economics.

The components of a fermentation medium are based on the nutritional requirements of the microbe. Microbes incorporate the medium into new biomass and the general composition of cells can be useful to determine their elemental requirements. The mass of microorganisms is measured in fresh and dry weight. The fresh weight is between 70 and 85% water. Dry weight is 15 to 30% of the fresh weight and typically consists of 50% proteins, 10-20% cell-wall materials, 10-20% RNA, 10% lipids, and 3-4% DNA. As a percentage of bioelements, the elemental composition of a microbe is roughly 50% carbon, 20% oxygen, 14% nitrogen, 8% hydrogen, 3% phosphorous, 1% sulfur, 1% potassium, 0.5% calcium, 0.5% magnesium, and 0.2% iron (Stolp, 1988). The first six of these macroelements (C, O, N, H, P, S) represent roughly 96% of the dry matter of many microbes.

The nutritional components required to fulfill the organism's requirements can be supplied by complex or defined medium sources. Complex or undefined media contain ingredients which have unknown or ill-defined specific composition. Examples include caseins, peptones, and yeast extract which provide a number of undefined vitamins and amino acids. Other examples of complex medium can be raw materials and wastes. The most often utilized raw material (complex medium) for fermentations are carbohydrates including sucrose, molasses, glucose and dextrin (Peters, 2007). At the industrial scale these complex raw materials are the typical fermentation carbon source.

In contrast to complex media, a defined medium has a chemical composition that is well known. Complex medium (as a raw material or a waste product) may be less expensive but there can be a number of advantages for developing and utilizing a chemically defined media. Some of those potential advantages are (Zhang & Greasham, 1999):

- equal or higher fermentation productivity.

- enhanced process consistency.
- better control and monitoring.
- improved scale-up.
- simplified downstream processing.

It is important to emphasize that there are potentially many benefits to the overall fermentation process by choosing a defined medium. These benefits can outweigh the material price advantage of using a raw or complex medium.

The "development" of the components in a fermentation medium is the pursuit of performance improvement. As a process, it endeavors to determine the nutritional factors, growth, production, operational conditions, and downstream processing for a specific micro-organism as influenced by the medium. Developing the composition of the fermentation medium is inseparably linked to improving the overall performance of fermentation. It is therefore a process critically important for product concentration, yield and productivity (Kennedy & Krouse, 1999). Notably, the process is laborious, expensive, open ended, time consuming and involves many experiments (Kennedy & Krouse, 1999).

Prior to medium design there are two important topics to be addressed. For scenarios where the microbial strain is not known; what will be the effect of the medium design on strain selection. When the microbial strain is known but the medium is undeveloped; what is the effect of strain selection on medium design. Knowledge of specific needs of an individual strain can then be incorporated into the design and improvement.

A second important predevelopment question is defining the target variable for improvement. Target variables can be productivity, cost, yield, or some other criteria. Setting a clearly defined goal and appropriate target level is essential before beginning the design process (Kennedy & Krouse, 1999). As noted previously, medium improvement is a process that is open ended and has no ultimate solution, and stopping at a defined target level can avoid a lot of unnecessary effort.

1.2.2 Initial development and Formulation

In developing a fermentation medium, the aim is to provide for the needs of the microbe. To begin the improvement process an initial medium is needed. There are numerous methods to determine the general components necessary for the fermentation medium. One method is to

determine the medium by evaluating the composition of microbial cells. For applications where the medium is primarily used to produce microbial cells (biomass) the components can also be estimated by stoichiometry(Atkinson & Mavituna, 1983). Although there are certainly differences between bacteria, yeast and fungi, the elemental composition of most microorganisms is quite similar (Greasham & Herber, 1997).

As discussed earlier, microbial cells are comprised of 70-85% water, with 96% of solids as macroelements (C, H, O, N, P, and S) by dry weight. Some generalizations are required when using the chemical composition of the microbe to roughly formulate the initial medium requirements. A carbon source and concentration can be used as a starting point because in conditions without a carbon source, growth is not expected. The concentration of carbon can be estimated by noting that cells are composed of 50 % carbon, and the conversion of glucose to cell mass is roughly 50%. In general terms the production of a cells of 10 g/l (dry cell weight) will require a minimum of 20 g/l glucose. Nitrogen can be provided as ammonium ion form as it is the preferred nitrogen for almost all bacteria (Merrick & Edwards, 1995). Traditionally the initial medium development is conducted in shake flasks without pH control. In addition to the carbon and nitrogen sources of the initial medium, a pH of 6-7.5 with buffering is supplied for bacteria.

A classic approach initiates the medium development by using a group of components (Glucose, NH_4, Mg, Na, K, Cl, SO_4, PO_4) that are the basic requirements of heterotrophs (Moat, 1979). Using this approach as a starting point for medium improvement, a crude selection of trace elements is usually sufficient to provide a satisfactory level of growth (Zhang & Greasham, 1999).

However, it is not always necessary to design and formulate a medium from inception. One often utilized starting point for medium development is to “borrow” a medium that has been previously used to produce the same or similar micro-organism. The borrowed medium is then improved further. In section 3.1 a new medium is developed using this technique, using a borrowed medium as a starting point for improvement.

1.2.3 Improving the Medium

There are a number of techniques to improve fermentation medium performance. As a general strategy they can be separated into three categories; procedural, design, and optimization.

1.2.3.1 Procedural

The procedural strategies to improve fermentation medium include borrowing, component swapping, biological mimicry and one-at-a-time (Kennedy & Krouse, 1999). Borrowing consists of using a medium from other work that appears to have promise. Component swapping is a useful screening technique which 'swaps' one component for another and evaluates the differences in performance.

One-at-a-time is the traditionally most popular improvement procedure. It is simple, easy and convenient. Performance improvements are pursued by changing a single variable and holding the others at a fixed point. In Kennedy and Krouse (1999):

- Its major detriments are that optimum values can be missed, component interactions are ignored, and it involves a large number of experiments.
- The significant advantage of the technique is that the effects of the components can be directly observed graphically and does not require statistical analysis.

A systematically structured one-at-a-time procedure can include the functions dilute, delete, and concentrate. The performance of the fermentation is evaluated by diluting, deleting, and concentrating the individual component. Other components are held at a fixed point. As is the case with one-at-a-time techniques, it is a labor intensive process. When conducted for each component of the medium, some interactions are observable, and optimums can be framed within in a range. In section 3.1, a systematically structured one-at-a-time procedural improvement is followed, using dilute, delete, and concentrate variations.

1.2.3.2 Experimental design

Experimental design was developed as a structured planning (design) phase for testing variants, replicates, and controls. Rather than testing only one factor, a multiple of factors are chosen and experimentally evaluated at the planned intervals or ranges. The theory of the procedure is that changing one factor at a time is less efficient than changing multiple factors at a time.

One reason for using the experimental design approach is to reduce the number of experiments to answer a particular question. However, the technique does require a degree of process knowledge to select factors and intervals. For systems with little or no process

knowledge, a screening phase is needed to determine the important factors to be evaluated. The overall efficiency of experimental design is therefore relative to prior knowledge.

Examples of experimental design employed for medium improvement include full factorial, partial factorial, Plackett and Burman, Hadamard, orthogonal arrays, orthogonal latin rectangles, central composite and Box-Behnken designs (Kennedy & Krouse, 1999).

For a given question the experiments are conducted according to the design examples above, and the results are further processed. Data from the experiments are then coupled to a mathematical optimization technique. The combination of the two techniques is used for predicting potential improvements. In regard to a fermentation medium the two techniques are used to predict the composition of a medium with improved performance. It is of course necessary to determine the merits of the prediction with additional experimentation. The media development in section 3.1 will follow a procedural (empirical) media improvement rather than experimental design improvement.

1.2.3.3 Optimization

Optimization of the fermentation medium refers to determining the nutrients and concentrations that will support the best growth or production of a specific product (Zhang & Greasham, 1999). Traditionally, optimization has been conducted using shake-flasks and using a one-factor-at-a-time-approach where improvements are pursued by changing a single variable and holding the others at a fixed point. It is a labor intensive and time consuming method and is not able to determine a true optimum.

Although the one-factor-at-a-time-approach is not efficient, it is easy and convenient. It can be an appropriate method, especially in less complex or dynamic applications, like producing viable biomass (cells), and primary metabolites. A more robust optimization method may be needed when pursuing optimization for secondary metabolites, enzymes, recombinant proteins, or other factors that are produced in small concentrations, and at specific phases of growth. To approach these complex systems medium designers have utilized numerous optimization techniques. Examples include response surface methodology, steepest ascent, canonical analysis, multiple linear regression, Gauss-Seidel, modified Rosenbrock, Nelder-Mead simplex, artificial neural networks, fuzzy logic, genetic algorithms, pulsed continuous culture, and stoichiometric analysis (Kennedy & Krouse, 1999).

In a discussion of the many types of optimization it is also useful to consider the highly practical suggestion by Kennedy and Krouse (1999) that medium optimization is different from mathematical optimization. An optimum is not possible in medium design and it is possible there is always another better medium. Therefore it is perhaps better to use the term improved medium, rather than optimum or optimized. In section 3.1 the media development is conducted via procedural (empirical) improvement rather than optimization.

1.2.4 The point of diminishing return

As noted previously, the task of medium improvement can be open ended with no true optimum endpoint. It is not possible to guarantee that a better medium will not be produced in the future. In practice then, in a task that has no ultimate solution, an important decision point is to know when to stop the improvement process. For example, Kennedy et al. (1994) describe that within the process of medium design there exists a "development curve". The development curve is a pattern of improvement that could be modeled using the number of media tested along with performance indicators such as productivity or cost. Results showed a characteristic kinetic curve described by an exponential rise leading to a plateau. The plateau could be reached by trialing only a small number (less than 20) of media. As the authors suggest, "when the plateau emerges it is time to do something radically different or stop". By plotting the medium development curve "a laboratory can avoid a large amount of wasted effort" (Kennedy & Krouse, 1999).

1.2.5 Functionality

The fermentation focus for this project is the development of the medium, and the production of the organism. There can be a temptation to consider medium development as a mathematical design process that can be optimized to achieve a maximum value. Design is certainly a more efficient way to understand complex systems. However, the temptation is to focus on design, strategy, and optimization and forget the final purpose. The following quotation from (Stanbury *et al.*, 1995) highlights this point:

> *"it must be recognized that efficiently grown biomass produced by an optimized high productivity growth phase is not necessarily best suited for its ultimate purpose".*

Stated another way; by focusing singularly on the production of large biomass it is possible to lose the *functionality*, or ultimate purpose, of the microbe being produced. To improve the fermentation of microbes, in a practical way, therefore requires a functional development process.

To summarize this fermentation introduction; the development of a growth medium for functionality includes the designation of the functionality target (ultimate purpose), planning the improvement, performing the work, confirming the target activity at each step, looking for the plateau, and knowing when to stop. When functionality is important, it can be better to have a lesser quantity that works well, than a high density that does not.

In section 3.1 the results of the development of a new growth medium are described which targets functionality. The concepts from this fermentation introduction are utilized and applied therein.

1.2.6 Medium development for *Pseudomonas* sp. strain ADP

The production of either liquid inoculants or formulation products requires the fermentation of the microbial strain to a useful cell concentration. *Pseudomonas* sp. strain ADP is the best characterised organism for the mineralization of atrazine (Govantes *et al.*, 2010). Therefore the starting point of fermentation for this work involves the standard bacterial culture for producing *Pseudomonas* sp. ADP (P.ADP) (Mandelbaum *et al.*, 1993; Mandelbaum *et al.*, 1995). The medium was utilized for the isolation and characterization of the strain. The medium yields a colony forming unit (cfu) in the range of 1×10^9. An 8.4×10^8 cfu/ml productivity was reported by (Shapir *et al.*, 1998b). For the purposes of this thesis productivity higher than 1×10^9 cfu/ml is considered a practical fermentation priority. To the author's knowledge a higher yielding medium containing atrazine, or an intermediate of atrazine, has not been developed. Fermentation technology is available to produce cell concentrations in the order of $>10^{10}$ colony forming units (cfu)/ml.

Throughout this thesis the standard medium for *Pseudomonas* sp. ADP will be termed (MB) as it was the bacterial culture described by Mandelbaum et al. (1995) when the strain was characterized. MB medium contains 1g/l sodium citrate and 100 mg/l of atrazine as the carbon source and nitrogen source respectively. The atrazine component was fully utilized within 24 hours of growth (Mandelbaum *et al.*, 1995). In subsequent literature this medium has been harvested at 24h growth post inoculation (Shapir & Mandelbaum, 1997; Shapir *et*

al., 1998a; Silva *et al.*, 2004) . A standard growth curve with *Pseudomonas* sp. ADP for the medium DSMZ which also contains 1g/l sodium citrate and 100 mg/l of atrazine (see appendix) as the carbon source and nitrogen source respectively has been presented (Wyss *et al.*, 2006). The growth parameters and scale up parameters of the *Pseudomonas* sp. ADP strain and MB medium have been conducted (Biglione *et al.*, 2008). Kinetic parameters of μ_{max} and K_s were determined to be 0.14 (± 0.012) h^{-1} and 1.88 (± 1.80) mg/L respectively using 125 ml flasks with 50 ml working volume of medium. After a scale up to a spherical stirred tank batch reactor of 300 ml working volume, the μ_{max} and K_s were determined to be 0.12 (± 0.009) h^{-1} and 2.18 (± 0.47) mg/L respectively. Biglione (2008) confirms the work of Mandelbaum (1995) that the atrazine is removed completely from the atrazine medium within roughly 30 h. For the current project, the process of developing a culture medium with higher cell productivity began with the medium MB

The development of a culture medium with higher cell yield is challenged by the reports that *Pseudomonas* sp. ADP will lose its ability to metabolise atrazine (atz-) in the presence of complex laboratory media such as LB (De Souza *et al.*, 1998a). In an attempt to make an educated hypothesis for a medium type which retained the functional pathway of the strain, a metabolic review was conducted.

The catabolism of atrazine is accomplished by six enzymatic steps encoded by genes *atz*ABC and *atz*DEF. The genes are held on a 108-kbp plasmid pADP-1 (De Souza *et al.*, 1998a; Martinez *et al.*, 2001). The plasmid is resistant to genetic manipulation and is "remarkably instable" (Govantes *et al.*, 2010) and forms mutants unable to catabolise atrazine (atz-). Plasmid instability is due to rearrangements of the *atz* genes on the plasmid and growth in conditions of non-selective media (De Souza *et al.*, 1998b; Garcia-Gonzalez *et al.*, 2003).

Pseudomonas sp. ADP transforms atrazine to hydroxyatrazine, hydroxyatrazine to cyanuric acid, and cyanuric acid to carbon dioxide and ammonia (Wackett *et al.*, 2002). The operon *atz*ABC is responsible for catabolising atrazine into cyanuric acid and is constitutively expressed. The operon *atz*DEF completes the catabolism from cyanuric acid to ammonia and carbon dioxide, and is regulated by a complex cascade circuit (Govantes *et al.*, 2010). Cyanuric acid ($C_3H_3N_3O_3$, 129.08 g/mol) s-Triazine-2,4,6(1*H*,3*H*,5*H*)-trione is the central intermediate of atrazine metabolism, and its presence promotes both groups of atrazine degradation genes for *Pseudomonas* sp. ADP (Garcia-Gonzalez *et al.*, 2005).

The activities of bacteria are known to be controlled by their nitrogen source (Merrick & Edwards, 1995). Growth on atrazine of *Pseudomonas* sp. ADP is a nitrogen limited state (Cheng *et al.*, 2005) and the degradation of atrazine is increased in nitrogen limited soil (Sims, 2006). Researchers have reported that atrazine mineralization is regulated by nitrogen and nitrogen availability (Abdelhafid *et al.*, 2000; García-González *et al.*, 2007). The enzymes utilized for atrazine degradation are elevated under nitrogen limited conditions (Cheng *et al.*, 2005). Likewise, the activity of *Pseudomonas* sp. ADP against atrazine has been demonstrated to be controlled by the nitrogen source cyanuric acid (Garcia-Gonzalez *et al.*, 2005; Garcia-Gonzalez *et al.*, 2003). In terms of growth and biomass, cyanuric acid as nitrogen source has as good a growth rate as ammonium (Neumann *et al.*, 2004). The growth rate on cyanuric acid is also comparable to nitrate (García-González *et al.*, 2007).

Based on a metabolic review of *Pseudomonas* sp. ADP, cyanuric acid appears to be a key metabolic intermediate of atrazine degradation and offers a potential metabolic control location for atrazine functionality. In addition to controlling the strains metabolism, the growth and biomass levels appear comparable to conventional fermentation nitrogen sources. Therefore the metabolic intermediate cyanuric acid was selected as the nitrogen source control mechanism for culture medium development.

Cyanuric acid was also selected to address the plasmid instability of the strain. The plasmid is prone to loss of functionality (atz-) when grown in conditions of non-selective media (De Souza *et al.*, 1998b; Garcia-Gonzalez *et al.*, 2003). From the literature it appears cyanuric acid may potentially serve as a selective pressure component.

From an industrial perspective cyanuric acid appears to be an appropriate substrate for large scale production, especially in comparison with the other atrazine inducing compounds such as biuret and serine. Cyanuric acid is non-toxic and commonly available as a swimming pool chemical with commodity pricing, and a high level of chemical purity. The role of cyanuric acid as a swimming pool additive is to stabilize the chlorine from ultraviolet degradation.

While this strain is a well-researched model organism, research in medium improvement to increase the cell productivity and functionality for this strain has not been previously conducted. The fermentation work of this thesis proposes to improve the cell productivity of the culture medium (higher than 1×10^9 cfu/ml) and also retain the target functionality by replacing atrazine with cyanuric acid.

1.3 Formulation

Formulation is a step-wise process of combining an active ingredient with various chemical and physical substances to produce an enhanced final product. The significance of formulation is described in a review of bacterial inoculants as 1) the industrial "art" of converting a promising laboratory-proven bacterium into a commercial field product, and 2) the crucial issue of inoculants which can determine the success or failure of a biological agent (Bashan, 1998). The formulation of these beneficial microbes (bacterial inoculants) is the process of combining the viable whole cells (the active ingredient) with other non-active ingredients (excipients) to produce a preparation which exhibits stable shelf life and efficacy.

In this context, the aim of Formulation science is able to provide accuracy, stability and protection to biological products. Controlled release techniques for active ingredients and biologicals are common in pharmaceutical, human, and animal health science. However, there are few publications where formulation and controlled release have been applied to enhance the performance of biological agents for bioremediation purposes.

Traditional inoculant products include *Rhizobium* for legumes, inoculants for food and drink fermentation, probiotics and silage. Agricultural uses include plant protection by pest and pathogen control, plant growth stimulation by seedling inoculation and hormone production, the improvement of soil structure, and the increased availability of nutrients such as nitrogen and phosphorous. In the environmental market, microbial inoculants for bioremediation are employed to remove contaminants in soil, water, and wastes. For agriculture the use of probiotics is common.

By carefully considering the ultimate end use of the product the formulator can provide the best possible combinations of the biological agent, its robustness, its activity, and its delivery. Figure 1 provides an example of formulation development for seeds. A considerable test for the formulator is to be cost effective while achieving delivery and efficacy. Producing a highly concentrated agent may appear economic but there may be serious negative effects in stability and efficacy. The best end use combinations can require compromises between microbe production methods in fermentation and microbe stability outcomes in formulation.

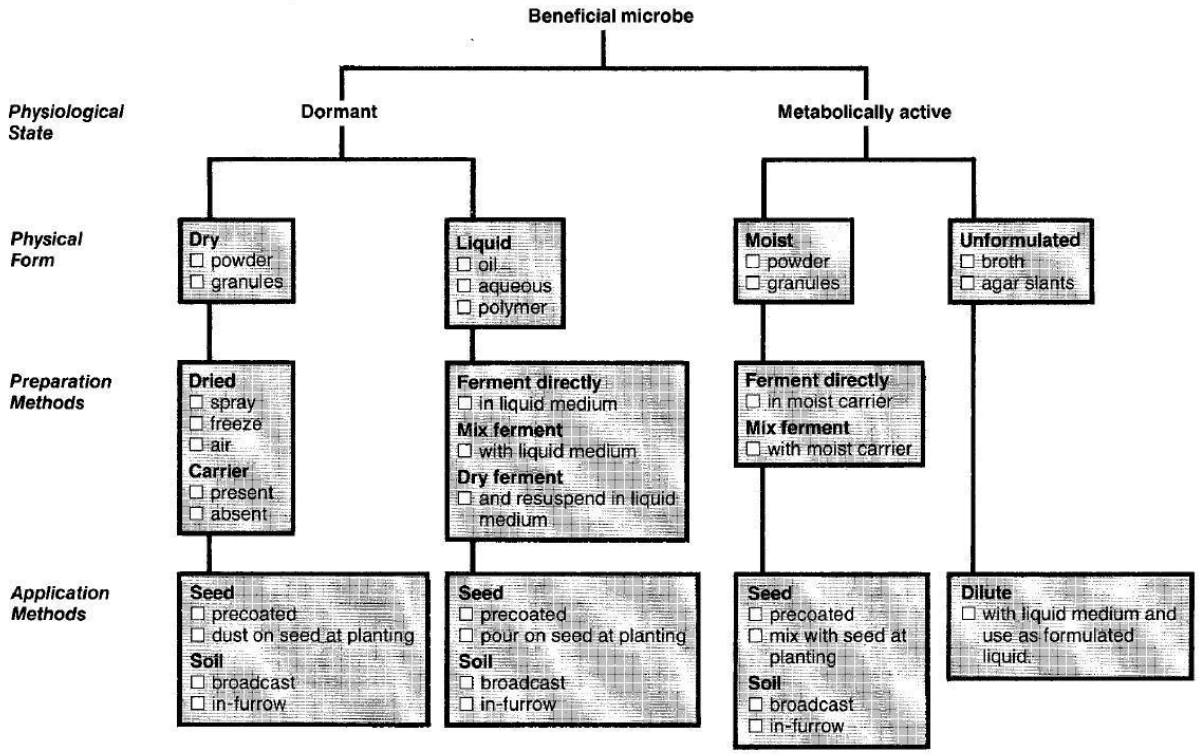


Figure 1. Overview of formulation of microbes for seeds (from Paau 1988).

In general, microbial agents display stability and delivery challenges, with perhaps the only predictably stable bacteria being Gram-positive spore formers (Emmert & Handelsman, 1999). Such challenges can be overcome or partially addressed by formulation (Bashan, 1998; Lewis & Papavizas, 1991). Even if stability is a minor issue, for example as with spore forming bacteria like *Bacillus* spp. or *Streptomyces* spp. (Brar *et al.*, 2006; Emmert & Handelsman, 1999; Navon, 2000), the need for effective delivery may present a limitation requiring formulation to address.

To be identified as a candidate agent a microbe must express a suitably specific functionality. Candidates may be initially constrained by a lack of robustness and this challenge can often be overcome or at least suitably addressed by formulation. While at first inspection formulation may simply appear to be the act of combining and suitably mixing a list of ingredients, the need to understand how each component of the mixture may interact together and, most importantly, with the formulated microbe cannot be ignored (Castro *et al.*, 1995; Dubey *et al.*, 2009; Johnson *et al.*, 2001; Rajkumar *et al.*, 2008; Terefe *et al.*, 2009; Wiyono *et al.*, 2008; Zhang *et al.*, 2003). However, to date analytical methods that can provide understanding of the mechanisms by which microbes interact with various materials leading

to either stabilisation or microbe death have yet to be suitably developed and validated. The determination of compatibility for each formulation ingredient, its concentration, its compatibility with other components, and each microbe is primarily an empirical process (Gill & Ballesteros, 2000; Jaronski, 2010; Trevors *et al.*, 1993).

1.3.1 Microbes and formulation

For plant protection the few microbes that do not present significant formulation challenges are *Bacillus* spp. or *Streptomyces* spp. The remaining biocontrol microbes are vast in number and differ in characteristics from one type of microorganism compared to another. This wide range of factors often requires unique formulation solutions in order to overcome problems of stability, and delivery for a specific target.

The production methods, products, and formulations for plant protection are perhaps as varied and numerous as the potential microbes for plant disease control and plant establishment targets. As an example, in 2007, a review of myco-insecticides and myco-acaricides identified 171 products worldwide (Faria & Wraight, 2007). The use of microbial agents for plant protection is driven by the emergence of new or expanded restrictions placed upon current chemical control agents (Bashan, 1998; Gerhardson, 2002; Lewis & Papavizas, 1991). Microbial agents for plant protection come under the broader classification of biocontrol, which also includes the use of predators and parasites such as insects.

An example of a biocontrol agent is *Serratia entomophilia* (Enterobacteriaceae). The stabilisation of *S. entomophilia* was reported (Johnson *et al.*, 2001). This work ultimately led to the development of Bioshield™ (Figure 2), a product for the control of New Zealand grass grub (*Costelyta zealandica)* that coats *S. entomophilia* onto zeolite granules. It is worthwhile comparing Bioshield to an earlier product it replaced, Invade®, which was developed in the late 1980s, and early 1990s. Invade® was a liquid applied using a modified seed drill at a rate of 1 litre/ha diluted to 100 litres with non-chlorinated water to deliver 4×10^{13} bacteria/ha. It required refrigerated storage at 4°C due to a less than 7 day stability at 20°C. Bioshield on the other hand is a granule applied at 30 kg/ha using a seed drill, delivering 4×10^{13} bacteria/ha and maintaining stability at 20°C for 180 days.

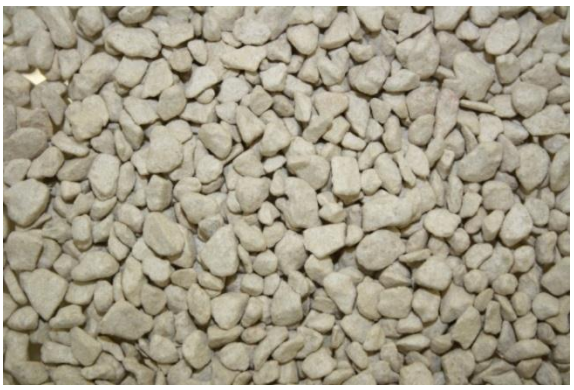


Figure 2. Bioshield for the control of New Zealand grass grub (*Costelyta zealandica*) containing stabilised *S. entomophilia* coated onto zeolite granules (Bunt & Swaminathan, 2010). Zeolite granules are ~2mm-4mm.

1.3.2 Stability

The term stability encompasses a wide range of meanings for microbial formulations, including stability during production and harvest of the microbe, stability during processing or compounding of a product, stability during storage and also stability during use. The key formulation stability indicator is microbe viability, which requires that the microbe can be cultured in order to enumerate. Enumeration by viable culturability is by far the most common, essentially exclusive, means to measure the quality of microbes that have been produced and compounded as a final product of formulation. In the absence of a method of enumeration, alternative bioassay methods to indicate an agent's activity are very difficult to validate. It is important here to emphasize that viable culturability of the formulation is a dual indicator of both quality and quantity, whereas measuring the quantity of cells present may not distinguish living from dead individuals. The key aspect of stability is viability: the microbe can be cultured and enumerated across an expanse of time. As a working definition, *stability is survival over time*.

After a microbial culture has been formulated, a number of factors may lead to a loss of viability, such as dehydration, heat inactivation, excessive moisture, ultraviolet (UV) radiation and the presence or absence of oxygen. The mechanisms by which many of these may lead to a loss in viability are well known, while for some of these parameters the mechanisms are still unclear. Membrane damage of *Lactobacillus plantarum* has been shown to be caused by dehydration but not thermal inactivation (Lievense *et al.*, 1994), while oxidation of *L. bulgaricus* cell membrane lipids has been reported to be proportional to the unsaturated/saturated fatty acid ratio (Castro *et al.*, 1995).

To maintain or enhance microbe survival some physical conditions can be avoided or minimized. For example, heat and UV during compounding should be minimised or avoided. Exposure to oxygen may need to be controlled for obligate anaerobes. Likewise, the importance of moisture and desiccation throughout the manufacturing process is critical for cellular survival. The optimum final product moisture content is often highly dependent upon species and sometimes even strains (Castro *et al.*, 1995; Champagne *et al.*, 1996; Kurtmann *et al.*, 2009; Vaamonde & Chirife, 1986).

Ultimately a formulation is a preparation which aims to minimize any difficulties or limitations associated with handling in order to maintain the viability of a microbe during storage, delivery and use. Some examples of simple formulation methods are to freeze, lyophilize or spray dry the microbial culture. The aim of these formulations is to provide a balance between the extremes of freezing and desiccation in order to achieve maximum microbial stability. Avoiding these extremes may not always be easily achieved or can require a compromise in other parameters that affect the yield and viability of microbes. Depending on the microbe and method, there may be a loss of microbial viability in the order of 1 to 5 logs from the freeze, lyophilize and spray dry process (Bunt & Swaminathan, 2010). If losses of this magnitude are acceptable the delivery of the biological agent may be conducted with appropriate thawing of the product, dilution with an appropriate vehicle such as chlorine free water, and direct spray application.

Once a microbe is applied to a surface it faces a new challenge as desiccation may lead to a rapid decline in viability. As such, the formulations also need to evaluate and provide for in-use stability. A *Lactobacillus* spp. culture on a glass slide that is left to desiccate under normal conditions will lead to complete cell death within 24 hours (Figure 3). By incorporation in a gel formulation, viability can be improved (Figure 4).

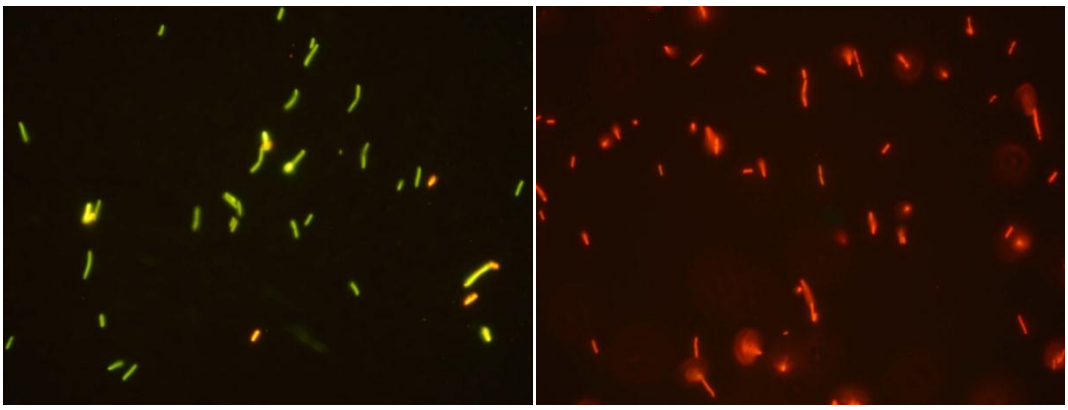


Figure 3. View of LIVE/DEAD BacLight™ stained 10 µl sample of Lactobacillus spp. culture on a glass slide (left) initially and (right) after 24 hours desiccation at room temperature. Green indicates bacteria with an intact cellular membrane (viable) while red indicates a compromised cellular membrane (Bunt & Swaminathan, 2010).

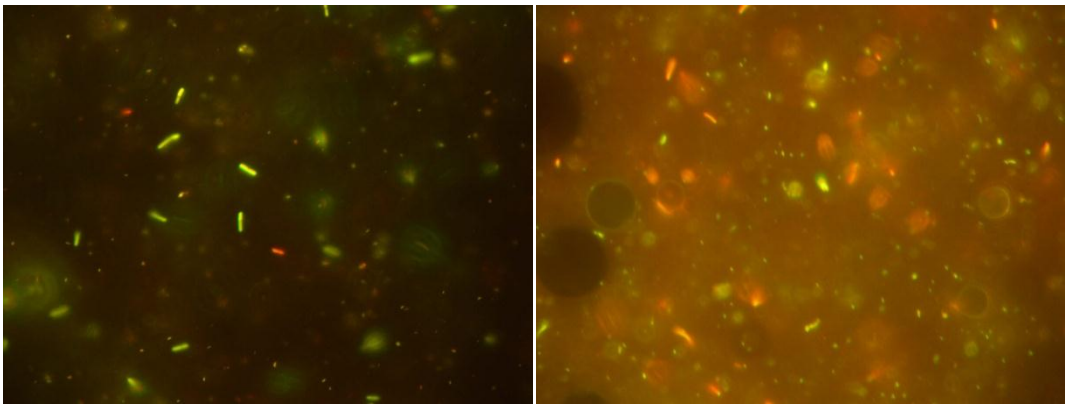


Figure 4. View of LIVE/DEAD BacLight stained 10 µl sample of *Lactobacillus* spp. in a gel (a) initially or (b) after 24 hours desiccation at room temperature. Green indicates bacteria with an intact cellular membrane (viable) while red indicates a compromised cellular membrane (Bunt & Swaminathan, 2010).

Water activity is a prime factor associated with microbe stability and is described as a dimensionless quantity representing the energy status of the water in a system and defined as the vapour pressure of water above a sample divided by that of pure water at the same temperature (Stolp, 1988). The relationship between temperature and water activity on survival of a freeze-dried *Lactobacillus* spp. stored in vacuum-sealed foil laminate bags is shown in Figure 5 (Bunt & Swaminathan, 2010). As temperature or water activity increased, survival was reduced. For example, at 25°C, as water activity increased from 0.24 to 0.34,

survival reduced from 35 to 25%, respectively over 6 months. Conversely, at 0.24 water activity, as temperature increased from 25 to 37°C, survival reduced from 35 to 20%, respectively. At 37°C and 0.34 water activity, survival was only 10% over 6 months.

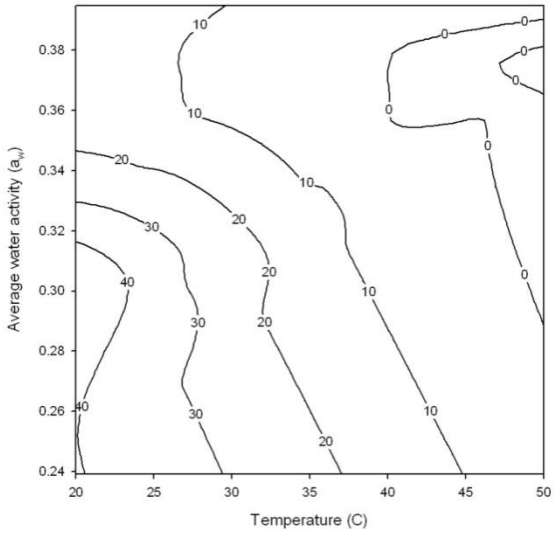


Figure 5. The effect of temperature and water activity on viability of *Lactobacillus* spp. when stored in vacuum-sealed foil laminate packaging. Contour lines are survival (%) over 6 months (Bunt & Swaminathan, 2010).

1.3.3 Formulation and delivery

The term delivery encompasses the process from the time the product is prepared for use, its actual use or application, and the minimum time period after application during which it is required to be active. Broadly, formulations can be either defined as one of two types: solid or liquid. Solid formulations rely upon a physical construct to stabilise and provide delivery. The microbe will be either homogenously dispersed through a monolithic matrix or located as a discrete layer in or on a substrate. It is now emerging that microbe interaction with the surroundings is one mechanism by which stability can be achieved (Bjerketorp *et al.*, 2006; Schoug *et al.*, 2006).

An often overlooked aspect of formulation is that once the product is formulated, the ingredients used may make characterization or analysis of microbes difficult. When *Lactobacillus* spp. is dispersed in a simple gel formulation it may be difficult to identify under the microscope. Over time this may become more problematic, particularly if trying to distinguish between live and dead microbes and background artifacts due to components of the formulation (Figure 4).

There are many papers and reports describing alginate encapsulation or variations of this type of formulation (Bashan, 1998; Dubey *et al.*, 2009; Lewis & Papavizas, 1991). The technique appears simple, involving a suspension of a microbe in a solution of sodium alginate that is formed into droplets or emulsified. Adding this to a solution containing polyvalent ions (usually Ca^{2+}) induces gelling by cross linking of the alginate, thereby forming a semi-solid bead. This bead may be hardened further by the addition of polyvalent cationic polymers (e.g. polylysine or chitosan). Additional processing may also be included, such as extracting Ca^{2+} from the interior of the hardened bead to liquefy the core (usually using citrate), leaving the hardened shell containing free microbes. This method and its modifications have been successfully used to produce beads or hollow shells, and there are numerous reports using alginates for this purpose. However, it has met with very little commercial success (if any) and attempts to scale-up the process have tended to encounter process and cost problems (Bashan, 1998).

There are also many methods to produce coatings or monolithic matrices, such as pelletization, granulation and film coating (Bunt & Swaminathan, 2010). Some techniques require a seed or core onto which layers are applied, allowing for a construct to be "put together" from base materials into the final form (an example of such a formulation is shown in Figure 6). Using a seeding core offers a number of advantages and can be used to dictate and control the final shape and mechanical properties. Along with maintaining microbe viability, solid formulations must also be suitable for delivery which includes the provision of mechanical robustness (or suitable friability) to ensure that the formulation maintains its integrity (Brar *et al.*, 2006) during delivery (e.g. a seed drill or spreader).

Figure 6. Granules (~5mm) for delivery of microbial agents (Bunt & Swaminathan, 2010).

Another delivery format type is wettable powders containing dried bacteria, which can be considered a solid formulation up to the time of mixing with water. A wettable powder formulation may be required to maintain microbe stability and the physical properties of the mixture, its viscosity and pouring characteristics, become most important once dispersed in water. Such formulations for spraying do allow for very high concentrations of microbe to be applied, but this can have disadvantages. For example, phytotoxicity has been reported when using *Bacillus firmus* for control of root-knot nematode infestation of tomato plants (Terefe *et al.*, 2009). Additionally, the water used to prepare the final product before application must be carefully considered. Chlorinated water may have a detrimental impact upon microbe viability and therefore the means to produce large volumes of non-chlorinated water may be required (Johnson *et al.*, 2001). Such limitations should be identified early in product development.

The non-active ingredients of a formulation (excipients) can play a major role influencing the storage stability of microbes. It is not always clear whether this is due to chemical, physical, or processing properties and the contributing factors may be difficult to identify. The sample pH, ionic strength, changes in moisture content, and the fermentation method may individually or collectively produce a microbe with poor stability.

The age of a delivered formulation is also important. Often samples are produced close to or immediately prior to field testing. This test-product might not be the same after weeks or months of storage. To address the impact of age on formulation delivery, samples of test-product should be retained under normal conditions until at least the end of field testing and then analysed for microbial content.

1.3.4 Performance of formulated inoculants

Remediation of a pentachlorophenol (PCP) contaminated soil was described using *Pseudomonas* sp UG30 encapsulated in κ-carrageenan (Cassidy *et al.*, 1997). Cells were encapsulated in a κ-carrageenan formulation using clay and skim milk components, to respectively enhance bead strength and viability. Soil loading densities of 10^8 cfu/g dry soil were used. Bacterial survival in soil was not part of the study. Results of contaminant removal were enhanced for encapsulated cells compared to free cells (64.7±0.3% PCP mineralized), and also compared to indigenous microbes stimulated with fertilizer (less than 50% PCP mineralized) over 30 weeks. Repeated inoculation of soil with the encapsulated cells produced a faster initial rate of removal. However, the advantage of a faster rate was temporary and by 12 weeks the single inoculation of encapsulated cells reached equivalent

levels of PCP mineralization (64.8±1.9%). Importantly, the authors suggested that bioremediation may be possible with only one soil application using a formulated encapsulation technique.

A slow-release inoculant was developed for soil and liquid remediation of atrazine using encapsulated *Rhodococcus erythropolis* NI86/21 (Vancov *et al.*, 2005). Alginate encapsulation was amended with bentonite, activated carbon or skimmed milk. Bentonite amended alginate produced the greatest removal of atrazine. The stability of the formulation was limited and viability could not be maintained after 21 days of storage at 4°C.

A recovery step was added to the above encapsulated cells of *Rhodococcus erythropolis* NI86/21 by soaking the beads in nutrient broth overnight (Vancov *et al.*, 2007). Using the recovery step immediately after encapsulation extended the storage stability to 6 months, declining 1 log, when stored at 4°C. In sterile soil slurry, the formulation removed 20 ppm atrazine in 3 weeks. For non-sterile soil slurries, atrazine was still present after 6 weeks.

For the remediation of gasoline contaminated aquifers, a consortia of degraders was encapsulated with a gellan gum and canola oil gel (Moslemy *et al.*, 2002). In liquid microcosms, the encapsulated cells rapidly degraded 90% of gasoline hydrocarbons within 5-10 days at 10°C. Free cells without the encapsulation accomplished the same level of removal but took up to 30 days. When encapsulated cells were exposed to toxic contaminant levels a reduced lag phase was observed indicating a protective effect by the gel matrix. However, results were less clear in saturated sterile soil microcosms. The authors report that mixing was insufficient to distribute the gasoline from the interface into the soil. Their findings illustrate that formulation for bioremediation has limits and the proximity to the target is fundamentally critical for the formulation to perform.

The above examples demonstrate that the formulated microbial products can be produced and successfully remove contaminants. Some specific examples utilizing *Pseudomonas* sp. strain ADP are reviewed below.

1.3.5 Formulation of *Pseudomonas* sp. strain ADP

There are few investigations of immobilization, encapsulation, or a structured formulation attempt using *Pseudomonas* sp. strain ADP. Entrapment of the strain was reported using a procedure that combined Ca-alginate encapsulation and the sol-gel glass process (Rietti-Shati

et al., 1996). The immobilization of the *Pseudomonas* sp. strain ADP cells appeared to cause the loss of atrazine activity. Aging the gel at 4°C for 4 days caused a further loss of activity and the addition of nutrients to the gel was proposed. Nutrients were supplied by immersion of the sol-gel discs in atrazine medium (containing NH_4Cl as a replacement for atrazine). The addition of additional nutrients produced atrazine degradation activity. However the entrapped bacteria were not stable and could not be revitalized (100% loss of viability) after 75 days (10 weeks) stored at 4°C.

A more recent approach to bioremediation is reported in which *Pseudomonas* sp. strain ADP was encapsulated in electrospun microtubules (Klein et. al 2009). Electrospinning is a method of producing polymer based fibers of ultrafine diameters (~10's to 100's of nanometers). The authors report that some activity was retained in the encapsulated material and it may show promise as a platform in the future. Starting with 10^9 cells, survival during the spinning process was low, with 1-2 orders of magnitude lost immediately. Extended shelf life stability was not included in the study.

Numerous studies have used *Pseudomonas* sp. strain ADP bacterial culture as an inoculum for atrazine bioremediation. Fewer studies have attempted to encapsulate or immobilize the stain as a technique to enhance the performance of the strain in the soil environment. We are not aware of previous studies with this strain using a formulation approach to stabilize the agent, produce an extended shelf life at ambient temperatures, or maintain the functionality of the microbe to degrade atrazine at the point of delivery.

The present author concludes that a formulation is needed which prevents the loss of viability and functionality of the strain. Liquid biological inoculants are generally not stable at room temperature and have short term (weeks) stability when stored at 4°C. The ideal formulation would consists of a small solid carrier which could be delivered with common agricultural equipment such a seed drill to an accurate target depth in the soil. The ideal formulation would also provide a minimum of 10 weeks storage at 25°C. This level of stability is a generalized supply-chain time frame for a product to be manufactured, inventoried, purchased and shipped to its final location prior to use. This supply-chain time line is therefore the minimum time frame thought to be needed for the approach to have a clear practical application as an industrial product.

This work therefore proposes to produce a shelf stable formulation which exhibits strong functionality to degrade atrazine. The formulation utilizes an immobilization technique

developed by AgResearch Limited (Johnson *et al.*, 2001). The approach consists of a biopolymer gel which is produced to encapsulate the microbial cells of the liquid bacterial culture. The mixture of gel and encapsulated cells are then applied to a solid carrier material (zeolite granules) which acts as a carrier for the formulation. A further description of this technique is presented in Materials and Methods (Chapter 2).

1.4 Aims

Fermentation

The aim of the fermentation work proposes to improve the cell productivity of the culture medium (higher than 1 x 10^9 cfu/ml) and also retain the target functionality by replacing atrazine with cyanuric acid.

Formulation

The aim of formulation is to develop immobilization methods which maximize cell survival, stability, and functionality. The formulations will be developed to optimize the shelf life (10 weeks) stability of the strain at 25°C.

In situ

An immobilized formulation will be introduced into sterile soil and evaluated for survival and functionality. The survival and functionality of liquid bacterial culture and the formulation granule will be compared. Additionally, the functionality and dispersal (cfu) levels from the granule into the sterile soil will be compared to liquid bacterial culture. The aim is to deliver in situ a formulation which improves the survival of the inoculant over the application of liquid bacterial culture.

2 Materials and Methods

2.1 Materials

2.1.1 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 medium and agar were purchased from Merck, Darmstadt, Germany. For the formulations work, Xanthan gum was purchased from Danisco, China. Lupi Extra Virgin Olive Oil (Italy) was purchased from local food supply retailers.

2.1.2 Zeolites

Zeolites are crystalline, hydrated aluminosilicates that contain alkali and alkaline-earth metals. Their structure is based on a three dimensional honeycomb negatively charged porous network of silica-oxygen tetrahedral. The negative charges are balance with exchangeable cations of calcium, magnesium, potassium and sodium. The survival of *Pseudomonas* spp on zeolite as well as other air dried mineral powders has been reported for use in plant pathology biocontrol (Dandurand et al. 1994).

A panel of eight zeolite types was evaluated. Five are New Zealand (NZ) zeolite, and three are Australian (AUS) zeolite. The eight zeolite types are designated Z1-BC, Z1-BDW, Z1-B1, Z1-CG, NZ-1, AU-FM, AU-ZB, and AU-II.

Of the eight initial zeolites, two distinct types of 2-6mm zeolite granules were selected for rigorous comparison. Type I is predominantly mordenite and clinoptilolite (Blue Pacific Minerals, New Zealand) and designated NZ-I. Type II is clinoptilolite with minor amounts of mordenite (Zeolite Australia, Australia) and is designated AU-II. The cation exchange capacity for the Australian and New Zealand zeolites are reported as 120 and 100 meq/100g respectively. Australian zeolite is much harder at 7 Mohs than the New Zealand zeolite at 5 Mohs.

Type NZ and AU were selected for comparison because the two zeolites have distinct physical differences visually, texturally, and in terms of material density. NZ-1 and AU-II

were selected because they are of the same size (2-4mm) which is suitable for agricultural seed drilling equipment.

The AU-II zeolite was generously shipped by Dr. Anwar Sunna from Australia to New Zealand. To meet the strict importation requirements of New Zealand the material was washed and certified to be heat sterilized prior to shipping.

2.1.3 Soil

A Templeton loam soil and was collected from the AgResearch Farm in Lincoln, Canterbury, New Zealand. Moisture properties of Templeton loam are a wilting point of 14 % water content and a field capacity of 30%. The soil was separated through a 2mm sieve.

A portion of the soil was sent to Schering-Plough of Upper Hutt, Wellington New Zealand, to be sterilized by gamma irradiation. The sterilized soil was generously donated to the project by Céline Blond. Sterility of the irradiated soil was confirmed by plating onto LB-Miller agar. One gram of soil was mixed with 9 ml of phosphate buffer diluent (10^{-1} dilution) and no colonies were observed after 48 hours incubation at 30°C. Prior to being used in the experiments the water activity of the sterilized soil was measured in triplicate (mean a_w = 0.527).

2.2 Methods

2.2.1 Microbial culture

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 cells were 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.

2.2.2 Confirmation of *Pseudomonas* sp. strain ADP (DSM 11735)

Partial sequencing of the 16S rRNA gene was carried out to confirm identity of the strain, *Pseudomonas* sp. strain ADP (DSM 11735). Well isolated colonies of DSM 11735 were suspended in 500 µl of sterile nanopure water and used as a template for PCR with 16S primers U16a and 1087R (Wang & Wang, 1996). The PCR product was sent for sequencing at the Allan Wilson Centre, Massey University, Palmerston North, in New Zealand. Forward and reverse sequences were aligned into a single contig using Sequencher. The contig was pasted into NBCI/BLASTn and matched to the nucleotide database.

PCR conditions were 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 mM Primer, 1.25U Taq (Thermoprime, Abgene) and 1x buffer in 50 µl final volume including 4 µl of template. The primers used were U16a (AGAGTTTGATCCTGGCTC) and 1087R (CTCGTTGCGGGACTTAACCC) as described by Wang and Wang (1996). The PCR programme was run on an Eppendorf Mastercycler Gradient PCR machine at 94°C for 2 min, then 30 cycles of 94°C for 30 sec, 50°C for 45 sec and 70°C for 1 min. PCR product size was confirmed by visualisation on an electrophoresis gel (0.8% agarose in 0.5x TBE, run for 30 min at 100 V). The Roche High Pure PCR Product Purification kit was used for product clean up.

2.2.3 Colony type morphology and identification

The culture received from DSMZ was plated and noted to have highly variable colony morphology, especially when grown on atrazine agar. Large and small colonies appearing on atrazine agar were individually sequenced using the method and primers above. Complimentary sequences from each type were aligned using BLAST (bl2).

2.2.4 Pre-culture

Pre-cultures were prepared by resuspending the contents of a culture stock microcentrifuge tube using 1 ml from a vial containing 15 ml sterile LB broth and transferring the resuspension 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 culture medium were inoculated with 1 ml (1% v/v) of pre-culture.

2.2.5 Cultivation of *Pseudomonas* sp. strain ADP (DSM 11735)

Shake flasks (500ml) were used to develop an alternative growth medium, and to produce the cells needed for formulation stability trials and soil inoculation. In medium development

experiments the inoculated flasks (per 2.2.4) were sampled across a profile period to produce a population growth profile. A sample of the initial conditions, Time= Zero, was taken after the flasks were inoculated with the pre-culture and mixed. Further samples were taken at time points across the growth profile period to include the logarithmic phase and stationary phases of growth. Flasks were incubated at 30°C on a shaker (200 rpm) and were enumerated by serial dilution to quantify viable cell counts. For the production of the inoculum for formulation and soils, the inoculated flasks (per 2.2.4) were sampled at Time= Zero and 24h, at the time of harvest.

2.2.6 Serial dilution and plate count

Viable cell count enumeration was performed by sampling and serial dilutions. Sample processing was done in real time without sample bulking or storage prior to processing. One millilitre of sample was pipetted from a well-mixed suspension to a dilution tube containing 9ml of the diluent. A 0.1 M phosphate buffer solution was used as the diluent fluid. Each tube was vortexed for approximately 7s prior to removing 1ml of the sample for transfer to the next dilution tube containing 9ml diluent. This process was repeated for each tube in the dilution series. At least three dilution tubes in series were plated, e.g. 10^{-4}, 10^{-5}, 10^{-6}.

The drop plate (DP) method was used for plating (Herigstad *et al.*, 2001). Triplicate samples of 10 µl were removed from a dilution tube and dropped onto LB agar plate. Application of the drops was located from left to right in parallel, roughly 1cm from the agar edge, with roughly 2cm separating each drop. In the conventional drop plate technique all the countable colony units are within the area covered by a 10 µl drop, or roughly 5-10mm. Due to the physical limitation of space in the drop, the DP method can be prone to difficult resolution. Examples include when colony forming units are overlapping, when plates become overgrown (exhibit more luxuriant growth) in the standard time period of incubation, or when multiple organism types are present.

A tilted variation of the DP method was utilized whereby immediately after application of the drops, the plate is tilted roughly 60 degrees (from horizontal). By tilting, gravity is used to gently elongate the 10 µl drop down the plate surface, producing three parallel lines of colony forming units. Elongation of the sample drops is essentially a no-contact spreading method. Using the tilted variation in conjunction with the DP method increases the resolution of the individual colony forming units by spreading them across a longer area. In practice, identifying the colonies individually is less uncertain.

After the drops on the agar are dried, the plates are inverted and incubated at 30°C for 24 h prior to counting. Colonies were counted and expressed as colony forming units (cfu) per milliliter (ml) or gram (g). For the traditional spread plate (SP) method, plates are counted which produce from 30-300 colonies. A standard for the DP method, corrected for the volume plated, counts the plate which produces 3 to 30 colonies per 10 µl drop of sample dispensed (Herigstad *et al.*, 2001). The concentration of microbes is given by $c = F \cdot z/v$ (Niemelä, 2003), where c is the estimated microbial content per unit weight or volume of sample, F is the dilution factor (reciprocal of the dilution), z is the number of colonies observed, and v is the volume of the test portion (in ml of the final dilution).

In serial dilution plate counts, there can be countable plates at multiple dilutions for an individual sample. For example, a plate at the highest dilution range may contain 3 colonies and the next lower dilution plate for the same series may contain 24 colonies. Traditionally, the technician will count colonies at only one dilution in the dilution series (Herigstad *et al.*, 2001). As a matter of standard practice in this project, all countable plates were counted and recorded. With few exceptions, the colony count value from the less dilute (higher concentration) plate was utilized for calculating the cfu/ml value. The cfu/ml of each plate was the mean of the three counted drops.

The selection of the less dilute plate as a source of count data is important to highlight. Count data from the less dilute plate commonly resulted in a lower calculated cfu/ml value than the calculated value of the next higher dilution plate. Utilizing the counts from lower dilution plates produced lower cfu/ml values and there was less variation between the three counts on a given plate. From a statistical perspective, producing consistent cfu counts with little variation between the means of duplicate samples is the goal. Such an aim is challenging using microbiological viable count methods. Viable count methods utilize the term ‘distribution uncertainty’ to describe the inevitable random scatter of counts that appear in microbial suspensions (Niemelä, 2003). In terms of viability, numerical scatter may not be a direct indication of inaccuracy for the measurement. Although cfu count scattering is expected, efforts to minimize the effect are statistically relevant and supports the reasoning, as described above, for utilizing the less variable number sets.

For the purpose of this project, the statistical distribution of the number of microorganisms in a test sample is non-normal or nonparametric. This interpretation is in keeping with microbiological viable count methods, specifically the drop plate method described above

which uses the Poisson distribution (Herigstad *et al.*, 2001). A Poisson distribution considers the majority of the uncertainty to be associated with the randomness in the sample rather than randomness in the technique and the mean and the variance are considered equivalent (Fuentes-Arderiu; Herigstad *et al.*, 2001; Niemelä, 2003).

2.2.7 Optical density

A Helios Gamma Spectrophotometer was used, NC: 9423 UVG 1700E. The equipment was switched on and allowed to warm up for at least 30 minutes prior to use. Optical density was measured with the wavelength set to 600nm. The spectrometer was zeroed with 0.1 M phosphate buffer solution.

Samples were processed as undiluted, 1:5 dilution, and 1:50 dilution with measurements recorded for each sample period. To produce the 1:5 dilutions, 1 ml of sample was added to 4 ml of 0.1 M phosphate buffer solution. One ml of the 1:5 dilution samples was then added to 9 ml of 0.1 M phosphate buffer solution to produce a 1:50 dilution.

2.2.8 Herbicide utilization

Throughout this study it was important to verify that the bacterium had retained the ability to utilize the herbicide atrazine. A clearing zone technique (Mandelbaum *et al.*, 1995) was utilized as both a quality control and also as a measure of functionality. Atrazine agar plates were prepared with an atrazine concentration of 1000 mg/l (Appendix 6.1.5) using a Nufarm Flowable Atrazine™ suspension. The Nufarm product is a formulated agricultural grade atrazine with high solubility was and superior to analytical grade atrazine for producing atrazine agar plates of a consistent composition.. It is also the type of atrazine which would be likely found in agricultural settings and spill sites. Triplicate 10 µl drops from a sample were applied to the plate and incubated at 30°C for 72 h. Cultures which produced a clear zone in the agar underneath the area of sample application were considered to have retained the ability to utilize atrazine.

The zone of clearing was semi-quantitatively scored from 0 to 3. A zero score indicates that no clearing zone was observed. For plates having only slight observable clearing, a score of 1 was given. Clearing zones larger than 1, but not clear under the full sample area were given a score of 2. Full and clear zones under the sample area were scored as 3.

In example E of Figure 7, the area in which the 10µl drops were applied is surrounded by an additional area (~3mm halo) of clearing. A score of three with a ~3mm halo in the

surrounding medium is the highest (observed) degree of functionality using the clearing zone method. A score of three indicates the highest magnitude of atrazine utilization and is the initial magnitude of functionality expressed from a freshly grown flask of *Pseudomonas* sp. strain ADP. In practical usage, the magnitude of the scoring scale is roughly separated by one order of magnitude, and can be observed by serial dilution. For example, at the 1×10^{-1} dilution level the sample may score a three, and the next dilution level 1×10^{-2} expresses a score of two, and so on. Although a linkage of order of magnitude (scoring) to cell numbers is noted here, it is not quantified.

For purposes of determining the functionality of liquid bacterial cultures, formulated bacterial cultures, and time trial experiment samples, each were applied to atrazine activity agar plates at the 1×10^{-1} dilution level. Functionality was measured using concentrated material (10^{-1}) from samples having high enumeration levels (10^{8} to 10^{10}). To clarify, in each dilution series for cell enumeration, the first dilution tube (10^{-1}) was used to determine functionality. In using concentrated material to determine functionality (from all sample types), this method delivers a large population to the agar for each analysis. Therefore, the differing levels of clearing, and the resultant functionality over time are due to metabolic ability. For the current method, functionality is a measure of the atrazine degradation capability of the cells rather than their number.

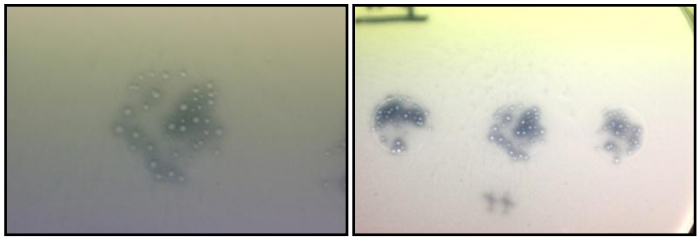
The usefulness of the current method is threefold:

- Designate an initial functionality level of three (3) shown in example E (figure 7).
- In production experiments, to confirm that that functionality is retained.
- In time trial experiments, to gauge the degree of functionality retention over time.

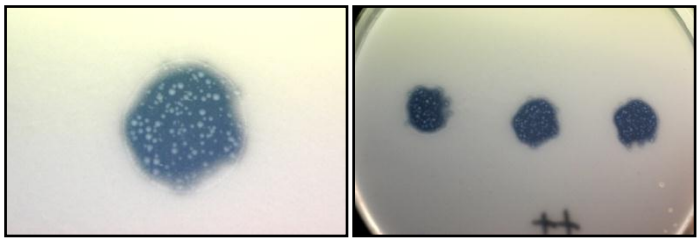
A.

B.

C.



D.



E.

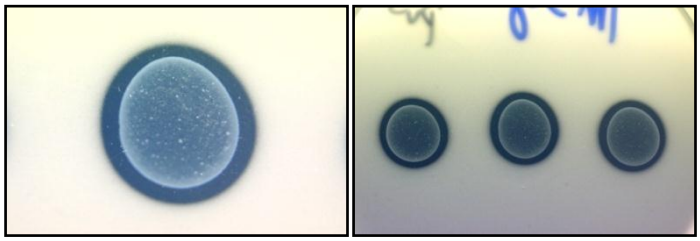


Figure 7. Examples of scoring the clearing zones in atrazine agar plates, A=0, B=1(low), C=2(low end), D=2(high end), E=3. Atrazine activity plates from liquid bacterial culture and formulated granules were conducted with triplicate drops (from each sample) at the 1 x 10^{-1} dilution level.

Due to the kind assistance of the biometrician Chikako Van Koten, it was possible to determine the level of confidence for the scoring method in Figure 7. For semi-quantitative

rank scoring, the highly conservative Fishers Exact Test was recommended for determining the level of confidence between the ranks. Using Fishers Exact Test a 90% confidence interval was calculated. Alternative statistical methods, Kruskal Wallis and Two factor Anova, produced less conservative levels of confidence ($p<0.05$). For the purposes of this thesis work the more conservative confidence analysis ($p=0.10$) is used to describe the clearing zone method. A 90% significance level also aligns with practical observation. The scoring scale is roughly separated by one order of magnitude, and can be observed by serial dilution where the cfu number is reduced by 90% with each ten-fold dilution.

2.3 Formulation methods

For the formulation work and storage trials developed in this chapter, the bacterial culture is produced by flask cultures, without pH control, and harvested after 24 hours. The methods of microbial culture, pre-culture, flask culture, and serial dilution are as described in Section 2.2. Examples of herbicide utilization are shown in section 2.2.8 where the zones of clearing are scored from 0 to 3 (Figure 7).

2.3.1 Production of the biopolymer gel

A biopolymer base was created by mixing xanthan gum and extra virgin olive oil (EVOO) together until homogenous. The components of the base, xanthan gum and EVOO, are described in section 2.1 and utilized a 1:1(w/w) ratio. The flask bacterial culture (section 2.2.5) to be encapsulated was added to the base, mixed vigorously until homogenous to produce a gel, and allowed to stand for 30 minutes at 4°C. The ratio (w/w) of xanthan, EVOO, and bacterial culture was 1:1:23g.

The gel produced was applied directly to the zeolite type and mixed together. Using a sterile stainless steel spatula and gentle tumbling, the material was distributed evenly onto the zeolite surface, thereby immobilizing the encapsulated cells onto the zeolite. A 4% (w/w) gel application was used to inoculate100 g of zeolite carrier.

Enumeration of gels (cfu/g) were conducted by transferring 1g of sample to a 120ml screw cap vial and adding 99g 0.1M phosphate diluent. The encapsulated cells were extracted from the gel by placing the 120 ml vials on a wrist-shaker for 10 minutes. After extraction, samples were serially diluted, plated, enumerated and scored for functionality per Section 2.2.

2.3.2 Enumeration of formulations on zeolite

Enumeration of zeolite samples (viability) was conducted by transferring 1g of sample to a 35ml vial and adding 9g 0.1M phosphate diluent. The immobilized cells were extracted from the zeolite surface by placing the 35ml vials on a wrist-shaker for 10 minutes. After extraction, samples were serially diluted, plated, enumerated per section 2.2 and scored for functionality per section 2.2.8.

2.3.3 Viability transfer

The transfer of cells through the formulation process stages were measured from the flask, to the encapsulation gel, and onto the immobilized gel and carrier formulation. Bacterial cultures of LB, MB, and IM (in triplicate flasks) were produced (Section 2.2.5), encapsulated in biopolymer gel (Section 2.3.1) and immobilized onto zeolite (Section 2.3.3.1). Enumeration was conducted at each process stage (Section 2.2.6, 2.3.1, and 2.3.2). Log scale enumeration data was converted to a linear scale and the percent recovery of the transfer process was calculated.

2.3.3.1 Ten week storage trials of formulations applied to NZ-I and AU-II zeolite

The viability, stability, and functionality of *Pseudomonas* sp strain ADP applied to zeolite was examined in triplicate 10 week storage trials conducted at 25°C. LB, MB, and IM bacterial culture was immobilized onto NZ-I or AU-II zeolite carrier with and without the application of an encapsulating gel onto the zeolite surface. The 20 variants of the storage stability trials were:

1. LB bacterial culture immobilized on NZ-I zeolite
2. LB bacterial culture immobilized on sterilized NZ-I zeolite
3. LB bacterial culture encapsulated in a gel and immobilized on NZ-I zeolite
4. LB bacterial culture encapsulated in a gel and immobilized on sterilized NZ-I zeolite
5. LB bacterial culture immobilized on AU-II zeolite
6. LB bacterial culture immobilized on sterilized AU-II zeolite
7. LB bacterial culture encapsulated in a gel and immobilized on AU-II zeolite
8. LB bacterial culture encapsulated in a gel and immobilized on sterilized AU-II zeolite
9. MB bacterial culture immobilized on NZ-I zeolite
10. MB bacterial culture immobilized on sterilized NZ-I zeolite
11. MB bacterial culture encapsulated in a gel and immobilized on NZ-I zeolite
12. MB bacterial culture encapsulated in a gel and immobilized on sterilized NZ-I zeolite

13. MB bacterial culture immobilized on AU-II zeolite
14. MB bacterial culture immobilized on sterilized AU-II zeolite
15. MB bacterial culture encapsulated in a gel and immobilized on AU-II zeolite.
16. MB bacterial culture encapsulated in a gel and immobilized on sterilized AU-II zeolite.
17. IM bacterial culture immobilized on AU-II zeolite
18. IM bacterial culture encapsulated in a gel and immobilized on AU-II zeolite
19. IM bacterial culture immobilized on pre-wetted AU-II zeolite
20. IM bacterial culture encapsulated in a gel and immobilized on pre-wetted AU-II zeolite

For each variant the bacterial culture was produced according to Section 2.2.5. At harvest (Time=24h) the bacterial culture was enumerated, the optical density was measured and the functionality was scored per section 2.2.8. A portion of the harvested *Pseudomonas* sp. strain ADP was encapsulated in a biopolymer gel. Production of the gel, its components, ratios, and application to the zeolite are described in section 2.3.1. The gel produced from the bacterial culture was enumerated (Time=Zero) per 2.3.1 and the functionality was scored per section 2.2.8.

A beaker was prepared for each zeolite type containing 100g of zeolite. Each beaker received a 4% (w/w) inoculation of bacterial culture or gel respectively. After inoculation, the beakers were stirred to evenly distribute the inoculum onto the zeolite surface, transferred in 10 g aliquots to 70 mL HDPE screw-cap containers, and closed tightly for storage. Ten samples for each variant (one for each week) were stored at 25°C and sampled weekly for 10 weeks. The variants were enumerated (Time=Zero) per 2.3.2 and the functionality was scored per section 2.2.8

At each weekly sampling, the water activity, enumeration and functionality were measured. Water activity sampling and measurement are described in 2.3.4.1. Extraction of the samples from the zeolite is per section 2.3.2. After extraction, samples were serially diluted, plated, enumerated per 2.2 and scored for functionality per section 2.2.8.

A 10 week trial for each variant was repeated in its entirety as a true triplicate, on three occasions, separated in time.

2.3.4 Physical Properties

2.3.4.1 Water activity assay

Water activity (a_w) is a prime factor of microbe stability, and is described as a dimensionless quantity representing the energy status of the water in a system and defined as the vapor pressure of water above a sample (P) divided by that of pure water (P0) at the same temperature where a_w =P/P0. Multiplication of the water activity by 100 provides the relative humidity of the atmosphere in equilibrium with the sample or R.H. (%) = 100 x a_w.

The importance of water activity in a microbial context is its ability to measure the water that is available to a microbe; or water bioavailability. The lower the water activity the less water is biologically available to the microbe for survival.

Water activity was measured using a Decagon AquaLab Series 3TE. To operate, the unit was switched on and allowed a warm-up period of 30 minutes prior to sample analysis. Three standards (0.250, 0.500, and 0.760) were used to confirm the calibration of the unit. Material to be analyzed was added, up to half full, in a plastic sample cup. The sample cup was gently inserted into the unit to avoid splashing and the analysis was commenced.

2.3.4.2 Zeolite moisture sorption

One primary role of the zeolite (in this work) is to act as a rigid construct to apply a microbial agent. It is likely that the zeolite carrier may also influence the moisture availability of the applied cells. In this section the moisture characteristics of the zeolite to sorb moisture was examined by drying, weighing, soaking, and reweighing the zeolite. Removing the free water from the zeolite, prior to weighing, in a consistent standard method proved challenging, and was therefore conducted using five water removal methods.

For each method the zeolite were washed to remove any dust, dried in an 80°C oven, weighed in triplicate, and then soaked in distilled water for 48 hours. The free or excess water from the soaked zeolite was removed by one of five methods and the sample was reweighed. The five manual methods of water removal were:

1. Using tweezers to shake the excess water from each zeolite piece. Sample size was six pieces of 2mm zeolite.
2. Blotting the zeolite (1-2 seconds) with a paper towel to remove excess water. Sample size was 1 gram dried sample for each type.

3. Pouring the zeolite into a sieve (425μm) and allowing ~15 seconds to drain the free liquid. Apply a paper towel directly to the sample (1-2 seconds). Sample size was 40ml by volume per sample type.
4. Sieve the zeolite (as in # 3) and blot the bottom of the sieve with a paper towel (indirect blotting for 1-2 seconds). Sample size was 40ml by volume per sample type.
5. Sieve the zeolite (as in #3) only with no paper towel. Sample size was 40ml by volume per sample type.

Five NZ zeolite types and three AUS zeolite types were examined using an electronic moisture analyzer to assess total water absorption. Total moisture content (% w/w) was measured with an electronic moisture analyzer, KERN MLS_N version 2.0 (Kern & Sohn Gmbh, Germany). Excess water was removed from the soaked sample material by method three and four (direct or indirect blotting) to remove excess water and dried by the moisture analyzer which recorded the cumulative weight loss.

2.3.4.3 Zeolite moisture sorption isotherm

Water sorption isotherms were prepared (Decagon Devices AquaSorp, USA) by plotting the water content (% w/w) against water activity (aw). Both adsorption and desorption curves were prepared for NZ-I and AU-II zeolites to confirm the results of section 2.3.4.2.

2.3.4.4 Appearance and friability of zeolite

Samples of each zeolite were photographed and visual observations made. The friability of the zeolite (e.g. tendency to shed mass) was assessed (Erweka TAR friability tester, Germany) at 25 rpm for 2 minutes and calculated as the percent mass loss from the zeolite granules.

2.3.5 Microscopy

2.3.5.1 Raman spectroscopy

Raman spectroscopy is a non-destructive, quantitative, and qualitative analysis for solids, liquids, and gases. Raman analysis has a high chemical specificity, and is able to probe non-absorbing and turbid complex matrices (Buckley & Matousek, 2011).

Raman spectra analysis was conducted to compare AU-II and NZ-I zeolite regarding water content and its distribution. The investigation was conducted to confirm the adsorption and absorption results of section 2.3.4.2.

The NZ-I and AUS-II zeolites were coated in xanthan gel produced (per 2.3.1) from LB bacterial culture (per 2.2.5). Samples were split or cut to obtain cross sections. The cut faces were then placed on the Renishaw XY motorized stage of the Renishaw System 1000 Raman microprobe. Raman spectra were recorded along measured steps of cross sections of each zeolite sample. The excitation line was 488nm provided by a Spectra Physics air cooled argon ion laser at a source power of 12 mW; the power at the sample was approximately 1mW and the laser spot size was 2um. The spectra were recorded in single spectrograph mode and a HNF filter was used to remove the Rayleigh scattered lines. Spectral resolution was 4 cm^{-1}. The spectra were recorded in backscattered mode between 4000 cm^{-1} and 200 cm^{-1}.

2.3.5.2 Scanning Electron Microscopy (SEM) characterization

Visual assessment of the surface of NZ-I and AU-II zeolite was performed by scanning electron microscopy (Philips XL30S FEG, Netherlands). Images of zeolite types were collected before and after coating with LB bacterial culture containing *Pseudomonas* sp. strain ADP and after coating with LB bacterial culture gelled with xanthan gum (per section 2.3.1). Samples were sputter coated with gold for 2 minutes (Quorum Technologies Polaron SC 7640 sputter coater, England) and viewed at an accelerating voltage of 5 kV.

2.4 In situ methods

The methods of microbial culture, preculture, flask culture, and serial dilution are as described in 2.2. Examples of herbicide utilization are shown in 2.2.8 where the zones of clearing are scored from 0 to 3.

In the results section it is important to note the difference in cell densities applied. Ideally the soil samples would be inoculated with roughly the same density of cells. However as noted in section 3.1, MB is a lower yielding media and produces roughly 1 log less cfu/ml than LB or IM. It was decided that rather than concentrate or dilute each media to a uniform density, the bacterial cultures would be used in the condition they were harvested. Using an unconcentrated bacterial culture is relevant for industrial applications and does not introduce additional unknown impacts due to concentration or dilution.

2.4.1 Physical Properties

The methods of water activity are as per discussed 2.3.4.1. Its importance to the bioavailability of water and microbial viability is discussed.

2.4.2 Survival delivered as a bacterial culture into soil

The first aim of in situ assessment for this project is to quantify the survival of liquid bacterial cultures applied into sterile soil. By utilizing a sterile soil the competitive and predatory aspects of the soil biology can be removed and the degree of survival of the inocula is then evaluated in terms of the physical and chemical properties of the soil. Candidates who perform well in response to the physical and chemical challenges of sterile soil are then suitable for advancement to field (non-sterile) soil trials.

An irradiated Templeton loam was used as the sterile soil matrix. The three culture medium types from the fermentation and formulation phase were used (LB, MB, and IM). Inoculum of these medium were applied to the sterile soil and evaluated for survival and functionality over a 10 week period. The culture stock, pre-culture, inoculation and flask growth are described in Methods 2.2. Flasks of LB, MB, IM (100 ml Medium in 500 ml flask) were produced containing *Pseudomonas* sp. strain ADP harvested in late log phase.

Samples of 25g sterile soil were transferred to nine HDPE 50ml screw-cap containers. Soil samples were pre-wetted with distilled water (5 ml/25g), stirred, and stored for 24 h at 15°C. Pre-wetting of the soil was conducted to allow soil to reach moisture equilibrium and also to lessen the osmotic shock to the inoculum. The purpose of this experiment is intended to evaluate the biological performance of the bacterial culture inoculum rather than its general response to desiccation. Thus the experiment provides an optimal amount of moisture to the soil thereby minimizing the chemical and physical challenges related to dehydration. In this way the biological performance can be isolated and evaluated on its own merits.

To provide optimal water content for the soil trial, a scoping experiment was conducted to raise the soil moisture content of the sterilized soil up to its field capacity of 30%. This level was measured to correspond to a water activity of 0.995. Using these results for guidance, the design of the soil trial includes 25 g dry soil, 5 ml water, and 1 ml inoculum to achieve a water content of ~24% water. For a Templeton loam soil the wilting point is 14 % water content and 30% is its field capacity. At a water content of ~24% the water activity (aw) is < 1% different from the 0.995a_w value of 30% measured in the scoping experiment.

Each of the three culture media was evaluated in triplicate (9 jars). Pre-wetted soil samples were inoculated with 1ml of culture medium, stirred with a flame sterilized spatula, and stored at 15°C.

Analysis of the soil was conducted as described in the Methods section 2.2. Samples were taken aseptically, serially diluted in duplicate and applied in triplicate (drop plate method) on LB-Miller agar for enumeration. Functionality was assessed by clearing zone method on atrazine containing agar (section 2.2.8). The water activity (a_w) of the soils was also measured at each time point (per 2.3.4.1). Analysis was conducted at the time of harvest for the flasks, at Time=Zero for the soils, and at week 2, 5, 7, and 10.

2.4.3 Survival delivered as an immobilized formulation in soil

The second aim of in situ assessment for this project is to quantify the survival of the immobilized formulation in sterile soil. As was done in the bacterial culture experiment (section 2.4.2) the inoculation is conducted into a sterile soil to remove the competitive and predatory aspects of soil biology. The degree of survival of the formulation over time is then assessed in terms of its competence to the physical and chemical properties of the soil. Candidates who perform well in response to the physical and chemical challenges of sterile soil are then suitable for advancement to field (non-sterile) soil trials.

An irradiated Templeton loam was used as the sterile soil matrix. The three culture medium types fromthe fermentation and formulation phase were used (LB, MB, IM). Bacterial culture from the media were encapsulated in a polymer gel and applied to a prewetted AU-II zeolite carrier. Dry zeolite was pre-wetted with a 2% (w/w) 0.1M sterile phosphate buffer solution. The formulation consisting of the bacterial culture encapsulated in a gel and applied to the AU-II zeolite was delivered into the sterile soil and evaluated for survival and functionality over a 10 week period.

The culture stock, pre-culture, inoculation and flask growth are described in Methods section 2.2. Flasks of LB, MB, IM (100 ml Medium in 500 ml flask) were produced containing *Pseudomonas* sp. strain ADP harvested in late log phase per 2.2.5.

Samples of 25g sterile soil were loaded in nine HDPE 50 ml screw-cap containers. Soil samples were pre-wetted with distilled water (5 ml/25g), stirred, and stored for 24 h at 15°C. Pre-wetting of the soil was conducted to allow soil to reach moisture equilibrium and also to

lessen the osmotic shock to inoculated microbes. The purpose of this experiment is intended to evaluate the biological performance of the formulation rather than its general response to desiccation. Thus the experiment provides an optimal amount of moisture to the soil thereby minimizing the chemical and physical challenges related to dehydration. In this way the biological performance can be isolated and evaluated on its own merits.

To provide optimal water content for the soil trial, a scoping experiment was conducted to raise the soil moisture content of the sterilized soil up to its field capacity of 30%. This level was measured to correspond to a water activity of 0.995. Using these results for guidance, the soil trial is designed to achieve a water content of ~24% water which includes 25 g dry soil, 5 ml water, and the inoculum. For a Templeton loam soil the wilting point is 14 % water content and 30% is field capacity. At a water content of ~24% the water activity (aw) is < 1% different from the 0.995a_w value of 30% measured in the scoping experiment.

Each of the three culture media was evaluated in triplicate (9 jars). Pre-wetted soil samples were inoculated with 5 g of the formulation, stirred with a flame sterilized spatula, sealed, and stored at 15°C.

An additional processing step in this experiment was the separation of the sample into its zeolite and soil fractions. At each analysis time point the jars were stirred and a sample was transferred to a sterile cup used for measuring water activity (section 2.3.4.1). The water activity data represents soil and zeolite portions combined. After the water activity measurements were completed, the sample comprised of both zeolite and soil was placed in a sterile petri dish. Using sterile tweezers the zeolite granules were physically separated from the soil. The zeolite and soil fractions were each enumerated to assess survival of both materials and whether a transfer of viable microbes from the zeolite delivery system into the soil fraction was occurring.

Enumeration was conducted as described in Methods Cultures section 2.2. Samples were taken aseptically, serially diluted in duplicate and applied in triplicate (drop plate method) on LB-Miller agar for enumeration. Functionality was assessed by clearing zone method on atrazine containing agar (section 2.2.8).

Survival, functionality, and water activity were measured at each time point. Analysis was conducted at the time of harvest for the flasks, at Time= Zero, and at week 2, 5, 7, and 10.

3 Results

3.1 Fermentation Results

The aim of the fermentation work was to improve the productivity (number of cells) of the culture medium and also retain the target functionality, by replacing atrazine with cyanuric acid. Medium development is notably a process that is laborious, expensive, open ended, time consuming and involves many experiments (Kennedy & Krouse, 1999). A brief overview of the development process is described in Section 3.1.1, 3.1.2, and 3.1.3. A step-by-step description and their data are not shown here. The process was procedural (empirical) and the resultant medium, the improved medium (IM), is presented in Section 3.1.4 with statistics in 3.1.5.

3.1.1 Initiation Phase

The initiation phase of the medium development for this research began with a medium borrowed from Mandelbaum (1995) and designated MB. A prototype medium was developed to replace the atrazine component in the MB medium with cyanuric acid. The prototype medium was compared to reference medium DSMZ 465i Medium and MB for production of cells. The effects of autoclaving on atrazine and cyanuric acid were examined for the potential to create microbial utilization problems during medium development. Prototype medium growth profiles were conducted in stirred and static flasks to examine oxygen preferences. Yeast extract was added to the medium components.

3.1.2 Concentrating the Components

The next phase of medium development increased the concentrations of citrate, cyanuric acid, yeast extract, and the mineral component (MM) by four times (4x) the previous levels. Interactions between media components and concentrations can produce uncertainty in culture medium optimization processes and these experiments allowed interactions and uncertainties to be better observed. These experiments served as a medium sized jump in concentration levels before the much larger increases during the optimum range phase.

3.1.3 Examination of the optimal range

A new medium was developed named by the ratio 4:4:1:4 which consisted of citrate (4g/l), cyanuric acid (4g/l), yeast extract (1g/l) and mineral medium (MM) components (4 times the original MB salt component levels). These four primary components were examined in separate evaluations, across a range of concentration levels, to produce a final improved

medium with improved cell productivity and functionality. Concentration range evaluations were conducted for citrate (4 to 40 g/l), cyanuric acid (4 to 12 g/l), yeast extract (0 to 20 g/l), and MB salt components (0 to 8 times) the original levels of Potassium phosphate dibasic, Potassium di-hydrogen orthophosphate, Magnesium sulphate heptahydrate, and Sodium chloride, Calcium chloride.

3.1.4 Selection of the Improved Medium

The production and selection of the best performing prototype medium, variant 20:6:1:4, concluded the medium development phase of the research. In further phases of research the 20:6:1:4 growth medium is utilized and designated as the Improved Medium (IM). Table 2 lists the composition of the Improved Medium. There may exist some further opportunities to perform a designed optimization of the cyanuric acid, citrate, yeast extract, and salt variables. The work presented here bracketed the optimal range. For future investigators this is an appropriate starting point for a designed experimental optimization, and may illuminate interactions within these parameters.

Table 2. Improved Medium (IM) composition.

Component	g/l
Sodium Citrate	20
Cyanuric acid	6
Yeast extract	1
Potassium phosphate dibasic	6.4
Potassium di-hydrogen orthophosphate	1.6
Magnesium sulphate heptahydrate	0.8
Sodium chloride	0.4
Calcium chloride	0.08

3.1.5 Significance

To state the significance (p value) of the flask media development process from the starting point of MB medium to the production of the IM medium, the count data (cfu) from each medium type was analyzed using Generalized linear modelling (GLM) with negative binomial distribution. Count data of six flasks for each media (n=6) after 24 hours growth (post inoculation) are shown in Figure 8 and were compared as a media specific negative binomial

distribution. The IM media was used as the intercept and showed a significant difference in cell numbers between the MB media type ($p<0.001$).

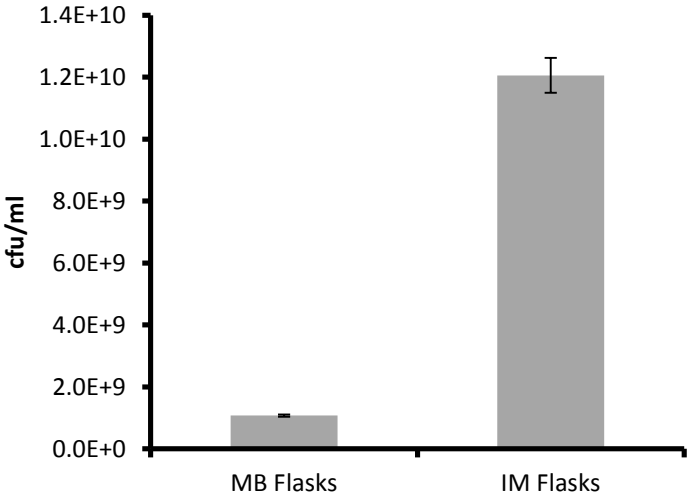


Figure 8. Flask populations for MB and IM media at the late log phase harvest point of 24 hours. Error bars are standard error of the mean (n=6) of six flasks of each media type. The difference in cell productivity between MB and IM media was significant ($p<0.001$).

Note the 24h cfu data used for calculating the p-value is separate from the maximum cfu count. The 24 hour time point was used to calculate the significant difference between media types as it is the culture harvest point (late log phase) in formulation development (Results Section 3.2). Previous investigators have also used the 24 hour harvest point for MB medium (Mandelbaum *et al.*, 1995; Shapir & Mandelbaum, 1997; Shapir *et al.*, 1998a; Silva *et al.*, 2004) Biglione (2008) confirms the work of Mandelbaum (1995) that the atrazine is removed completely from the atrazine medium within 24-30 h.

3.1.6 Summary

An overview of the process of cell productivity improvement for *Pseudomonas* sp. strain ADP is shown in Figure 9. The culture medium component ratios are citrate (g/l), cyanuric acid (g/l), granulated yeast extract (g/l), and 0,1,4, or 8 times the original MB salt component levels (Potassium phosphate dibasic, Potassium di-hydrogen orthophosphate, Magnesium sulphate heptahydrate, Sodium chloride, Calcium chloride). Maximum cell productivity of the final improved medium was ~2 x 10^{10} and is an improvement from the initial ~1x 10^{9} cells of the MB starting medium. The overall improvement of the medium is twice one full log scale, or a 20 fold increase in colony forming units. In percentage terms, the increase in cells is 2000%. The stepwise optimization improvement from 4:4:1:4 to 20:6:1:4 increased the

cells by roughly double from ~1x 10^{10} to 2 x 10^{10}. Further opportunities may exist to increase the cell productivity by performing a designed optimization.

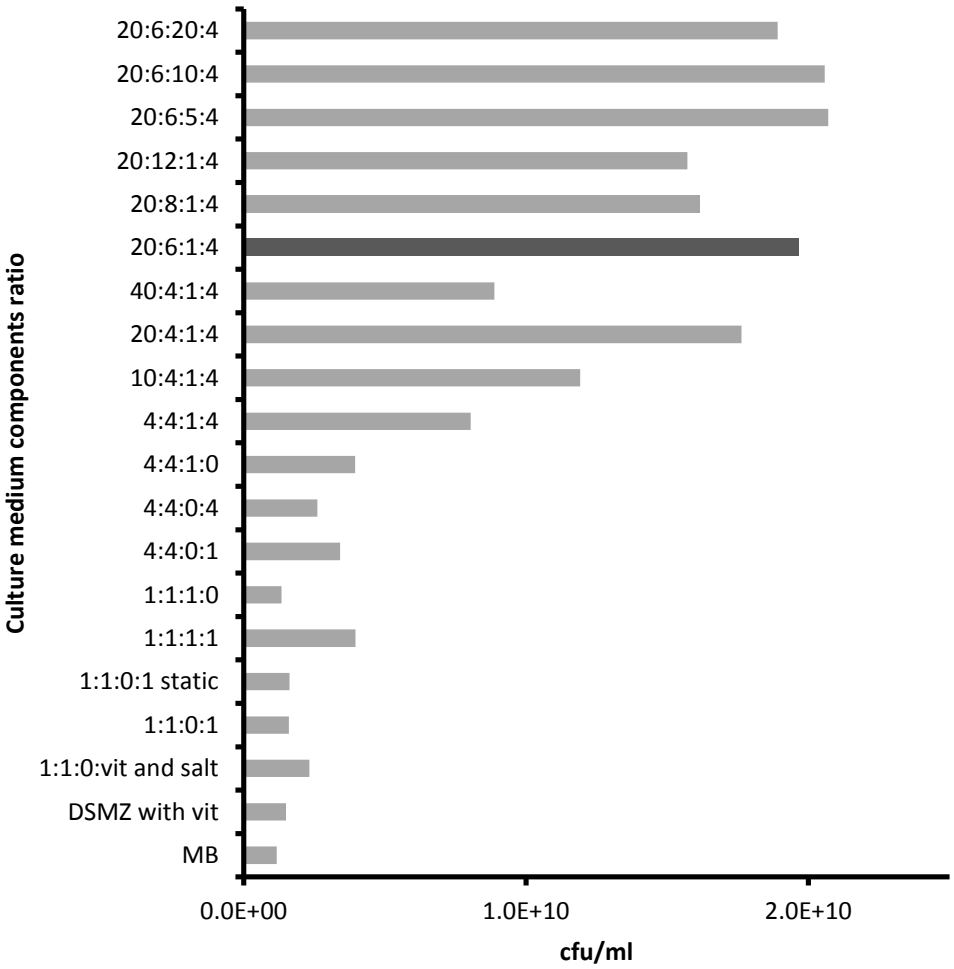


Figure 9. General overview of improvement in cells (cfu/ml). The starting point is the model medium (MB) and development is shown through the evaluation of the optimal range and the selection of the improved medium (dark bar).

The aim of the fermentation work was to improve the cells productivity of the culture medium and also retain the target functionality, by replacing atrazine with cyanuric acid. This outcome was accomplished.

3.2 Formulation Results

The aim of formulation was to develop immobilization methods which maximize cell survival, stability, and functionality. The formulations were developed to optimize the shelf life (10 weeks) stability of the strain at 25°C.

3.2.1 NZ zeolite

Throughout the formulation work NZ and AU zeolite types were evaluated. In section 3.2, physical, chemical, biological, and time frame characteristics were examined. NZ zeolite showed poor results in numerous stability trials and was discontinued in the last stages of the development process.

3.2.2 Physical properties of zeolite

An examination of the physical properties of the zeolites was conducted to investigate possible explanations for the stability differences between NZ and AU zeolite types.

3.2.2.1 Zeolite moisture adsorption

Five NZ zeolite types and three AU zeolite types were examined using an electronic moisture analyzer to assess total water absorption. Total moisture content (% w/w) was measured with an electronic moisture analyzer, KERN MLS_N version 2.0 (Kern & Sohn Gmbh, Germany). Excess water was removed from the soaked sample material by method three and four of section 2.3.4.2 (direct or indirect blotting), to remove excess water and dried by the moisture analyzer which records the cumulative weight loss.

The absorption characteristics of the zeolite types were distinct when measured as percent moisture after soaking, and samples were blotted to remove adsorbed surface water content (Figure 10). As a group, all zeolites from the NZ type had a significantly higher capacity to absorb water. The moisture content for all NZ samples was in the 40% range. In contrast, the zeolites for the AU group had much lower water content, in the 7% range. The results show a clear physical difference between the NZ and AU zeolite types to absorb water with NZ zeolite retaining over 5 times the amount of moisture (by weight) than the AU zeolites.

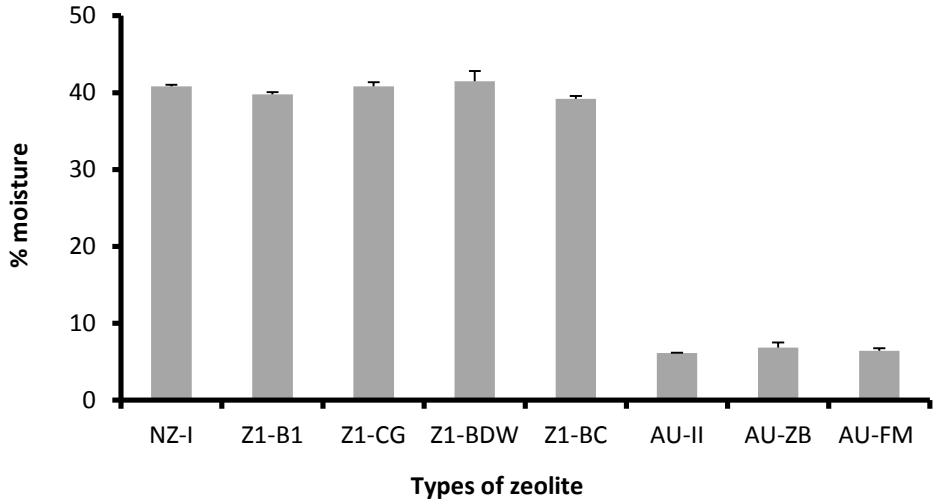


Figure 10. Moisture analyzer profile of zeolite types. Error bars are the standard error of the mean (n=3).

In the results above, the samples were prepared by direct towel blotting prior to weighing. Absorption of the zeolites was examined by removing the adsorbed (surface) water content.

The difference between absorbed and adsorbed water content was examined by removing excess water via direct and indirect blotting, prior to being dried by the moisture analyzer. In Figure 11 the moisture analysis for NZ zeolite is compared. For both direct and indirect water removal methods the percent moisture remaining in the NZ zeolites was similar. It appears that the moisture content of the NZ zeolite is absorbed internally, rather than adsorbed externally.

In contrast to the NZ results, when AU zeolite is prepared by direct and indirect towel blotting to remove the adsorbed (surface) water content the results are distinctly different (Figure 12). For AU zeolite, direct contact with the towel surface removed more than double the moisture content. These results show a distinct difference between the water content absorbed into AU zeolite and the water adsorbed onto AU zeolite. The adsorbed moisture content of AU zeolite appears to be proportionally equivalent, or greater, on the surface of the material rather than absorbed internally.

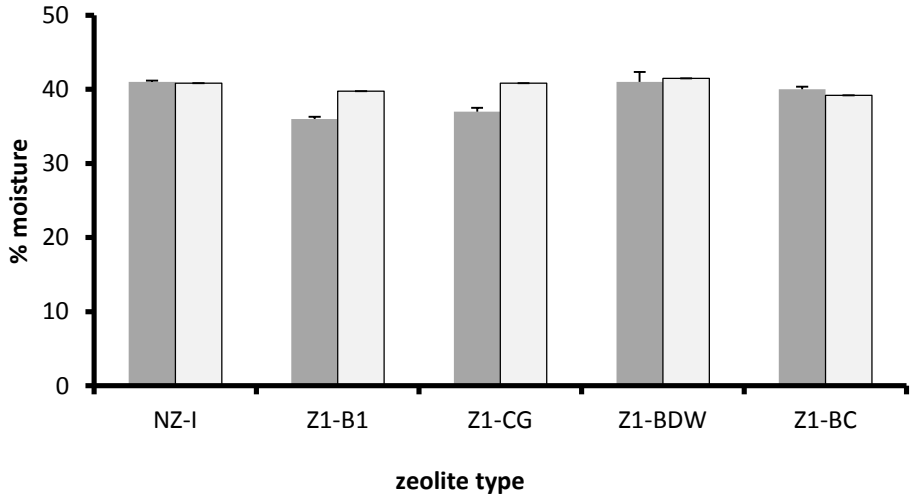


Figure 11. Comparison of two methods of free water removal for a panel of NZ zeolite. Symbols are (■) towel under sieve, and (□) towel blotted. Error bars are standard error of the mean (n=3).

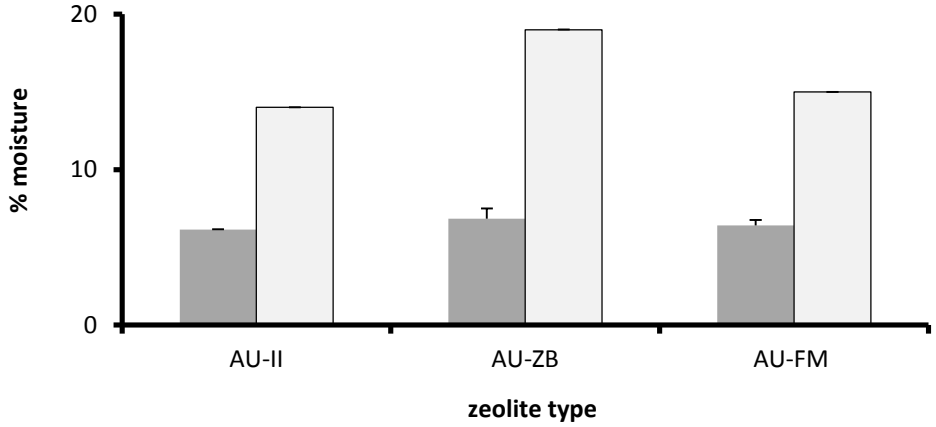


Figure 12. Comparison of two methods of free water removal for a panel of AU zeolite. Symbols are (■) towel blotted and (□) towel under sieve. Error bars are standard error of the mean (n=3).

The two types of zeolite that will be utilized in further formulations are NZ-1 and AU-II. These zeolite types have the same size (2mm) and are suitable for agricultural seed drilling equipment. From the moisture profiles (Figure 10) both NZ-1 and AU-II appear to representative of their groups.

The two types of zeolite that were utilized in further formulations (NZ-1 and AU-II) were compared using four methods of water removal (Figure 13). For the pre-soaked AU-II zeolite

a broader range of percent (%) moisture data was observed depending on the method of free water removal prior to measurement. Methods which targeted the water accumulated on the zeolite surface (e.g. shaking or towel contact) removed larger volumes of water from the AU-II material. These results are supportive of the earlier results which suggested an adsorptive accumulation of water on AU-II material. A working concept is therefore strengthened that water primarily accumulates on the surface of AU-II. In contrast, the excess water removal method had little effect on the moisture content of the NZ-I. These results support the earlier work and suggest that water is strongly absorbed into NZ-I, rather than adsorbed, and accumulates within the zeolite rather than on the surface as in the AU-II.

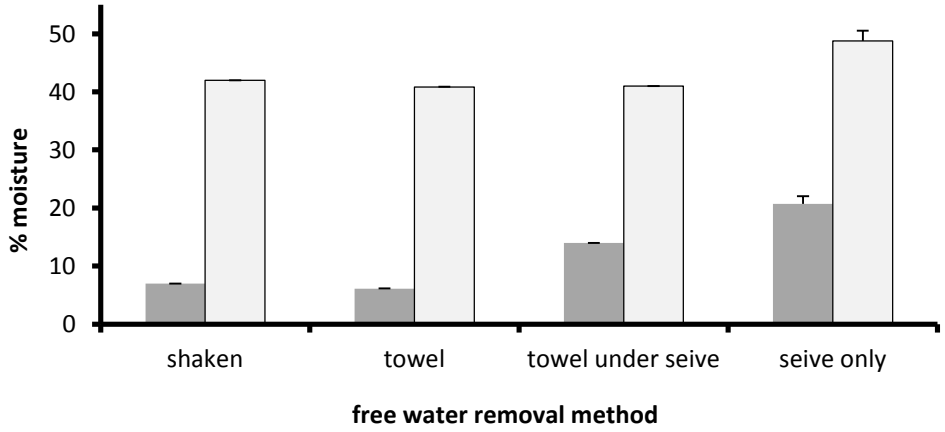


Figure 13. Comparison of percent (%) moisture measurements using four water removal methods for the two zeolite types used in further formulations. Symbols are (■) AU-II, and (□) NZ-I zeolite types. Error bars are standard error of the mean (n=3).

The results of this section indicate distinct differences in the moisture characteristics for NZ and AU zeolite types. Moisture content (by weight) was five times greater for NZ zeolites than AU zeolites. The NZ zeolite appears to carry a larger reservoir of water content internally. Zeolites from the AU group have little internal absorption, and appear to accumulate the available moisture on the surface of the material.

3.2.2.2 Zeolite moisture sorption

Water sorption isotherms were prepared (Decagon Devices AquaSorp, USA) by plotting the water content (% w/w) against water activity (a_w). The two types of zeolite generate two distinct types of moisture sorption isotherm curves (Figure 14). Zeolite type NZ-I has a type 2 sorption isotherm. Zeolite type AU-II has a type 1 sorption isotherm. Both isotherm types

are typical of surface monolayer adsorption. In regard to water levels sufficient to enable microbial stability, the far right points of the isotherms (the 'wet end') are important. There is a clear separation of moisture content for the zeolites after a water activity of ~0.40 with the NZ-I continuing to gain moisture content whereas the AU-II is at a plateau level. In percentage terms, at the wet end, the moisture content of NZ-I is roughly double AU-II. It is interesting to highlight that although the moisture (by weight) is roughly double for NZ-I zeolite, the definition of water activity would suggest that both zeolites have equivalent "availability" at the wet end (a_w >0.9).

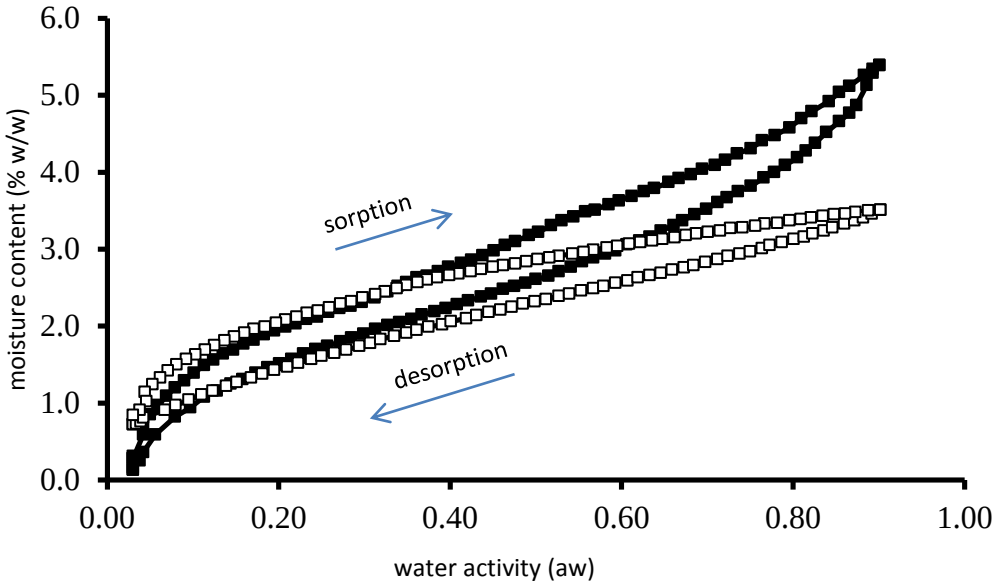


Figure 14. Comparison of moisture sorption isotherms for two types of zeolite, Symbols are (■) NZ-I and (□) AU-II zeolite types. Error bars are standard error of the mean (n=3) and within symbols. For each type, sorption is the top data line, and desorption is the bottom line.

The isotherm results support the results from Section 3.2.2.1, and demonstrate the zeolite types NZ-I and AU-II have separate and distinct moisture holding characteristics.

3.2.2.3 Appearance and friability of zeolite

Samples of each zeolite were photographed and visual observations were made (Figure 15 and 16). Friability (e.g. tendency to shed mass) was assessed (Erweka TAR friability tester, Germany) at 25 rpm for 2 minutes and calculated as the percent mass loss from the zeolite granules.

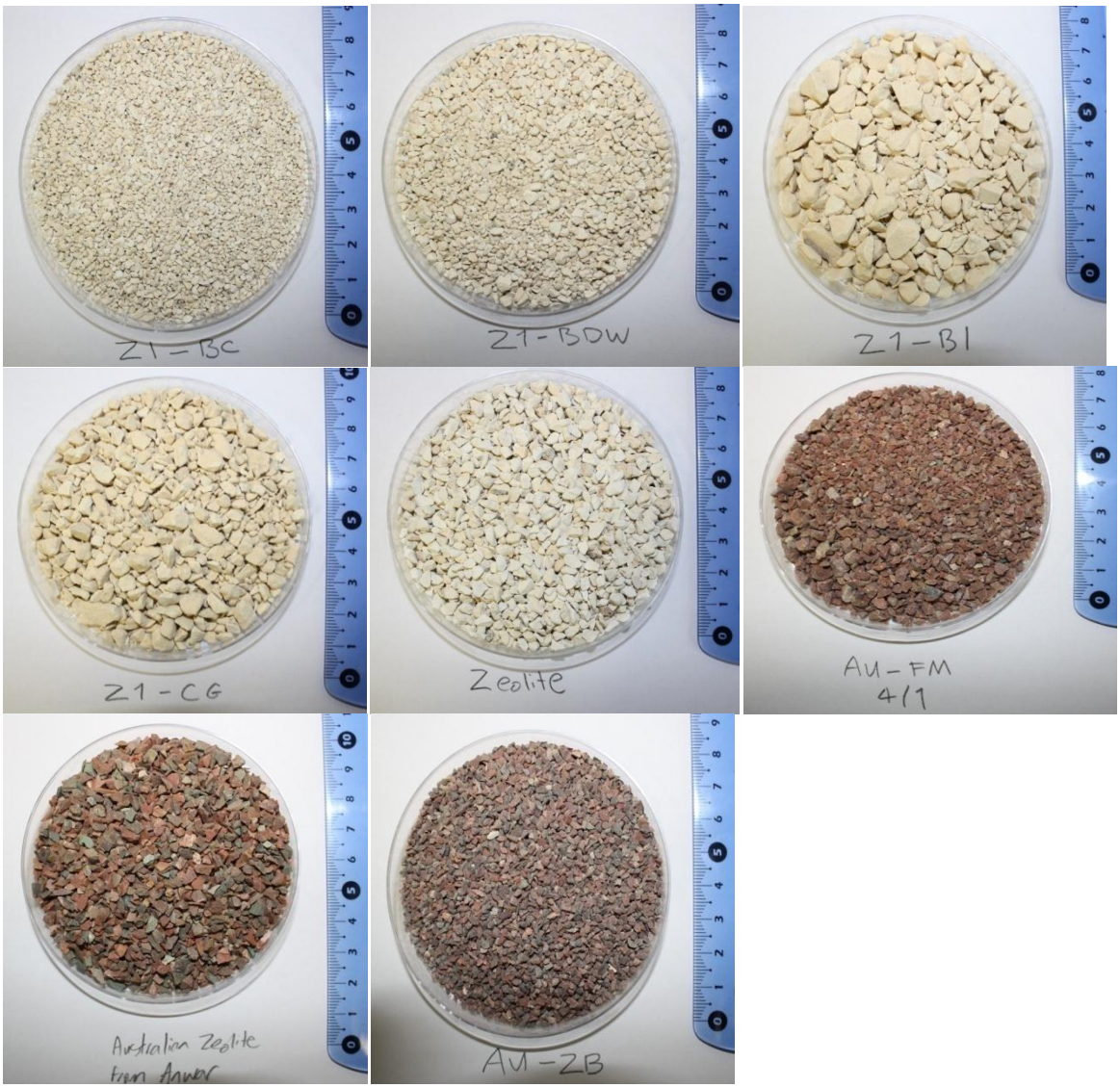


Figure 15. Appearance of eight zeolite types.

Figure 16. Visual comparison of two types of zeolite. The Australian (AU-II) zeolite is left and New Zealand (NZ-I) zeolite is shown on right. Granule size is 2-4mm.

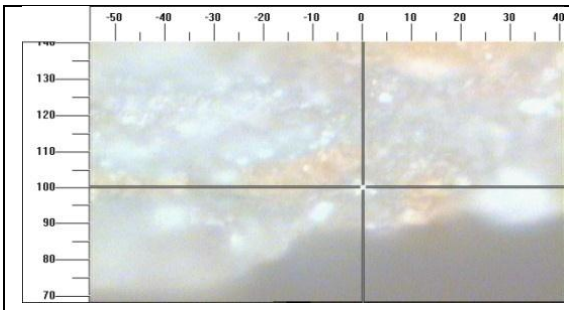
The New Zealand zeolite was found to be very friable compared to the Australian zeolite losing 5% mass compared to no loss respectively. This result supports earlier observations that AU-II was denser by volume than NZ-II, and was also less prone to breaking apart by sieving and mixing methods during handling.

3.2.2.4 Raman spectroscopy of zeolite

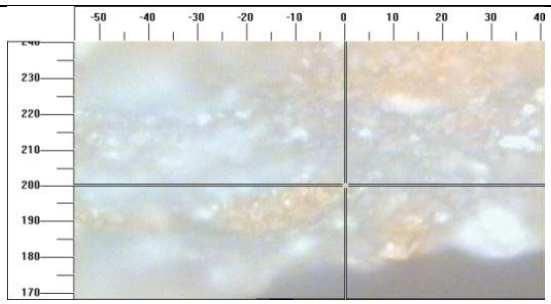
Raman spectra analysis was conducted to compare the water content and its distribution within the zeolite. Raman spectra analysis is described in section 2.3.5.1.

A. Australian Zeolite

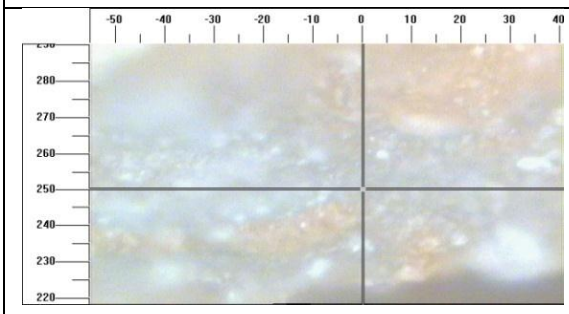
The AU-II zeolite contained more than one phase as can be seen in the optical images in Figure 17, and also from the Raman spectra in Figure 18. Spectra of the gel coating and zeolite areas close to the gel coating exhibited high fluorescence resulting in weak spectra; therefore the fluorescent background was subtracted from the spectrum to obtain the spectra shown in Figure 18. The bacterial gel coating can be seen on the edge of the cross section in Figure 19 and is approximately 20 – 30 um thick.



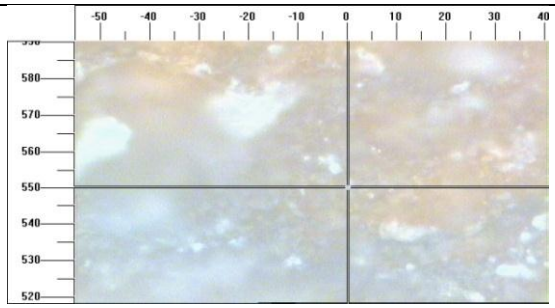
AU-II zeolite 1: 10 um from edge



AU-II zeolite 5: 20 um from edge



AU-II zeolite 6: 25 um from edge



AU-II zeolite 8: 50 um from edge

Figure 17. Optical images (500x magnification) of some analysis points of the cross section of the AU-II zeolite.

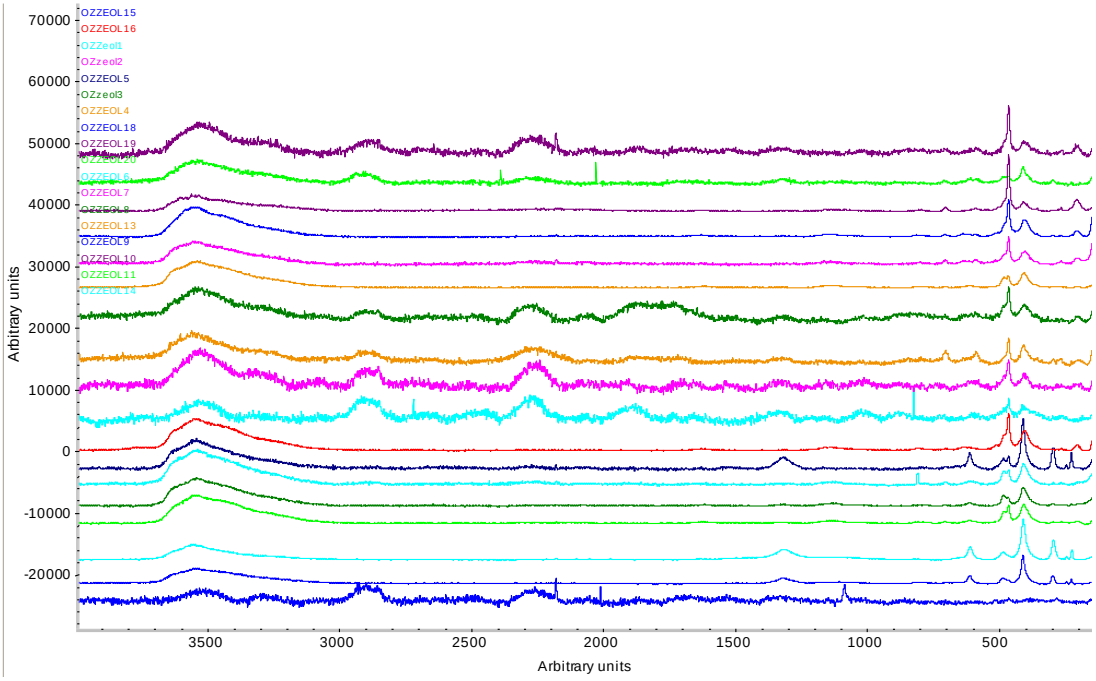


Figure 18. Raman spectra of the cross section of AU-II zeolite sample. The areas analysed for water content are indicated by colored lines. From approximately 3700 to 3100 arbitrary units corresponds to the H2O band while 350 to 550 arbitrary units corresponds to the mineral band.

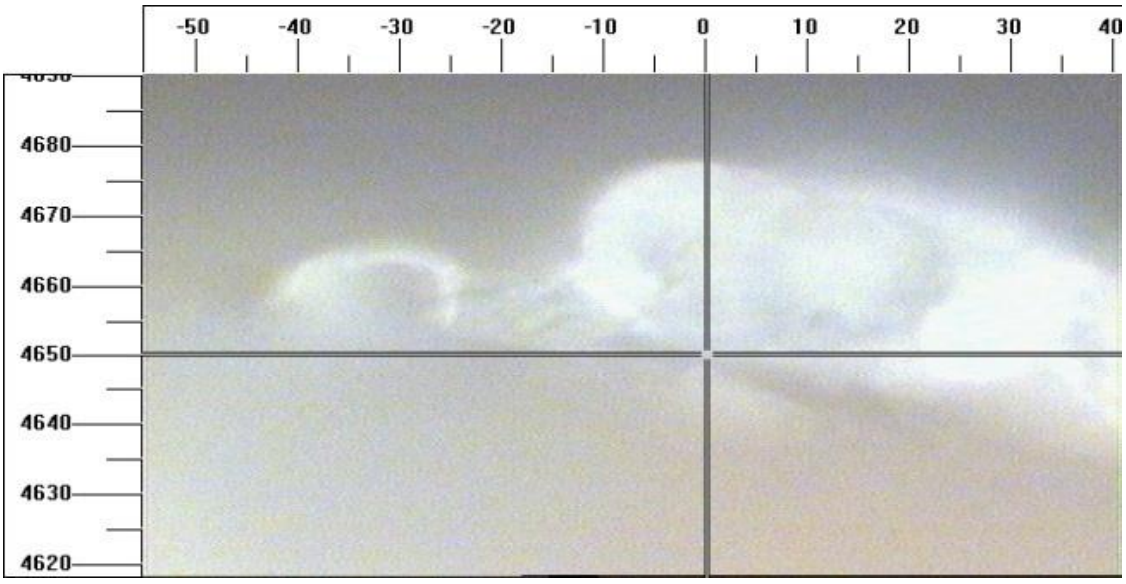


Figure 19. Optical image at 500 x magnification of gel coating on edge of cross section of AU-II zeolite.

In order to obtain a quantitative estimate of the amount of water at each point analysed, the area under the OH stretch region (3700 – 3100 cm^{-1}) was normalised by dividing this area by

the total area of the major mineral bands between 550 – 330 cm^{-1}. It was not possible to measure the area of a single band because of the different minerals present in the zeolite. The normalised water content at each point along the cross section is shown below in Figure 20.

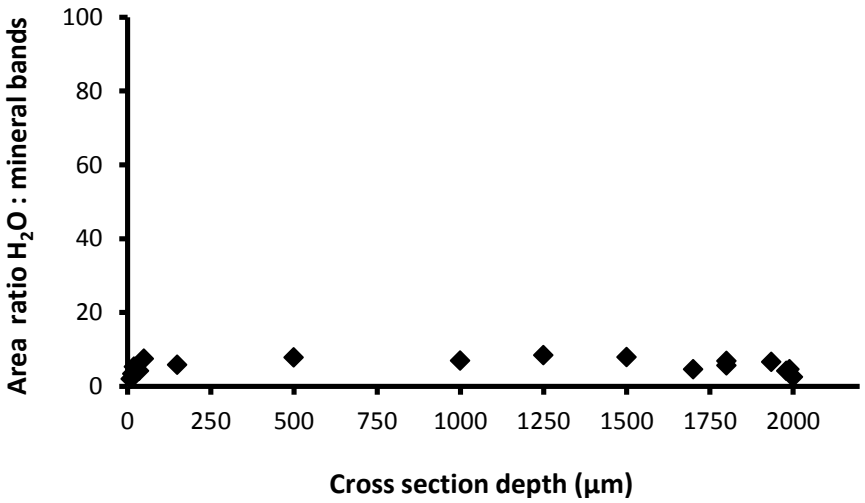


Figure 20. Normalised water content across a 2mm cross section of an AU-II zeolite sample.

B. New Zealand Zeolite

An optical image of the NZ-I zeolite sample at 200x magnification is given in Figure 21. The bacterial gel coating on the edge of the cross section was approximately 5 – 10 um thick as can be seen in the optical image at 500x magnification in Figure 22.

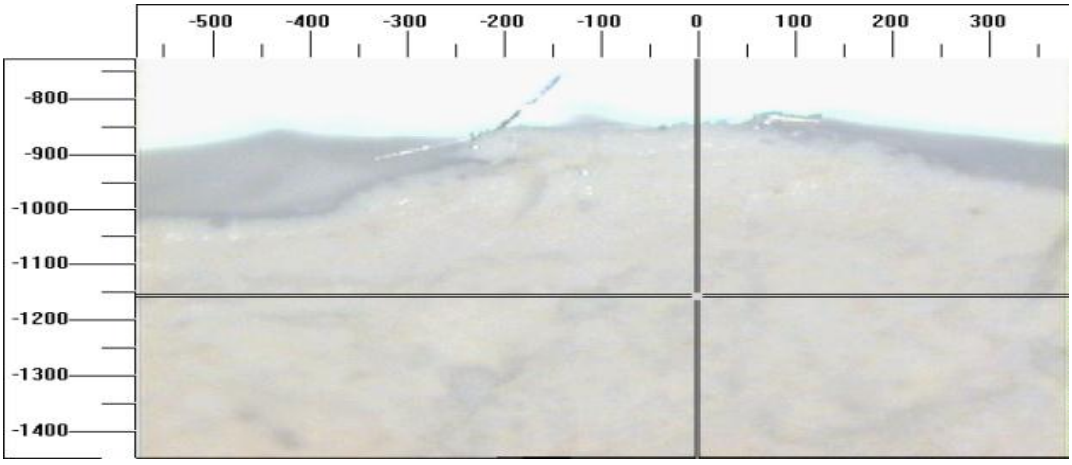


Figure 21. Cross section of the NZ-I zeolite at 200x magnification.

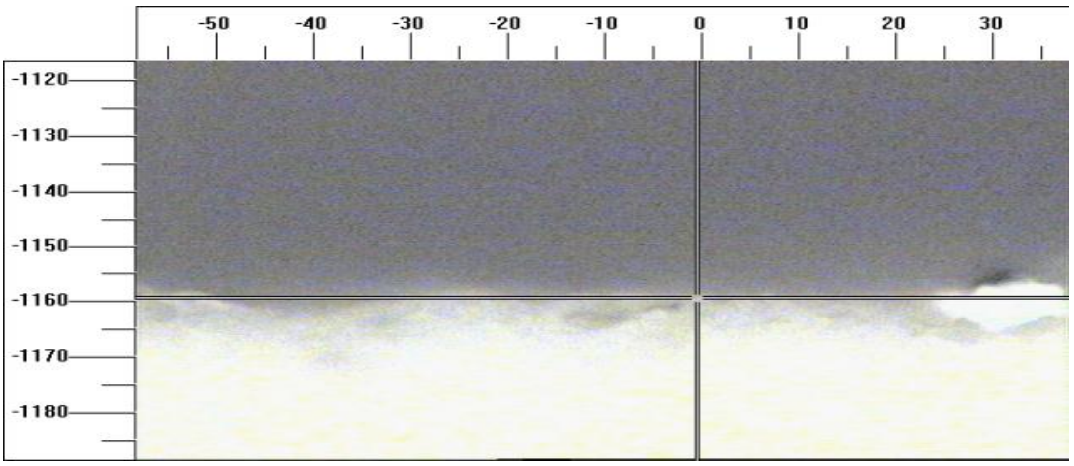


Figure 22. Optical image of the edge showing the bacterial gel coating on the cross section of the NZ-I zeolite sample (500x magnification).

The Raman spectra recorded at various points along the 1.4 mm cross section are given in Figure 23. The spectra of the gel coating and zeolite areas close to the gel coating exhibited high fluorescence resulting in weak spectra; therefore the fluorescent background was subtracted from the spectrum to obtain the spectra shown (in Figure 23).

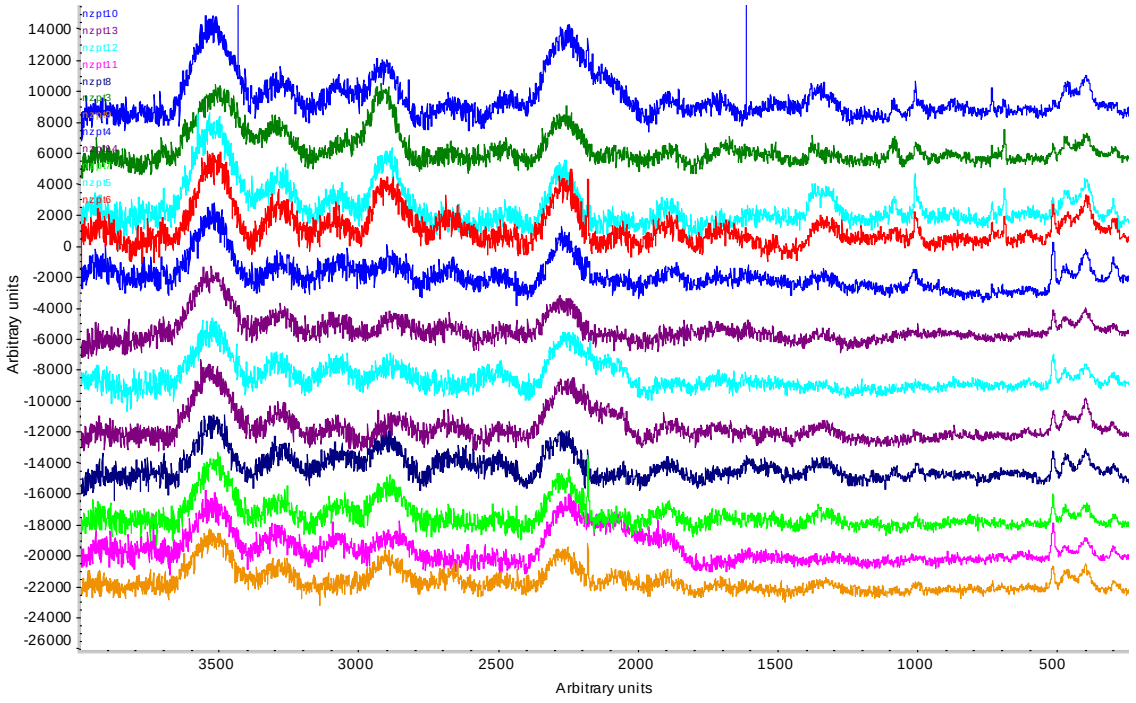


Figure 23. Raman spectra of the cross section of a NZ-I zeolite sample. The areas analysed for water content are indicated by colored lines. From approximately 3700 to 3200 arbitrary units corresponds to the H2O band while 500 to 600 arbitrary units corresponds to the Si band.

In order to obtain a quantitative estimate of the amount of water at each point analysed, the area under the OH stretch region (3700 – 3200 cm^{-1}) was normalised by dividing this area by the area of the Si mode at 521 cm^{-1}. The normalised water content at each point along the cross section is shown below in Figure 24.

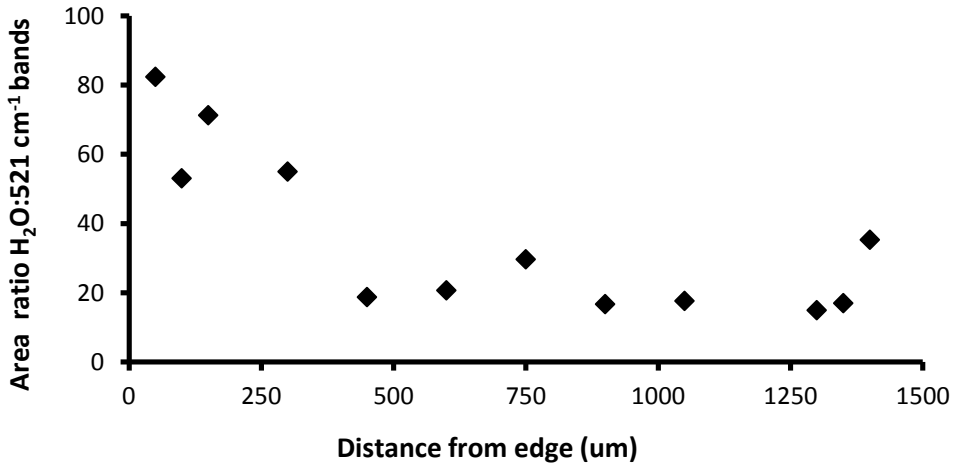


Figure 24. Normalised water content across a 1.4 mm cross section of NZ-I sample.

The primary conclusions of the Raman spectra analysis comparing AU-II and NZ-I zeolite are regarding water content and its distribution. The AU-II zeolite contains little water, whereas the NZ-I zeolite retains a large magnitude of water. These results agree the findings in section 3.2.2.1 and 3.2.2.2 which evaluated moisture content levels. A significant additional benefit of the Raman spectra is the quantification of the water levels inside the zeolite granules (Figure 25). Water is not stored internally with AU-II zeolite. In contrast, NZ-II zeolite contains a reservoir of internal water. Overall, the water content of NZ-II zeolite was up to 4 times higher than AU-II internally and up to 11 times higher externally. This conclusion is especially relevant for understanding the unique water retention capabilities of these two distinctly different zeolite types, and how water levels may affect the stability results for cells applied to zeolite surfaces.

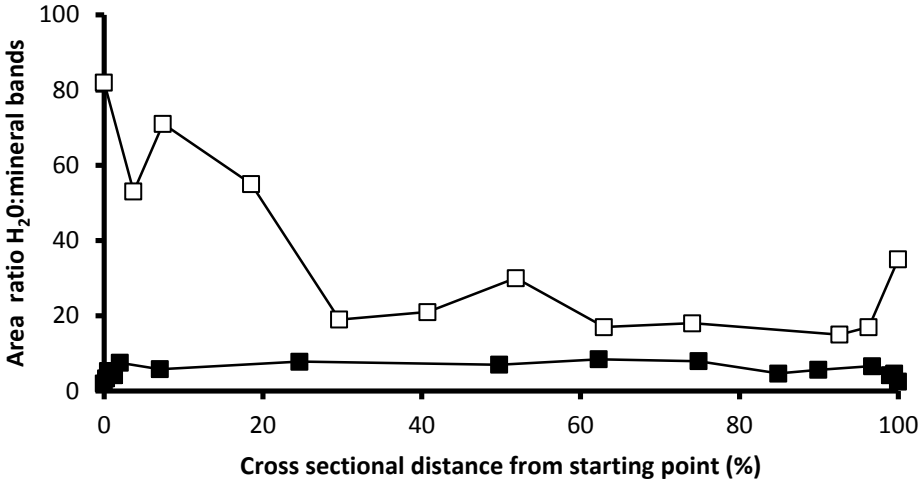
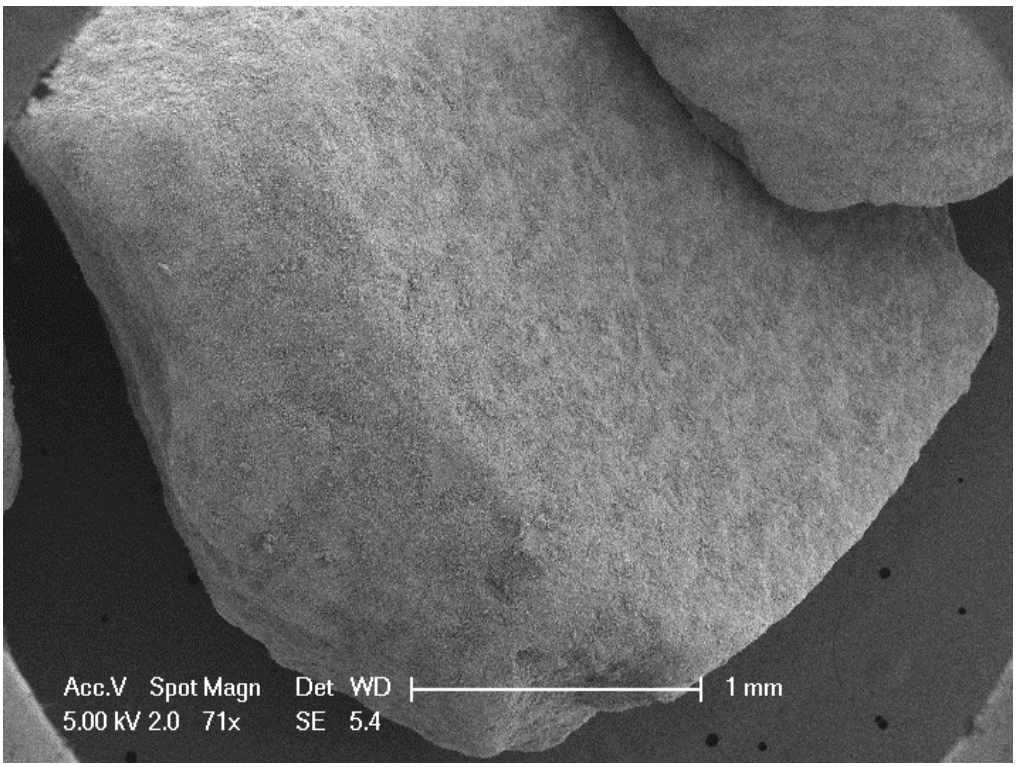


Figure 25. Comparison of normalised water content for NZ-I (□) and AU-II zeolite (■).

3.2.2.5 Cryo-SEM and SEM characterization of formulations

Scanning electron microscopy was conducted to visually assess the surface of NZ-I and AU-II zeolite. SEM and Cryo-SEM images are shown in Figure 26-30. Zeolite types NZ-I and AU-II are magnified with and without the biopolymer gel coating. Cryo-Sem images of gel coating fractures and gel emulsion features were conducted to indicate the spatial characteristics of *Pseudomonas* sp. strain ADP within the formulation matrix.



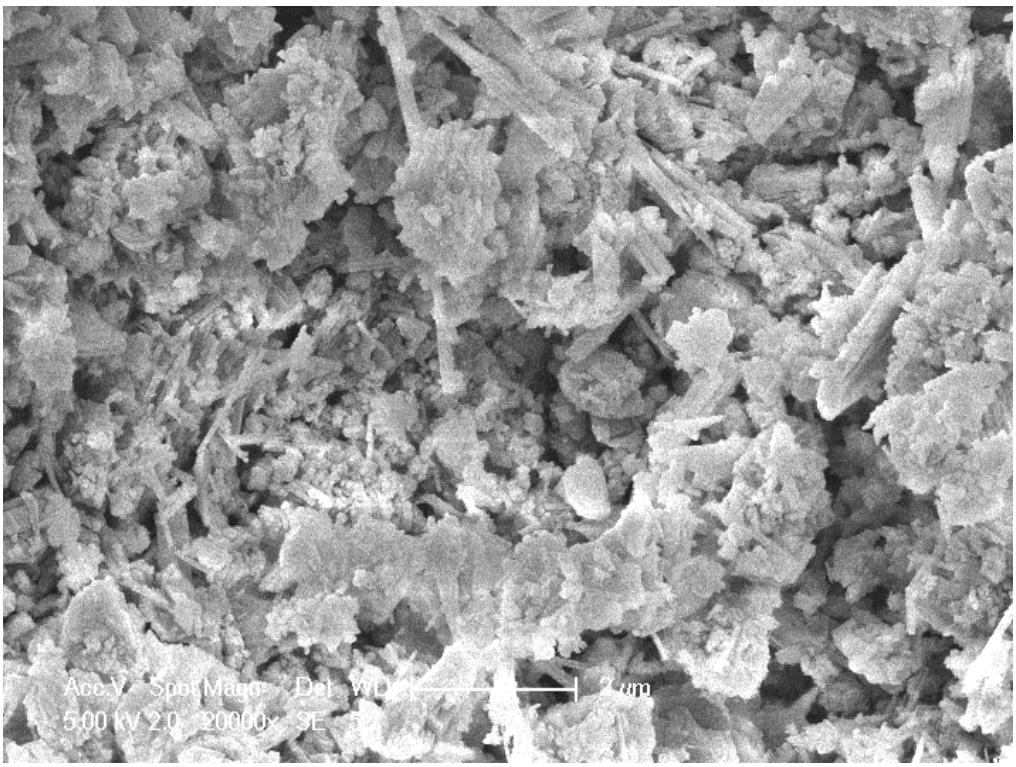
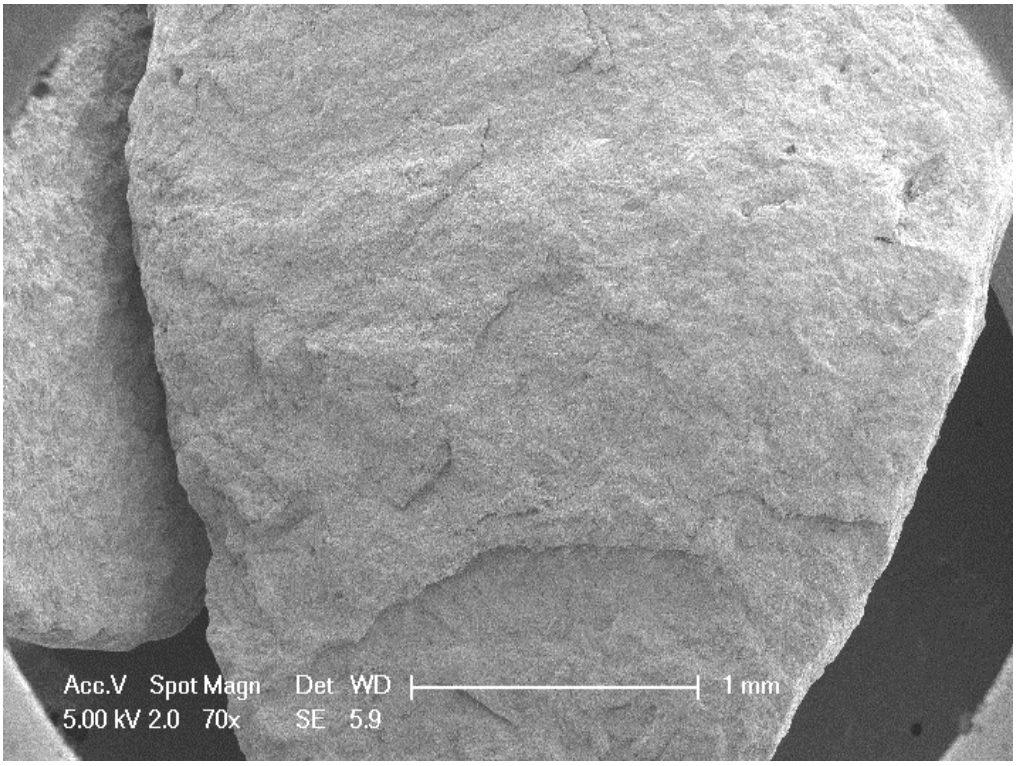


Figure 26. SEM images of New Zealand zeolite (NZ-I). Magnification is 71X (top) and 20000x (bottom).



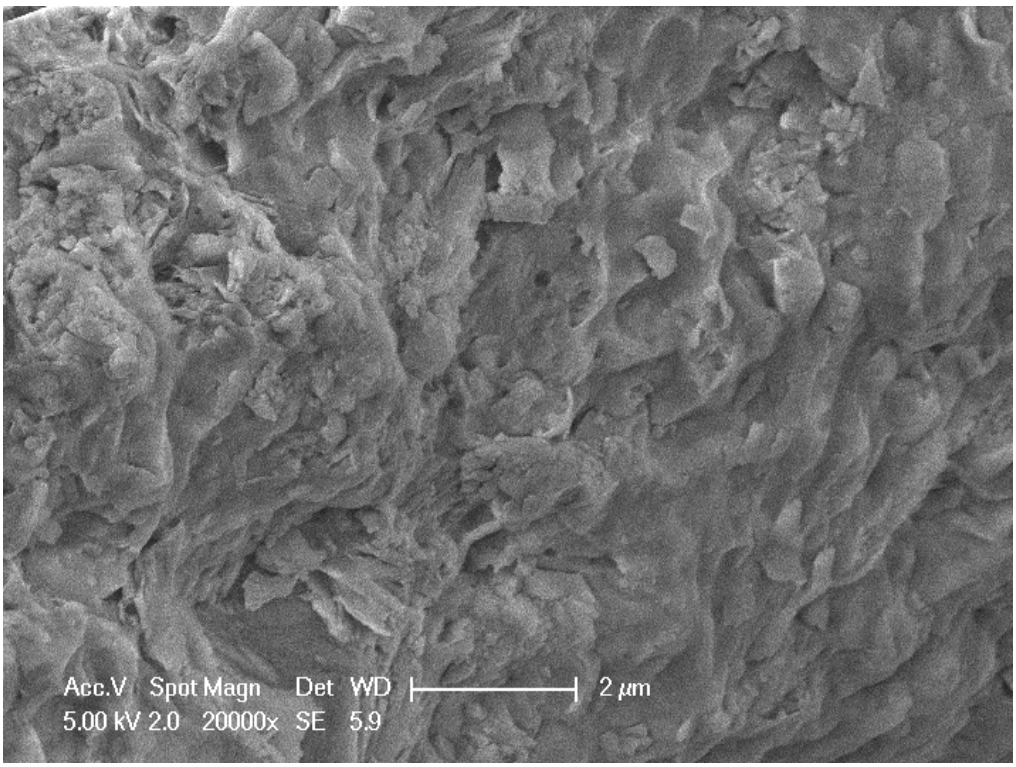
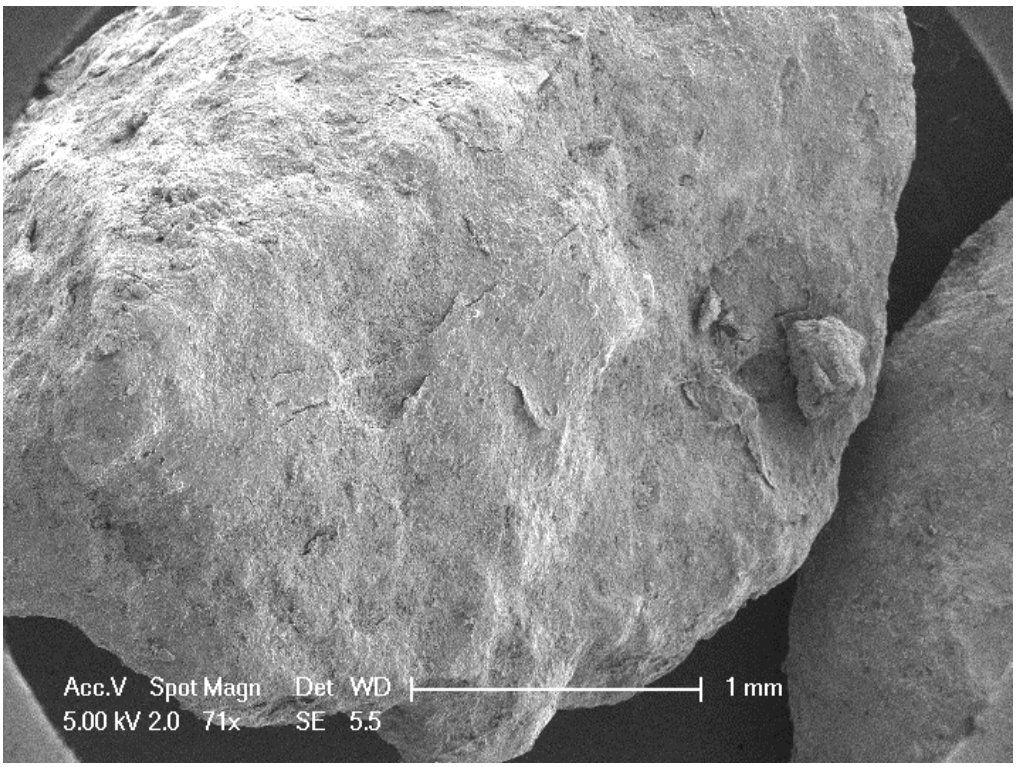


Figure 27. SEM images of Australian zeolite (AU-II). Magnification is 70X (top) and 20000x (bottom).



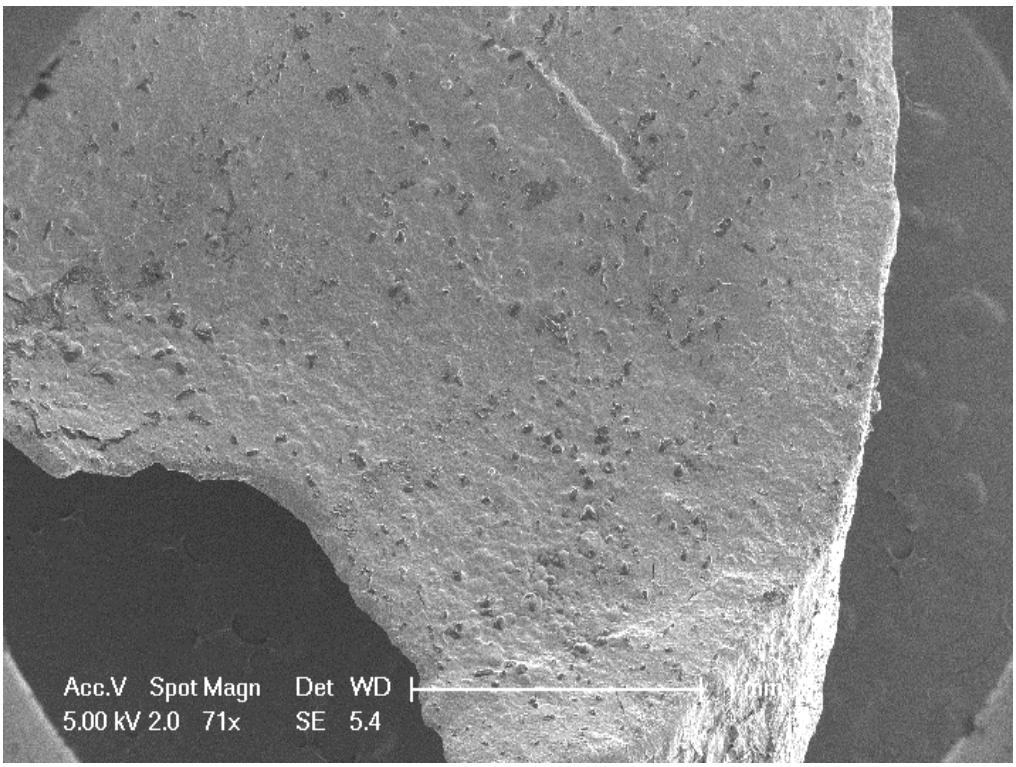
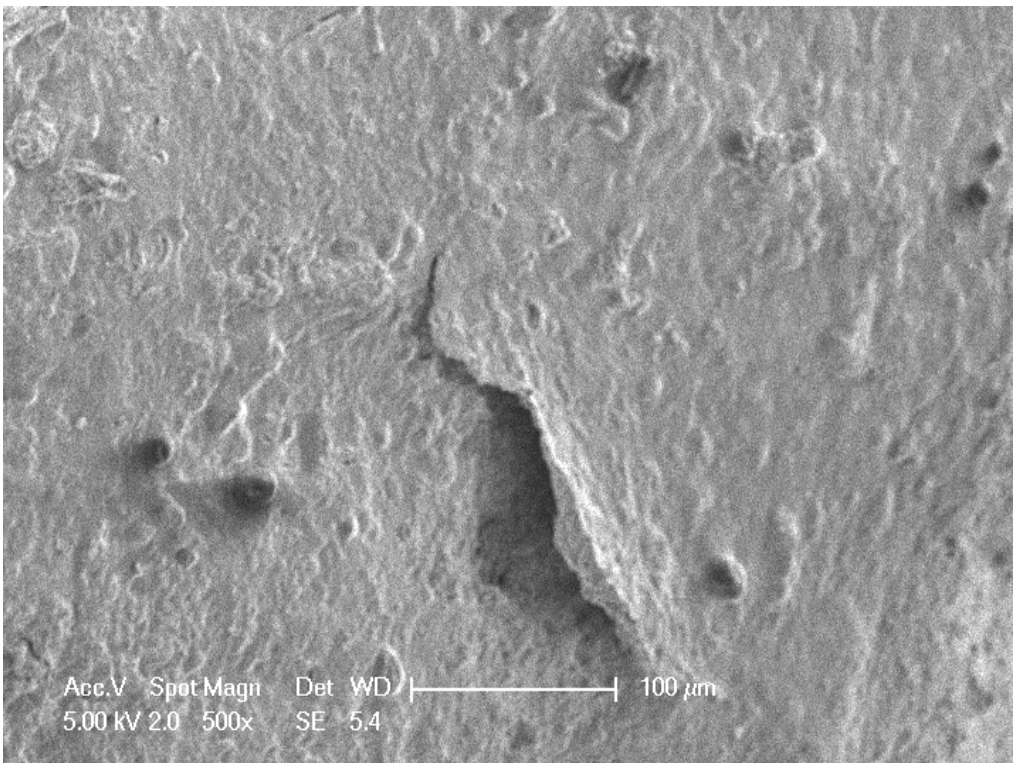
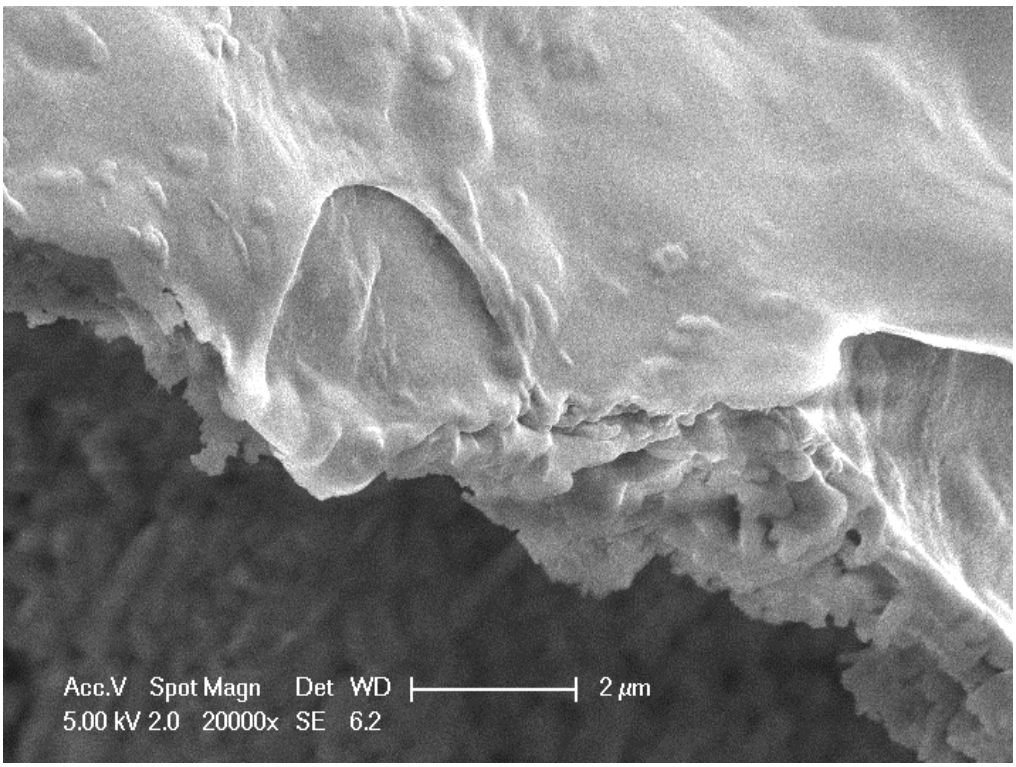


Figure 28. SEM images of zeolite types coated with biopolymer gel. Magnification is 71x for NZ-I (top) and AU-II (bottom).





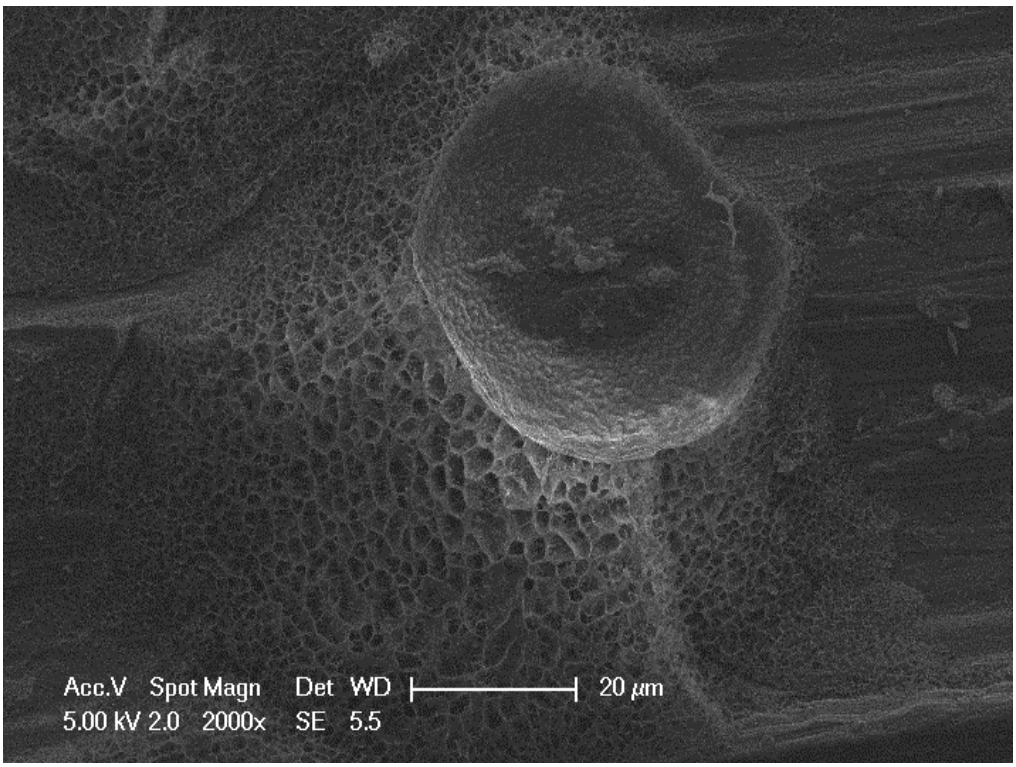


Figure 29. Cryo-SEM images of (top) a fracture in the gel coating and (bottom) a 20000x magnification of the gel fracture with cells visible.

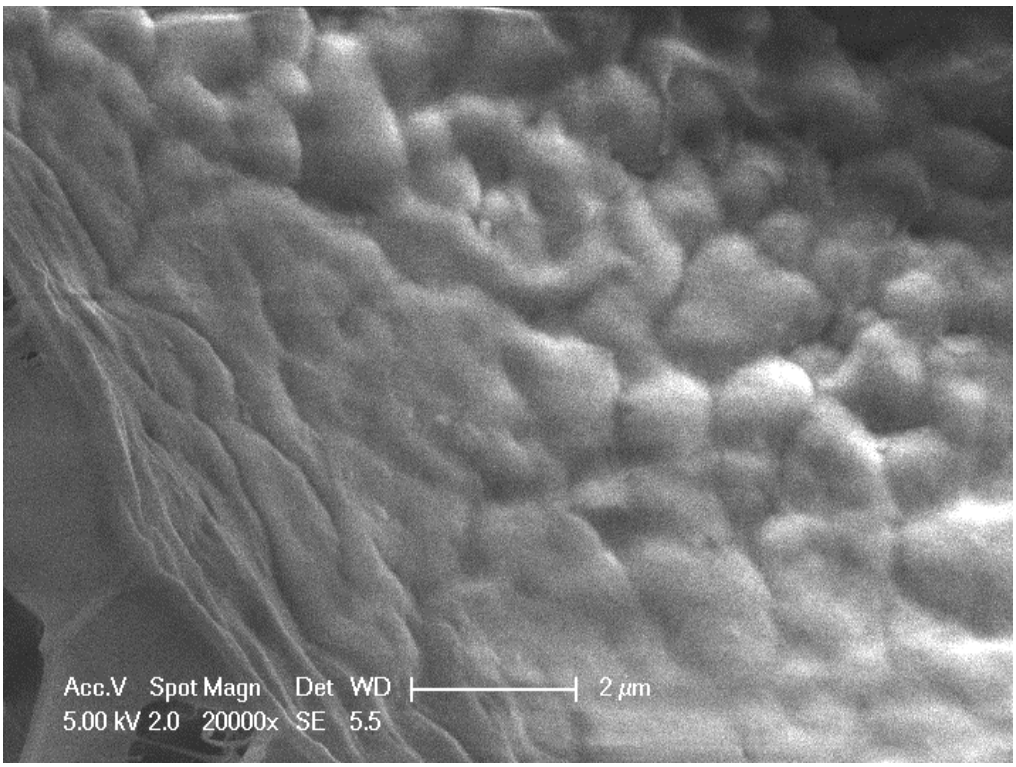


Figure 30. Cryo-SEM images of (top) the biopolymer gel with cells lining interior of an emulsion feature and (bottom) cells imbedded in biopolymer gel of an emulsion feature.

As discussed in section 2.1.2, the NZ and AU zeolite types were selected for comparison because the two zeolites have distinct physical differences visually, texturally, and in terms of material density. The visual assessment of zeolites by SEM confirms the NZ zeolite is more rugged and porous than AU zeolite. The film formed on zeolite following coating with gel and imbedded bacteria were clearly observed by SEM. This observation supplements earlier work which suggests that AU has a greater general density and has less capacity to absorb water.

3.2.3 Bacterial culture stability

The liquid bacterial culture contains the cell suspension to be used in the formulation. A first stage of formulation assessment was determining whether the bacterial culture had any inherent stability in its "as-produced" liquid state. Stability was assessed by enumerating the process stages from production, to harvesting, and during storage of the liquid bacterial culture at 4°C and 25°C. These evaluations were conducted to confirm the instability of LB, MB, and IM liquid bacterial culture.

At 4°C the stability of the LB bacterial culture was short term (Figure 31). Over half of the cells was lost in the first two weeks, and 90% was lost after week 4.

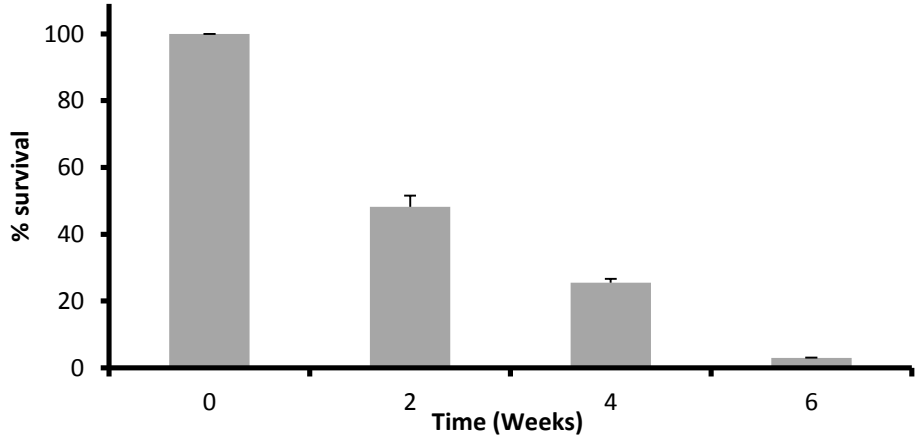


Figure 31. Percentage survival of liquid bacterial culture stored at 4°C. Error bars are the standard error of the mean (n=2).

When three media types are produced at flask scale and stored at 4°C, all three media types lose viability within weeks (Figure 32). Both MB and IM cultures do not show any inherent stability at 4°C and were below the range of enumeration plating by week 4. These results suggest that liquid bacterial culture storage stability at 4°C is short term, and of the three media types, LB bacterial culture retains some viability in refrigerated conditions.

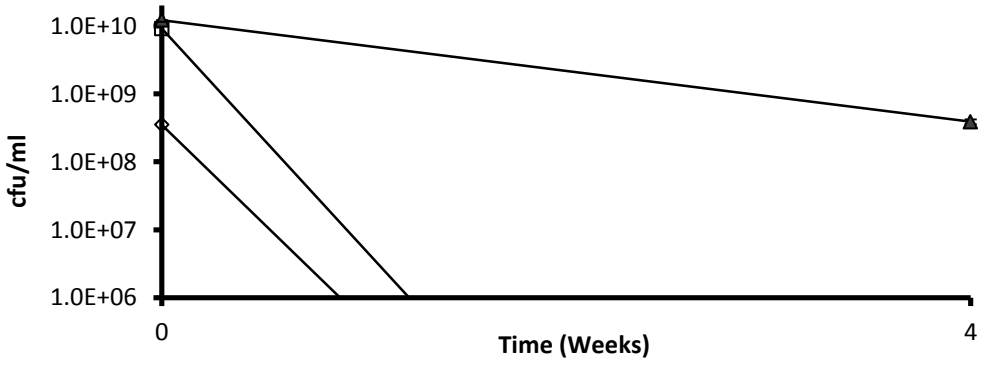


Figure 32. Viability of the bacterial culture for three media types stored at 4°C. Symbols are (▲) LB, (□) IM, and (◊) MB media types. Error bars are standard error of the mean (n=2) and are within symbols.

The percent survival of LB medium was examined at 4°C and 25°C (Figure 33). At 4°C the stability of the LB bacterial culture was short term, with over half of the cells lost in the first two weeks. The stability at 25°C was worse, with ~80% off the cells lost within 1 week. These results demonstrate that the stability of the agent is poor when stored as a liquid at

ambient temperatures. When stored as a liquid at refrigerated temperatures the survival is better but viability is quickly lost and stability remains poor.

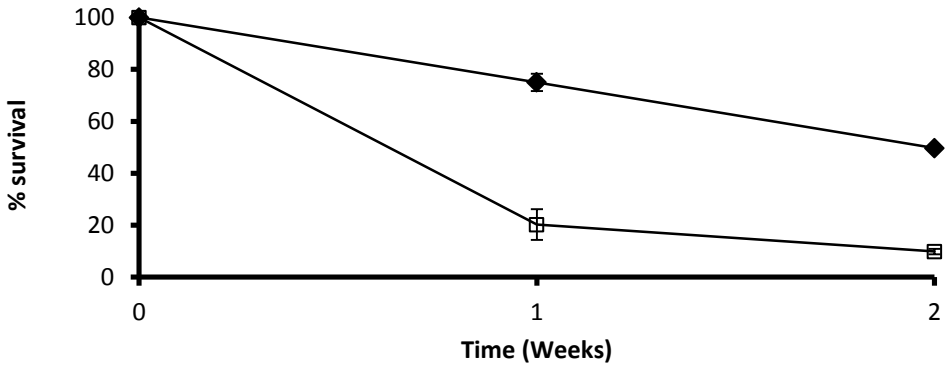


Figure 33. Percentage survival of LB liquid bacterial culture stored at (♦) 4°C and (□) 25°C. Error bars are standard error of the mean (n=2).

3.2.4 Stability at 4°C and 25°C in open and closed containers

The survival of *Pseudomonas* sp. strain ADP was assessed when applied to zeolite. In the following experiments the biopolymer gel described in section 2.3.1 was produced using LB medium. The cells were applied to the two types of zeolite (NZ-I and AU-II) at 4%w/w and stored in open and closed containers at both 4°C and 25°C. Results showed that a sealed storage container retained the level of water available (water activity) and confirmed that packaging is an important issue for retaining moisture levels at ambient temperatures. These evaluations were conducted to select storage conditions and are supplemental to the development of a stable formulation.

When using LB medium with biopolymer and stored in open or closed containers at 4°C the results are similar. For all samples types the viability of the cells was reduced over one full log (Figure 34). A benefit was noted in samples stored in closed containers for both zeolite types.

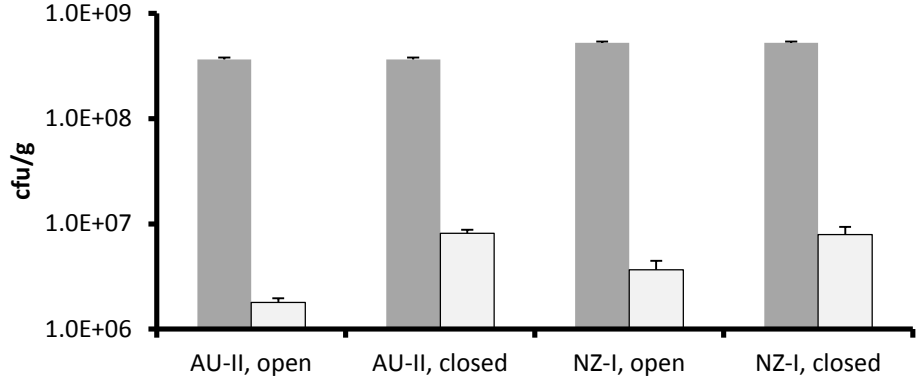


Figure 34. Enumeration of zeolite/gel formulation stored at 4°C for 24 hours in open or closed containers. Symbols are (■) Time equals zero and (□) 24 hours after storage. Error bars are standard error of the mean (n=2).

Some possible explanations for the poor stability at 4°C are osmotic shock, incompatibility with the biopolymer or zeolite, or a reduction in culturability. A short term reduction in culturability has been observed for inoculants applied to carrier materials by the Microbial Products Group at AgResearch Ltd (personal communication).

The water activity of the samples was conducted at Time=Zero and 24 hours later (Figure 35). For samples in closed containers and also the NZ-I/open variant the water activity was consistent in the 4°C storage conditions for the short term of 24 hours. The water activity of the AU-II/open variant was most affected by the storage and indicates a substantially reduced level of water available to the immobilized cells.

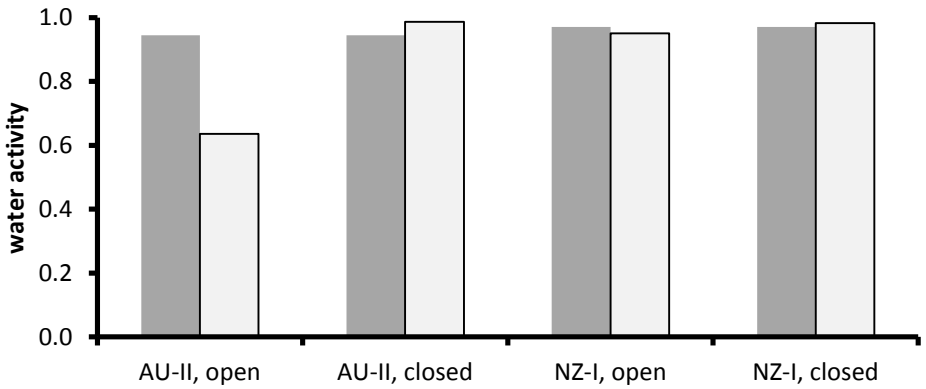


Figure 35. Water activity of zeolite/gel formulation stored at 4°C for 24 hours in open or closed containers. Symbols are (■) Time equals zero and (□) 24 hours after storage (n=1).

Stability at ambient temperatures is a primary goal of this work and in Figure 36 gels applied to zeolites were stored at 25°C for two weeks. As in the 4°C trial, LB bacterial culture is encapsulated in the biopolymer gel and stored in open or closed containers. In comparison to the short storage time at 4°C, the two week storage at 25 °C is a greater stability challenge (to the cells) and the results are more distinct. The only variant demonstrating stability for the two week incubation was immobilized onto AU-II zeolite and stored in a closed container. These results suggest that all zeolites are not equal and zeolite type has a high degree of importance in the process of producing a stable microbial formulation.

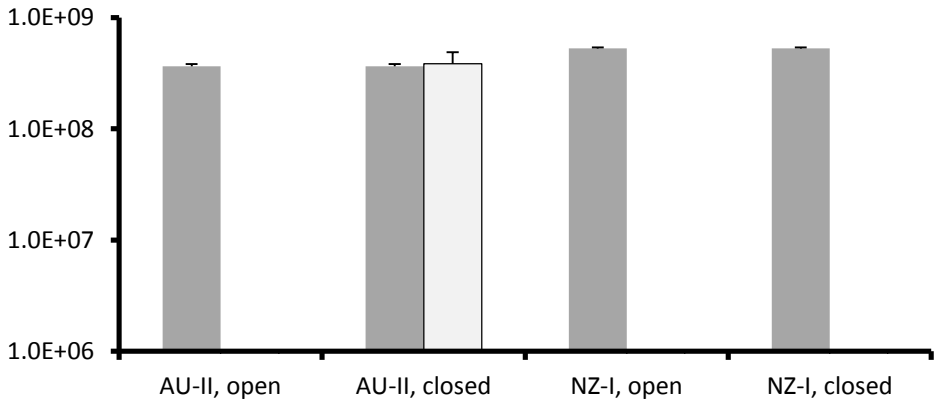


Figure 36. Enumeration of zeolite/gel formulation stored at 25°C for 2 weeks in open or closed containers. Symbols are (■) Time equals zero and (□) 2 weeks after storage. Error bars are standard error of the mean (n=2).

The water activity of the samples was conducted at Time=Zero and 2 weeks later (Figure 37). Water activity was retained in closed containers stored at 25°C for the 2 week evaluation period.

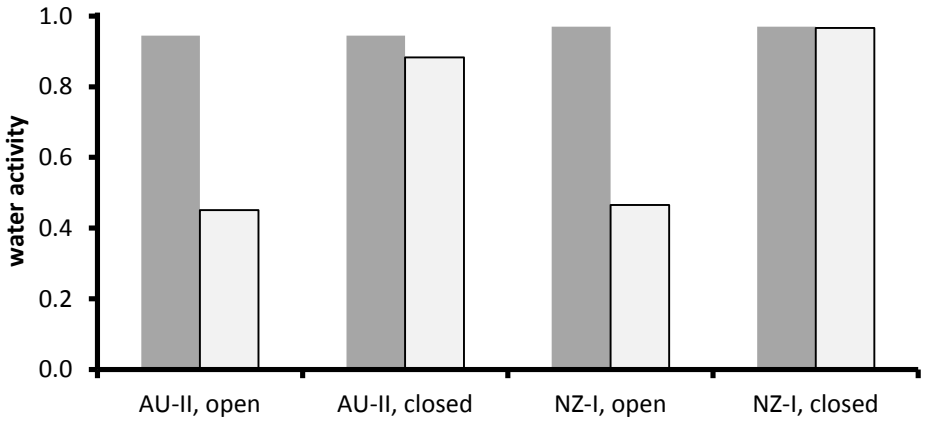


Figure 37. Water activity of zeolite/gel formulation stored at 25°C for 2 weeks in open or closed containers. Symbols are (■) Time equals zero and (□) 2 weeks after storage (n=1).

In open containers stored at 25°C, a large loss of water activity occurs within 24 hours (data not shown). Both open container variants were strongly affected by the longer incubation period of two weeks. These results suggest that storage and packaging are especially important issues for retaining moisture levels at ambient temperatures. A sealed storage container retained the level of water available.

The IM medium was scaled-up to 2L and the bacterial culture from the bioreactor was applied to zeolite, stored at 4C and 25C, and evaluated over 10 weeks for stability and functionality. These evaluations were a pre-formulation assessment of the bioreactor produced material using pH control and confirmed (data not shown) that a functionality benefit was conferred by storage on zeolite.

3.2.5 Viability transfer

An assessment of the formulation process was conducted. LB, MB, and IM bacterial culture (in triplicate flasks) were encapsulated in biopolymer gel and immobilized onto zeolite. Enumeration was conducted at each process stage to measure the transfer of cells from the flask, to the encapsulation gel, and onto the immobilized gel and carrier formulation (MB and IM are shown in Figure 38).

A ~1 log reduction of cells was measured after the encapsulation gel was immobilized onto the zeolite carrier. To clarify, this reduction is not a loss of viability but rather a proportional dilution, due to the materials balance of the formulation. To 100grams of zeolite, 4grams of gel are added. By mass balance, applying a 4% gel (w/w) to the zeolite dilutes the cells (as cfu/g) by ~1log.

A general transfer metric was calculated using mass balance proportions by dividing the viable cfu's from the zeolite by bacterial culture cfu's and multipling by 100. For LB and MB a value of 3% was calculated. For IM a value of 7% respectively was calculated. A 4% value would indicate 100% transfer efficiency (from a 4% gel). Using this calculation the process of immobilization is considered to be efficient with no substantial loss of viability observed from the flask to the immobilized formulation. The transfer of cells from the gel to the zeolite was conserved.

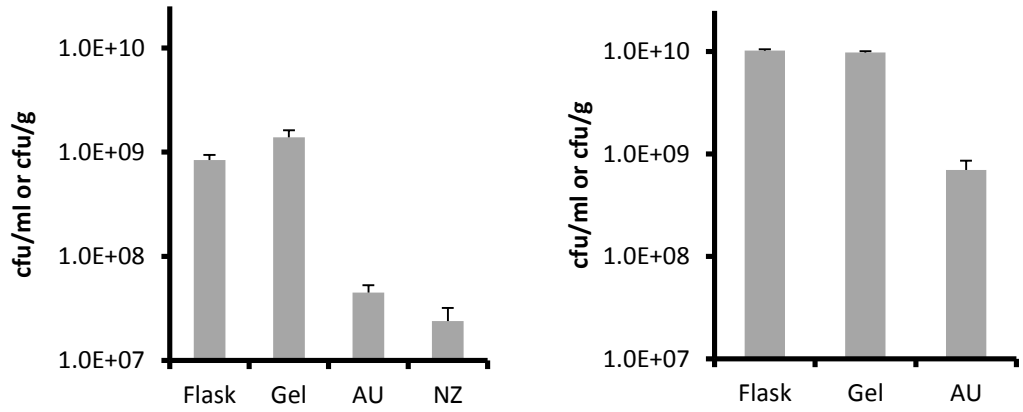


Figure 38. Transfer of viability from bacterial culture , to the encapsulation gel, and to the immobilized gel and carrier formulation for MB (left) and IM media (right). Error bars are standard error of the mean (n=3).

For LB, MB, and IM bacterial culture the percent recovery of the cells transferred from gel to zeolite carrier is shown in Table 3. Percent recovery was calculated by dividing the actual cfu by the theoretical cfu value (from the gel) and multiplying by 100. The percent recovery of the matrix transfer from gel to carrier was therefore considered to be greater than 98%. For all media the process difference of the transfer was +/- 0.3 log, which is within the accuracy of the enumeration (plate count) method.

Table 3. Calculation of - log values of viability (pV) for cfu data from three media types transferred from the gel to the AU-II zeolite. The % recovery is the difference between the theoretical and experimental values of cfu transfer.

Bacterial culture/Gel	cfu	-log	difference	% recovery
LB/Gel	1.62E+10	10.21		
theoretical 4 % transfer	6.48E+08	8.81		
actual transfer	4.29E+08	8.63	0.2	98.0
MB/Gel	1.39E+09	9.14		
theoretical 4% transfer	5.57E+07	7.75		
actual transfer	4.50E+07	7.65	0.1	98.8
IM/Gel	9.79E+09	9.99		
theoretical 4% transfer	3.92E+08	8.59		
actual transfer	6.97E+08	8.84	0.3	102.9

For three media types, LB, MB and IM, the transfer of cells from the flask to the gel, and from gel to the zeolite was conserved. The formulation technique was efficient and no substantial loss of viability was observed from the flask to the immobilized formulation. The formulation technique was effective irrespective of the media type. With the exception of initial cell concentrations of the flask, the type of medium does not appear to be important for the freshly produced formulation. For three media types there appears to be little, if any, practical difference in terms of cells transferred.

3.2.6 Ten week storage trials at 25°C

The viability, stability, and functionality of *Pseudomonas* sp strain ADP applied to zeolite was examined in triplicate 10 week storage trials conducted at 25°C. LB, MB, and IM bacterial culture was immobilized onto NZ-I or AU-II zeolite carrier (described in 2.3.3.1) with and without the application of an encapsulating gel onto the zeolite surface. The 20 types of variants examined in the storage stability trials were described in section 2.3.3.1.

The performance of IM grown cells with and without the biopolymer gel, and with and without pre-wetting of the zeolite was compared (Figure 39). These results indicate a substantial benefit is gained using the formulation gel. Numerical stability of the cells is maintained for 10 weeks in gel encapsulated samples. The consistency of viability (robustness) is improved in the gel formulated samples and is highlighted by the small error bars in the figure. Pre-wetting of the zeolite (AU-II) appears to have some utility with both unformulated and formulated types. In terms of stabilization, gel encapsulation is more influential than pre-wetting.

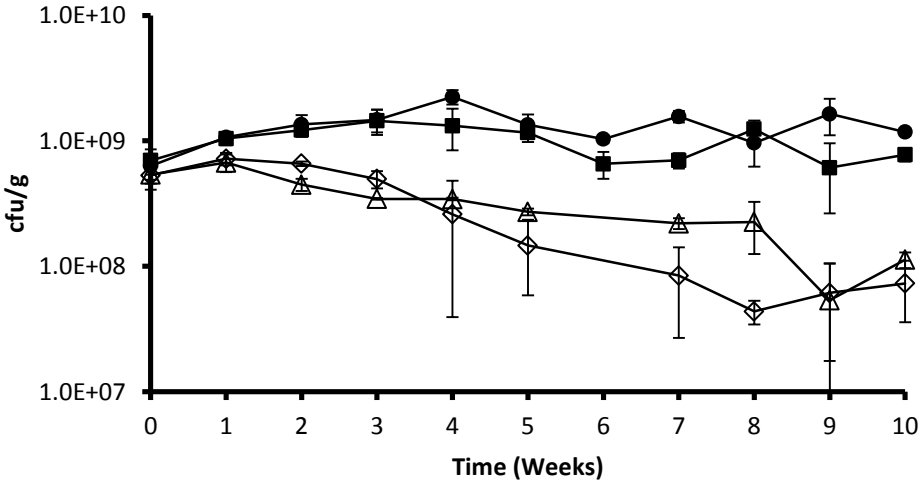


Figure 39. Enumeration of formulation stored at 25°C for 10 weeks in closed containers. Symbols are (◊) IM bacterial culture on AU-II zeolite, (■) IM bacterial culture encapsulated

in a biopolymer gel on AU-II zeolite, (Δ) IM bacterial culture on pre-wetted AU-II zeolite, and (●) IM bacterial culture encapsulated in a biopolymer gel on pre-wetted AU-II zeolite. Error bars are standard error of the mean (n=3).

The functionality of IM grown cells with and without the biopolymer gel, and with and without pre-wetting of the zeolite was compared (Figure 40). Functionality, as the ability to create clearing zones in the atrazine agar was maintained for 10 weeks in all IM samples. More importantly, the functionality was retained at full strength in gel encapsulated samples.

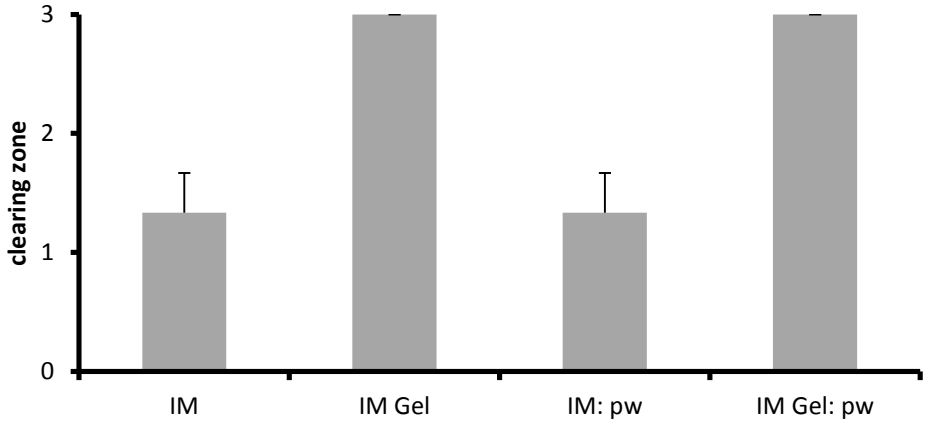


Figure 40. Functionality assay of atrazine utilization after 10 weeks storage at 25°C. Error bars are standard error of the mean (n=3) (p=0.10).

3.2.6.1 Numerical stability comparison of formulations

To address the degree of numerical stability discussed in the 10 week trials, the cfu data was converted to $\log_{10}$ values. The decline in log magnitude was calculated by subtracting the $\log_{10}$ of week 10 from the $\log_{10}$ of week zero. A log decline and the percent difference of the $\log_{10}$ decline, from time zero to week 10, for each formulation type is shown in Table 4. Stability is strongly separated between formulations with the gel encapsulation, and those without, irrespective of media type. For each media type, the gel formulations were more stable by an order of magnitude. Using data from Table 4, the percent difference of log decline is shown in Figure 41 and indicates a clear stabilization benefit is gained using the formulation gel for storage at ambient temperature.

Table 4. Log decline of formulation types in storage for 10 weeks. Formulations are immobilized on AU-II zeolite stored at 25°C (n=3).

	LB	LB/ Gel	MB	MB/ Gel	IM	IM Gel	IM: pw	IM Gel: pw
Log decline of formulation	1.1	0.1	1.1	0.2	0.9	0.0	0.7	-0.3
Log decline % difference	13.3	0.8	15.6	2.1	10.4	-0.5	8.1	-3.0

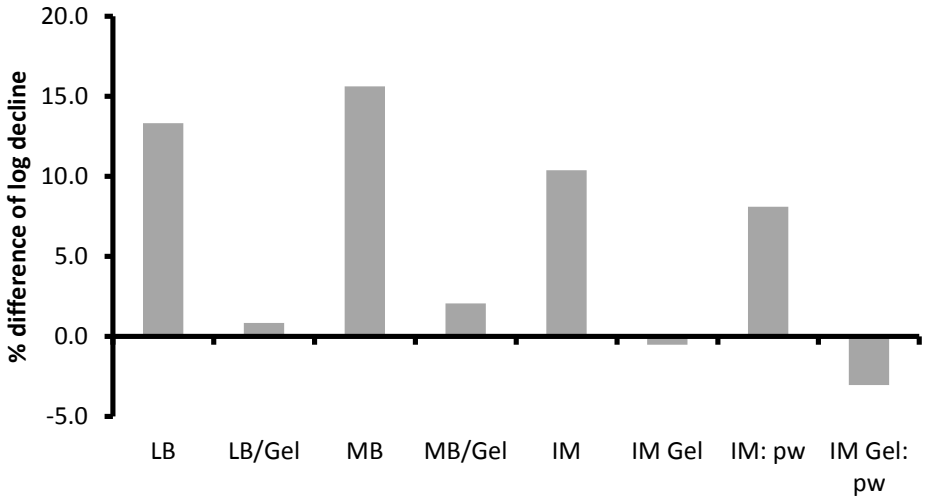


Figure 41. Percent difference of log decline for formulation types in storage for 10 weeks. Formulations are immobilized on AU-II zeolite stored at 25°C (n=3).

3.2.7 Significance

To state the significance (p value) of the formulations development process, a survival ratio was calculated for each formulation type by transforming ($\log_{10}$) week 10 count data divided by week 0 data ($\log_{10}$ t10/t0). Survival ratios are shown in Figure 42. Formulation stabilities were compared using a t-test (two samples assuming unequal variances, hypothesised mean difference =0, two-tailed test at 95%). For formulations applied to Australian (AU-II) zeolite there was no significant difference between the stability of MB and IM format. There was no significant difference between MB and LB, or MB/Gel and LB/Gel formats so the LB variants are not shown. However, a significant difference ($p<0.05$) was observed when MB was compared to IM/Gel ($p=0.015$), IM:pw ($p=0.038$), and IM/Gel:pw ($p=0.004$). The gel

component was significant ($p<0.05$) to 10 week stability and prewetted (IM:pw) AU-II zeolite was observed to be statistically less stable than IM/Gel ($p=0.018$) and IM/Gel:pw ($p=0.001$).

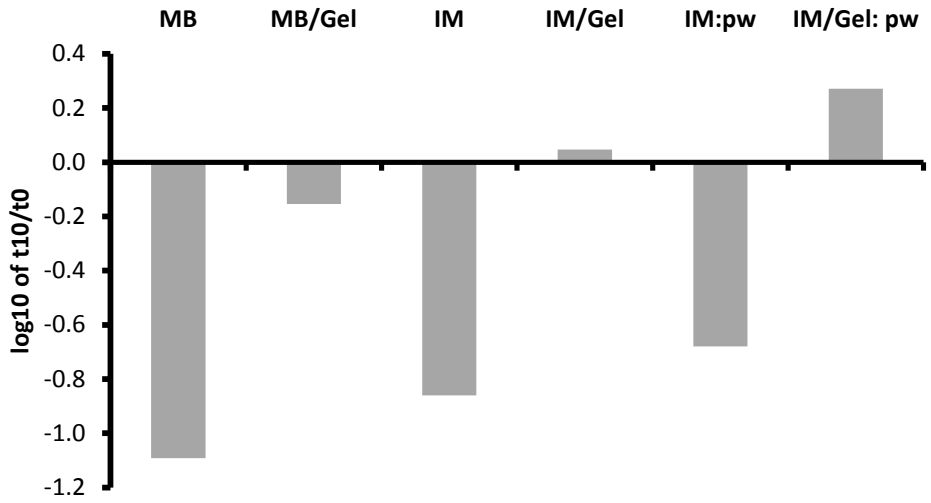


Figure 42. Survival ratio of formulation types in storage for 10 weeks (n=3). Formulations are immobilized on AU-II zeolite stored at 25°C.

The general aim of formulation work was to develop immobilization methods which maximize cell survival, stability, and functionality. The formulations were developed to optimize the shelf life (10 weeks) stability of the strain *Pseudomonas* sp. ADP at 25°C. The specific aim was to produce a stable formulation that retained (or increased) its cell concentration and functionality for the full 10 week storage period at 25°C.

The results of this work accomplished room temperature stability at 25°C, shelf life stability for the full 10 weeks storage, and the retention of maximum functionality.

3.3 In situ Results

3.3.1 Performance of liquid bacterial culture in soil

The first in situ assessment quantified the survival of liquid bacterial cultures applied into sterile soil. Results of inoculating the bacterial culture of three media types (LB, MB, IM) into sterile soil are shown in Figure 43. Viability of *Pseudomonas* sp. strain ADP is preserved for the full ten weeks for all media types. Media type LB produced a consistent viability for the term of the trial and remained within ~0.5 log of the initial cell number. Both MB and IM media types demonstrated a greater than 1log increase in cells within the sterile soil.

There appears to be some disadvantage conveyed to the cells grown in LB media to colonize the sterilized soil matrix and also increase in number. Although the LB bacterial culture was stable and retained its viability, there was not a marked growth phase as observed for the IM and MB bacterial culture types.

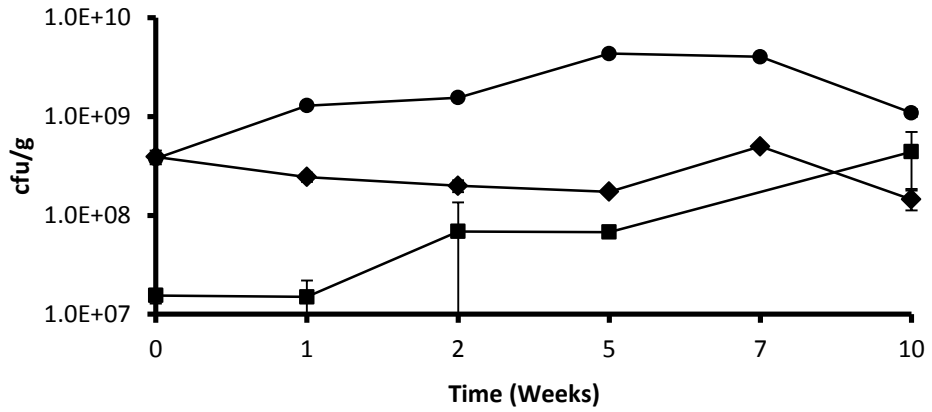


Figure 43. Enumeration of three bacterial culture types inoculated into sterile soil and stored at 15°C. Symbols are (■) MB, (♦) LB, and (●) IM. Error bars are standard error of the mean (n=3).

In the sterilized soil microcosm there appears to be sufficient spatial capacity for populations in excess of $1x10^8$. By providing sterile conditions and ample moisture the strain was capable of achieving high levels of cells in soil. These results agree with previous researchers which note that some inoculants introduced into sterile soil do not exhibit a decline in population magnitude (van Veen *et al.*, 1997). In sterile soil, the cells can increase due to a lack of biotic inhibitions.

For all media types, the viability was retained for the full ten weeks (Figure 44). In contrast, functionality levels appear to be separate from cell numbers, and are maintained at maximum levels only in the IM bacterial culture. The functionality levels of utilizing atrazine for the three bacterial cultures (LB, MB, IM) are shown in Figure 44 after inoculation into sterile soil for 10 weeks. All bacterial cultures started (Time=Zero) at maximum functionality levels. LB retained a low level of functionality while the MB was unable to produce clearing zones at week 10.

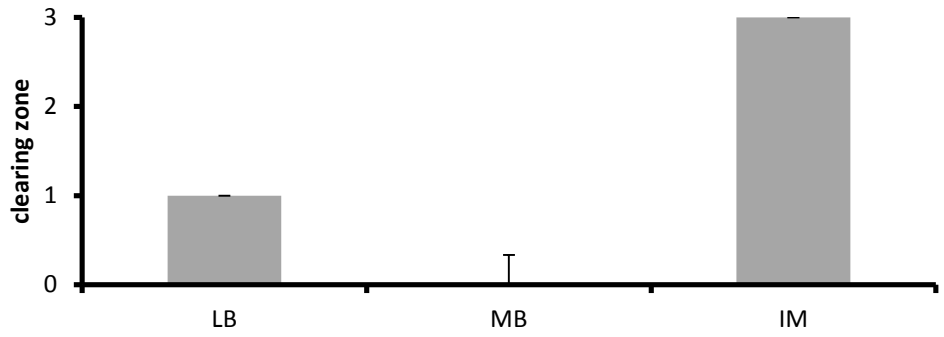


Figure 44. Functionality of bacterial culture types inoculated into sterile soil and stored at 15°C for 10 weeks. Error bars are standard error of the mean (n=3) (p=0.10).

As a bacterial culture inoculated into sterile soil, the IM bacterial culture showed the highest performance in both cells and functionality. LB maintained its cells but its functionality was reduced. The media type MB does not appear to be practical for retaining the ability of *Pseudomonas* sp. strain ADP to degrade the target component atrazine.

The author speculates that the MB medium was generally limited or exhausted in nutrients at the time the inoculum (flask) was harvested. When the free cells of the inoculum were applied to the soil, their activity adapted to more nutrient rich surroundings. As a liquid bacterial culture applied to soil, the process of cell attachment may be a transition phase where cells evaluate and respond to their new possibilities. Using this "evaluate and respond" concept, MB cells located a new nutrient source in the soil capable of expanding its density, while discarding its capability to utilize atrazine. The LB bacterial culture is a rich medium and the population did not respond (up or down) to the new conditions. However, without a selective agent (atrazine or cyanuric acid) in the media or soil there was a lowering of functionality after 10 weeks. The IM bacterial culture is also a nutrient dense medium and its population responded by expanding its population density in the available spatial capacity of the sterilized soil. In further speculation, the expanding IM bacterial culture population was able to retain the functionality to utilize atrazine due to the presence of the selective agent cyanuric acid during the transition phase of attachment.

3.3.2 Performance of immobilized formulation in soil

The second aim of in situ assessment for this project quantified the survival of the immobilized formulation in sterile soil. Results of delivering immobilized formulations produced from three bacterial culture types (LB, MB, IM) into sterile soil are shown (Figure

45). Granules of AU-II zeolite were manually separated from the soil and their cfu's were enumerated. The presence of the formulation produced stable viability for the full test period of 10 weeks, irrespective of media type. A 2 log increase in cells was observed for the MB type. Formulations on the AU-II zeolite separated from sterile soil appear to be capable of a loading capacity of between $2x10^9$ and $4x10^9$ regardless of media type or bacterial culture delivery concentration. The loading capacity appears to be stable in the sterile soil.

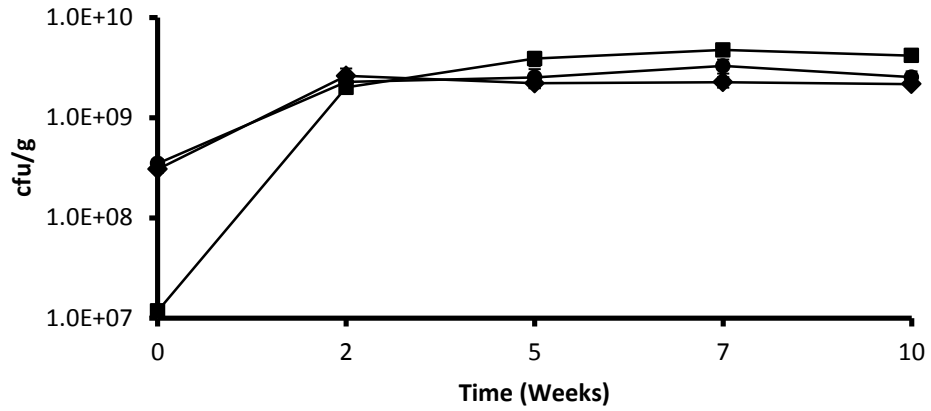


Figure 45. Enumeration of the AU-II zeolite carrier separated from soil. Formulations were inoculated into sterile soil and stored at 15°C. Data symbols are (■) MB, (♦) LB, and (●) IM. Error bars are standard error of the mean (n=3) and are within symbols.

The soil fraction separated from the zeolite granules was also enumerated. The results from the soil fraction are shown in Figure 46. For all media types the cell numbers of the soil increased after a single inoculation of the formulation. *Pseudomonas* sp. strain ADP is clearly transferred from the formulation into the surrounding soil. A two log increase of cells from the starting population was produced by the MB type. As was noted for the zeolite carriers, there appears to be a stable threshold concentration. After ten weeks storage at 15°C the threshold of the soil fraction was ~1x 10^9 regardless of media type. The loading capacity appears to be stable in the sterile soil.

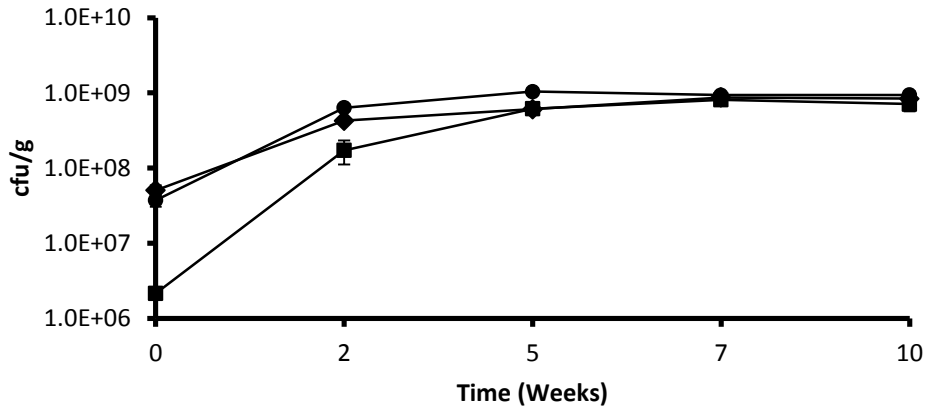


Figure 46. Enumeration of the soil separated from the AU-II zeolite carrier. Data symbols are (■) MB, (♦) LB, and (●) IM. Error bars are standard error of the mean (n=3) and are within symbols.

An explanation for the population transfer into the soil in Figure 46 could be the shedding of the gel from the carrier into the soil during mixing. The carriers were wet with gel when applied into the wet soil matrix at Time=Zero, and mixed, to produce the data points at that time period. Therefore cells shed at the first mixing event (Time=Zero) should be accounted for at that event. The author speculates that if shedding is the explanation it is perhaps a useful format for in situ dispersal at a desired depth. Maximum cfu/g levels were achieved by week 5 which corresponds to three mixing events (or dispersals).

In a further observation, cells dispersed into the soil from the inoculum carriers (Figure 46) do not decrease the population levels of the carriers in Figure 45. The author speculates that the population levels on the carriers increased to the loading capacity and was maintained there. Cells dispersed (shed) into the soil were replaced on the "mother colony". Both carrier and soil populations expand vigorously to threshold levels within two weeks.

The functionality levels of the three formulations (LB, MB, IM) delivered into sterile soil and stored at 15°C are shown in Figure 47. AU-II zeolite granules were manually separated from the soil and assayed. Irrespective of media type the formulation retained the maximum level of functionality and produced robust clearing zones for the full test period of 10 weeks. Results show that all cell densities (Figure 45) are equivalent at week 10 and functionality levels (Figure 47) are also equivalent and at the maximum level. The positive contribution of the formulation to functionality expression is demonstrated in Figure 47 when compared to

bacterial culture alone (Figure 44) in which only one media (IM) retained robust atrazine functionality.

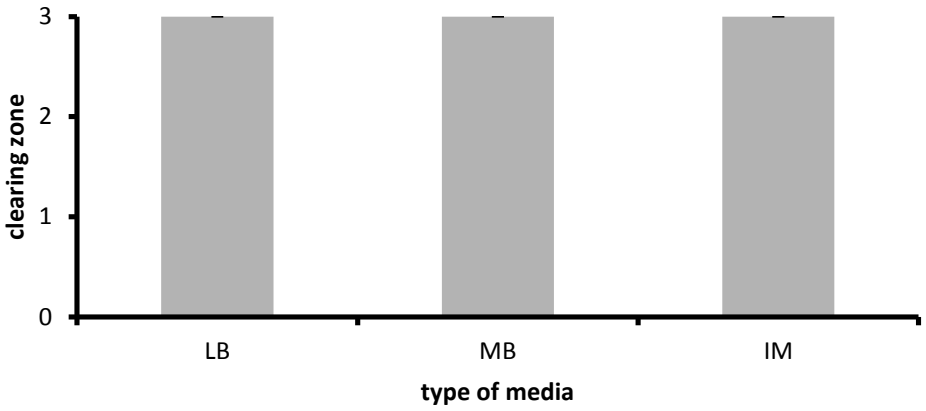


Figure 47. Functionality of zeolite carrier separated from soil after 10 weeks. Error bars are within symbols (n=3) (p=0.10).

One of the more important observations of this research is shown in Figure 48. For the soil fraction manually separated from the formulations fraction, the levels of functionality in the soil exhibited the maximum level of atrazine utilization by production of clearing zones. It was expected that functionality would be retained on the formulated AU-II carrier, but was not known whether the functionality would be stable when conveyed to the surrounding soil. As shown in Figure 48, the maximum level of functionality to utilize atrazine has been transferred to the soil surrounding the zeolite carrier and is stable for 10 weeks.

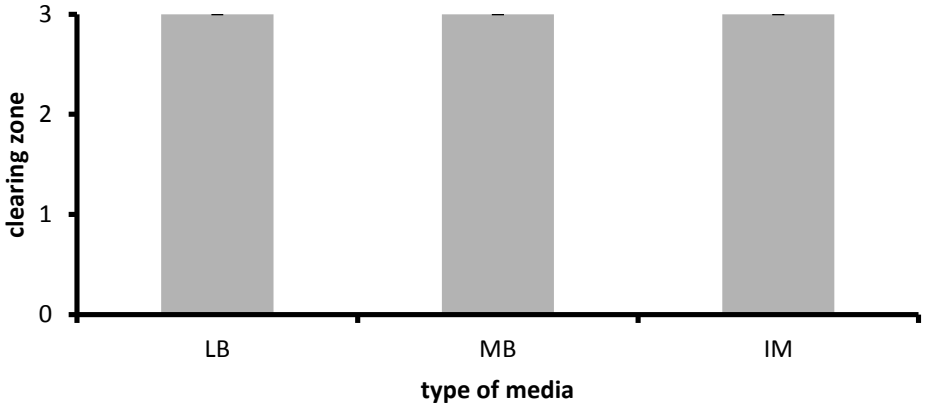


Figure 48. Functionality of the soil separated from the zeolite carrier after 10 weeks. Error bars are within symbols (n=3) (p=0.10).

To address the mechanisms which may produce the functionality phenomenon in Figure 47 (formulation) and Figure 48 (soil) in contrast to Figure 44 (bacterial culture) the author offers a summary of previous speculations (3.3.1 and 3.3.2). For free-cells, attachment is a transition point where cells evaluate and respond. Based on the evaluation, a response is determined. Functionality of the transitioning cells is affected by the presence of selective pressures at that time, which are coded and expressed at the plasmid level. Plasmids and their functions are triggered by cell attachment. The plasmid is the anchor point of functionality for the conditions that were experienced in that time and space (memory). A biofilm is produced at the attachment point which retains those plasmid functions (conservation of memory). The biofilm expands to its threshold level and that threshold is maintained.

Building from these earlier considerations, the functionality in soil expressed in Figure 48 could perhaps be viewed as:

- Cells shed from the "mother colony" are likely aggregates rather than free-cells.
- These clusters of cells may be able to retain their biofilm functionality (memory) for 10 weeks at the new dispersal point (soil) by retaining their cohesion as a biofilm and bypassing the transition phase of a new attachment.

These conclusions are supported by the work of (Sauer *et al.*, 2002)) who characterized five distinct stages from planktonic (free cells) to the development and ultimate dispersion of biofilm cells in *Pseudomonas aeriginosa*. The difference between of planktonic (free cells) and cells growing in a biofilm matrix was profound and gene products differed by as much as 70% in location and intensity (Costerton, 2007). Costerton (2007) concludes "Biofilm cells differ from planktonic cells with the same genotype because they express a different set of genes when they are growing in a matrix enclosed community associated with surfaces and interfaces". For *P. aeriginosa*, multiple phenotypes were displayed during biofilm development and may be important as a mechanism to control biofilm growth (Sauer *et al.*, 2002).

The results and conclusions of the in situ phase of work point to the critical importance of retaining functionality e.g. delivering a microbial product which expresses a desired trait. Inconsistency has hampered biological products in general. As a means of preprogramming or controlling the functionality of a microbial product there appears to be a substantial advantage for delivering microbes (and their full functionality) as a biofilm.

3.3.3 Significance

To state the significance (p value) of stability for the formats delivered to the soil matrix, a survival ratio was calculated for each type by transforming ($\log_{10}$) week 10 count data divided by week 0 data ($\log_{10}$ t10/t0). Survival ratios of cells (from IM medium) delivered as a bacterial culture, as a formulated AU-II zeolite granule, and the cells dispersed into soil from the granule are shown in Figure 49. The stability of the formulations delivered into soil were compared using a t-test (two samples assuming unequal variances, hypothesised mean difference =0, two-tailed test at 95%). As In situ trials for each variant were conducted three times (n=3) the t-test considered three independent survival ratios ($\log_{10}$ t10/t0) for each variant.

The delivery of a formulated AU-II zeolite granule was superior for 10 week survival compared to using a bacterial culture delivery ($p<0.05$) into the soil matrix. As a dispersal method, survival of cells dispersed into soil from the granule were significantly improved compared to cells dispersed as a bacterial culture ($p<0.05$). The survival ratio of cells dispersed from the granule was a larger magnitude of increase than on the granule itself ($p<0.05$).

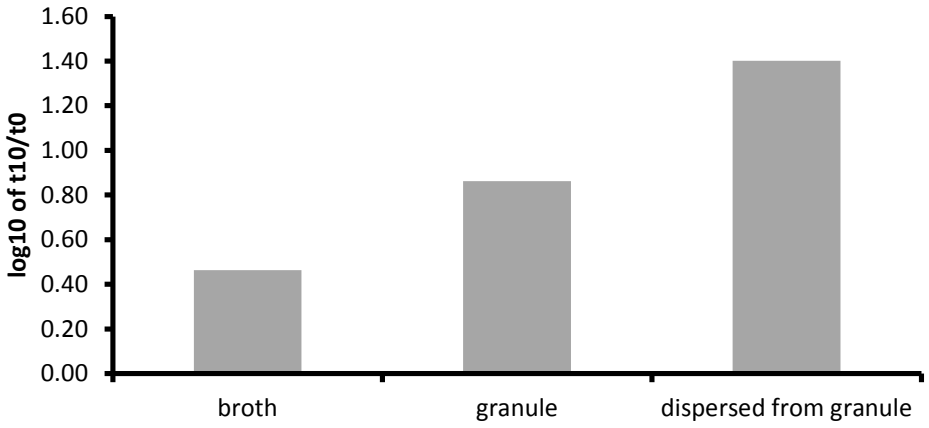


Figure 49. Survival ratio of bacterial culture and granule delivery formats delivered to sterile soil (mean of n=3) after 10 weeks stored at 15°C. All cells were produced with IM medium. The dispersed from granule column is the survival ratio of cells dispersed by mixing into the soil surrounding the granule.

3.3.4 Summary

An immobilized formulation was introduced into sterile soil and evaluated for survival and functionality. The aim was to deliver a formulation in situ to improve the survival of the

inoculant over the application of liquid bacterial culture. The survival and functionality of liquid bacterial culture and the formulation granule were compared. Additionally, the functionality and dispersal (cfu) levels from the granule into the sterile soil were compared to liquid bacterial culture.

The results of the in situ work suggest that the development of a formulated inoculant constitutes an improvement when compared to liquid bacterial culture for the delivery of *Pseudomonas* sp. strain ADP. The strains survival in sterile soil was improved. The dispersal of the strain into the surrounding soil was enhanced. The functionality of both the formulation and the dispersed microbial agent was retained at the maximum level. As a means of preprogramming or controlling the functionality of a microbial product there appears to be a substantial advantage for delivering microbes (and their full functionality) as a biofilm.

4 Discussion

To contribute to the methodology of the bioremediation science, *this thesis is about inoculant production and formulation*. This research considered bioaugmentation from a formulation perspective in an attempt to combine production, performance, protection, and delivery. This approach attempted to:

- Utilize a microbial agent.
- Develop a medium and produce an inoculant.
- Encapsulate the inoculant in a protective coating.
- Immobilize the inoculant on a carrier.
- Deliver the agent into soil.

The overall aim was to produce and formulate a bacterial culture with greater stability and functionality characteristics that conventional liquids inoculums. Liquid inoculums are inherently unstable and are in need of improvement. To achieve these aims the work was conducted in three sections; Fermentation, Formulation, and In situ.

4.1 The aims

4.1.1 Fermentation

The aim of the fermentation work was to improve the cell productivity of the culture medium and also retain the target functionality, by replacing atrazine with cyanuric acid. This outcome was accomplished (section 3.1.6).

4.1.2 Formulation

The general aim of formulation work was to develop immobilization methods which maximize cell survival, stability, and functionality. The specific aim was to produce a stable formulation that retained (or increased) its cells and functionality for the full 10 week storage period at 25°C. These outcomes were accomplished (section 3.2.7).

4.1.3 In situ

An immobilized formulation was introduced into sterile soil and evaluated for survival and functionality. The survival and functionality of liquid bacterial culture and the formulation granule were compared. Additionally, the functionality and dispersal (cfu) levels from the granule into the sterile soil were compared to liquid bacterial culture. The aim was to deliver a

formulation in situ to improve the survival of the inoculant over the application of liquid bacterial culture. These outcomes were accomplished (section 3.3.4)

4.2 The hypotheses

At the outset of this research a combination of fermentation and formulation work was proposed for producing a microbial inoculant. The inoculant was to be developed and delivered toward a bioremediation task. There were two initial hypothesis used to support the research proposal. Both of these hypotheses were tested. Both were proven to be false.

Firstly, cell density was perceived (at the time) to be the primary driver of the project. Essentially the production of more cells would deliver more active agents to the task. To achieve this aim, three phases were proposed: a medium development process, followed by a high cell density cultivation optimization, followed by a scale up to 2L bioreactor with further optimization.

Medium development is notably a process that is laborious, expensive, open ended, time consuming and involves many experiments (Kennedy & Krouse, 1999). Unsurprisingly the majority of the research time for this project was consumed by medium development, empirical optimization to locate the optimization range, and scaling up to the 2L bioreactor scale. The medium that was developed in this work is an improvement over the reference medium MB. However, there was no significant difference in the production of cells when compared to LB ($p=0.89$).

In terms of productivity the fermentation hypothesis was not well conceived. More importantly, with hindsight, the functionality expression of the strain could possibly have been controlled without the devotion of time and resources toward medium development, optimization, and scaling up to the 2L bioreactor scale. Simply adding the inducing substrate (cyanuric acid) into the LB medium, or into the formulation gel itself, may have been a more effective, and certainly more efficient approach to improve cultivation productivity and inducer controlled functionality.

The second major hypothesis was that we could use NZ-I zeolite and the biopolymer gel immobilization method (Johnson *et al.*, 2001) to stabilize the bioremediation reference strain *Pseudomonas* sp. strain ADP. Previous immobilization methods with this strain had been unstable (Klein *et al.*, 2009; Rietti-Shati *et al.*, 1996).

When NZ-I zeolite was used as a carrier for this strain, the material proved unusable at low gel application rates (4% w/w) and was fully replaced with a zeolite type AU-II in order to achieve the aims of the project. This was unexpected, as the biocontrol agent *Serratia entomophilia* was stabilized using NZ-I zeolite (Johnson *et al.*, 2001). However, the previous work (Johnson *et al.*, 2001) was conducted using a more dense microbial load. The positive outcome from replacing the NZ-I with AU-II zeolite was a roughly ten- fold lower rate of gel application to the zeolite surface. From a delivery perspective, and also an economic stance, AU-II allows ten times more formulated zeolite product to be made with the same amount of gel.

4.3 The gaps in research

The work in this thesis fills in a number of gaps in the available research. For producing *Pseudomonas* sp. ADP a productive medium was not available which ensured functionality, a formulation method did not exist, stability and functionality of the strain needed to be developed, and formulations applied in situ were not available. These gaps are presented in the following subsections and discuss the improvements made in this work.

4.3.1 Fermentation

The starting point of fermentation for this work involved the standard bacterial culture for producing *Pseudomonas* sp. ADP (P.ADP) (Mandelbaum *et al.*, 1993; Mandelbaum *et al.*, 1995). The medium yields a colony forming unit (cfu) in the range of 1×10^9. An 8.4×10^8 cfu/ml productivity was reported by (Shapir *et al.*, 1998b).

The flask media development process from the starting point of MB medium to the production of the IM medium was calculated in terms of significance (p value). The difference in cell productivity between the starting point MB media type and the IM type was significant ($p<0.001$). When the productivity (cfu/ml) of IM was statistically compared to LB there was no significant difference ($p=0.89$). However, *Pseudomonas* sp. strain ADP will lose its ability to metabolise atrazine (atz-) in the presence of complex laboratory media such as Luria-Bertani (LB) (De Souza *et al.*, 1998a) or alternate nitrogen sources such as ammonium, nitrate, and urea (Garcia-Gonzalez *et al.*, 2003). The IM medium produced in this work is an improvement which also retains full functionality.

In the current work the productivity of the growth medium was improved by 20 fold. An increase in colony forming units was demonstrated from the initial ~1x 10^9 cells of the MB starting medium, to the maximum cells of the final improved medium at ~2 x 10^{10}.

In terms of productivity, a single 2L batch of cells cultivated with the improved medium (IM) will deliver the same cell count as twenty 2L cultivations using the MB medium. This constitutes a substantial improvement in the production of a *Pseudomonas* sp. strain ADP which fully retains its functionality.

4.3.2 Formulation

In section 3.2, physical, chemical, biological, and time frame characteristics were examined. By following the factors which produce a strong formulation the essential needs of the microorganism were more clearly prioritized. In terms of viability, stability, and functionality, the inoculant performance was most affected by the choice of zeolite carrier. Encapsulation in the biopolymer gel provided numerical stability and also retained the functionality of the formulation. Pre-wetting provided a slight benefit in the absence of an encapsulation gel.

For formulations applied to Australian (AU-II) zeolite there was no significant difference between the stability of cells from media type MB, LB, or IM. However, a significant difference ($p<0.05$) was observed when cells with no formulation (MB) were compared to gel encapsulated, prewetted zeolite and encapsulated on prewetted zeolite formats, respectively IM/Gel ($p=0.015$), IM:pw ($p=0.038$), and IM/Gel:pw ($p=0.004$). The gel component was significant ($p<0.05$) to 10 week stability and prewetted (IM:pw) AU-II zeolite was observed to be statistically less stable than IM/Gel ($p=0.018$) and IM/Gel:pw ($p=0.001$).

In the following three examples, the performance of the formulation from the current work is compared to previous investigations using bioremediation agents, and specifically some previous works to immobilize *Pseudomonas* sp. strain ADP.

A slow-release inoculant was developed for soil and liquid remediation of atrazine using encapsulated *Rhodococcus erythropolis* NI86/21 (Vancov *et al.*, 2005). Alginate encapsulation was amended with bentonite, activated carbon or skimmed milk. The stability of the formulation was limited and viability could not be maintained after 21 days of storage

at 4°C. Using a recovery step immediately after encapsulation extended the storage stability to 6 months, declining 1 log, when stored at 4°C.

- Presented in this thesis, viability was retained without loss for 10 weeks at 25°C.

Entrapment of *Pseudomonas* sp. strain ADP was reported using a procedure that combined Ca-alginate encapsulation and the sol-gel glass process (Rietti-Shati *et al.*, 1996). The immobilization of the *Pseudomonas* sp. strain ADP cells appeared to cause the loss of atrazine activity. Aging the gel at 4°C for 4 days caused a further loss of activity and the addition of nutrients to the gel was proposed. Nutrients were supplied by immersion of the sol-gel discs in atrazine medium (containing NH_4Cl as a replacement for atrazine). The addition of additional nutrients produced atrazine degradation activity. However the entrapped bacteria were not stable and could not be revitalized (100% loss of viability) after 75 days (10 weeks) stored at 4°C.

- In comparison, the results described in this thesis do not cause a loss of functionality, the cells are stable at 4°C, but more importantly 25°C, and the formulation is stable without significant loss for 10 weeks.

Encapsulation of *Pseudomonas* sp. strain ADP was reported using electrospun microtubules (Klein et. al 2009). The authors report that some activity was retained in the encapsulated material and suggested it may show promise as a platform in the future. Transfer of cells to the microtubules was low. Starting with 10^9 cells, survival during the spinning process lost 1-2 orders of magnitude immediately. A discussion of extended shelf life stability was not included in the study.

- The results from the current thesis describe the complete transfer of cells to the encapsulation gel and carrier, no loss of viability from the immobilization process, and extended shelf life of at least 10 weeks at ambient temperature.

The primary formulation results were:

- The efficient transfer of cells to the encapsulation gel and carrier.
- No loss of viability from the immobilization process.
- The retention of maximum functionality.
- An extended shelf life of at least 10 weeks at ambient temperature.

These accomplishments are improvements to the techniques available to encapsulate and immobilize *Pseudomonas* sp. strain ADP. As a technique to produce stable functional

inoculants, the work presented here demonstrates an approach that is simple, practical, effective, and robust. All formulation objectives were accomplished.

4.3.3 In situ

The methods of immobilization and encapsulation have had little application in bioremediation or bioaugmentation (for contamination).

The primary results from inoculating the formulation into sterile soil were:

- Viability was retained in the soil for the full test period of 10 weeks.
- Functionality was retained at the maximum level for the full test period of 10 weeks.
- The dispersal of the strain into the surrounding soil from the formulation was improved over liquid bacterial culture inoculation.
- The functionality of both the formulation and the dispersed microbial agent was retained at the maximum level.
- As a means of preprogramming or controlling the functionality of a microbial product there appears to be a substantial advantage for delivering microbes (and their full functionality) as a biofilm.

We are not aware of previous studies with this strain using a formulation approach to stabilize the agent, maintain the functionality of the microbe, and disperse both microbial agent and its functionality into the surrounding soil at the point of delivery. These accomplishments are improvements to the techniques available. The In situ objectives were accomplished.

4.4 The wider literature

To incorporate the findings of this research into a wider context the following sections are provided.

The formulation system developed in this research is generally described as a solid support delivery which is coated with a gel to create an artificial biofilm. From the results shown in Chapter 3, there is potential that solid support delivery of bacteria may also be translated to other systems.

- This is a contribution to the delivery of bacterial cultures in general

In the wider literature there are a great variety of other beneficial microbes and problems to be addressed. Bioaugmentation has uses other than bioremediation and there are a variety of potential applications for augmentation of microbial inoculants. Traditional inoculant products include *Rhizobium* for legumes, probiotics, silage, and inoculants for food and drink fermentation. Agricultural uses include plant protection by pest and pathogen control, plant growth stimulation by both seedling inoculation and hormone production, and the improvement of both soil structure and the increased availability of nutrients such as nitrogen and phosphorous (Bashan, 1998; Paau, 1988; van Veen *et al.*, 1997). The use of microbial agents for plant protection is driven by the emergence of new or expanded restrictions placed upon current chemical control agents (Bashan, 1998; Gerhardson, 2002; Lewis & Papavizas, 1991). The potential role of microorganisms, for these and other beneficial processes, is limited by their survival and proliferation in soil (Bashan, 1998; van Veen *et al.*, 1997).

- It would appear that the methods described here may provide a useful starting point, or perhaps a model, for the improvement of these and existing biocontrol agents.

For *Pseudomonas* sp. strain ADP, functionality to mineralize atrazine is controlled at the plasmid level and is positively enhanced by the presence of the formulation.

- The system used here may be transferable to other stability and delivery applications where bacterial processes are controlled at the plasmid level.

Biofilm research may offer a preview of the mechanisms that will be needed for biocontrol agent delivery to be consistently accomplished. An ultimate goal in biofilm research is to control and/or eliminate the formation of naturally self-determined biofilms. For *Pseudomonas aeriginosa*, multiple phenotypes were displayed during biofilm development and may be important as a mechanism to control biofilm growth (Sauer *et al.*, 2002). Five distinct stages were characterized from planktonic (free cells) to the development and ultimate dispersion of biofilm cells in *P. aeriginosa*. By measuring proteins produced (genes expressed) biofilm cells differed from planktonic cells because they expressed a different set of genes when grown in a matrix enclosed community associated with surfaces and interfaces (Costerton, 2007).

- In this thesis we have used a biopolymer formulation in which an inoculum is simply mixed into a gel and applied directly to the surface of the zeolite with no special equipment, drying, temperatures, or secondary re-growth steps required. It is a simple model system consisting of a carrier, a artificial biofilm, and a

known starting point to measure the process of biofilm maturation. Such a method may be useful for investigating the multiple biofilm development stages and their characteristic variation in gene expression over time.

Lastly, to address the difficulties of characterizing, quantifying and evaluating bioaugmentation research, Vogel (1996) suggests using standardized parameters for evaluating a bioaugmentation inoculum in soil. In section 1.1.3 a case was made that bioremediation, especially bioaugmentation, is designed to fail without a coherent, and scientific, methodology. A standard inoculum type does not exist in the literature. Importantly, a *stable* control inoculum type does not exist in the literature. More importantly, a *stable* control inoculum type has not been applied and quantified in lab and field settings. The primary, essential building block is absent in bioremediation. There is no stable seed from which to develop a methodology. It is therefore useful to begin the process of creating a standard.

- Without a system of standards the notions of accountability, expectations, and success are not possible. This thesis has contributed to that process.

4.5 Future directions

For future researchers interested in continuing this work, the following subsections offer some suggestions.

4.5.1 Fermentation

After completing the formulation and soil phases of this work it became clear that the type of media is of low importance for producing an effective formulation with high viability and functionality. Optimization of media appears to be a pursuit which may be shifted to some later stage in the development of microbial products. Therefore, in hindsight, it does not seem worthwhile to suggest an investment of more valuable time in future research for media development. As noted in 4.2, simply adding the inducing substrate (cyanuric acid) into the LB medium may have been a more effective and certainly more efficient approach.

4.5.2 Formulation

The method of formulation used in this work was successful but could be further developed. Specifically relevant are improvements that produce room temperature stability beyond the 10 week study period of this work. While 10 weeks is sufficient as a minimum standard of

stability, a longer period such as six months (26 weeks) would be of interest to the commercial sector.

Encapsulation using the biopolymer gel could be perhaps improved with layering of various materials. The gel was prone to desiccation outside of sealed containers and may benefit from an additional coating external to the gel. A layering of multiple gel coating may also be useful to increase the overall cell loading of the individual zeolite granules.

The NZ-I zeolite is a particularly interesting area of investigation. It was determined that the NZ-I zeolite retained 5 times more water that the AU-II zeolite yet had little ability to maintain microbial viability (section 3.2.1). A working hypothesis was formed that the highly absorptive interior of the NZ zeolite effectively removed biologically available water from the microbial cells which lead to desiccation and loss of viability. Future work could investigate various liquid media, buffers, and levels of pre-wetting the NZ-I zeolite to produce results more similar to the AU-II zeolite.

Due to its friable and absorptive character the NZ-I zeolite may also be useful as an external layer to the gel encapsulation. A powdered NZ-I zeolite applied as an external shell may benefit the formulation by absorbing and holding moisture from the surrounding soil. One specific investigation could compare the performance of a powdered NZ-I zeolite to bentonite as an external shell to the formulation.

4.5.3 In situ

In this work, the performance of a formulated microbial inoculant was evaluated against the physical and chemical challenges of a sterile soil. Biological challenges of soil such as predation and inhibition have not been addressed here. The next logical evaluation for the formulation is outside the laboratory at the level of a live field soil trial.

To make a contribution toward understanding the survival of the inoculant in the context of soil microbiology requires the examination of various soil types across environmental, climate, and distance (proximity) scenarios. The soil environment itself is a driver in soil microbiology and a variety of pH, organic matter, water content, as well as organic and inorganic phosphorous levels should be necessarily examined. Climatic scenarios could include numerous options of wetting and drying cycles and temperature fluctuation and durations. Scenarios of proximity and the solubility of the target add a further level of investigatory rigor.

To address the difficulties of characterizing, quantifying and evaluating bioaugmentation research, Vogel (1996) suggests using standardized parameters for evaluating a bioaugmentation inoculum in soil. They are:

1. Pollutant characteristics

   Bioavailability, concentration, and microbial toxicity.
2. Soil physico-chemical characteristics

   Humidity, water content, organic matter content, clay matter content, and pH.
3. Microbial ecology

   Presence of predators, interspecies competition.
4. Microbiology

   Presence of co-substrates, genetics of relevant organisms, enzyme stability and activity.
5. Methodology

   Inoculation concentration, method of inoculation, the presence/absence of indigenous activity, and inoculum heterogeneity.

In the current work a decision was made that such an examination was certainly needed, but was outside the constraints of this project. The current project had met its original aims successfully and therefore the investigative work of this research project was concluded.

Further work could certainly include an evaluation of the formulation produced in this thesis at field scale in a live soil environment. It is important that the formulation is examined in actual field soil conditions rather than in the laboratory. The dynamic swings of temperature and moisture cycles are necessary to determine whether a formulated microbial product is stable in real-world conditions.

Field soil conditions are also necessary to confirm whether a formulation is capable of protecting the microbial agent from the indigenous biology. The plate counts conducted in this study are insufficient at the live soil level due to the presence of unknown organisms at unknown concentrations. A more robust method would be required to enumerate only the strain type that was inoculated. Molecular techniques are likely the appropriate option to identify the *Pseudomonas* sp. strain ADP levels over time. Tracking and enumerating the plasmid copy number from the strain may also lead to observations regarding the conjugation or transfer of plasmids to the indigenous community.

For the formulation to be considered a commercially useful product it must demonstrate the ability to consistently remove atrazine from the soil. Therefore future research could include an analysis of the removal kinetics of atrazine from various soil types and conditions. Atrazine has six steps of degradation and a rigorous analysis could include the intermediate compounds from these steps and their concentrations. *Pseudomonas* sp. strain ADP fully degrades atrazine to CO_2 (Wackett *et al.*, 2002),which would be advantageous for complete removal of the contaminant in field conditions. An investigation of the intermediate products could confirm whether that is the case when a formulated product is delivered to the complex ecology of live field soil.

5 Appendix

5.1 Media

5.1.1 Mandelbaum et al. Medium (MB)

Atrazine media *(per liter):*

1.6 g K_2HPO_4

0.4 g KH_2PO_4

0.2 g $MgSO_4$ x $7H_2O$

0.1 g NaCl

0.02 g $CaCl_2$

1 g sucrose (the sucrose was removed in 1995 version)

1 g sodium citrate

2.5 ml atrazine stock solution

20 ml salt stock solution

20 ml vitamin stock solution

pH is adjusted to 7.3

Final concentration of atrazine= 100ppm (0.46mM)(0.1g/L)

Atrazine stock solution *(from 1995 publication)*

Concentrated stock solution, 500mg/ml in methanol.

Sonicated to reduce crystal size.

Stored unfiltered at room temperature in the dark.

Salt stock solution *(per liter of DI water)*

2.5 g EDTA

11.1 g $ZnSO_4$

5.0 g $FeSO_4$

1.54 $MnSO_4$ x H_2O

0.4 g $CuSO_4$ x $5H_2O$

0.25 g $CO(NO_3)_2$ x $6H_2O$

0.18 g $Na_2B_4O_7$ x $10H_2O$

5.0 ml [H_2SO_4]

Filter sterilized and stored at 4°C.

Vitamin stock solution *(per liter of DI water)*

5mg Thiamine-HCl

2 mg Biotin

2mg Folic acid

10 mg Nicotinamide

10 mg Pyridoxine-HCl

Filter sterilized and stored at 4°C.

5.1.2 DSMZ 465i Medium

Composition per liter:

Na_2HPO_4 x $2H_2O$	3.5g
Na-citrate	1.0g
KH_2PO_4	1.0g
$MgCl_2$ x $6H_2O$	0.1g
$CaCl_2$	0.05g
Atrazine solution	10 ml
SL-4 (Trace elements)	1.0ml

Adjust pH to 7.25

Preparation: Add components except atrazine to 990ml distilled water. Autoclave the media for 15 minutes (121°C at 15 psi). Cool to room temperature. Aseptically add 10 ml atrazine solution.

Atrazine solution

Preparation: Add 100mg Atrazine in 10 ml methanol. Sonicate to reduce particle size.

SL-4 Solution per liter:

EDTA	0.5g
$FeSO_4$ x $7H_2O$	0.2g
SL-6 (Trace elements)	100 ml

Preparation: Add components to distilled water and bring volume to 1.0 L.

***SL-6 Solution per liter**:*

H_3BO_3	0.3g
$CoCl_2$ x $6H_2O$	0.2g
$ZnSO_4$ x $7H_2O$	0.1g
$MnCl_2$ x $4H_2O$	0.03g
Na_2MoO_4 x H_2O	0.03g
$NiCl_2$ x $6H_2O$	0.02g
$CuCl_2$ x $2H_2O$	0.01g

Preparation: Add components to distilled water and bring volume to 1.0 L. Adjust pH to 3.4.

5.1.3 Improved Medium

Composition per liter:	g/l
Sodium Citrate	20
Cyanuric acid	6
Yeast extract	1
Potassium phosphate dibasic	6.4
Potassium di-hydrogen orthophosphate	1.6
Magnesium sulphate heptahydrate	0.8
Sodium chloride	0.4
Calcium chloride	0.08

Preparation: Add components to distilled water and bring volume to 1.0 L.

Autoclave the media for 15 minutes (121°C at 15 psi).

5.1.4 RS medium

Composition per liter:

40 g raw sugar

2 g NPK 1:1:1

- 0.68 g NH_4NO_3
- 0.45 g KCl
- 0.86 g Na_2HPO_4

2 g Urea

10 g Granulated Yeast Extract

Preparation: Add components to distilled water and bring volume to 1.0 L.
Autoclave the media for 15 minutes (121°C at 15 psi).

5.1.5 Atrazine agar (NuFarm)

Composition per litre:
2.0 g Sodium Citrate
1.6 g K_2HPO_4
0.4 g KH_2PO_4
0.2 g $MgSO_4$ x $7H_2O$
0.1 g NaCl
0.02 g $CaCl_2$
10g agar
Preparation: With stirring bar, add components to distilled water and bring volume to 1.0 L.
Autoclave the media for 15 minutes (121°C at 15 psi). Aseptically add 2ml Nufarm Flowable. Atrazine™ suspension (100ppm)

Stir well and pour into plates.
Store face down at 4°C.

5.2 Buffers and Solutions

5.2.1 Phosphate buffer (0.1 M)

65 ml solution A
35 ml solution B

Make up to 1 L with distilled H_2O and sterilise by autoclaving for 15 minutes (121°C at 15 psi).

Solution A

174.18 g K_2HPO_4
Dissolve in 800 ml distilled H_2O. Adjust volume to 1 L and sterilise by autoclaving for 15 minutes (121°C at 15 psi).

Solution B

136.09 g KH_2PO_4

Dissolve in 800 ml distilled H_2O. Adjust to 1 L and sterilise by autoclaving for 15 minutes (121°C at 15 psi).

5.3 *Pseudomonas* sp. strain ADP gene typing sequence

Contig:

CGATTCCCGAAGGCACCCYCGCATCTCTGCARGATTCCGGACATGTCAAGGCCAG
GTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGC
CCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACT
TATCGCGTTAGCTGCGCCACTAAGATCTCAAGGATCCCAACGGCTAGTCGACATC
GTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCA
CCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCTAT
ATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTAGT
CAGGCAGTTATGGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCATCTT
ACCAAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCC
TTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTGGTAAC
GTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCTCCCAACTTAAAGTGCTTTAC
AATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATT
GTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCA
GTGTGACTGATCATCCTCTCAGACCAGTTACGGATCGTCGCCTAGGTGAGCCATT
ACCCCACCTACTAGCTAATCCGACCTAGGCTCATCTGATAGCGTGAGGTCCGAAG
ATCCCCCACTTTCTCCCGTAGGACGTATGCGGTATTAGCGCTCCTTTCGGAACGTT
GTCCCCCACTACCAGGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTGAATC
C

The strain was placed in the AgResearch culture collection, number AGR3432.

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