
Microbial CaCO₃ Precipitation for the production of Biocement

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“Upon touching sand, may it turn to gold”

Anon. (Greek proverb)

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Microbial CaCO₃ precipitation for
the production of biocement.

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Subject headings: Urease / Calcium
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Declaration

I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any university.

Victoria Whiffin

Summary

The hydrolysis of urea by the widely distributed enzyme urease is special in that it is one of the few biologically occurring reactions that can generate carbonate ions without an associated production of protons. When this hydrolysis occurs in a calcium-rich environment, calcite (calcium carbonate) precipitates from solution forming a solid-crystalline material. The binding strength of the precipitated crystals is highly dependent on the rate of carbonate formation and under suitable conditions it is possible to control the reaction to generate hard binding calcite cement (or Biocement). The objective of this thesis was to develop an industrially suitable cost-effective microbial process for the production of urease active cells and investigate the potential for urease active cells to act as a catalyst for the production of Biocement.

The biocementation capability of two suitable strains was compared. *Sporosarcina pasteurii* (formally *Bacillus pasteurii*) produced significantly higher levels of urease activity compared to *Proteus vulgaris*, however the level of urease activity was variable with respect to biomass suggesting that the enzyme was not constitutive as indicated by the literature, but subject to regulation. The environmental and physiological conditions for maximum urease activity in *S. pasteurii* were investigated and it was found that the potential urease capacity of the organism was very high (29 mM urea.min⁻¹.OD⁻¹) and sufficient for biocementation without additional processing (e.g. concentration, cell lysis). The regulation mechanism for *S. pasteurii* urease was not fully elucidated in this study, however it was shown that low specific urease activity was not due to depletion of urea nor due to the high concentrations of the main reaction product, ammonium. pH conditions were shown to have a regulatory effect on urease but it was evident that another co-regulating mechanism existed. Despite not fully exploiting the urease capability of *S. pasteurii*, sufficient urease activity to allow direct application of the enzyme without additional processing could still be achieved and the organism was considered suitable for biocementation.

Urease was the most expensive component of the cementation process and cost-efficient production was desired, thus an economic growth procedure was developed for large-scale cultivation of *S. pasteurii*. The organism is a moderate alkaliphile (growth optimum pH 9.25) and it was shown that sufficient activity for biocementation could be cultivated in non-sterile conditions with a minimum of upstream and downstream processing. The cultivation medium was economised and expensive components were replaced with a food-grade protein source and acetate, which lowered production costs by 95%. A high level of urease activity (21 mM urea hydrolysed.min⁻¹) was produced in the new medium at a low cost (\$0.20 (AUD) per L).

The performance of urease in whole *S. pasteurii* cells was evaluated under biocementation conditions (i.e. presence of high concentrations of urea, Ca²⁺, NH₄⁺/NH₃, NO₃⁻ and Cl⁻ ions). It was established that the rate of urea hydrolysis was not constant during cementation, but largely controlled by the external concentrations of urea and calcium, which constantly changed during cementation due to precipitation of solid calcium carbonate from the system. A simple model was generated that predicted the change in urea hydrolysis rate over the course of cementation. It was shown that whole cell *S. pasteurii* urease was tolerant to concentrations of up to 3 M urea and 2 M calcium, and the rate of urea hydrolysis was unaffected up to by 3 M ammonium. This allowed the controlled precipitation of up to 1.5 M CaCO₃ within one treatment, and indicated that the enzyme was very stable in spite of extreme chemical conditions.

A cost-efficient cementation procedure for the production of high cementation strength was developed. Several biocementation trials were conducted in order to optimise the imparted cementation strength by determining the effect of urea hydrolysis rate on the development of strength. It was shown that high cementation strength was produced at low urea hydrolysis rates and that the development of cementation strength was not linear over the course of the reaction but mostly occurred in the first few hours of the reaction. In addition, the whole cell bacterial enzyme had capacity to be immobilised in the cementation material and re-used to subsequent applications, offering a significant cost-saving to the process.

An industry-sponsored trial was undertaken to investigate the effectiveness of Biocement for increasing in-situ strength and stiffness of two different sandy soils; (a) Koolschijn sand and (b) 90% Koolschijn sand mixed with 10% peat (Holland Veen). After biocementation treatment, Koolschijn sand indicated a shear strength of 1.8 MPa and a stiffness of 250 MPa, which represents an 8-fold and 3-fold respective improvement in strength compared to unconsolidated sand. Significantly lower strength improvements were observed in sand mixed with peat.

In combination, trials of producing bacteria under economically acceptable conditions and cementation trials support the possibility of on-site production and in-situ application of large field applications.

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Introduction

1.1 Introduction

Calcite (CaCO_3) is one of the most common and widespread minerals on Earth, constituting 4% by weight of the Earth's crust. It is naturally found in extensive sedimentary rock masses, as limestone, marble and calcareous sandstones in marine, freshwater and terrestrial environments (Hammes and Verstraete, 2002; Klein and Hurlbut, 1999). Bacterial contribution to these extensive formations had been suspected for some time (Drew, 1910) but remained controversial until recent investigations involving the microbial pathways and the required precipitation conditions, indicated that bacteria have the potential to far exceed the abiotic contribution to calcium carbonate deposition in most environments on Earth (Castanier *et al.*, 2000b). In addition to this, the huge limestone formations on the sea bottom have to a large extent resulted from great thicknesses of calcareous material from pelagic skeletal organisms (such as coccolithophores and foraminifera) (Morse, 2003; Klein and Hurlbut, 1999). It has been shown that the cellular organelles that are largely responsible for the production of carbonate shells in eukaryotic organisms, are the mitochondria (or chloroplasts). These organelles are widely considered as primitive endosymbiotic bacteria, which further supports the bacterial contribution to carbonate precipitation (Castanier *et al.*, 2000b).

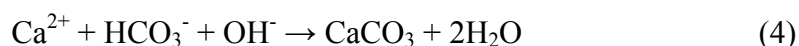
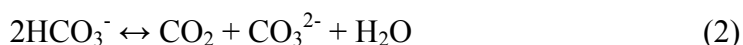
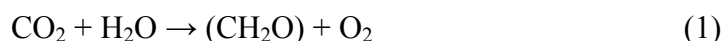
The precipitation of calcium carbonate is governed by four parameters; (1) the calcium concentration, (2) the carbonate concentration, (3) the pH of the environment (which affects carbonate speciation and calcium carbonate solubility) and (4) the presence of nucleation sites (Hammes and Verstraete, 2002). Carbonate precipitation may theoretically occur in natural environments by increasing the concentration of calcium and/or carbonate in solution or by decreasing the solubility of calcium and/or carbonate. Calcite precipitation may come about abiotically by evaporation or shifts in temperature or pressure, or biotically through the action of microorganisms. Bacterial cells have themselves been shown to be excellent nucleation sites for growing minerals (authigenic)

during the formation of rock (Ferris *et al.*, 1986; Ferris *et al.*, 1987), with many studies confirming the precipitation of calcite on the bacterial cell surface (Fujita *et al.*, 2000; Hammes *et al.*, 2003c; Warren *et al.*, 2001). As there is no shortage of nucleation sites in bacterial cultures, the first three parameters of calcium concentration, carbonate concentration and pH are key for microbial carbonate precipitation (MCP).

1.2 Microbial Carbonate Precipitation (MCP)

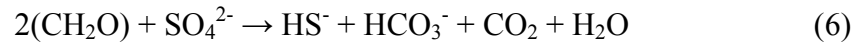
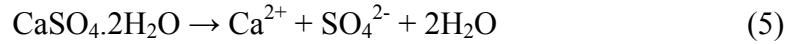
Microbial carbonate precipitation (MCP) has gained interest in the last 20 years, particularly with regard to the potential role marine systems may play as ‘carbon sinks’ for the increasing global production of CO₂. Three main groups of organisms exist that can induce MCP through their metabolic processes; (i) photosynthetic organisms such as cyanobacteria and algae that remove CO₂, (ii) sulphate reducing bacteria that are responsible for the dissimilatory reduction of sulphate and (iii) several organisms that are involved in the nitrogen cycle (Castanier *et al.*, 1999; Hammes and Verstraete, 2002).

The most common form of MCP in aquatic environments is caused by photosynthetic organisms (McConnaughey and Whelan, 1997). The metabolic processes of algae and cyanobacteria utilise dissolved CO₂ (Eqn 1), which is in equilibrium with HCO₃⁻ and CO₃²⁻ (Eqn 2). The removal of CO₂ induces a shift in this equilibrium, and results in an increase in pH (Eqn 3) (Ehrlich, 1998). When this reaction occurs in the presence of calcium ions, calcium carbonate is produced (Eqn 4) (Hammes and Verstraete, 2002).

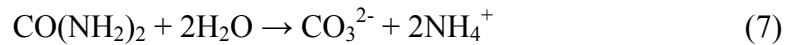


Calcite can also be precipitated by heterotrophic organisms, by the production of carbonate or bicarbonate and modification of the environment to favour precipitation (Castanier *et al.*, 1999). The abiotic dissolution of gypsum

(CaSO₄·2H₂O) (Eqn 5) provides an environment that is rich in both sulphate and calcium ions. In the presence of organic matter and absence of oxygen, sulphate reducing bacteria (SRB) can reduce sulphate to H₂S and release HCO₃⁻ (Eqn 6) (Castanier *et al.*, 1999; Ehrlich, 1998; Wright, 1999). If H₂S then degasses from the environment, this results in an increase in pH and favours the precipitation of calcium carbonate (Eqn 4) (Castanier *et al.*, 1999).



MCP can also be induced by organisms involved in the nitrogen cycle, via ammonification of amino acids, nitrate reduction and the hydrolysis of urea. The simplest of all of the mechanisms described for MCP is the hydrolysis of urea by the enzyme urease, which results in the production of carbonate ions in the presence of ammonium (Eqn 7). Calcite is readily precipitated under these conditions, in the presence of calcium.



Urease activity is widespread amongst bacteria and this has been the approach used most often for applied MCP for the production of calcite (Fujita *et al.*, 2000; Mobley and Hausinger, 1989; Stocks-Fischer *et al.*, 1999).

1.3 MCP by way of urea hydrolysis

Urea hydrolysis is the most easily controlled of the carbonate generating reactions, with the potential to produce high concentrations of carbonate within a short time. A number of applications involving MCP have been previously attempted. To date, most of the published work has focussed on MCP for purposes other than strength development.

MCP has been investigated for the solid-phase capture of the divalent radionucleotide, strontium⁹⁰ (⁹⁰Sr²⁺). The precipitation of ⁹⁰SrCO₃ was desired to prevent the spread of radionucleotide contamination in the subsurface via

groundwater movement (Fujita *et al.*, 2000; Warren *et al.*, 2001). The authors' approach was to enrich the indigenous microbial population and promote MCP by addition of high concentrations of urea and low concentrations of calcium (Table 1.1). All groundwater samples collected during the study tested positive for urease activity, indicating that urease activity was common and widespread in the environment (Fujita *et al.*, 2000; Mobley and Hausinger, 1989). Successful capture of strontium⁹⁰ (⁹⁰Sr²⁺) was achieved by substitution of calcium with strontium in the calcium carbonate crystal matrix (Warren *et al.*, 2001). It was found that the rate of calcite precipitation was directly linked to the rate of urea hydrolysis and that the primary means by which microorganisms promoted calcite precipitation was by alkalinisation of the precipitation environment (Fujita *et al.*, 2000).

Table 1.1: Reaction conditions reported in the literature for the production of CaCO₃ via microbial production of carbonate from urea hydrolysis.

Purpose	Urea (mM)	Ca ²⁺ (mM)	Urease Activity (mM urea.min ⁻¹)	Reference
Sr ⁹⁰ sequestration	333	25	0.045	(Fujita <i>et al.</i> , 2000)
Sr ⁹⁰ sequestration	330	0.025	0.042	(Warren <i>et al.</i> , 2001)
Removal of Ca ²⁺ from wastewater	16	14	0.293	(Hammes <i>et al.</i> , 2003c)
Removal of Ca ²⁺ from wastewater	8	15	0.032	(Hammes <i>et al.</i> , 2003a)
Stone remediation	333	12 - 50	0.190	(Stocks-Fischer <i>et al.</i> , 1999)
Stone remediation	66	25	0.041	(Bachmeir <i>et al.</i> , 2002)
Portland cement remediation	333	50	n/s	(Ramachandran <i>et al.</i> , 2001)
Plugging of rock pores	333	0.025	n/s	(Gollapudi <i>et al.</i> , 1995)
<i>Biocementation</i>	<i>1500</i>	<i>1500</i>	<i>4 - 18</i>	<i>The present study (Chapters 6 and 7)</i>

n/s = not stated

MCP has also been used to facilitate the removal of Ca²⁺ from industrial wastewaters. MCP via urea hydrolysis was a preferred alternative to adding Na₂CO₃ because of the low cost of urea and the precipitation of CaCO₃ on the

bacterial cell surface. The presence of calcium presents severe scaling problems due to precipitation of CaPO_4 , CaCO_3 and $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ in downstream plant facilities (Hammes *et al.*, 2003a; Hammes *et al.*, 2003c). Up to $0.5 \text{ g.L}^{-1} \text{ Ca}^{2+}$ was removed by addition of low concentrations of urea, representing 90% of the calcium present in the tested wastewater (Table 1.1). The urease activity of certain bacterial isolates was increased up to 10-fold in the presence of 30 mM calcium, indicating the calcium can enhance urease activity in certain species. In addition, variation in crystal growth rates generated calcite crystals that were morphologically different from each other, suggesting that the shape and size of calcite crystals could be controlled by control of the rate of urea hydrolysis (Hammes *et al.*, 2003b)

In the recovery of heavy oil from oil fields, where water is more readily removed than the viscous oil, the ability to selectively plug porous rock to focus pumping energy in oil rich zones is highly desirable (Hart *et al.*, 1960; Lappin-Scott *et al.*, 1988; MacLeod *et al.*, 1988). The potential of MCP for selective plugging of sand has been recognized (Ferris and Setehmeir, 1992) and investigated by mixing bacteria with sand prior to packing into cores and then applying a continuous gravity flow of urea/calcium/carbonate ions through the core (Gollapudi *et al.*, 1995) (Table 1.1). A 50% reduction in flow rate was achieved after 45 hours, with full plugging resulting after 120 hours, suggesting that the degree of plugging and hence remaining permeability of the treated material could be controlled by the extent of the treatment. Stocks-Fischer *et al.* (1999) further investigated the microbial plugging of sand cores and showed that the microorganism directly participated in calcite precipitation by providing a nucleation site (on its surface) and by creating an alkaline environment, which favoured calcite precipitation. At high biomass concentrations, the rate of CaCO_3 precipitation per cell decreased and the authors hypothesised that the calcite ‘coating’ over the cell surface limited urea diffusion (Stocks-Fischer *et al.*, 1999).

None of the aforementioned applications considered the strength of the precipitated calcite. For cementation purposes, the binding strength between the particles and cementation quality of the precipitated calcite are important. Only one study considers the cementation potential of MCP via urea hydrolysis,

investigating the effect of biomass concentration (and hence rate of urea hydrolysis) on the compressive strength of porous Portland cement mortar cubes (Ramachandran *et al.*, 2001). Sporulating urease-producing bacteria were mixed with cement and sand, and set into 5 cm cube moulds. After 24 hours, the cubes were de-moulded and placed into a urea/calcium solution for 28 days (Table 1.1). No appreciable difference in the total amount of calcite could be detected between the control and the bacterially-treated cubes but a 24% improvement in compressive strength was observed in cubes treated with low concentrations of bacteria (7.6×10^3 cells.ml⁻¹). High concentrations (7.6×10^7 cells.ml⁻¹) of bacteria produced no improvement in strength. This suggests that slow rates of calcite formation imparted more strength than high rates, but the study was not conclusive due to the lack of difference in the amount of deposited calcite before and after treatment.

All of the above approaches come from the general conception in the literature, that the primary means by which microbes promote calcite precipitation is by metabolic processes that increase alkalinity (Castanier *et al.*, 2000a; Ehrlich, 1998; Fujita *et al.*, 2000). While this may be true for most natural systems, where the rate of calcite precipitation is low, it does not necessarily represent the only role that microbes can play in a controlled environment. The hydrolysis of urea generates carbonate ions at a 1:1 molar ratio (Eqn 7). If a high concentration of urea is present, the carbonate concentration can be significantly increased, which is one of the key parameters for calcite precipitation. The hydrolysis of urea is ideal for high rates of MCP because it not only provides an alkaline pH, but also generates a readily available supply of carbonate.

1.4 MCP for consolidation of porous media

MCP by way of other processes than urea hydrolysis has been investigated for the purposes of stone consolidation. Tiano *et al.* (1999) evaluated a biologically-mediated calcite precipitation treatment on limestone samples for the preservation of monumental stones, and assessed the impact on pore volume, strength (shearing strength) and colour. The limestone samples were wetted and brushed with a bacterial suspension, which was fed with a nutrient medium every 24

hours. The pore volume (i.e. the amount of water that could be absorbed into the stone) was reduced by approximately 60% after treatment – approximately half of this reduction was due to calcite precipitation and the other half due to pore obstruction by the growth of non-calcite producing contaminant microorganisms. Despite the precipitation of calcite inside the stone matrix, no significant difference in strength was shown after treatment. The preservation of the aesthetic appearance of monumental stones was an important consideration in this study and no appreciable colour difference was detected in the areas of calcite precipitation. There were however black and red patches along the sides of all samples (including controls) due to the growth of airborne fungal contaminants (Tiano *et al.*, 1999).

In addition to the approach used by Tiano *et al.*, (1999), two application methods for the regeneration of calcite in monumental stones have been patented (Adolphe *et al.*, 1990; Castanier *et al.*, 1995; Le Metayer-Levrel *et al.*, 1999). The first involved protecting the surface of the stone by spray application of bacteria and nutrients, followed by the numerous applications of nutrient solution only. The inventors of this process stated that calcite was cemented “a few micrometers thick” but the actual number was not specified (Castanier *et al.*, 2000a). Rodreguez-Navarro *et al.*, (2003) have further extended the depth of cementation to approximately 500 μm , by soaking the limestone samples in the cementation media, and this is the greatest depth of cementation currently reported. As cementation is very thin and confined to the surface of the stone, this type of application is more suitable as a preventative rather and restorative treatment, and is not suitable for cementation of unconsolidated material.

A second approach for the purposes of filling holes or cracks in stones, involved creating a “biological mortar” consisting of crushed limestone powder and a bacterial paste (pelleted bacterial cells after most of the culture liquid had been removed) which can then be packed into the crack or hole (Castanier *et al.*, 1995). This application involved the inclusion of an antifungal agent to prevent the growth of fungal contaminant organisms.

1.5 Calcite In-situ Precipitation System (CIPS)

In addition to work published in the literature, an in-situ calcite cementation process, called the Calcite In-situ Precipitation System (CIPS) has been developed by Calcite Technology Pty Ltd (Perth, Australia). The CIPS treatment precipitates calcite inside existing matrix materials (such as sand) in such a way as to provide binding strength (Fig 1.1). CIPS can be used to cement destabilised materials in-situ, without the need for removal and the resulting mechanical properties of treated material are comparable with natural calcarenite (medium grained natural sediments of calcium carbonate) (Ismail *et al.*, 2002). The composition of the CIPS cementation solution is proprietary to Calcite Technology Pty Ltd.

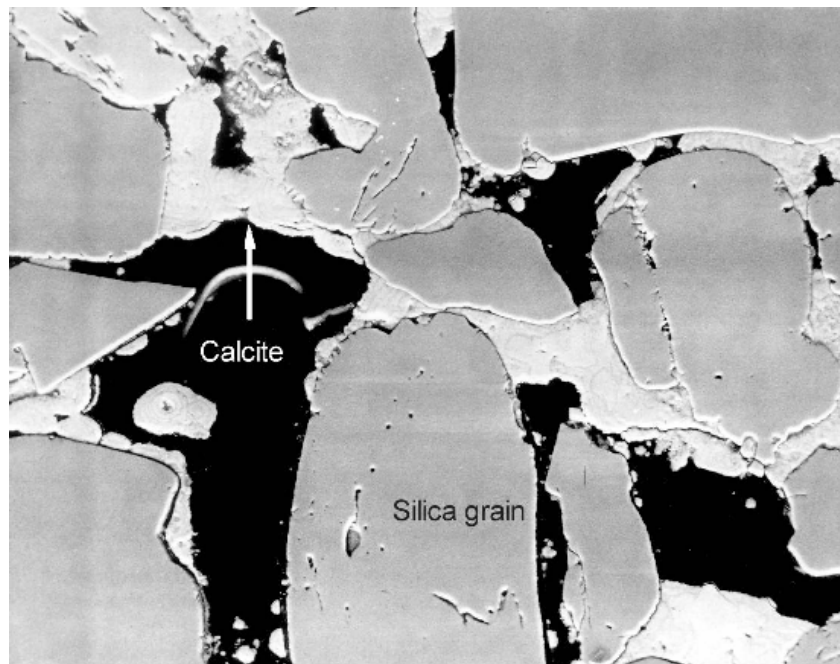


Figure 1.1: Cross-section of silica sand treated with the Calcite In-situ Precipitation System (CIPS). Note the calcite cement binding the contact areas of the grains together and that large pore spaces (black areas) remain after cementation (Image courtesy of Dr. Ed Kucharski, Calcite Technology Pty Ltd).

1.6 Important factors for cementation

As mentioned earlier, the precipitation of calcium carbonate in bacterial systems, where nucleation can occur on the bacterial cell surface, is governed by three parameters; (1) the calcium concentration (2) the carbonate concentration and (3) the pH of the environment (which affects carbonate speciation and calcium carbonate solubility) (Hammes and Verstraete, 2002).

In order for precipitation to take place, supersaturation of the precipitating species must exist (Randolph and Larson, 1988). Because the solubility product (K_{sp}) of calcite is extremely low ($3.3 \times 10^{-9} \text{ mol.L}^{-1}$ at 25°C (Sawada, 1998)), it is straightforward to achieve the supersaturation. Precipitation can be achieved by simply mixing together moderate concentrations of soluble Ca^{2+} and CO_3^{2-} ions. The crystals that form when the reaction happens rapidly are very small and powder-like with little cementation strength.

One of the prime factors controlling the rate of precipitation is the difference between the saturation concentration and the supersaturation concentration (Bodek *et al.*, 1988). In general, the higher the supersaturation concentration, the faster the rate and the smaller the crystals that form, as exemplified by the chemical system of mixing the carbonate and calcium ions together. The supersaturating product concentration and thus the degree of disequilibrium remains relatively low (compared to the chemical example), which allows larger crystals to form over an extended period with higher cementation strength.

The supersaturation level of carbonate ions can be further influenced by controlling the pH. The proportion of total carbonate that exists as CO_3^{2-} in solution at any time is highly dependent on the pH. Below pH 8, the carbonate concentration is very low (Fig 1.2), which means that if desired, the size of crystals that form can be increased by decreasing the pH or visa versa, crystal size can be decreased by increasing the pH.

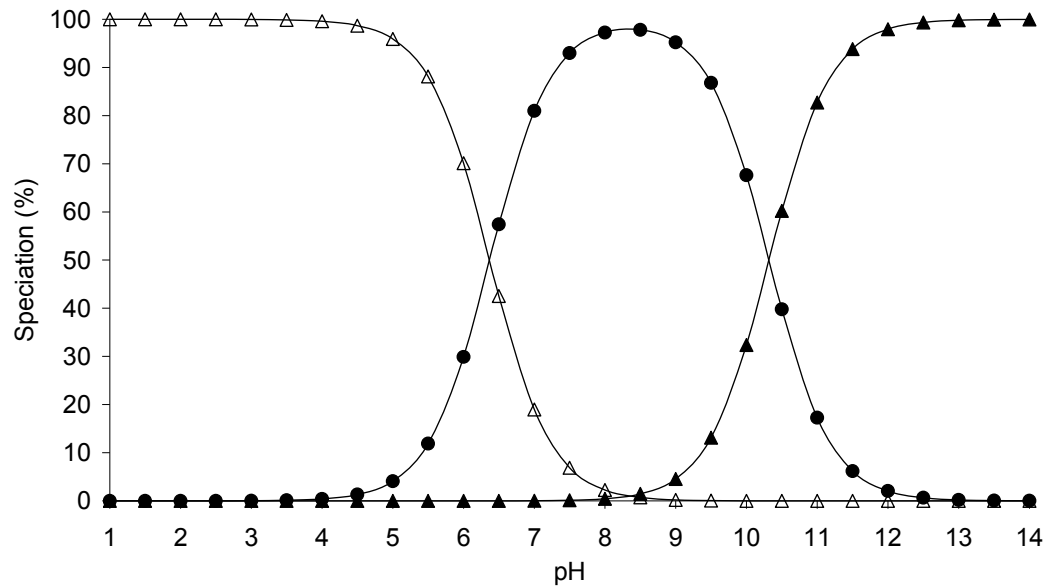


Figure 1.2: Chemical speciation of H_2CO_3 (Δ), HCO_3^- (\bullet) and CO_3^{2-} (\blacktriangle) at 25°C (Calculated from Atkins, 2002).

1.7 Approach of this study

The approach of the present study was to fuse together the feasibility of MCP already established in the literature with the applicability of the CIPS process. The product of this fusion was anticipated to be the development of a microbially mediated process that produced sufficient strength to set loose sand into a cohesive block with high strength properties. This process was envisaged to be useful in consolidation treatments of porous materials such as sand (Fig 1.3).

In order to achieve an increased degree of cementation, one clearly requires a greater amount of calcite to form, compared to the amount produced in the previous microbio-geological studies. Thus, a microbial urease is required that has a high tolerance to urea, calcium, ammonium and either nitrate or chloride (depending on the calcium salt used).

1.8 Suitable Sources of Urease

The commercial demand for urease is not high and currently, urease is only available in industrial quantities from Roche for use in the diagnostic and high technology specialist ceramics fields (Gauckler Th. and Baader, 1999; Roche, 2001). It is thus expensive and is of a higher purity than is required for

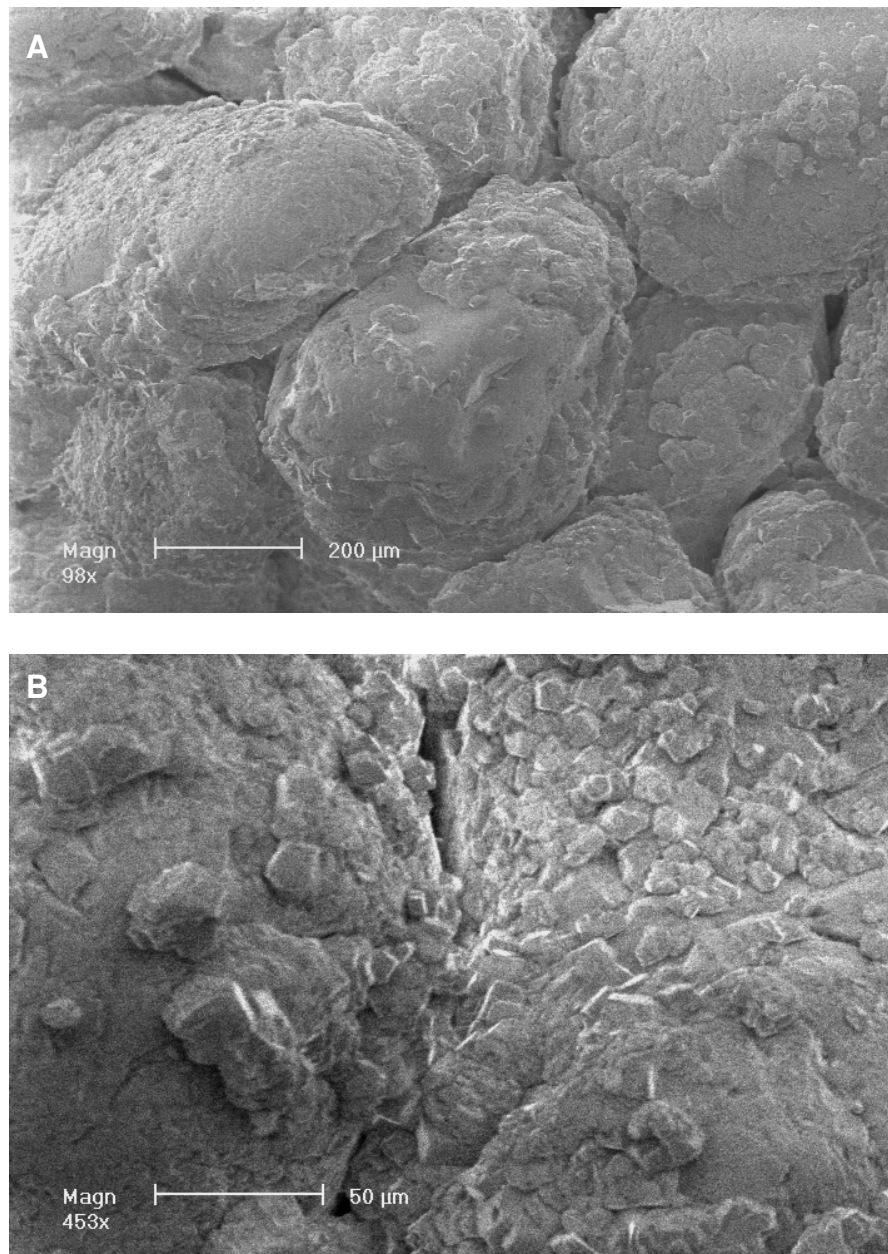


Figure 1.3: Scanning electron micrographs of <600 µm silica sand in the early stages of biocementation. A: Large pore spaces are still present after treatment, indicating that the porosity of the material is largely intact (98 x) B: Zoom in on contact between two grains and the bridging calcite that cements the grains together. Note the characteristic rhombohedral calcite crystal formation and increased amount of calcite precipitated where grains contact (453 x) (Source: the present study).

biocementation. The ability to produce urease is widespread amongst microbial populations and the enzyme has been well studied from a clinical perspective as it can indicate increased virulence properties in pathogenic bacteria (Collins and D'Orazio, 1993; Lee and Calhoun, 1997; Mobley *et al.*, 1995; Provorov and

Vorobyov, 2000) and as a general nitrogen volatilisation phenomenon in agricultural soils (Nielsen *et al.*, 1998; Pettit *et al.*, 1976; Sadeghi *et al.*, 1988; Sloan and Anderson, 1995; Zantua and Bremner, 1977). The urease capability of organisms outside of these two areas is not well investigated. In general, four modes of regulation exist for the synthesis of urease in microbial systems (Mobley and Hausinger, 1989; Mobley *et al.*, 1995);

- (i) *Constitutive* – where a constant enzyme activity is expressed per cell, independent of external conditions.
- (ii) *Inducible* – where a background level of enzyme activity is expressed per cell which can be induced by the presence of an inducer molecule (e.g. urea) or other environmental condition.
- (iii) *Repressible* – by the presence of ammonia or ammonia precursors including urea. This synthesis is de-repressed (i.e. enzyme activity increases) under nitrogen limiting conditions.
- (iv) *Developmental* – where an organism in different developmental stages (e.g. swarming versus non-swarming) has variable expression of urease (Falkinham III and Hoffman, 1984).

An ideal microbial source of urease for biocementation must be tolerant to high concentrations of urea and calcium. The organism should also have a high level of urease activity that is either constitutively produced (i.e. a constant amount of enzyme is expressed per cell) or can be reliably induced.

Urease-producing bacteria can be divided into two distinct groups according to their urease response to ammonium; those whose urease activity is not repressed (listed in Table 1.2) and those whose urease activity is repressed (e.g. *Pseudomonas aeruginosa*, *Alcaligenes eutrophus*, *Bacillus megaterium* (Kaltwasser *et al.*, 1972b) and *Klebsiella aerogenes* (Friedrich and Magasanik, 1977)). In *K. aerogenes*, the presence of ammonium inside the cell induces the production of glutamine, which prevents further hydrolysis of urea (Mulrooney *et al.*, 2001). Because high concentrations of urea are hydrolysed during biocementation, only those microorganisms whose urease activity is not repressed by ammonium are useful.

Table 1.2: Microorganisms with urease activity that is not repressed in the presence of ammonium.

Microorganism	High activity	Not repressed by NH_4^+	Not Pathogenic or GM
<i>Sporosarcina pasteurii</i>	✓	✓	✓
<i>Proteus vulgaris</i>	Unknown	✓	Moderately
<i>Proteus mirabilis</i>	Unknown	✓	x
<i>Helicobacter pylori</i>	✓	✓	x
<i>Ureplasmas (Mocillicutes)</i>	✓	✓	x

As well as meeting the needs for biocementation, the organism must also meet the needs for safe environmental application. In order to safely release an organism into the environment, it must be non-pathogenic, non-genetically modified, and not contain any transferable elements that may increase the pathogenicity of environmental strains (e.g. antibiotic resistance). Considering both biocementation and environmental constraints, two organisms have potential as sources of urease for biocementation; *Sporosarcina pasteurii* and *Proteus vulgaris* (Table 1.2).

The moderately alkaliphilic organism *S. pasteurii* (formerly known as *Bacillus pasteurii* (Yoon *et al.*, 2001)) is a commonly found in the soil, sewage and urinal incrustations (Sneath, 1986). *S. pasteurii* has a unique mechanism for the formation of ATP which involves the coupling of ATP generation with urea hydrolysis. The generation of ATP is controlled by proton motive force (Δp) which is the sum of the transmembrane pH gradient (ΔpH) and the charge gradient or membrane potential ($\Delta \psi$):

$$\Delta p = \Delta \text{pH} + \Delta \psi$$

Neutrophilic organisms generate ATP from the chemiosmotic proton gradient generated by pumping protons out of the cell from the electron transport chain. This proton concentration gradient (high outside/low inside) drives protons back into the cell through the ATP-synthase, resulting in ATP generation (Prescott *et al.*, 1993). The environment for alkaliphilic growth is high pH (low proton concentration) outside and lower pH (high proton concentration) inside the cell,

resulting in a reversed ΔpH with the tendency for protons to move from inside to outside the cell – opposite of the normal direction for ATP generation. To combat this alkaliphiles have developed two alternative means to increase proton motive force and drive protons into the cell for ATP generation:

1. Alkalinisation of the cytoplasm, resulting in a reduced ΔpH – this is only tolerated within a small pH range.
2. Efflux of a cation other than H^+ , resulting in an increase in the $\Delta\psi$

If the charge separation across the membrane ($\Delta\psi$) is large enough, proton motive force becomes sufficient to drive the influx of protons into the cell, against the concentration gradient. In the case of *S. pasteurii*, the type of ions that are effluxed to increase $\Delta\psi$, depends on the growth history of the culture. For cells grown at low urea concentrations (15 mM), the ions that can be transported out of the cell to increase $\Delta\psi$, have been shown to be K^+ , Na^+ or NH_4^+ . Cells grown on high urea concentrations (300 mM) however, cannot use K^+ or Na^+ but only NH_4^+ to drive ATP generation (Jahns, 1996) (Fig 1.4). Because this organism has a high level of urease activity, urea is only present at the beginning of batch cultivation and will be fully hydrolysed to NH_4^+ and CO_3^{2-} within the first few hours. After urea is depleted from the medium, NH_3 is influxed into the cell via passive diffusion through the membrane.

Interestingly, the pH optimum for *S. pasteurii* growth (9.25) is also the half dissociation constant (pK_a) of the $\text{NH}_3/\text{NH}_4^+$ equilibrium where NH_3 and NH_4^+ exist in equal proportions (Fig 1.5). It is fair to assume that the growth optimum for the organism is also the ATP-generating optimum, suggesting that NH_4^+ and NH_3 are required in equal proportions outside of the cell for maximum ATP generation, but as yet this is not clearly understood in the proposed mechanism (Fig 1.4).

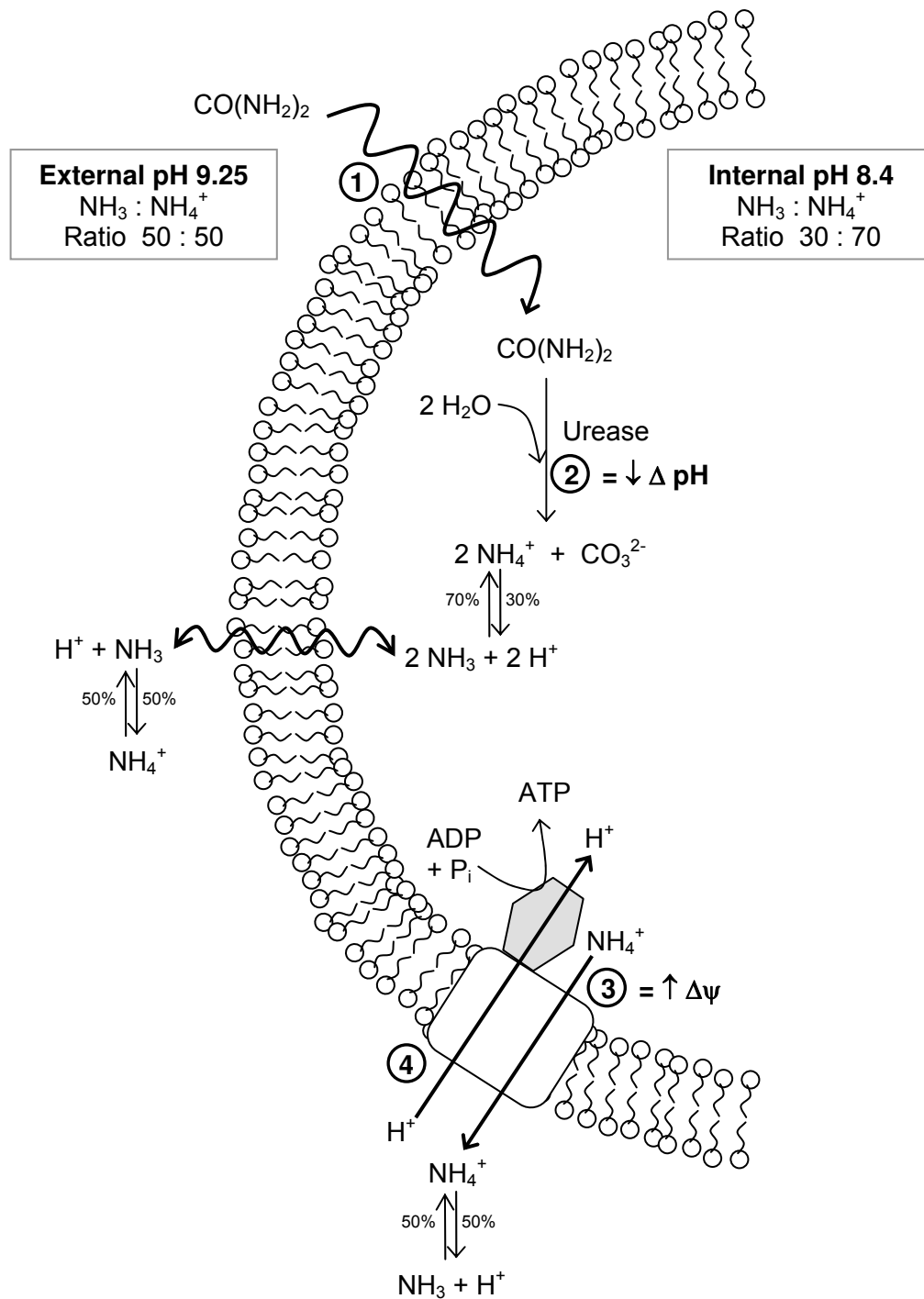


Figure 1.4: Coupling of urea hydrolysis and ATP generation in *S. pasteurii* as suggested by Jahns (1999). Reaction ①: urea diffuses into the cell according to the concentration gradient. ②: Urea is hydrolysed by urease which results in alkalinisation of the cytoplasm to pH 8.4 and a decrease in ΔpH (difference in pH between inside and outside of the cell). ③: Ammonium ions are removed from the cell according to the ammonium concentration gradient, which results in an increase in $\Delta \psi$ (membrane potential – difference in charge between inside and outside of the cell). ④: The increased membrane potential is reversed by driving in protons against the concentration gradient into the cell, which results in the generation of ATP.

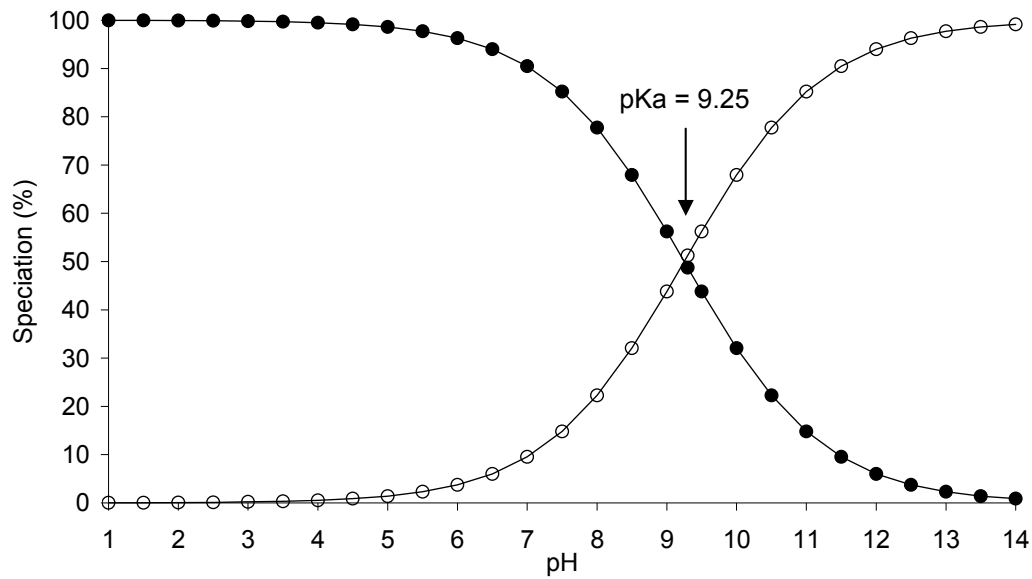


Figure 1.5: Chemical speciation of Ammonia (NH₃) (○) and ammonium (NH₄⁺) (●) according to pH. Note the pK_a of ammonia (pH where the two species exist in a 50:50 ratio) is 9.25, which is the growth optimum of *S. pasteurii*.

P. vulgaris is a soil-inhabiting organism which is particularly common in decomposing matter. The main role of urease for this organism is to provide a source of ammonium which can then be directly assimilated into biomass via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway or by the action of glutamate dehydrogenase (GDH) (Tyler, 1978).

1.9 Derivation of Thesis Objectives

The feasibility of microbial calcite precipitation is well established in the literature. Most of the previously published work has focussed on microbial calcite precipitation for purposes other than strength development (Bachmeir *et al.*, 2002; Bang *et al.*, 2001; Castanier *et al.*, 1995; Fujita *et al.*, 2000; Gollapudi *et al.*, 1995; Hammes *et al.*, 2003a; Hammes *et al.*, 2003b; Hammes *et al.*, 2003c; Le Metayer-Levrel *et al.*, 1999; Stocks-Fischer *et al.*, 1999) and those that have considered strength have only achieved minor improvements that are useful only as consolidation or patching treatments for existing material (Castanier *et al.*, 2000a; Ramachandran *et al.*, 2001; Rodriguez-Navarro *et al.*, 2003; Tiano, 1995; Tiano *et al.*, 1999). No published work to date has described a sufficient degree of cementation to allow loose material to form rock, by microbial calcite

precipitation via urea hydrolysis. The CIPS process however, has shown that high degrees of calcite cementation – equal to that of natural sediments, are achievable within less than 24 hours (Ismail *et al.*, 2002). This indicates a novel area for application of microbial calcite precipitation.

It is clear that in order to achieve higher degrees of cementation, higher amounts of cement and thus, significantly higher concentrations of cementation reactants will be required. Equally, an enzyme with tolerance to high concentrations of urea, calcium, ammonium, nitrate and/or chloride will be necessary for successful cementation. The objectives of this thesis seek to provide “proof of concept” for generating high strength, economical cementation using microbial carbonate precipitation via urea hydrolysis.

1.10 Thesis Objectives

The specific objectives of this thesis are:

1. To determine if microbial carbonate precipitation can consolidate loose material using high concentrations of cementation reactants.
2. To identify a suitable source of bacterial urease and various environmental and physiological conditions for maximum urease activity.
3. To investigate the economic aspects of production by developing an economical growth procedure that is suitable for industrial-scale production.
4. To determine the most cost efficient biocementation procedure for obtaining strength.
5. To test the developed technology with a pilot-scale trial in order to establish “proof-of-concept” that high strength, economical cementation can be achieved using microbial carbonate precipitation via urea hydrolysis.

Investigation of suitable microbial sources of Urease

2.1 Introduction

Urea is the chief nitrogenous waste produced by vertebrates and is a major nitrogen resource in aquatic and soil ecosystems. In response to the widespread availability of urea in the environment and the universal requirement for nitrogen, a diverse section of the biota has evolved with the ability to hydrolyse urea, through the action of urease. Urease occurs in many bacteria, several species of yeast and a number of higher plants including jack beans (*Canvalia ensiformis*) (Dixon *et al.*, 1980), soybean leaf and seed (*Glycine max*) (Kerr *et al.*, 1983), pigweed (*Chenopodium album*) (El-Shora, 2001) and mulberry leaf (*Morus alba*) (Hirayama *et al.*, 2000).

Most organisms with ureolytic ability use urea as a source of nitrogen by actively transporting or passively diffusing urea into the cell cytoplasm, where urease hydrolyses urea releasing two ammonium molecules, which can then be directly assimilated into biomass via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway or by the action of glutamate dehydrogenase (GDH) (Tyler, 1978). To ensure this process is energy efficient, the production of urease in organisms such as *Pseudomonas aeruginosa*, *Alcaligenes eutrophus*, *Bacillus megaterium* (Kaltwasser *et al.*, 1972b) and *Klebsiella aerogenes* (Friedrich and Magasanik, 1977), is repressed by the presence of ammonium. There are however some exceptions to this regulation, such as *Proteus vulgaris*, which can produce urease even in the presence of high concentrations of ammonium (Mörsdorf and Kaltwasser, 1989).

Some specialist organisms exist that have additional uses for urease, beyond nitrogen assimilation. *Helicobacter pylori*, an inhabitant of the low pH gastric juices in the stomach, not only has intracellular but also extracellular urease that is located on the cell surface. The extracellular urease plays a protective role from the low pH environment of the stomach, by providing a microenvironment of

more neutral pH, generated from production of ammonium near the cell surface (Dunn and Grütter, 2001; Ha *et al.*, 2001; Marshall *et al.*, 1990). *Sporosarcina pasteurii* is another specialist organism that has a different use for urease, other than nitrogen assimilation. *S. pasteurii* is a moderately alkaliphilic organism with a growth optimum at pH 9.25. Alkaliphiles present a special problem for the generation of ATP due to a reversed chemiosmotic proton gradient. In neutrophilic organisms, ATP is produced from the proton motive force that is generated by pumping protons out of the cell from the electron transport chain. This generates a proton concentration gradient (high outside/low inside) and causes protons to be driven back into the cell through the ATP-synthase, resulting in ATP generation (Prescott *et al.*, 1993). When the external environment is highly alkaline, the proton concentration gradient is reversed and the gradient favours protons to be fluxed out of the cell, but not back into it. Alkaliphilic organisms must create a high membrane potential (charge difference across the membrane) by pumping out cations, to drive protons back into the cell against the concentration gradient (Ivey *et al.*, 1998) (Fig 1.4). For *S. pasteurii*, the effluxed cation used to create a high membrane potential to drive ATP synthesis is ammonium, which can be supplied directly as ammonium or indirectly as urea (Jahns, 1996).

As the biocementation reaction results in the generation of high concentrations of ammonium, only those bacterial sources where urease is not down regulated by the presence of ammonium are useful. These organisms include *Sporosarcina pasteurii* and *Proteus vulgaris*. For biocementation purposes, an ideal microbial source of urease has the following properties:

- High urease production capacity
- Ability to produce urease in the presence of ammonium
- High stability (robust)
- Consistent production (reliable)
- Does not require further down-stream processing prior to use in biocementation

The objective of this chapter is to compare and contrast the urease production capacity of two organisms, *Proteus vulgaris* and *Sporosarcina pasteurii*, for potential sources of urease to be used in the biocementation process.

2.2 Materials & Methods

2.2.1 Cell Storage

Both microorganisms were grown aseptically under batch cultivation conditions and stored as a working seed bank at -80°C in 15% (v/v) glycerol stabilised 1.8 ml cryogenic vials. A new vial was used as inocula for each experiment.

2.2.2 *Proteus vulgaris*

2.2.2.1 Batch cultivation

P. vulgaris (Murdoch University Culture Collection) was cultivated under batch conditions on 20 g.L^{-1} yeast extract, at 37°C .

2.2.2.2 Chemostat cultivation

Wastewater treatment sludge was de-protozoated by three consecutive freeze-thaw lysis treatments ($-20^{\circ}\text{C} \rightarrow +20^{\circ}\text{C}$) (Jouany and Senaud, 1979) (removal of protozoa was confirmed by microscopy after treatments) and was allowed to establish steady-state growth in a chemostat reactor over 14 days. The chemostat was fed with the following medium (g.L^{-1}): peptone (1.5), meat extract (1.5), NH_4Cl (0.43), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.3), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2), KH_2PO_4 (0.05), K_2HPO_4 (0.05), and the following trace elements (mg.L^{-1}): $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.54), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.30), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.24), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.31), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.38), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.24), $\text{Na}_2\text{SeO}_4 \cdot 10\text{H}_2\text{O}$ (0.25), H_3BO_4 (0.02), and $\text{NaWO}_4 \cdot 2\text{H}_2\text{O}$ (0.06) (Strous, 2000). The chemostat dilution rate was 0.04 h^{-1} , which allowed a 24 h residence time in the reactor. After the sludge culture had reached steady state after 14 days, a 50% (w/w) inoculum of *P. vulgaris* was introduced into the reactor and the urease activity in the chemostat was monitored for 48 hours.

2.2.3 *Sporosarcina pasteurii*

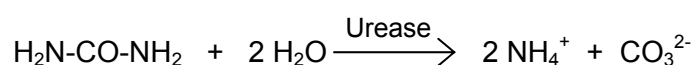
2.2.3.1 Batch Cultivation

S. pasteurii (ATCC 11859) was cultivated under batch conditions on 20 g.L⁻¹ yeast extract and 75 mM (NH₄)₂SO₄, pH adjusted before sterilisation to 9 with 4 M NaOH, at 28°C.

2.2.4 Analytical Methods

2.2.4.1 Urease Activity

The hydrolysis of urea liberates ionic products from non-ionic substrates according to the following equation:



The production of ionic species from non-ionic substrates generates an increase in overall conductivity of the solution, and the rate at which conductivity increases is proportional to the concentration of active urease present (Appendix A) (Grunwald, 1984; Hanss and Rey, 1971; Lawrence and Moores, 1972). Conductivity was recorded over three minutes under standardised conditions of 1.5 M urea at 25°C.

The rate of conductivity increase (mS.min⁻¹) was converted to urea hydrolysis rate (mM urea hydrolysed.min⁻¹) by relating conductivity measurements of test samples against standards containing purified urease from the same organism (Sigma Cat. No. U-7127), under standardised conditions of 1.5 M urea and 25°C (Appendix B). The effect of pH on *S. pasteurii* urease was accounted for by standardisation of all activities to pH 7 (Appendix C).

Specific activity was defined as the amount of urease activity per unit biomass and was calculated according to the following equation:

$$\text{Specific Urease Activity} = \frac{\text{Urease Activity (mM urea hydrolysed.min}^{-1}\text{)}}{\text{Biomass (OD}_{600}\text{)}} \quad \text{(mM urea hydrolysed.min}^{-1}\text{.OD}^{-1}\text{)}$$

2.2.4.2 Biomass Determination

Biomass was determined spectrophotometrically at 600 nm.

2.2.4.3 NH₄-N Analysis

Ammonium concentration was determined spectrophotometrically by a modified Nessler Method (Greenburg *et al.*, 1992). Samples were immediately centrifuged to remove cells and the resulting supernatant was transferred into a clean tube and frozen prior to analysis. The sample was thawed before dilution to be in the range of 0 - 0.5 mM. 2 ml of the diluted sample was mixed with 100 μ l of Nessler's reagent and allowed to react for exactly 1 minute before reading the absorbance at 425 nm. Absorbance values were compared to those from ammonium chloride standards measured under the same method (Appendix D).

2.3 Results

2.3.1 Investigation of *P. vulgaris* as a potential source of urease for biocementation

2.3.1.1 Urease activity and Biomass production

To determine the level of urease activity and biomass produced by *P. vulgaris*, a batch cultivation was conducted at pH 7. Urease activity was produced proportionally to biomass, suggesting constitutive expression of the enzyme. After 24 hours of incubation, the level of urease activity was 1 mM urea hydrolysed.min⁻¹ with a specific activity of 0.2 mM urea.min⁻¹.OD⁻¹ (Fig 2.1).

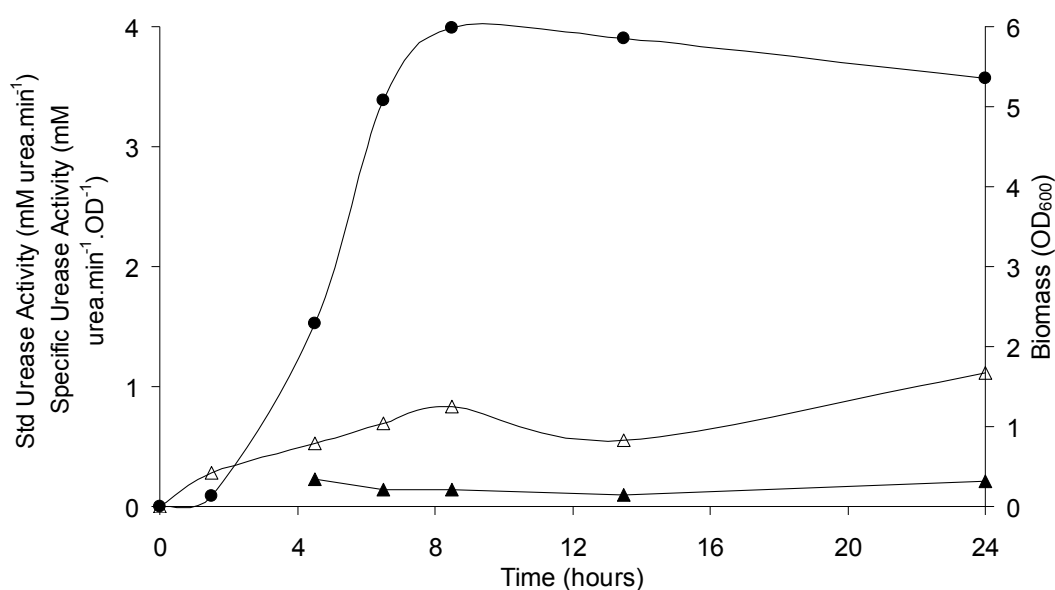


Figure 2.1: Urease activity (△), specific urease activity (▲) and biomass production (●) of *P. vulgaris* during cultivation. Cultivation was conducted at 30°C

2.3.1.2 Effect of pre-exposure to urea

The urease activity of other *Proteus* species have been shown to be activated in the presence of urea (D'Orazio *et al.*, 1996). To determine if the presence of urea could induce a higher level of urease in *P. vulgaris*, cells were exposed to different concentrations of urea for one hour prior to determining urease activity under standard conditions. Before determining urease activity, the cells were washed and resuspended in saline to remove any residual urea. A slight increase in activity was observed in all urea treated *P. vulgaris* cultures, compared to no exposure to urea, suggesting that *P. vulgaris* urease was not significantly induced in the presence of urea. A concentration-dependent effect was not evident (Fig 2.2).

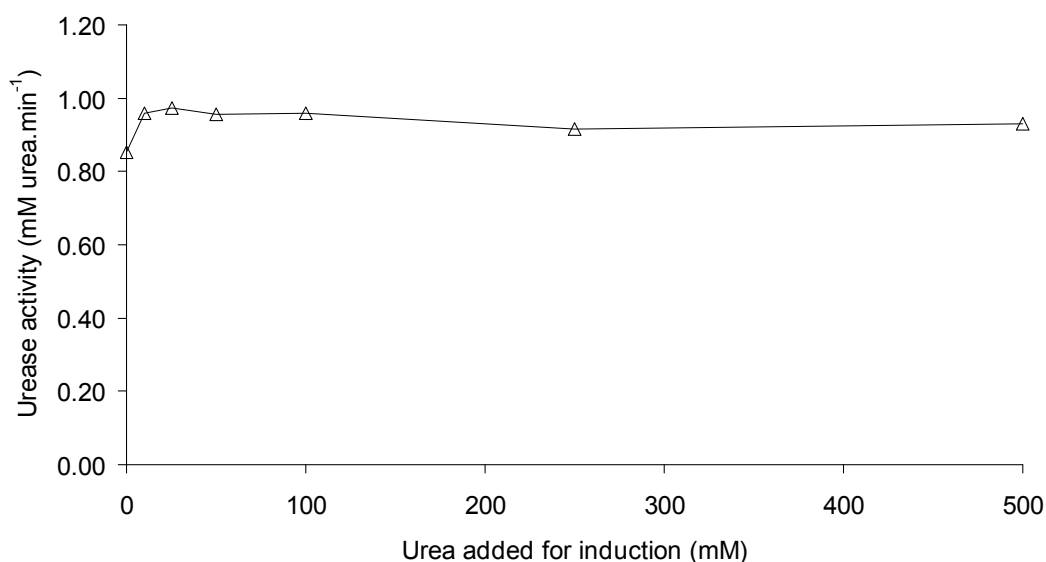


Figure 2.2: *P. vulgaris* urease activity (Δ) one hour after treatment with different concentrations of urea.

2.3.1.3 Effect of pH on urease activity

To establish the effect of pH on urease activity, *P. vulgaris* cells were washed and resuspended in various pH media and the urease activity was determined. Urease activity in *P. vulgaris* was unaffected by pH between the range of 7.25 - 8.5 (Fig 2.3).

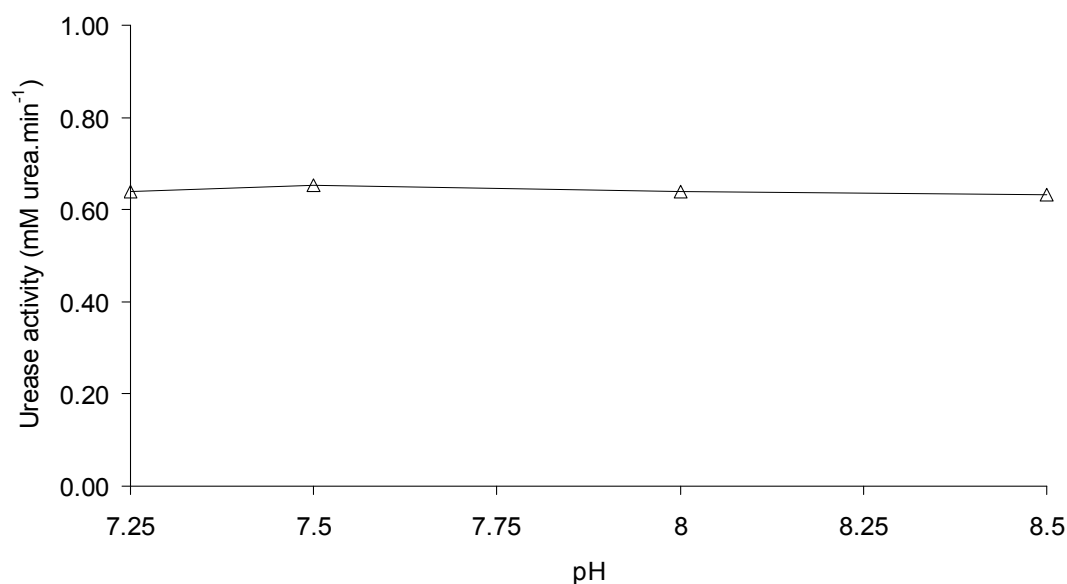


Figure 2.3: Effect of pH on urease activity of *P. vulgaris* at 25°C.

2.3.1.4 Enzyme in a non-sterile environment

The stability of the enzyme in a non-sterile environment is important for biocementation as the enzyme is required to work for up to 24 hours in-situ. To investigate the stability of *P. vulgaris* urease, a 50% inoculum was introduced into a pre-established wastewater treatment sludge chemostat (see methods 2.2.2.2) and the level of urease activity in the chemostat was monitored for 48 hours. The level of urease in the chemostat followed the wash out kinetics indicating that *P. vulgaris* did not establish in the chemostat. There was no evidence of enzyme degradation over 48 hours, indicated by the level of urease activity remaining above or equal to the predicted wash out level (Fig 2.4).

2.3.1.5 Meeting urease activity concentration needed for biocementation

The urease activity produced by *P. vulgaris* was approximately 10 times less than the required level of activity for biocementation, of 10 mM urea.min⁻¹. The usefulness of *P. vulgaris* as a source of urease for biocementation therefore depended on an economic process for concentration of the enzyme. Two possible routes for concentration existed:

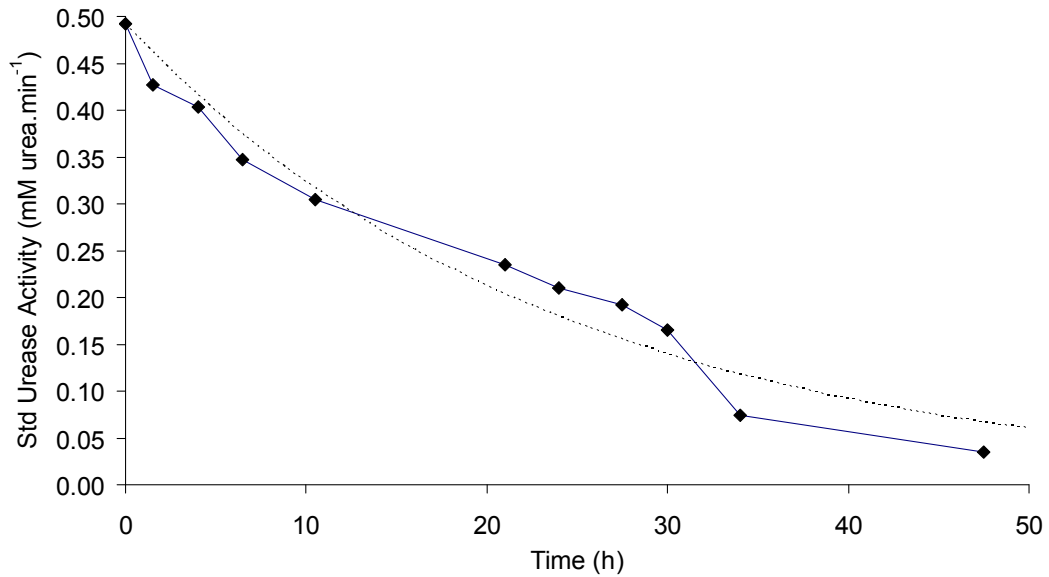


Figure 2.4: Survival of *P. vulgaris* urease activity in pre-established wastewater treatment sludge chemostat environment. Dashed line indicates wash out kinetics of an inert tracer.

- (1) Growing the cells to a higher cell density.
- (2) Concentration of the cells into a smaller volume after they have been grown.

To maintain an economical process, it was envisaged that cell cultivation for biocementation would have to be conducted under industrial conditions on the proposed biocementation site, without sterility. Under these conditions it was considered difficult to produce a high cell density culture that was 10-times more concentrated than a usual batch ($OD_{600} 5 \rightarrow 50$), thus concentrating the cells post-growth was the preferred route. Flocculants are routinely used in the wastewater treatment and processing industries for concentration of large volumes of cells. Several commercially available flocculants were tested for their effectiveness to flocculate cells from the suspension and move them into a concentrated fraction at the bottom of the vessel (Fig 2.5). The most effective flocculant at removing cells from the supernatant was Superfloc C-494 (Cyanamid) with 90% of the cells flocculated by addition of 90 mg.L^{-1} .

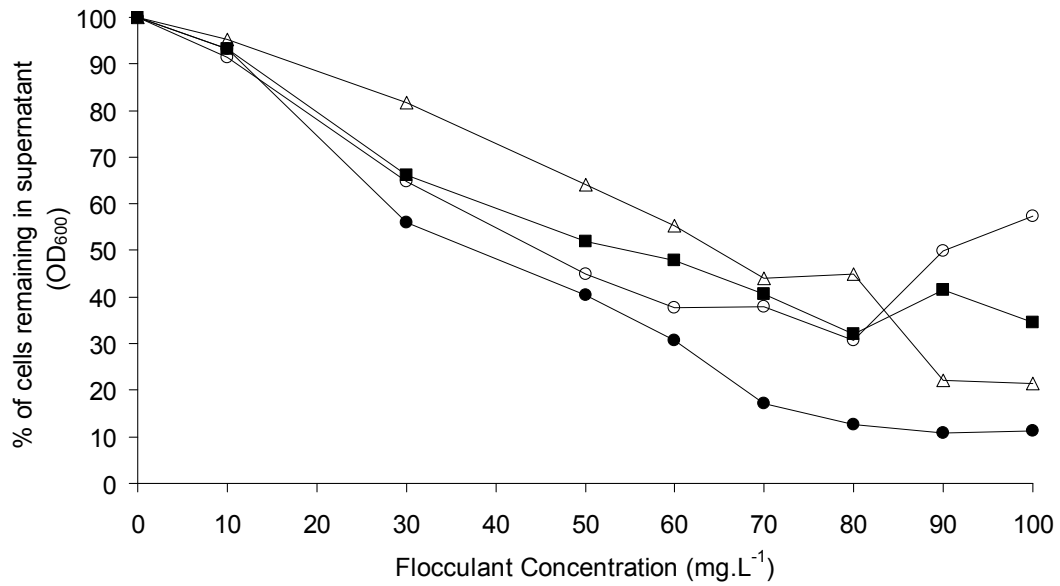


Figure 2.5: Effectiveness of several commercial flocculants for concentrating *P. vulgaris* cells. The tested flocculants were Superfloc C-496 (■) and Superfloc C-494 (●) (Cytec) and, Zetag 87 (△) and Zetag 92 (○) (Ciba Speciality Chemicals).

The level of concentrated urease activity in the pellet after flocculation was 9.6 mM urea.min⁻¹ and was sufficient for cementation however the distribution of urease activity after flocculation indicated that more than 50% of the activity was unaccounted for. This suggested that Superfloc C-494 had a negative effect on urease activity, possibly by inhibition, deactivation or masking the enzyme activity inside the flocculated pellet (Fig 2.6).

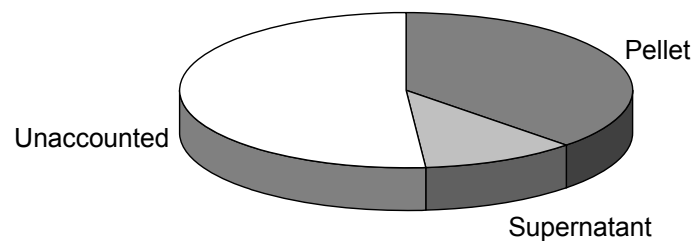


Figure 2.6: Distribution of urease activity after flocculation with 90 mg.L⁻¹ Superfloc C-494 (Cyanamid)

2.3.2 Investigation of *Sporosarcina pasteurii* as a potential source of urease for biocementation

2.3.2.1 Urease activity and Biomass production

The necessity of additional processing to produce a suitably high urease activity from *P. vulgaris* and the unexpected loss of activity after flocculation indicated that this organism had some limitations as a source of urease for the biocementation process. The potential for *S. pasteurii* to provide urease for biocementation was then investigated.

To determine the level of urease activity and biomass produced by *S. pasteurii*, a culture was grown at a starting pH of 7. After 24 hours of incubation, the level of urease activity was 13.7 mM urea hydrolysed.min⁻¹, with a variable specific activity between 3 – 9.7 mM urea.min⁻¹.OD⁻¹ (Fig 2.7). This level of urease activity was more than ten times higher than that produced by *P. vulgaris* under the same conditions (Fig 2.1).

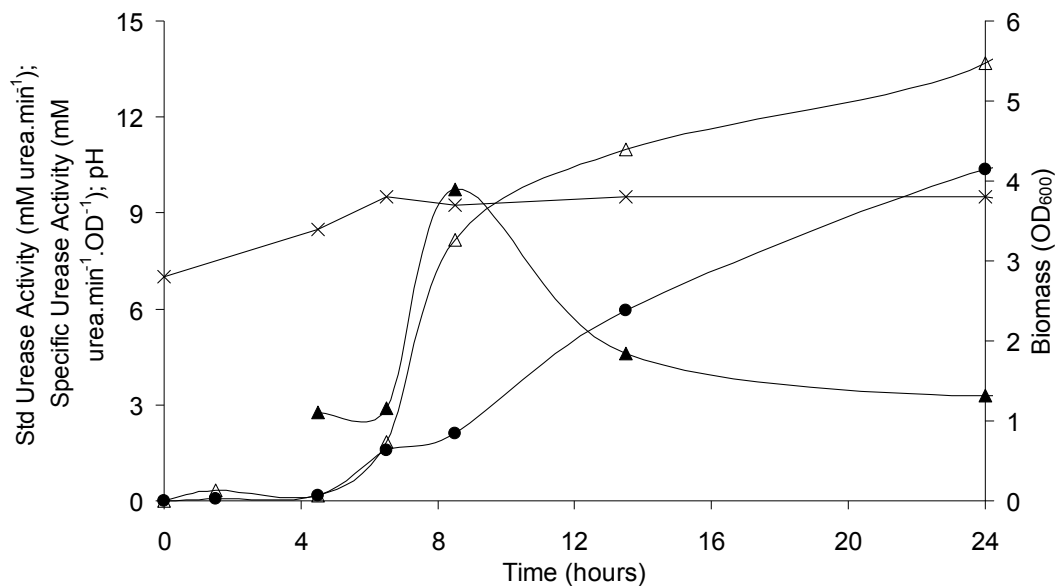


Figure 2.7: Urease activity (\triangle), specific urease activity (\blacktriangle), biomass production (\bullet) and pH (\times) during cultivation of *S. pasteurii* at 30°C with 20 g.L⁻¹ yeast extract and 10 g.L⁻¹ urea.

The observed lag phase in both growth and urease activity, corresponded to a pH shift due to the hydrolysis of urea (Fig 2.7). Interestingly, the amount of enzyme

present per cell (specific activity) was two-fold higher between 6.5 - 8.5 h than during the rest of the cultivation (Fig 2.7), which suggests that the enzyme was not constitutively regulated, but possibly inducible by some factor of the early growth environment. This phenomenon was observed in several batch cultivations of *S. pasteurii* (Fig 3.4, 3.5A, 3.6).

2.3.2.2 Enzyme variability

S. pasteurii has been described with the ability to constitutively express high levels of urease (Mörsdorf and Kaltwasser, 1989), however the variable specific urease activity indicated previously did not agree with this finding. To determine if a correlation existed between urease activity and biomass produced, urease activity was plotted as a function of biomass concentration for several cultivations of *S. pasteurii* (Fig 2.8). Few cultures produced levels of urease activity that were proportional to biomass. Within the same concentration of biomass, urease activity varied by more than a factor of ten, reaching as high as 29 mM urea.min⁻¹.OD⁻¹. This suggested that the cultivation conditions conducive to growth were not necessarily conducive to high urease production, and that urease activity in *S. pasteurii* was not constitutively regulated.

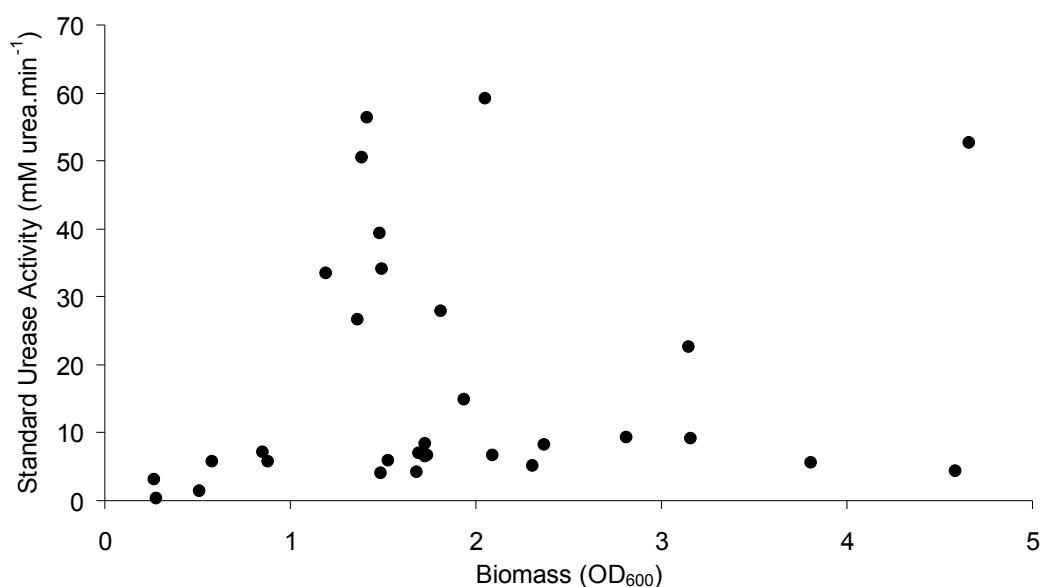


Figure 2.8: Urease activity versus biomass concentration for several cultivations of *S. pasteurii*, indicating enzyme variability.

2.3.2.3 Effect of pH on urease activity

One of the roles of urease plays for *S. pasteurii* is to increase the external pH to 9.25 thus creating an environment conducive to growth (Wiley and Stokes, 1962; Wiley and Stokes, 1963). To determine if pH conditions below the growth optimum could induce an increase in specific urease activity in *S. pasteurii*, cells were grown, removed from their medium and resuspended into new media at various pH conditions. Urease activity was determined immediately after inoculation into the new medium and again after 5 hours of incubation. Despite the same concentration of cells being added to the different pH media, a difference in urease activity was evident immediately after inoculation (Fig 2.9). There was insufficient time for the organism to have generated additional enzyme, and the variation in enzyme activity appeared to be a biochemical effect. To quantify this effect urease activity was determined in a wider range of pH conditions (Fig 2.10). pH had a significant biochemical effect on urease activity, which exhibited an optimum activity between pH 7 and 8 (Fig 2.9). This relationship was used to standardise all *S. pasteurii* urease activities to pH 7 (Appendix C), so that only real changes in urease activity could be interpreted and not changes in biochemical activity due to the enzyme being in different pH conditions.

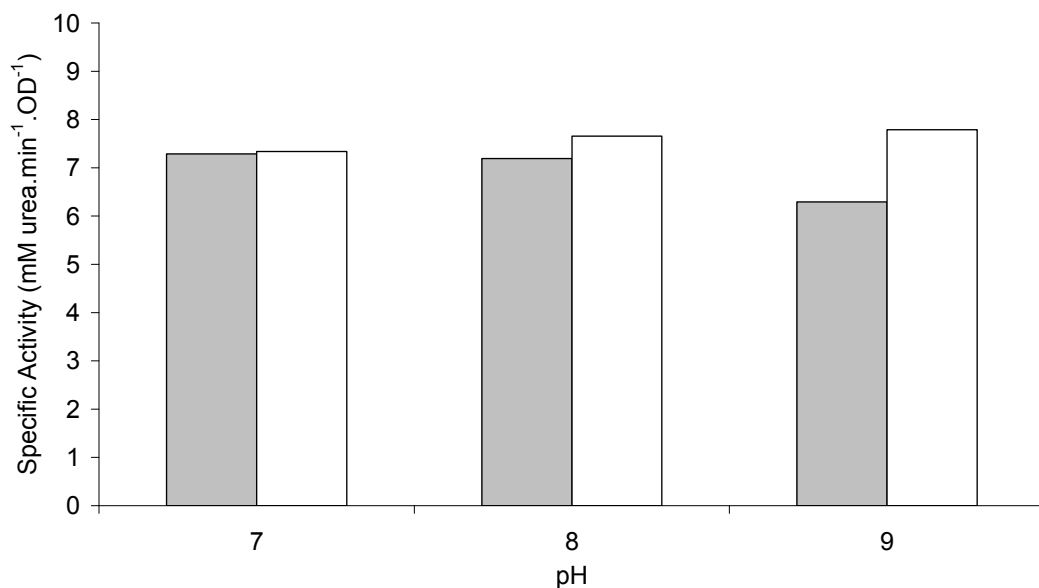


Figure 2.9: Initial specific urease activities immediately after inoculation into various pH media. Urease activity measured at each pH (■) compared with urease activity corrected for the biochemical pH effect (□), according to Appendix C.

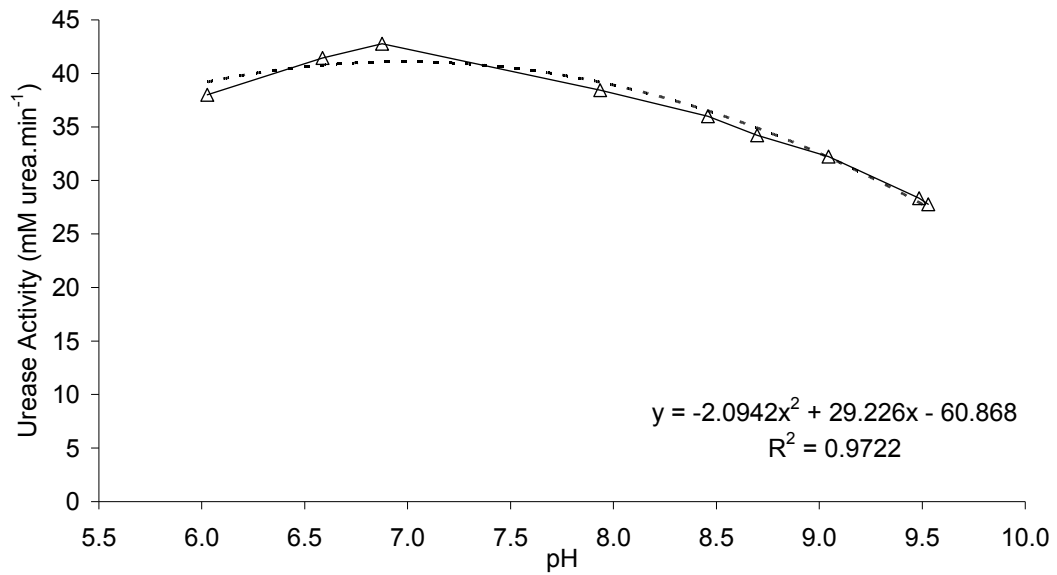


Figure 2.10: Effect of pH on urease activity of *S. pasteurii* at 25°C.

After five hours of incubation in different pH media, urease activity was measured and activities were standardised to pH 7 to allow comparison between the cultures (Appendix C) (Fig 2.11). High specific urease activities (> 5 mM urea.min⁻¹.OD⁻¹) were observed in all conditions. Only slightly higher specific urease activities were observed at pH 7 and 8 versus pH 9, suggesting that sub-optimum pH conditions did not substantially increase the amount of urease produced per cell.

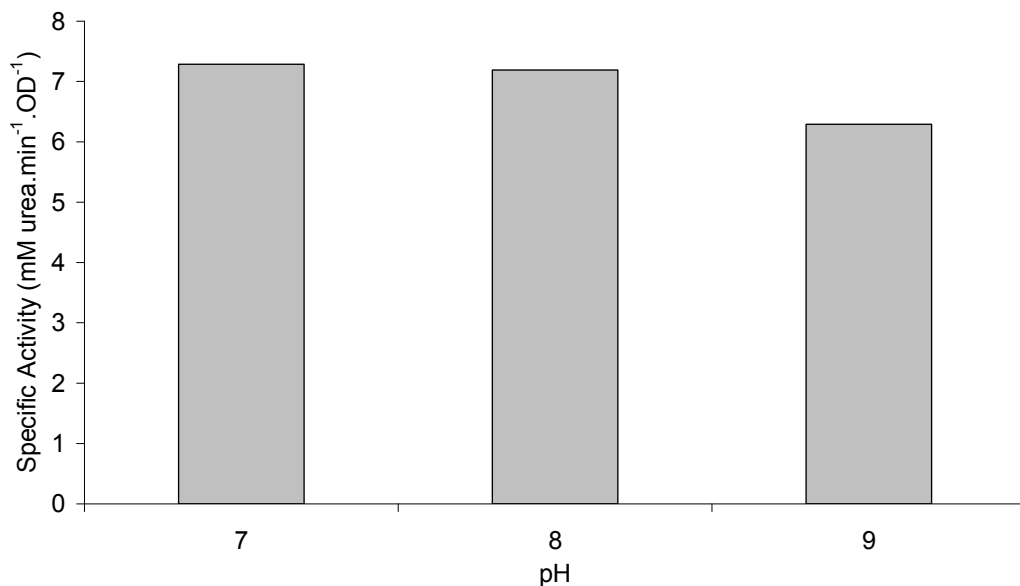


Figure 2.11: Effect of different pH conditions on standardised specific urease activity, after 5 hours of incubation.

2.3.2.4 Enzyme stability in a non-sterile environment

The cost of growth media sterilisation is in the order of \$0.46 – 0.66 per L (Acsion-Industries, 2002), which represents 2 – 3 times the cost of the medium ingredients (Table 4.3). Thus ability to cultivate the organism with a high level of urease activity, under non-sterile conditions was highly desirable to maintain low costs and flexibility of the process for on-site cultivation. To compare the level of urease produced in a non-sterile environment versus a sterile environment, several batch cultures were conducted with between 0 – 90% (w/w) contamination with wastewater treatment sludge microorganisms. The same level of *S. pasteurii* inoculum was added to each culture, with a variable percentage of contamination relative to the *S. pasteurii* inoculum. After 48 hours incubation, the level of urease activity in cultures with up to 50% contamination were similar or higher than the control culture (0% contamination), indicating that a high level of urease activity could still be obtained, even at a relatively high level of contamination (Fig 2.12). At contamination levels higher than 50%, the level of urease activity was adversely affected.

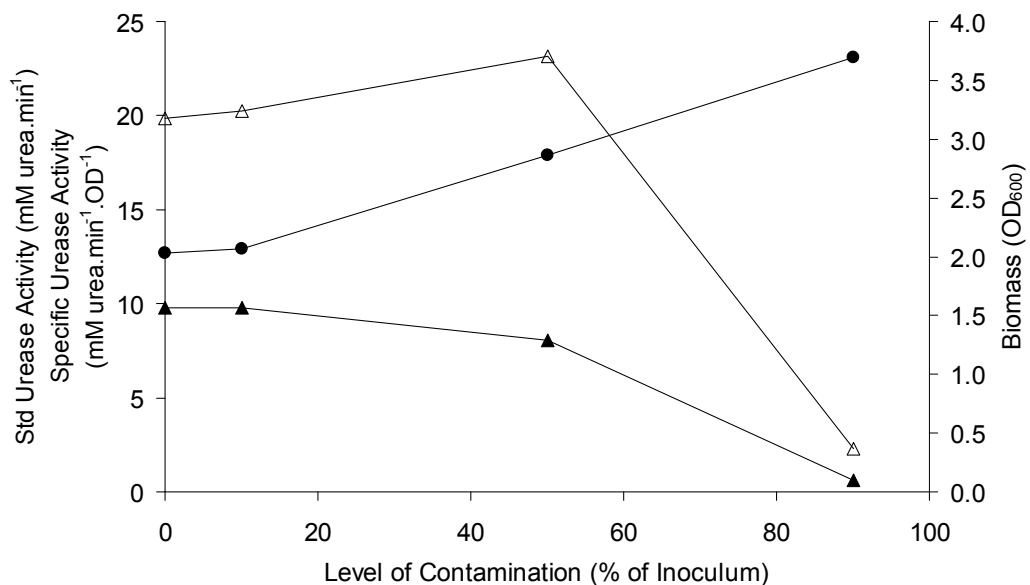


Figure 2.12: Urease activity (△), specific urease activity (▲) and biomass production (●) of *S. pasteurii* at different levels of contamination with wastewater treatment sludge microorganisms, after 48 hours incubation.

In some cases it may be desirable to store the enzyme or produce it off-site, which would require some level of stability of the enzyme, during transport or storage. To investigate the storage life-time for *S. pasteurii* urease, *S. pasteurii* cells were harvested from cultivation and stored in a non-sterile closed container at 4°C and enzyme activity was monitored over 25 days. The urease activity remained relatively stable over the monitored time, indicating that it was possible to store it in its unprocessed form for up to 25 days at 4°C (Fig 2.13).

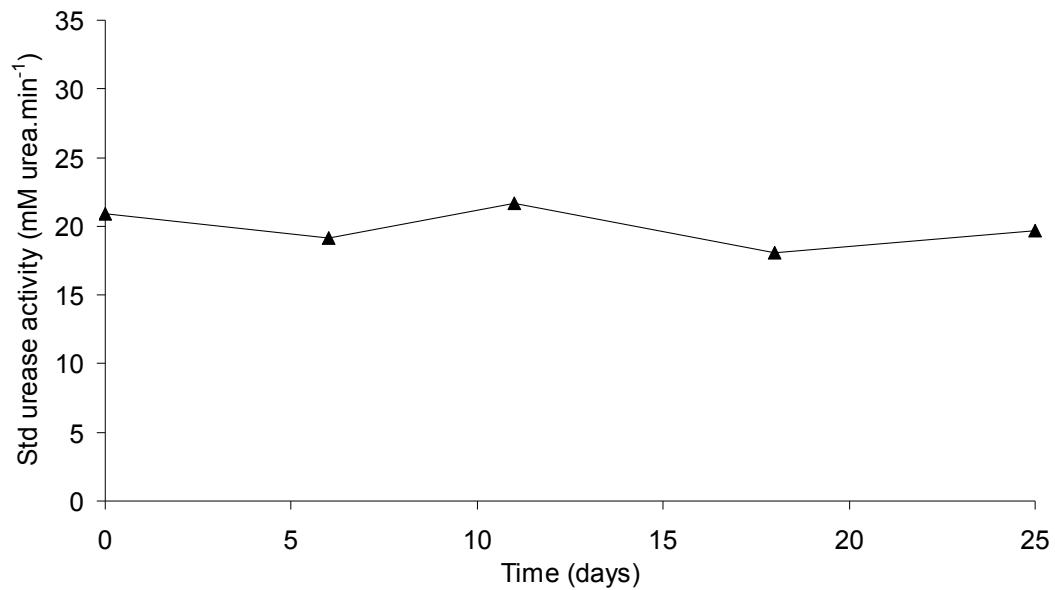


Figure 2.13: Stability of *S. pasteurii* urease stored in a closed vessel at 4°C.

2.4 Discussion

Under normal batch conditions *P. vulgaris* produced approximately 10-times less urease than the 10 mM urea.min⁻¹ activity required for biocementation (Fig 2.1). The desired enzyme activity for biocementation could still be obtained by concentrating the enzyme with a commercial flocculant, however the recovered urease activity was significantly lower than expected. Although flocculation was successful at capturing 90% of the cells in suspension (Fig 2.5), more than half of the activity was unaccounted for after flocculation (Fig 2.6). Alternatively, *S. pasteurii* produced a high level of urease activity that was sufficient for biocementation, without the need for additional processing. To value the impact of this difference, the cost to produce 100 L of urease with an activity of 10 mM

urea.min⁻¹ was determined (Table 2.1). *S. pasteurii* urease was significantly cheaper at \$90 per 100 L of enzyme versus \$1330 for *P. vulgaris*. The significant reduction in cost was largely due to the lower volume of media required.

Table 2.1: Cost for production of 100 L of urease for biocementation.

Component	Kg required	\$(AUD).kg ⁻¹	Component cost (\$)
<i>P. vulgaris</i> urease			
1000 L of culture			
20 g.L ⁻¹ Yeast extract	20	64	1280
0.09 g.L ⁻¹ Flocculant	0.09	9.20	1
Cost			1281
Final activity (mM urea.min ⁻¹)			9.6
Cost per 100 L of enzyme activity produced (\$. (mM urea.min ⁻¹) ⁻¹)			\$ 133
Cost per 100 L with an activity of 10 mM urea.min⁻¹			\$ 1 330
<i>S. pasteurii</i> urease			
100 L of culture			
20 g.L ⁻¹ Yeast extract	2	64	128
Cost			128
Final activity (mM urea.min ⁻¹)			13.7
Cost per 100 L of enzyme activity produced (\$. (mM urea.min ⁻¹) ⁻¹)			\$ 9
Cost per 100 L with an activity of 10 mM urea.min⁻¹			\$ 90

Although *S. pasteurii* produced clearly more urease per cell than *P. vulgaris*, urease production in this organism was less consistent and varied with respect to biomass concentration (Fig 2.7, 2.8). An explanation for the large difference in urease activity and nature of urease production (variable versus constitutive) between the two species, may be given by the role that urease plays for each organism. *P. vulgaris* uses urease to provide a constant low level of ammonium required for biomass growth. Organisms that use urease for purposes other than nitrogen assimilation generally have the ability to produce urease to high levels and/or variably to make the enzyme production energy efficient. For example, *Y. enterocolitica* and *M. morganii* normally exhibit low levels of urease, but urease can be induced to high levels under low-pH conditions, to aid survival during passage through the low pH gastric juices on their way to colonise the intestines

(Young *et al.*, 1996). *H. pylori* colonises the gut mucosa so requires a constant high level of urease for survival (Dunn and Grütter, 2001). In the case of *S. pasteurii*, the organism's ability to generate ATP is dependant on the availability of high concentrations of ammonium (Atlas, 1997; Jahns, 1996; Wiley and Stokes, 1962; Wiley and Stokes, 1963). From this, it could be reasoned that once sufficiently high concentrations of ammonium are present to enable ATP generation and the external pH is equal to the K_{sp} for ammonium (9.25), the ions could be cycled in and out of the cell, and the need for high urease activity would reduce to a level that was sufficient to sustain the growing population. Thus high urease activity is required during the early stages of growth to establish a reusable pool of ammonium ions and to increase the pH to 9.25, but after this lowered activities are necessary to prevent the pH from becoming too alkaline for survival.

To determine where the level of urease activity generated by these two organisms ranked amongst the wider population of urease producing organisms, the activities of several urease positive organisms were compared from the literature (Fig 2.14). The level of activity produced by the two chosen organisms was at the extremes of the urease positive organisms, with *P. vulgaris* ranked among the lowest producers and *S. pasteurii* among the highest.

For the purposes of biocementation, the three most important criteria (in no particular order) are (i) economic production of (ii) a high level of urease activity that (iii) can be generated in a non-sterile environment. *S. pasteurii* fitted these criteria more closely than *P. vulgaris* and was thus the preferred source of urease for biocementation. It has been previously established from literature that *S. pasteurii* urease is active in the presence of high concentrations of ammonium (Wiley and Stokes, 1962; Wiley and Stokes, 1963). Results from this investigation have established the following novel properties:

- *S. pasteurii* was able to be cultured in a non-sterile environment for up to two days, providing that the level of contamination did not exceed 50% (w/v) of the inoculum (Fig 2.13). It is thus desirable to inoculate non-sterile environments with a large inoculum.

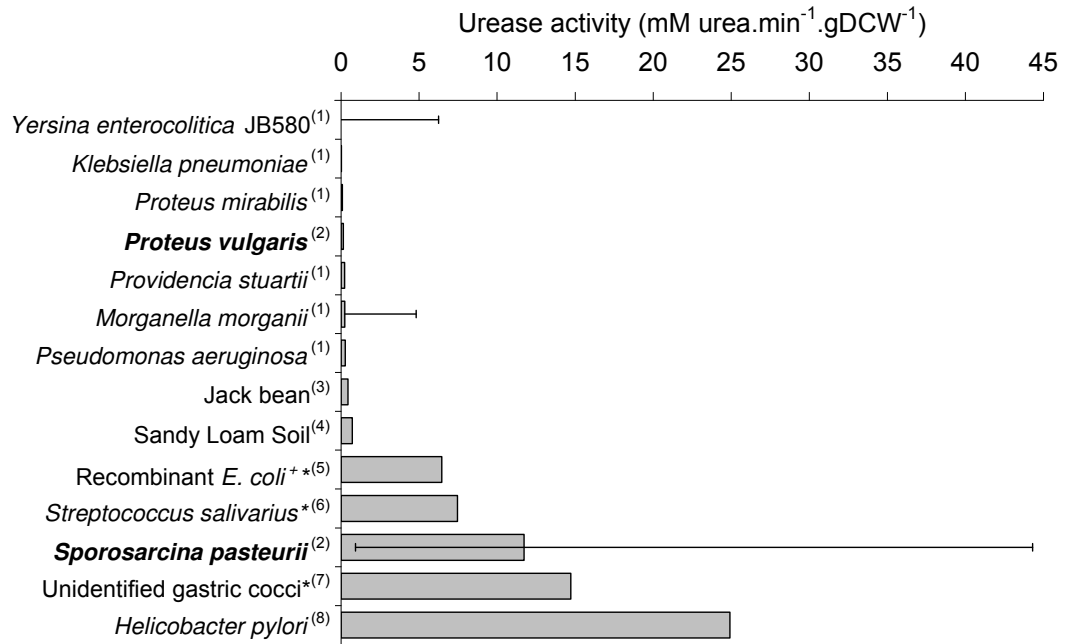


Figure 2.14: Comparison of urease activities of *P. vulgaris* and *S. pasteurii* against other urease positive organisms. Bars indicate the variability of urease activity under normal conditions of growth. Error bars indicate the reported variable range of urease activity, under inducible conditions. The reported urease activities for organisms marked with * were converted from activity.g protein⁻¹ to activity.g dry cell weight (DCW)⁻¹ according to the general protein content of prokaryotic organisms (55%) (Madigan *et al.*, 2000). + The recombinant *E. coli* was modified with the urease-coding gene from *Ureaplasma urealyticum*. Activities were sourced from ⁽¹⁾ (Young *et al.*, 1996); ⁽²⁾ the present study; ⁽³⁾ (Hanabusa, 1961); ⁽⁴⁾ (Pettit *et al.*, 1976); ⁽⁵⁾ (Neyrolles *et al.*, 1996); ⁽⁶⁾ (Li *et al.*, 2000); ⁽⁷⁾ (Lee and Calhoun, 1997); ⁽⁸⁾ (Hansen and Solnik, 2001).

- pH had a significant effect on *S. pasteurii* urease activity, which should be taken into account when the enzyme is applied under real conditions (Fig 2.9, 2.10)
- Higher specific urease activities were evident at the early batch cultivation stage, compared to the mid-late stages of growth and the resulting level of activity produced was not proportional with respect to biomass (Fig 2.7, 2.8). This suggested that *S. pasteurii* urease was not produced constitutively. The literature is somewhat divided on this subject, as Mörsdorf and Kaltwasser (1989) claim that *S. pasteurii* urease is constitutive, however *S. pasteurii* has also been reported to “lose its ureoclastic ability when maintained on synthetic media” (Sneath, 1986).

Before proceeding to biocementation trials, it was highly desirable to optimise *S. pasteurii* urease production by establishing the conditions that enable high specific urease activity.

Optimisation of Urease Production in *Sporosarcina pasteurii*

3.1 Introduction

In the previous chapter, it has been established that urease activity in *Sporosarcina pasteurii* is not proportional to the biomass concentration, during normal cultivation (Fig 2.7, 2.8). This suggested that urease activity in *S. pasteurii* is controlled by a regulatory mechanism. In order to achieve maximum and reproducible productivity, it is important to establish how the enzyme is regulated by the bacterium, and identify the parameters that maintain high specific urease activity. This also governs whether a two-step process can be used for producing urease, involving cultivation under optimum growth conditions followed by a change in conditions for optimum enzyme activity, or if optimum enzyme conditions are better maintained from the beginning of cultivation.

Regulation of enzyme activity is essential for energy efficient cell function. Many enzymes within the cell are produced constitutively; which means that they are produced under all growth conditions. Other enzymes are not required all of the time and their synthesis can be either turned “off” (repressed) or “on” (induced or de-repressed) according to the metabolites present (or absent). This kind of genetic control is regulated by the cell at the transcriptional level where messenger RNA (mRNA), which codes for the enzyme, is produced from the DNA template (Lewin, 1994; Ratledge, 2001) (Fig 3.1).

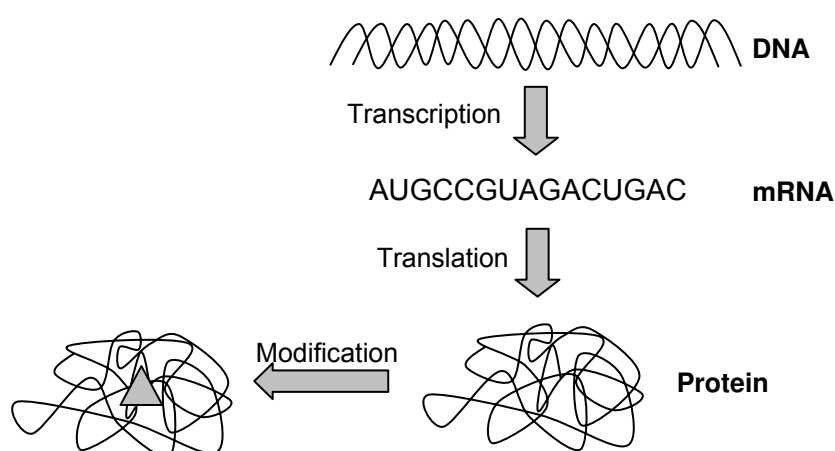


Figure 3.1: Regulation levels for enzyme activity by microorganisms. Enzymes can be regulated at the transcriptional level or the modification level (post-transcription).

Enzymes that are controlled at transcription (inducible/repressible) are usually repressed under normal conditions, which helps to conserve energy from unnecessary protein synthesis. The presence of an inducer, normally its substrate, can strongly induce an enzyme up to 1000-fold its level under non-induced conditions (Lowe, 2001).

As bacteria are not compartmentalised organisms, mRNA is both transcribed and translated in the same location, and the two processes are so closely linked that they occur virtually simultaneously (Lewin, 1994). Because of the close association of these two processes, the life-time of bacterial mRNA is substantially shorter than eukaryotic mRNA, and translation of any one mRNA molecule typically occurs for only a few minutes before it is degraded (Ratledge, 2001). This system allows tighter control over protein production, as the response time needed to stop protein synthesis after the gene is switched off is only a few minutes.

Enzyme activity can also be controlled after the enzyme has been synthesised, at the post-translational level (Lewin, 1994; Ratledge, 2001). Post-translational modifications are often carried out by other molecules and control the enzyme to be in either an active form or less active or inactive form. These effector molecules control enzyme modification and can either be promoters or inhibitors of enzyme activity (Freifelder, 1985).

With regard to enzyme regulation, the literature presents three general classes of ureases (Collins and D'Orazio, 1993; Mobley and Hausinger, 1989; Mobley *et al.*, 1995); (i) urease that can be repressed by high ammonium concentrations, and de-repressed by low ammonium concentrations, (ii) urease that can be induced by urea (enzyme substrate), and (iii) urease that is produced constitutively.

The first class includes those that are tightly regulated in conjunction with nitrogen availability. The synthesis of urease in these organisms, is repressed under conditions of high ammonium (or urea) concentration and de-repressed at low ammonium (or urea) concentration (Collins and D'Orazio, 1993; Mobley and Hausinger, 1989; Morsdorf and Kaltwasser, 1989; Mulrooney *et al.*, 1989).

Species in this class include *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Alcaligenes eutrophus* and *Bacillus megaterium* (Kaltwasser *et al.*, 1972a; Morsdorf and Kaltwasser, 1989).

The second class of ureases can be induced in the presence of urea (substrate induction). In this class of ureases, the concentration of ammonium plays no role in the regulation of urease. Instead, urease can be induced to activities that are 5- to 25-fold higher in the presence of urea, compared to non-induced levels (Jones and Mobley, 1987; Mobley *et al.*, 1991). Species that have been reported with substrate inducible urease include those from the genera *Proteus* and *Providencia*, *Salmonella cubana* and some *E. coli* strains (Mobley *et al.*, 1995; Morsdorf and Kaltwasser, 1989).

Thirdly, *Sporosarcina pasteurii*, *S. ureae* and *Morganella morganii* have been reported to constitutively produce urease (Kaltwasser *et al.*, 1972a; Mobley *et al.*, 1995; Morsdorf and Kaltwasser, 1989). The usage of the term constitutive has been somewhat variable in the literature over recent years. Freifelder (1985) defines constitutive as “without regulation”, and it has also been described as “produced constantly or in fixed amounts, regardless of environmental conditions” (Dorland, 2000). Ratledge (2001) however, leaves the definition open for interpretation regarding the effects of post-translational modifications on enzyme activity, by simply stating that it is “present under all growth conditions”. For the purpose of this study, the accepted definition of constitutive refers to a constant level of enzyme activity proportional to biomass concentration (i.e. constant specific urease activity).

A few examples where urease appears to be regulated in a manner outside of these three classes have been reported. Some species have shown developmental regulation of urease. *Proteus vulgaris* and *P. mirabilis* have been reported to generate elevated levels of urease during swarming behaviour (Falkinham III and Hoffman, 1984). Li *et al.* (2000) found that urease activity in the oral-dwelling bacterium *Streptococcus salivarius* was up to 130-times higher when grown in a biofilm, compared to liquid chemostat culture under similar conditions. pH has also been reported as having an effect on urease regulation in *S. salivarius*, with

urease activity elevated six-fold within an hour under lowered pH conditions after the addition of glucose (Li *et al.*, 2000).

The regulation of urease in *S. pasteurii* has been reported to be constitutive (Mörsdorf and Kaltwasser, 1989), which is contrary to the preliminary finding of this study that urease was not produced proportionally to biomass concentration (Fig 2.8). The objective of this chapter is to establish conditions that promote high specific urease activity and in doing so, generate new knowledge on the regulation of urease in *S. pasteurii*.

3.2 Materials & Methods

3.2.1 Microorganism

The microorganism used was *Sporosarcina pasteurii* (formally known as *Bacillus pasteurii* (Yoon *et al.*, 2001)) ATCC 11859. All cultures were inoculated from the -80°C glycerol frozen working seed bank (section 2.2.1) unless otherwise indicated.

3.2.2 Cultivation conditions

3.2.2.1 Ammonium YE medium

S. pasteurii was cultivated under aerobic batch conditions on 20 g.L⁻¹ yeast extract and 170 mM (NH₄)₂SO₄, pH adjusted before sterilisation to 9 (NaOH) at 28°C.

3.2.2.2 Urea YE medium

Urea YE medium cultivation was conducted under aerobic batch conditions on 20 g.L⁻¹ yeast extract and 170 mM CO(NH₂)₂. Medium pH was 7.5 after autoclaving. Urea was added post-autoclaving by 0.2 µm filter sterilisation to prevent chemical decomposition under autoclave conditions (DSMZ, 2003).

3.2.2.3 Acetate YE medium

Acetate YE medium was prepared under the same conditions as Ammonium YE medium, except the medium contained 10 g.L⁻¹ yeast extract, 100 mM NaCH₃COO and 170 mM (NH₄)₂SO₄, pH adjusted before sterilisation to 9.

3.2.2.4 pH-controlled experiments

pH-controlled experiments were conducted with an automated pH controller that continuously determined the pH of the culture liquid and added 3 M H₂SO₄ or 6 M NaOH via a peristaltic pump as required, in order to maintain the desired pH set-point.

3.2.2.5 Continuous (chemostat) cultivation

The chemostat reactor was a 1 L stirred tank reactor and was run at a dilution rate of 0.04 h⁻¹ (indicating that the reactor volume was fully replaced in 24 hours). The organism was grown on urea YE medium at 28°C, at a starting pH of 7.5, which increased to 9 within the first 2 hours, where it remained for the rest of the experiment.

3.2.3 Analytical Methods

Urease activity, specific urease activity, biomass and ammonium concentration were calculated as described previously (Chapter 2 - Section 2.2).

3.3 Results

3.3.1 Effect of urea on urease activity

3.3.1.1 Is urease produced only in the presence of urea?

As indicated in the previous chapter, the specific urease activity of *S. pasteurii* was variable between virtually 0 – 29 mM urea.min⁻¹.OD⁻¹ (Fig 2.8). This indicated that urease activity in *S. pasteurii* was not constitutive, as described in the literature, but regulated by some other mechanism (Mobley *et al.*, 1995; Morsdorf and Kaltwasser, 1989).

It was hypothesised that the presence of the enzyme substrate may be necessary to maintain a high level of specific urease activity (> 5 mM urea hydrolysed.min⁻¹.OD⁻¹). To determine if this were the case, *S. pasteurii* was grown with different ammonium sources; 170 mM of either autoclaved urea (containing both urea and ammonium), 0.2 µm filter sterilised urea (containing urea only), or filter sterilised ammonium sulphate (containing ammonium only) (Fig 3.2). Both autoclaved and

filter sterilised urea were tested because the autoclave process caused partial decomposition of urea to ammonium ions (DSMZ, 2003).

No growth or urease activity occurred in the control culture, which did not contain either ammonium sulphate or urea, indicating that ammonium or the ammonium precursor urea were essential for growth. Urease was produced in all other cultures to a similar level, indicating that the presence of urea was not necessary for the production of urease activity, providing that another ammonium source

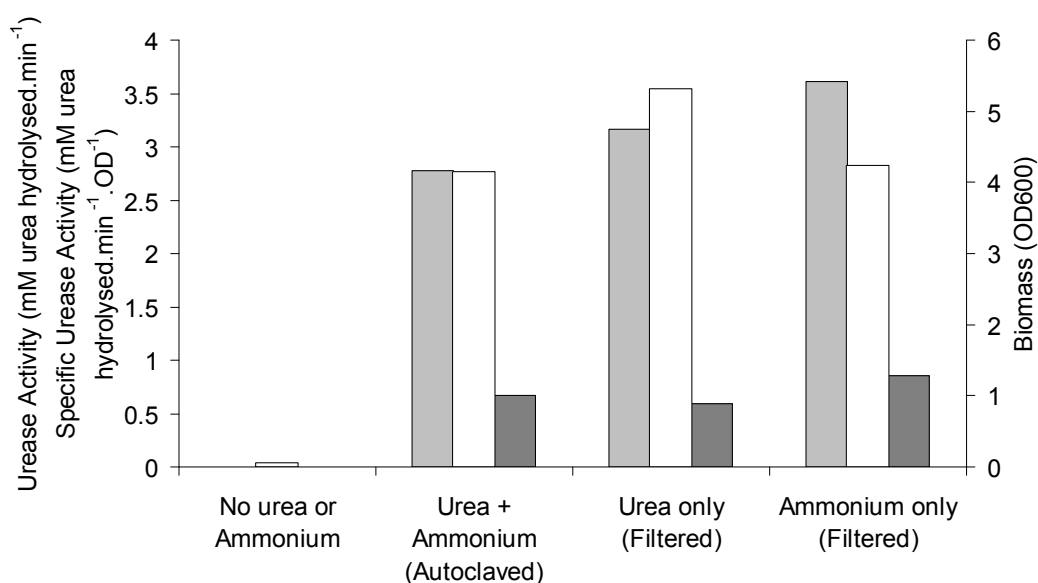


Figure 3.2: Effect of providing different sources of ammonium as either ammonium ions or urea or a mixture of both on urease activity (■), final biomass concentration (□) and specific urease activity (■). *S. pasteurii* was grown on 20 g.L⁻¹ yeast extract with 170 mM of the ammonium source added as indicated. All cultures were grown for 24 hours, at 30°C and at pH 9.

was available to the organism (Fig 3.2). The specific urease activity in all three test cultures was low (approximately 1 mM urea hydrolysed.min⁻¹.OD⁻¹) after 24 hours of incubation. It has been previously shown that specific urease activity was high at the beginning and decreased towards the end of the cultivation (Fig 2.7), which suggested that the depletion of urea may have been responsible for decreased specific urease activity.

3.3.1.2 Is the depletion of urea responsible for a decrease in specific urease activity?

To determine if the depletion of urea resulted in a decrease in specific urease activity, a batch culture was monitored for urea depletion. All of the added urea (110 mM) was degraded within 3-4 hours by a 10% inoculum (28-37 mM urea hydrolysed.h⁻¹). Urease activity increased until hour 6, after which it suddenly became stagnant (Fig 3.3).

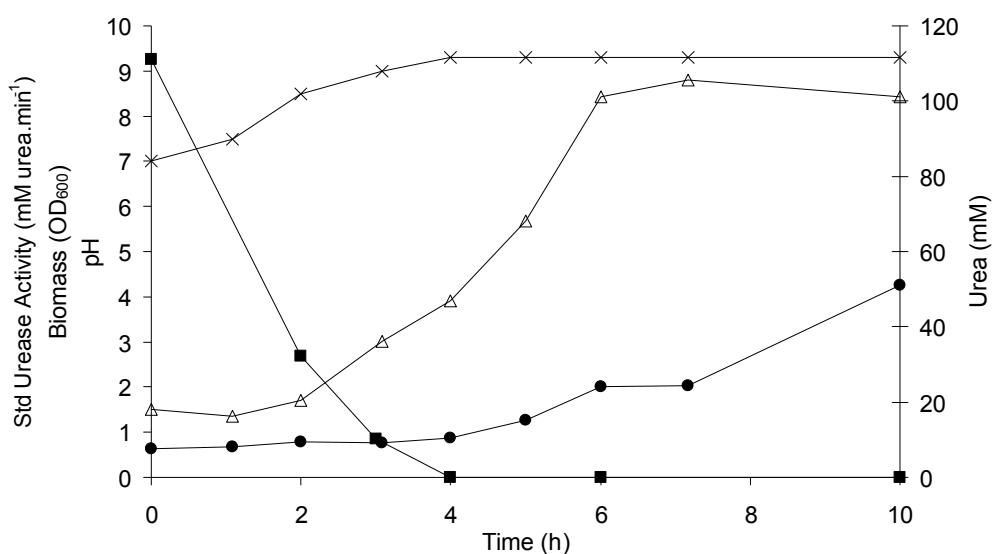


Figure 3.3: Effect of urea depletion on urease activity. Urea (■) is completely degraded within 3-4 h with no further change in pH (×) after this time. Urease activity (△) stagnated at 6 hours. Continued biomass (●) growth after this time indicated that sufficient nutrients were available.

Specific urease activity was matched to urea concentration to determine if the two events coincided (Fig 3.4). Specific activity remained at a high level (4.5 mM urea hydrolysed.min⁻¹.OD⁻¹) for several hours after the urea had been fully degraded. A significant decrease in specific activity was evident after hour 7 (4.3 to 2.0 mM urea hydrolysed.min⁻¹.OD⁻¹), which suggested that the decrease in specific activity was not due to urea depletion from the medium (Fig 3.4), however the time required to down regulate the enzyme was unknown, and a further experiment with varying concentrations of urea was required.

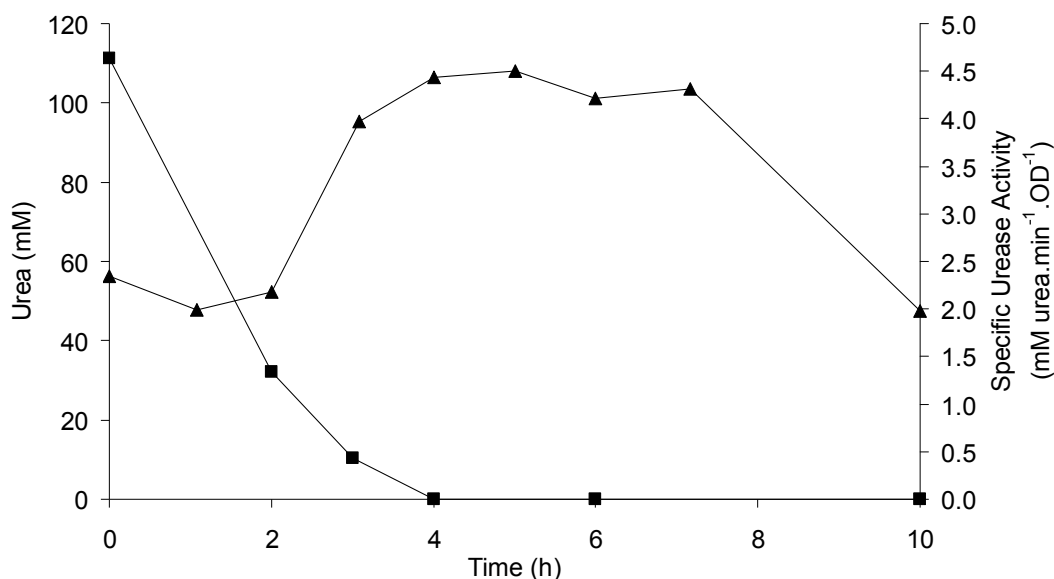


Figure 3.4: Effect of urea depletion on specific urease activity. Specific urease activity (▲) remains at an elevated level for 3 hours after urea (■) has been depleted from the medium.

3.3.1.3 Effect of urea concentration on specific urease activity

In order to determine whether the depletion of urea was responsible for the decrease in specific urease activity, it was necessary to further investigate the effect of urea concentration on the specific urease activity. *S. pasteurii* was grown with varying concentrations of urea, and specific urease activity was monitored for 10 hours (Fig 3.5). Different concentrations of ammonium were included to give the same final concentration of total ammonium in each culture (300 mM) (Fig 3.5) and the initial pH conditions were 9 in the 300 mM ammonium culture and 7.5 in all cultures that contained urea.

The maximum specific urease activities were observed in all cultures at 8 hours and were related to the amount of urea added, with high concentrations of urea resulting in high specific urease activity (Fig 3.5A). The time of urea depletion was not measured but it can be assumed that the different concentrations of urea were removed from the cultures at different times because urease activity was relatively similar in all cultures (Fig 3.5C). Specific urease activity decreased in all cultures at the same time, indicating that depletion of urea from the medium did not trigger the immediate decrease in specific activity.

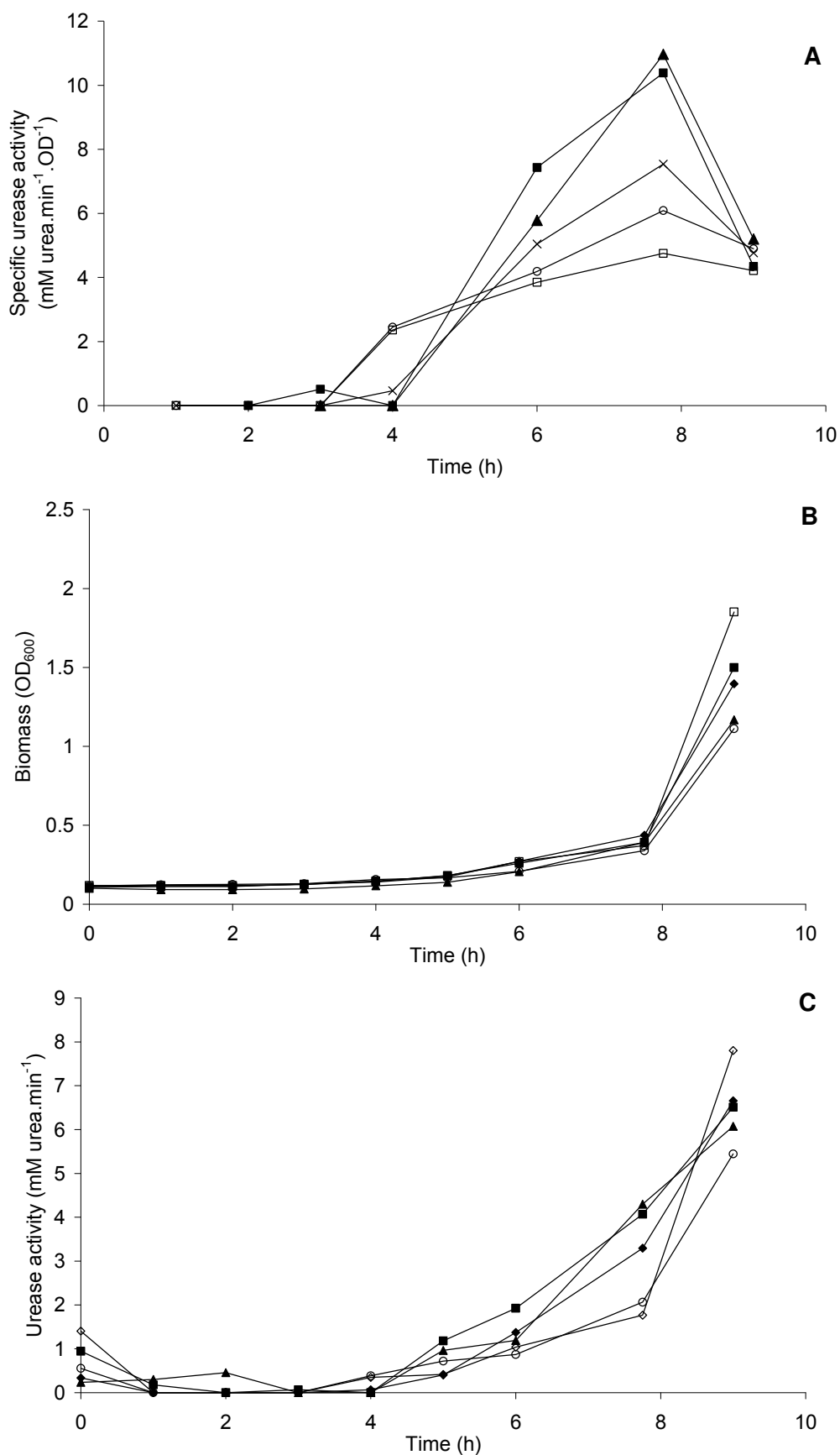


Figure 3.5: Effect of different starting urea concentrations on (A) specific urease activity, (B) biomass growth and (C) urease activity. Cultures contained NH₄⁺/Urea (mM); 300/0 (□), 200/50 (○), 150/75 (×), 100/100 (■) and 0/150 (▲) (1 mM urea = 2 mM NH₄⁺).

Interestingly, specific urease activity decreased to approximately 5 mM urea.min⁻¹.OD⁻¹ in all cultures at the same time as appreciable levels of growth commenced (Fig 3.5A and B). This suggested that pre-exponential growth conditions were more conducive to high urease activities, which was also observed in figures 2.7, 2.8, and 3.3. The lowered specific urease activities observed at higher biomass concentrations could be explained by the accumulation of a product(s), such as NH₄⁺, CO₃²⁻ or OH⁻ (pH), in the culture medium that caused urease activity to become repressed.

3.3.2 Effect of cell products on specific urease activity

To determine if a metabolic product built-up in the culture medium and resulted in repression of urease activity, cells from a grown culture were harvested by centrifugation, and then transferred into a new medium, of identical starting composition. Immediately on transfer of the cells to the new medium, specific activity and urease activity substantially increased (Fig 3.6). Within three hours of the transfer specific urease activity had increased to more than double the pre-transfer value, which indicated that if the condition or component was removed, a high level of specific urease activity could be quickly recovered (from 3.2 to 7.1 mM urea hydrolysed.min⁻¹.OD⁻¹) (Fig 3.6). After some time in the new medium, a decrease in specific urease activity (from 5.9 to 3.2 mM urea hydrolysed.min⁻¹.OD⁻¹) and a significant overall loss of total activity were observed (Fig 3.6), indicating that the component or condition again arose.

Having established that specific urease activity could be increased by replacing the old culture broth with new medium, it was then desirable to elucidate which component(s) of the transfer were responsible for the restoration of high specific urease activity. The transfer initiated several events, including a decrease in pH from 9.1 to 7.8, removal of ammonium and carbonate ions, and addition of urea. As carbonate is unlikely to have a repressive effect on urease activity and the replacement of urea can be excluded (Fig 3.4, 3.5), the possible repressors include pH, and ammonium concentration.

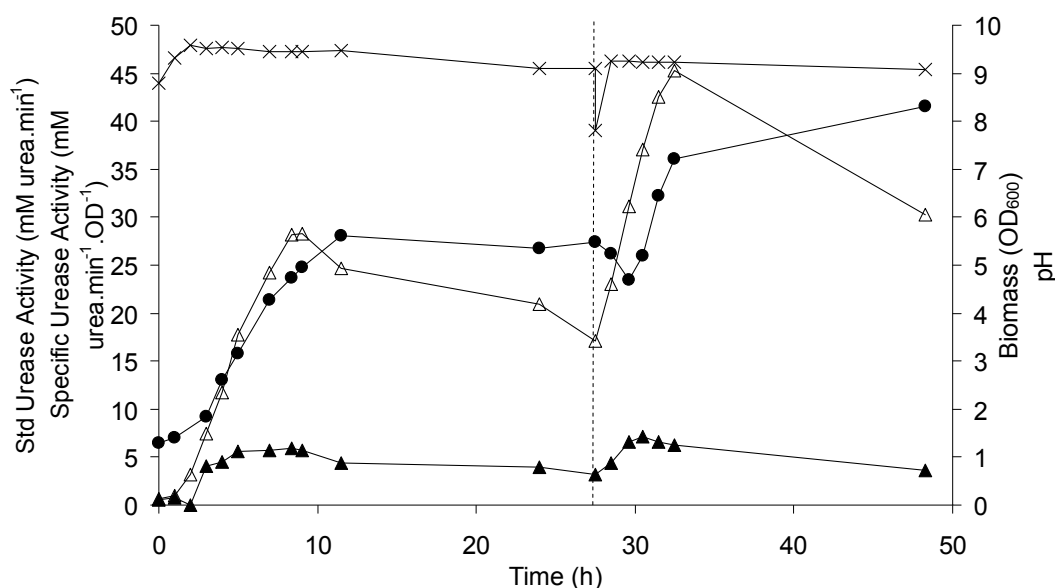


Figure 3.6: Effect of harvesting cells from a stagnated urease activity culture and transfer into a new medium (indicated by dashed line), on biomass (●), urease activity (△), specific urease activity (▲) and pH (×). Both first and second media were identical and contained 30 g.L⁻¹ Vegemite™ (commercially available concentrated yeast extract paste) and 170 mM urea, under cultivation conditions that were the same as for urea YE cultivation.

3.3.3 Effect of pH on specific urease activity

3.3.3.1 Effect of decreasing pH on specific urease activity

The effect of pH on urease activity has already been considered in the preliminary study by incubating washed cells in new media at different pH conditions (section 2.3.2.3). High specific urease activities (approximately 7 mM urea.min⁻¹.OD⁻¹) were observed under all conditions but no substantial increase in specific urease activity was observed at sub-optimum pH conditions (Fig 2.11). However, with knowledge that the specific urease activity could be significantly increased by transfer into a new medium (Fig 3.6), it was worthwhile to confirm possible pH effects under different conditions.

To investigate the effect of pH on specific urease activity, a pH-controlled experiment was conducted with cells that were grown under urea YE cultivation conditions to mid-exponential phase (5 hours). At this time the culture was given a concentrated yeast extract shot (20 g.L⁻¹ final culture concentration) to ensure sufficient nutrients were available for enzyme production. Immediately after this, the pH was changed from 9.2 to 8.5 at hour 5, and over the course of the

experiment the pH was changed in several steps down to 7.9 (Fig 3.7). Decreasing the pH, did not stimulate urease activity and a constant specific urease activity of 4 mM urea hydrolysed.min⁻¹.OD⁻¹ was maintained (Fig 3.7), indicating that lowering the pH below the growth optimum did not have a de-repressing effect on specific urease activity.

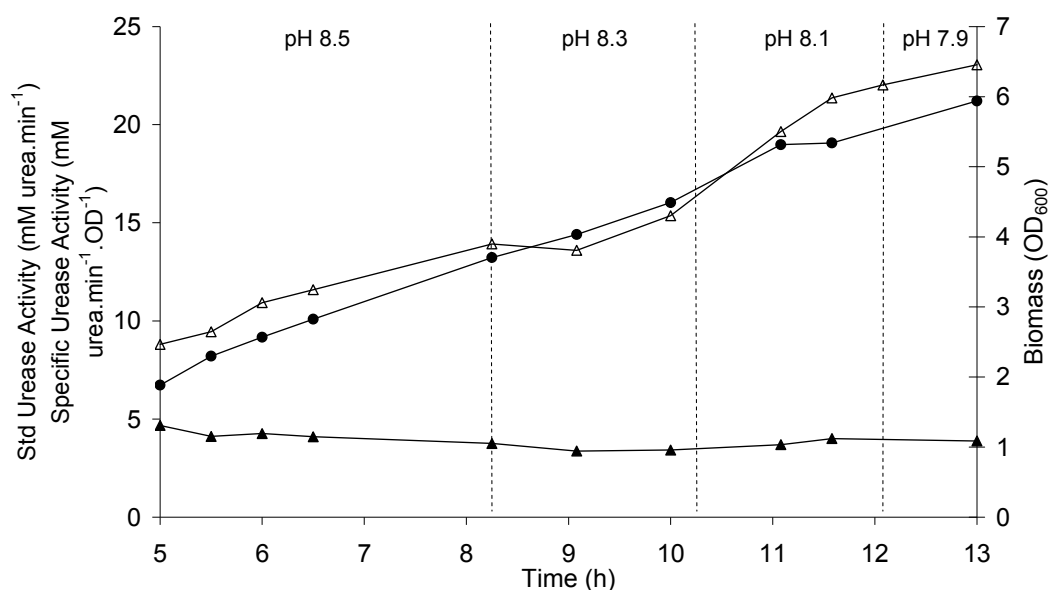


Figure 3.7: Effect of lowering pH in a mid exponentially grown culture on biomass (●), urease activity (△) and specific urease activity (▲). pH was changed from 9.2 to 8.5 at time zero, and *S. pasteurii* was cultivated of urea YE medium.

3.3.3.2 Effect of decreasing pH in the presence of urea on specific urease activity

The previous experiment was conducted in medium that initially contained urea, however by the time the pH changes were initiated, there was no remaining urea in the medium. A second experiment was conducted to investigate the effect of lowering pH in the presence of urea. The culture was grown for 24 hours and was given a concentrated yeast extract shot (20 g.L⁻¹ final culture concentration) prior to commencing the pH controller. Immediately after the 24 hour sample was taken, the pH was decreased from 9 to 7, which did not increase urease activity. 50 mM urea spikes were added immediately after sampling at hour 27, 28, and 29 (indicated by arrows), which also did not increase urease activity (Fig 3.8).

Lowering the pH to 7 in the presence of urea was more detrimental to urease activity, compared to lowered pH conditions without urea addition.

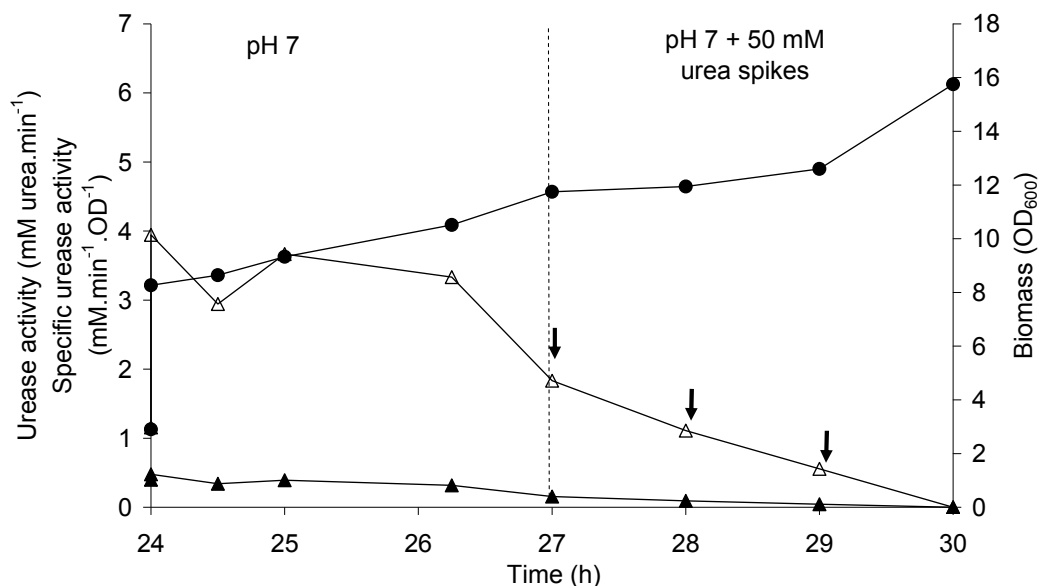


Figure 3.8: The effect of decreasing pH in the presence and absence of urea, on urease activity. Vertical arrows indicate that addition of 50 mM urea spikes. Cells were grown with ammonium YE medium. Biomass (●), urease activity (△) and specific urease activity (▲).

3.3.3.3 Effect of increasing pH on specific urease activity in a highly active culture

Conversely, increasing the pH did have an effect of specific urease activity. *S. pasteurii* is known to have a growth optimum at pH 9.25 (Fig 1.4) (Wiley and Stokes, 1962). In an attempt to improve growth and thus the overall urease activity of a highly active culture, pH was increased from 8 to 9.25. Increasing the pH to the optimum for growth did result in an immediate increase in growth, however this was accompanied by a stagnation of urease activity and a concomitant decrease in specific activity (Fig 3.9). This indicated that high pH conditions which were conducive to high growth resulted in a decrease in specific urease activity.

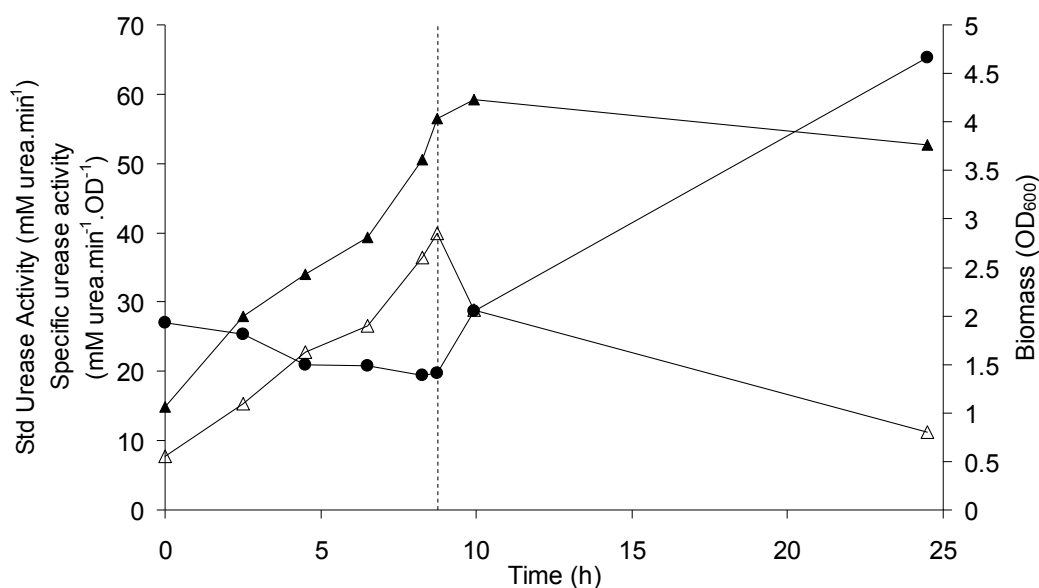


Figure 3.9: Effect of increasing pH to 9 (indicated by vertical dashed line) on the biomass concentration (●), urease activity (△) and specific urease activity (▲) of a highly active culture. Dashed line indicates where the pH was changed from 8 to 9 by addition of NaOH.

Interestingly, before the pH change this culture produced the highest specific urease activity ever measured, during the course of this study. This culture was one of six identical cultures, which were prepared by the same method, medium (urea YE medium) and growth conditions (25°C), however for an unknown reason this culture produced very slow growth and the pH stagnated at pH 8 (Fig 3.9 hours 24 - 33). All other cultures produced three times lower specific urease activities and there was no obvious reason why this one became highly active and the others did not. This indicated that the urease activity in this strain could be affected by small differences in culture conditions that could not be exploited for optimising the process.

3.3.4 Effect of ammonium concentration on specific urease activity

It has been established that harvesting cells from their old medium and transfer into a new medium could further increase specific urease activity (Fig 3.5). This suggested that a product that increases during cultivation was responsible for the observed decrease in specific urease activity. Ammonium was one of the major

products that increased during cultivation in urea containing medium. In order to investigate the effect of ammonium concentration on specific urease activity, *S. pasteurii* was grown in individual batch cultures containing different concentrations of total ammonium, supplied as $(\text{NH}_4)_2\text{SO}_4$. Urease activity was determined after 24 hours of incubation. All cultures were conducted at pH 9.25, where the chemical speciation of $\text{NH}_4^+/\text{NH}_3$ existed in equal proportions (Fig 1.5). Marginally higher urease activities were observed in cultures containing ammonium concentrations greater than 200 mM. The concentration of ammonium was found to have little effect on specific urease activity (Fig 3.10).

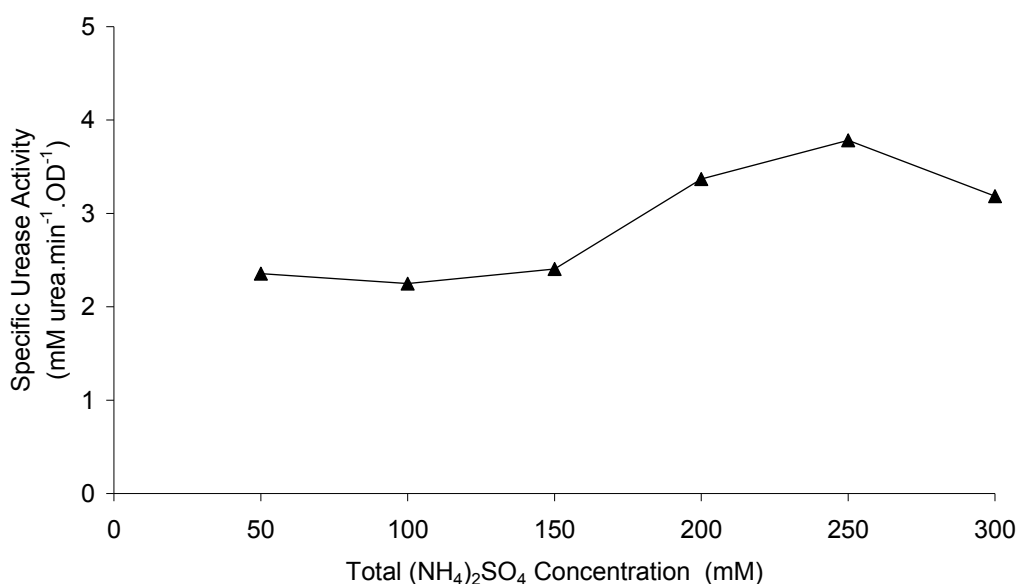


Figure 3.10: Effect of total ammonium concentration on specific urease activity of *S. pasteurii* at pH 9.

3.3.5 Continuous (chemostat) culture

In order to further elucidate the cultivation conditions that result in elevated levels of specific urease activity, continuous (chemostat) cultivation of *S. pasteurii* was attempted. Despite reaching a steady-state biomass concentration, significant loss of urease activity was evident (Fig 3.11), indicating that this method of cultivation was not suitable for urease production.

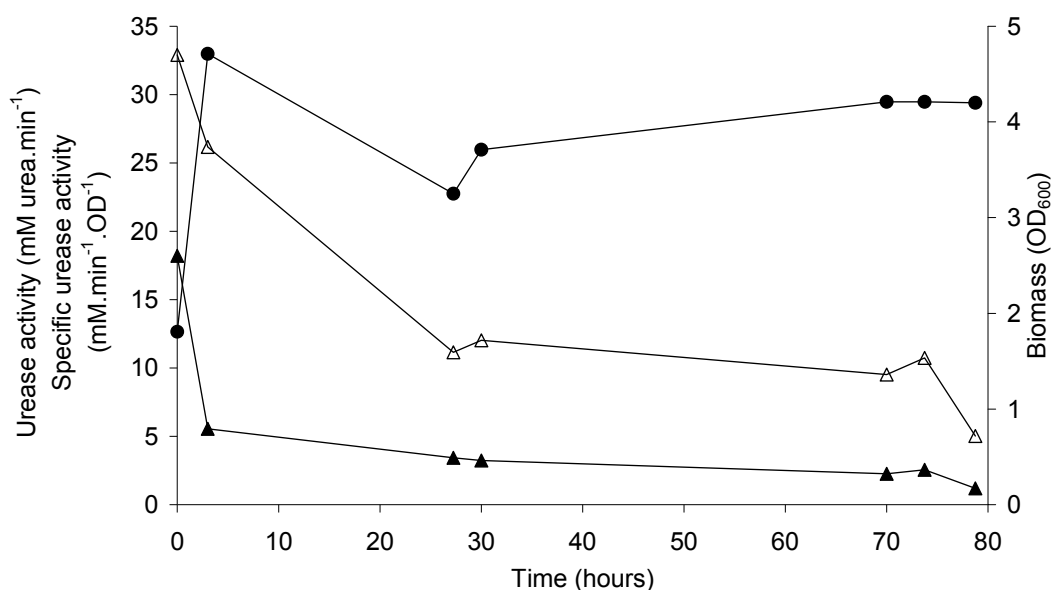


Figure 3.11: Attempted continuous (chemostat) cultivation of *S. pasteurii*. Steady state biomass concentration (●) could be achieved but urease activity (△) and specific urease activity (▲) decreased significantly.

3.3.6 Capacity for restoration of urease activity after loss

3.3.6.1 Attempted restoration by sub-culture into new medium

As the conditions that favour biomass growth, do not favour high levels of urease activity, a two-step cultivation process for *S. pasteurii* has been considered. In this system it has been proposed that the first step should be conducted in conditions favourable to biomass production and the second-step in conditions that favour enzyme production (induction). In such a process it would be expected that low urease activity cells would be produced in the first-step and subjected to conditions that favour urease activity. To investigate if high activity could be restored to a culture after a period of low activity, a 1% inoculum from three different sources was sub-cultured into urea YE medium. The three inocula sources were from (i) a highly active culture (taken before the activity decrease; Fig 3.9), (ii) a low activity culture and (iii) a normal -80°C frozen inoculum from the cell seed bank (section 2.2.1).

The high activity and frozen inocula produced typical levels of biomass, urease activity and specific urease activity under the cultivation conditions, indicating that the highly active state of the cells in the previous culture (Fig 3.9) was due to the cultivation conditions and not a change in the cell physiology. Despite

cultivation under the same conditions, the inactive inocula produced a low level of urease activity, which was approximately 90% lower than the other two cultivations (Fig 3.12). All cultivations were conducted under sterile-conditions and were checked by microscopy to ensure that there was no contamination. It appeared that the loss of urease activity was not able to be recovered in one subculture. This indicated a new aspect of urease productivity, that the history of the culture and inoculum can have a major effect on urease activity.

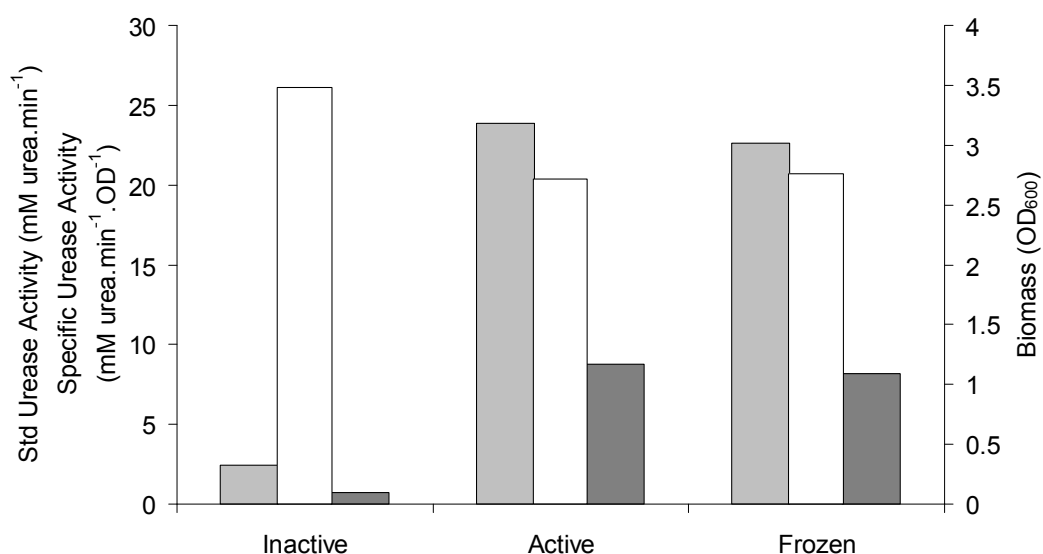


Figure 3.12: Cultivation of *S. pasteurii* under the same conditions, using inocula from three different sources. The inactive and active inocula were sourced from previous experiments, which had generated low and high final urease activities. The frozen inoculum was from the glycerol frozen -80°C cell seed bank that was the source of inocula for all other experiments. Urease activity (■), biomass (□) and specific urease activity (■).

3.3.6.2 Attempted restoration by additional subculture into new medium

The inactive culture from the previous experiment was subjected to several further successive subculturing steps to see if activity could be restored. Despite three additional subcultures, urease activity decreased further, suggesting that once urease activity had decreased to a low level, it was not evident that activity could be regained (Fig 3.13).

3.3.6.3 Reversibility of activity stagnation

An experiment was conducted to investigate if it was possible to restore urease activity to a low activity culture. After 30 h without any significant increase in urease activity, a moderate pH change from 9.5 to 9 and feed addition were made to see if urease activity could be increased. An increase in biomass was observed but no concurrent increase in urease activity resulted (Fig 3.14). At 50 h the pH was lowered from 9 to 7.5 by the automated pH controller. The pH controller overshoot the targeted pH, which resulted in a 15-minute period where the pH was between 7.5 and 5.5 before being reinstated to 7.5. Urease activity resumed at

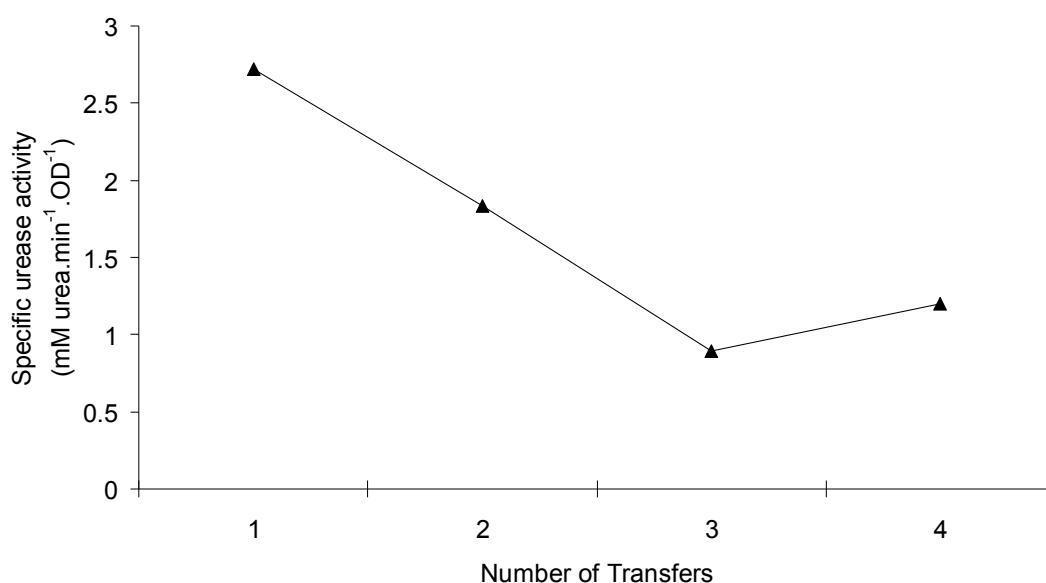


Figure 3.13: Effect of four consecutive subcultures of *S. pasteurii* into urea YE medium under optimum growth conditions, on specific urease activity. All cultures were grown for 24 hours before subculture. Each subculture involved transfer of 1% of the previous culture as an inoculum, and all cultures produced similar biomass concentration. All transfers were conducted under aseptic conditions. The initial pH of the culture was 7.5, which increased to 9.25 due to the hydrolysis of urea, within a few hours.

51.5 hours and increased to five times its stagnated level over a 20-hour period, which indicated that it was possible to increase the amount of active enzyme per cell after activity had stagnated, by a combination of pH change and new feed supply.

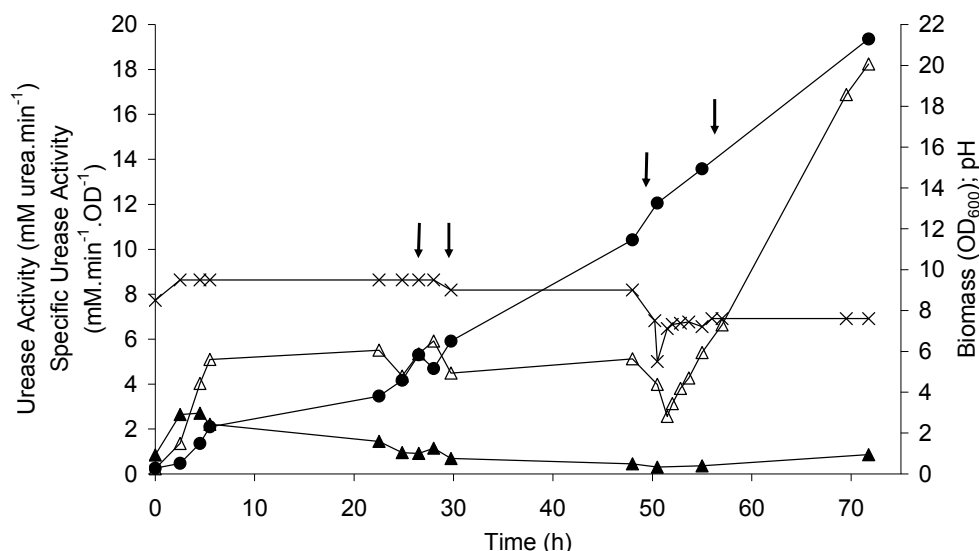


Figure 3.14: Cultivation profile of *S. pasteurii* demonstrating reactivation of enzyme activity in response to culture conditions. Biomass (●); urease activity (△); specific urease activity (▲); pH (×). *S. pasteurii* was grown at a starting pH of 8.5, on yeast extract and urea with concentrated feed/urea additions (indicated by vertical arrows).

It was considered that the 15 minute period where the pH controller over shot the desired pH, may have induced the increase in enzyme activity. To test this, the pH regime leading up to the increase in urease activity (Fig 3.14), was replicated in an additional experiment. No increase in urease activity was observed, indicating that parameters outside the control of the designed experiments were responsible for regulation of urease activity (Fig 3.15)

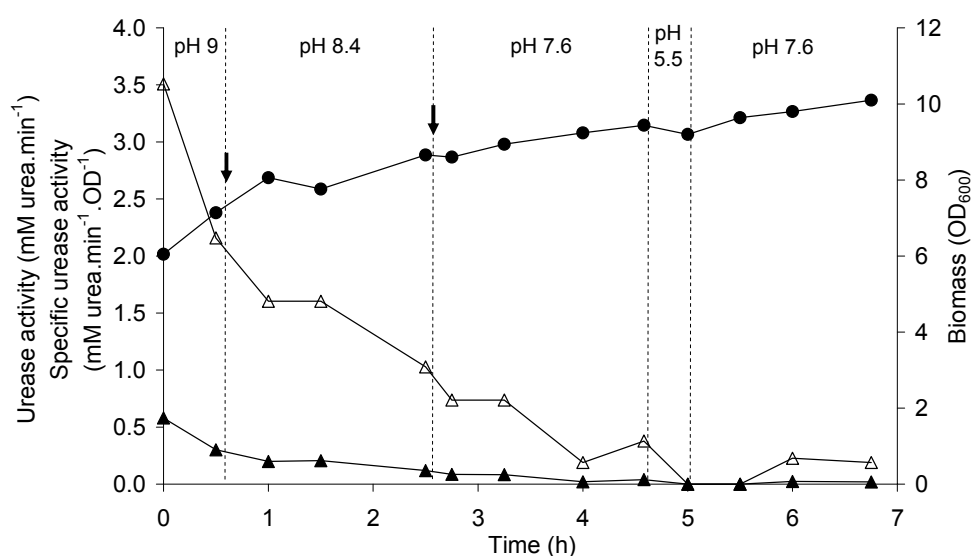


Figure 3.15: Replication of the pH regime followed in Figure 3.14, in order to determine if this event was responsible for increased urease activity. Biomass (●); urease activity (△); specific urease activity (▲);

3.4 Discussion

Biomass growth and the regulation of urease in *S. pasteurii* is not simple. No growth could be achieved in the absence of ammonium or the ammonium precursor urea, which conforms with the coupling of urea hydrolysis/ammonium presence and ATP-generation in *S. pasteurii* (Fig 3.2, 1.4) (Jahns, 1996). Urease activity was observed in all cultivations with either urea or ammonium, however the level of activity typically increased in the early stages of cultivation before becoming stagnant once an appreciable level of growth had been initiated (Fig 3.3, 3.5, 3.6, 3.9). This resulted in a decrease in specific urease activity and suggested that the available enzyme was divided between the growing cells with no further production of activity after growth had commenced.

The stagnation in urease activity could be immediately reversed by removing the cells from their medium and resuspending them into fresh medium, which indicated that an accumulating component in the medium during growth was responsible (Fig 3.6). Elucidation of the triggering factor that resulted in the activity stagnation was attempted. It was shown that the stagnation in activity (and subsequent decrease in specific activity) was not due to depletion of urea from the medium (Fig 3.4, 3.5).

It was considered that specific urease activity could be enhanced by provision of unfavourable conditions which could be made favourable by the action of urease.

The action of urease has two effects for *S. pasteurii*:

- (i) It hydrolyses urea to generate high concentrations of ammonium which are required for growth and energy generation.
- (ii) It increases the pH of the growth environment to 9.25 which is the optimum growth pH for this species.

The unfavourable growth conditions that can be made favourable by the action of urease are, lowering of the pH in the presence of urea, and low concentrations of ammonium. Lowering the pH in the presence of ammonium and urea were investigated and shown to have no effect on urease activity (Fig 3.7, 3.8). It should also be considered that as well as changing the alkalinity of the environment, lowering the pH also altered the speciation of ammonium (more

NH_4^+ /less NH_3) (Fig 1.5). If NH_4^+ was more repressive to urease than NH_3 , lowering the pH would increase this repressive effect, despite a favourable pH condition and urease would remain repressed. This hypothesis is supported by other evidence that suggested that ammonium could be repressive to urease. Stagnated urease activity could be increased by transfer of the cells into new medium (Fig 3.6). Ammonium was not present in the urea medium at inoculation and increased in concentration during the course of cultivation. It was also shown that the highest levels of specific activity prior to stagnation were observed in cultures that contained the lowest concentration of ammonium at inoculation (Fig 3.5A). To investigate this hypothesis, ammonium concentrations between 50 – 300 mM were tested, and marginally higher specific urease activities were evident at the higher concentrations, indicating that ammonium was not repressive within this concentration range. It is possible that the lowest concentration tested was maximally repressive, however the minimum ammonium concentration required for growth of *S. pasteurii* is 40 mM (Mörsdorf and Kaltwasser, 1989).

Increasing the pH to the growth optimum provided a favourable growth condition in which the action of urease was undesirable. In this case, urease activity was immediately stagnated and specific activity was dramatically reduced (Fig 3.9). This may have been due to the alkaline conditions, the increase in NH_3 concentration, the decrease in NH_4^+ concentration, or the initiation of growth. It is not clear exactly which of these factors or combination of factors were responsible for the stagnation of activity.

pH did have a regulatory effect on the level of urease activity expressed by *S. pasteurii* cells (Fig 3.9). However, if the only regulator for urease activity was pH, it would be expected that lowering the pH in the presence of suitable organic substrate, would result in an increase in specific urease activity. However, this was not evident (Fig 3.7, 3.8). It would also be expected that once the growth optimum pH had been attained, no further increase in specific activity would occur. However, this was not shown (Fig 3.6). These findings suggest that in addition to pH, another regulatory mechanism(s) exists to control the level of urease activity in *S. pasteurii*.

The capacity for regeneration of urease activity after loss was investigated, by subculture of inocula from different sources (low activity, high activity, normal cell bank inoculum) into new medium. The high activity inoculum showed a normal level of activity on subculture, indicating that the high level of activity shown previously was not due to a change in cell physiology but due to the cultivation environment (Fig 3.12). Conversely, the low activity inoculum maintained low activity. It was considered that this may have been due to the transfer of a repressive component in the medium with the cells (1% inoculum) and the cells were further subcultured with no improvement in activity, suggesting that the urease capability of the cell had been reduced (Fig 3.13). Further to this, an extended period of cultivation was undertaken and it was shown that some activity could be recovered however the level of specific urease activity was low ($1 \text{ mM urea} \cdot \text{min}^{-1}$) (Fig 3.14).

As the growth optimum pH was shown to be repressive to urease activity, a two-stage growth system seems preferred for maximum urease production (Fig 3.9). However, lowering pH did not result in an increase specific urease activity (Fig 3.7, 3.8) and the capacity for restoration of urease activity after loss was low (Fig 3.12, 3.13, 3.14). Chemostat cultivation in urea YE medium was shown to be an unsuitable method for the production of urease (Fig 3.11). Batch cultivation of *S. pasteurii* showed that high levels of overall activity ($28 \text{ mM urea} \cdot \text{min}^{-1}$, Fig 3.6 hours 0 – 10) could be achieved despite stagnation of specific urease activity. Thus batch cultivation of *S. pasteurii* is recommended until a clearer understanding of urease regulation is acquired.

The actual repressive mechanism was not fully elucidated in this study and requires further investigation. Despite not fully exploiting the urease capability of *S. pasteurii*, sufficient urease activity for biocementation could still be achieved and this organism was considered suitable for biocementation.

Economisation and Up-Scaling Issues

4.1. Introduction

The total cost of a biotechnology-based product includes labour, medium, cultivation operating costs, waste treatment and any downstream or upstream processing. In most processes, the medium ingredients are a major cost factor, ranging between 10 to 60% of the total operating costs (Kristiansen, 2001). After the initial savings achieved with economies of scale (buying in bulk quantity), the medium cost is a production cost that increases proportionally with the size of the scale up (Fig 4.1). Because of this, it is important to give due consideration to optimisation of the medium prior to scale up. Given that the biocementation process does not require ease of removal of medium components or use of a defined medium, we are able to look at a range of more economical components to replace the existing expensive analytical grade chemicals.

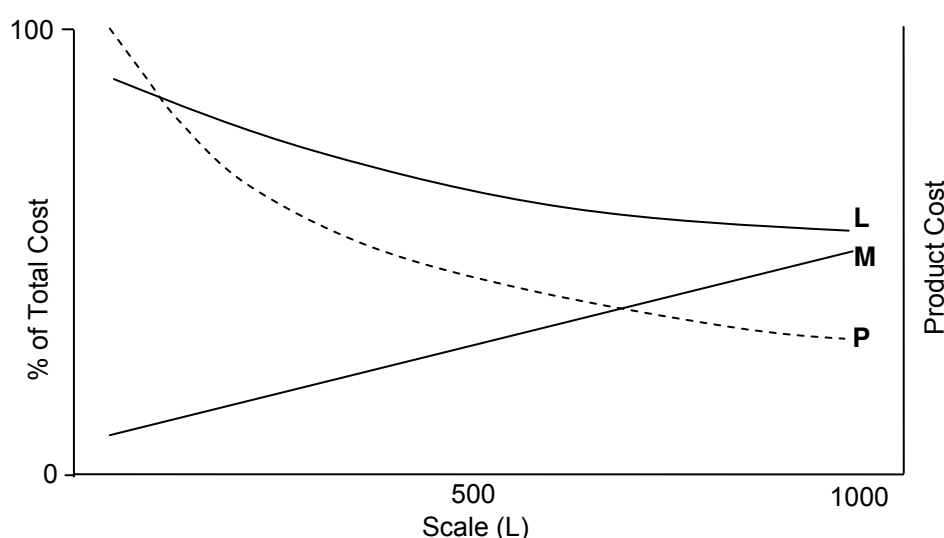


Figure 4.1: Effect of scale-up on the cost of cell culture. The overall product cost (P) and labour component (L) decreases with scale up but media costs (M) increase proportionally. (Adapted from Griffiths, 1986).

The cost of well-defined media is prohibitively expensive for large scale cultivations of microorganisms. Large scale growth media is typically complex,

and often consists of waste or by-products from the food or agricultural industries (Chaplin and Bucke, 1990; Prescott *et al.*, 1993). The reported nutritional profile for *S. pasteurii* indicated a high preference for protein-based media (Morsdorf and Kaltwasser, 1989; Wiley and Stokes, 1962). Thus the required media must have high available protein content. Several inexpensive alternative protein sources exist which could replace laboratory grade yeast extract and these are listed in Table 4.1.

Table 4.1: Possible protein sources for large-scale cultivation of microorganisms. Yeast extract is included for comparative purposes.

Protein Source	Description	Protein Content (%) Dry weight	Cost * (per kg or L) Manufacturer
Yeast Extract	Water soluble portion of autolysed yeast. Supplied as powder. Completely soluble.	66%	\$204 per kg (Becton Dickenson)
Corn Steep Liquor (CSL)	Concentrated aqueous liquid from steeping corn. Supplied as viscous liquid with suspended solids.	40%	\$36 per L (Sigma)
Torula Yeast	Autolysed food grade yeast. Supplied as a dried powder with suspended solids.	54% minimum	\$9 per kg (Cellulose Attisholz)
Vegemite™	Concentrated autolysed yeast paste. Supplied as a paste with suspended solids.	40%	\$3.60 per kg (Kraft)
Brewery Waste Yeast	Yeast from the brewing process. Whole non-lysed cells suspended in brewery waste.	54% minimum	\$2 per kg (Swan Brewery)
Sludge Biomass from WWTP	Concentrated sludge biomass from waste water treatment processing (WWTP). Whole heat killed non-lysed cells.	55%	Free

* All costs are given in Australian dollars (AUD) (including GST) and based on prices for 10 kg quantities (excluding delivery). It is to be expected that these prices would be significantly reduced when considering economies of scale.

Low cost substrates are generally subject to lowered quality control and reproducibility of the substrate supply. The effects of variable feedstock should be considered in relation to the application of the produced enzyme, where the consequences of different microbial performance from batch to batch will be encountered. If the consequences are likely to significantly reduce the efficiency of the process or application, it may be more economical to use a more expensive medium of reproducible composition (Chaplin and Bucke, 1990).

Another major concern for scale up of aerobic organisms is the reduced efficiency for supplying oxygen into large volumes of liquid. Oxygen is poorly soluble in water with saturation achieved at a low concentration of 8 mg.L^{-1} (0.25 mM). The major factor limiting the transfer of oxygen into liquid is slow diffusion across the gas bubble interface into the surrounding liquid (Prescott *et al.*, 1993). Thus the oxygen transfer rate can be improved by shearing bubbles into smaller sizes (i.e. increasing surface area to volume ratio), increasing the flow of air into a reactor, or increasing bubble residence time in the liquid. The larger the liquid volume, the more difficult it becomes to achieve high oxygen transfer rates.

The objective of this chapter is to develop an economical, industrial medium that is suitable for large-scale production of *S. pasteurii* and to investigate the effect of limiting oxygen supply on specific urease activity.

4.2. Materials & Methods

4.2.1. Cultivation conditions

4.2.1.1. Evaluation of low cost protein sources

The ability of *S. pasteurii* to use alternative protein sources was tested by cultivation in 20 g.L^{-1} of the protein substrate and $170 \text{ mM CO(NH}_2)_2$. Medium pH was 7.5 after autoclaving and urea was added post-autoclaving by $0.2 \text{ }\mu\text{m}$ filter sterilisation to prevent chemical decomposition under autoclave conditions (DSMZ, 2003). Cultivation was stopped after 48 hours.

4.2.1.2. Evaluation of low cost alternative substrate supplements

To test whether additional low cost supplements could be used as a substitute high cost yeast extract, *S. pasteurii* was cultivated under aerobic batch conditions on 10 g.L^{-1} yeast extract, $170 \text{ mM (NH}_4)_2\text{SO}_4$ and either 100 mM of defined substrates (ethanol, acetate, glucose, citrate, fumarate, succinate and fructose) or 10 g.L^{-1} of non-defined substrate (tryptone). In all cultures, pH was adjusted before sterilisation to 9 with 4 M NaOH , and cultures were grown at 28°C .

4.2.1.3. Optimisation of acetate as an alternative substrate supplement

Various concentrations of acetate were evaluated as a substitute for some of the protein component of the medium. Cultures were conducted under aerobic batch conditions on 3 g.L⁻¹ protein supplied as either 5 g.L⁻¹ yeast extract or 13.5 g.L⁻¹ Vegemite and 170 mM (NH₄)₂SO₄, pH adjusted before sterilisation to 9 with 4 M NaOH, at 28°C., Acetate was supplied as CH₃COONa at various concentrations listed in results section.

4.2.1.4. Cultivation of *S. pasteurii* under high and low oxygen conditions

The growth and urease activity response of *S. pasteurii* under low and high oxygen concentration cultivation were investigated. The oxygen mass transfer coefficient (k_{La}) was determined for each experimental set-up, by deoxygenating the system and measuring the oxygen transfer rate (OTR). The k_{La} was determined according to the equation:

$$k_{La} \text{ (h}^{-1}\text{)} = \text{OTR (mg O}_2\text{.L}^{-1}\text{.h}^{-1}\text{)} / (\text{Oxygen saturation deficit (mg O}_2\text{.L}^{-1}\text{)})$$

The high oxygen supply reactor had the capacity to supply oxygen at a rate that was 4.5 times faster than the low oxygen supply reactor. Each reactor was contained 20 g.L⁻¹ yeast extract and 10 g.L⁻¹ urea medium and cultivation was conducted for 26 hours.

4.2.2. Analytical Methods

Urease activity, specific urease activity and biomass were calculated as described previously (Chapter 2 - Section 2.2)

4.3. Results

4.3.1. Optimisation of an economical industrial medium

4.3.1.1. Evaluation of alternative low cost protein sources

For large scale production of urease, it was necessary to find an inexpensive substrate for the bacteria to grow on, that still produced a good level of urease activity. Several alternative protein sources were investigated to replace

laboratory grade yeast extract. A description of these sources is listed in Table 4.1.

Two of the alternative protein sources (Brewery waste yeast and WWTP sludge) were largely comprised of whole cells, which were inaccessible to the growing microorganisms. Initial attempts to lyse the cells were made by exposing them to 0.5 M NaOH for 20 minutes, followed by addition of H₂SO₄ to neutralise the cell extract to pH 8 (Schutte and Kula, 1990). This procedure produced an extract that did not sustain growth of *S. pasteurii* (data not shown) and despite there being scope for further investigation of other disruption methods, this task was deemed outside the scope of this study.

The pre-lysed protein sources (Corn steep liquor (CSL), Vegemite and Torula yeast) were compared to the laboratory-grade yeast extract for the production of urease activity. Biomass concentration measurements were difficult to determine reliably due to the presence of various concentrations of solids in the different media, hence these are not shown. The high levels of urease activity and activity yield per gram of substrate were produced in the yeast extract and Vegemite media (Fig 4.2).

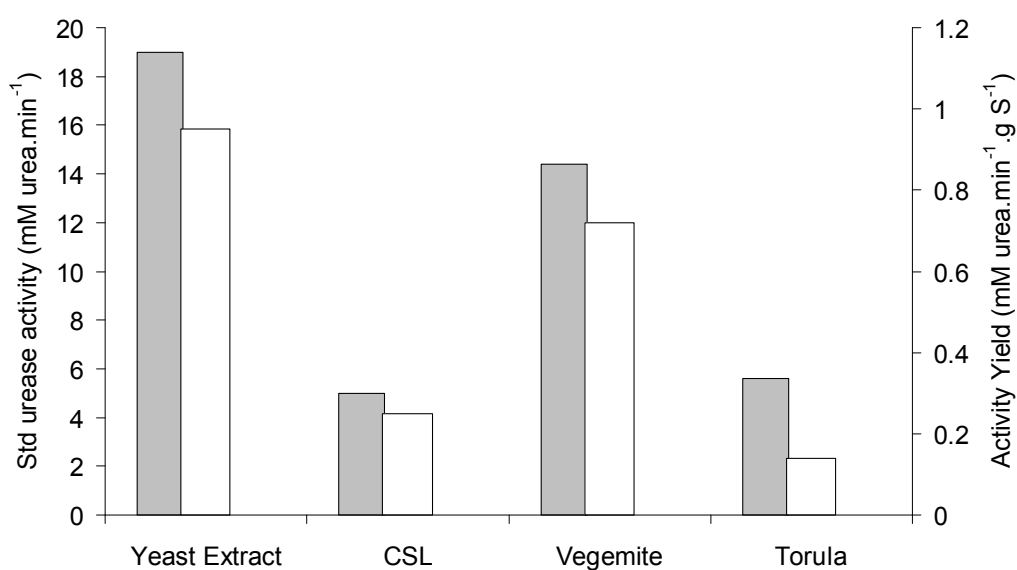


Figure 4.2: Effect of alternative protein sources on urease activity (■) and activity yield per gram of substrate added (□).

The cost of the protein component in the media was determined and compared on the basis of cost per activity produced per L of medium (all costs are shown in Australian Dollars (AUD)). Costs given are based on prices for 10 kg quantities and it is assumed that these costs would be significantly lower if considering economies of scale. The cost of producing the same amount of urease activity in Vegemite medium was 20-fold less than in yeast extract medium (Fig 4.3).

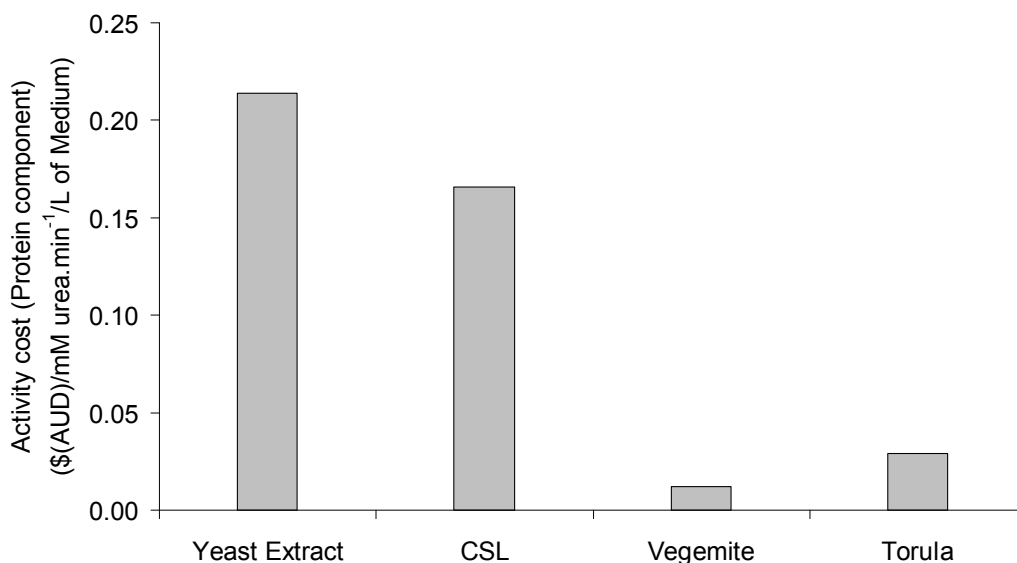


Figure 4.3: Activity cost for protein component of medium. Protein sources are yeast extract (YE), corn steep liquor (CSL), Vegemite (commercial yeast paste) and Torula yeast (commercial dry yeast powder). Costs are given in Australian dollars (AUD).

4.3.1.2. Optimising Vegemite™ concentration

In order to optimise urease production in Vegemite medium, several cultures were grown with various concentrations of Vegemite. Cultivations included urea and were conducted under the same conditions as section 4.2.2.1, except for the variable concentrations of Vegemite used.

The optimum level of urease activity was produced in the culture containing 75 g.L⁻¹ Vegemite (Fig 4.4). The higher concentration of Vegemite produced significantly less urease activity. Specific urease activity appeared to increase linearly in the cultures containing up to 75 g.L⁻¹ of Vegemite, but this observation is more likely due to a proportional increase in the presence of very small suspended solids that interfered with the optical density measurements. In this

case, more confidence can be drawn from the urease activity measurements (Fig 4.4).

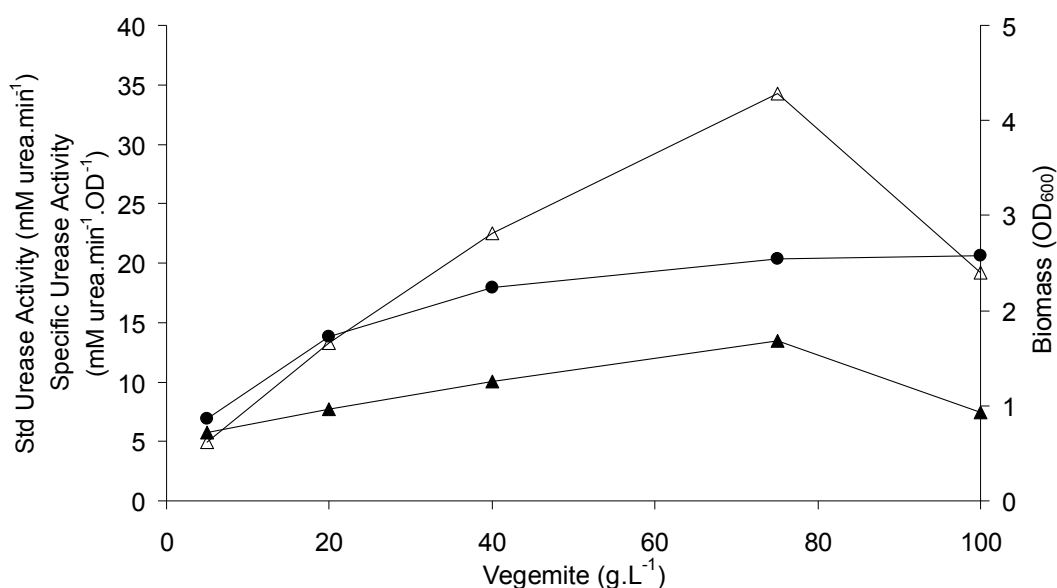


Figure 4.4: Effect of Vegemite concentration on urease activity (△), biomass (●) and specific urease activity (▲). Each culture was allowed to grow to completion before termination and the maximum values that were achieved have been plotted.

To determine which media offered the most economical urease production, the cost of each cultivation condition was compared on the basis of dollars per activity produced per L of medium. The protein cost of the same amount of enzyme activity is similar in the media containing up to 75 g.L⁻¹ of Vegemite, and is more than doubled in media containing 100 g.L⁻¹ of Vegemite (Fig 4.5).

4.3.1.3. Evaluation of low cost alternative substrate supplements

To further optimise the medium, several alternative substrates were investigated with a view to replace or substitute some of the protein component in the medium. The new substrates were tested for their ability to produce biomass and urease activity and all cultures were grown under alternative substrate cultivation conditions (section 4.2.2.2). Each test culture contained 10 g.L⁻¹ yeast extract and 170 mM ammonium sulphate plus either 100 mM of the defined substrates (Ethanol, acetate, glucose, citrate, fumarate, succinate or fructose) or 10 g.L⁻¹ of

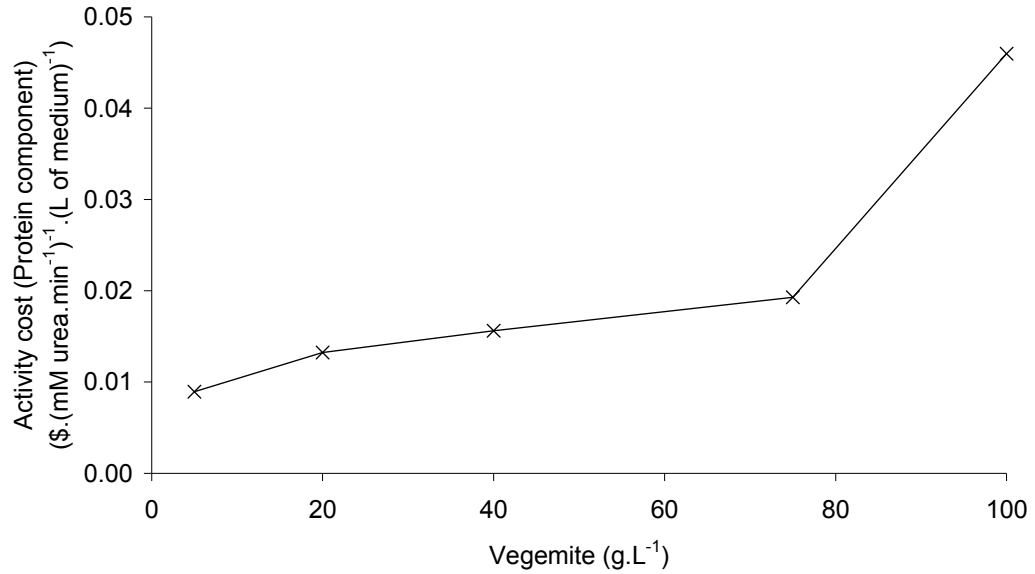


Figure 4.5: Cost of urease activity grown in various concentration Vegemite media. Prices are given in Australian dollars.

the non-defined substrate (tryptone). Yeast extract was used instead of Vegemite as the protein source, to give more accurate biomass measurements in the testing stage. The YE control contained 10 g.L⁻¹ yeast extract and 170 mM ammonium sulphate only.

Biomass production was highest on the yeast extract/tryptone medium, but the highest level of urease activity was produced with the yeast extract/acetate medium. In the yeast extract/acetate medium, urease activity was produced to a level that was 2.5-fold higher than the control (Fig 4.6). Ethanol, citrate, fumarate, succinate and fructose all produced less specific urease activity than the yeast extract control (Fig 4.7).

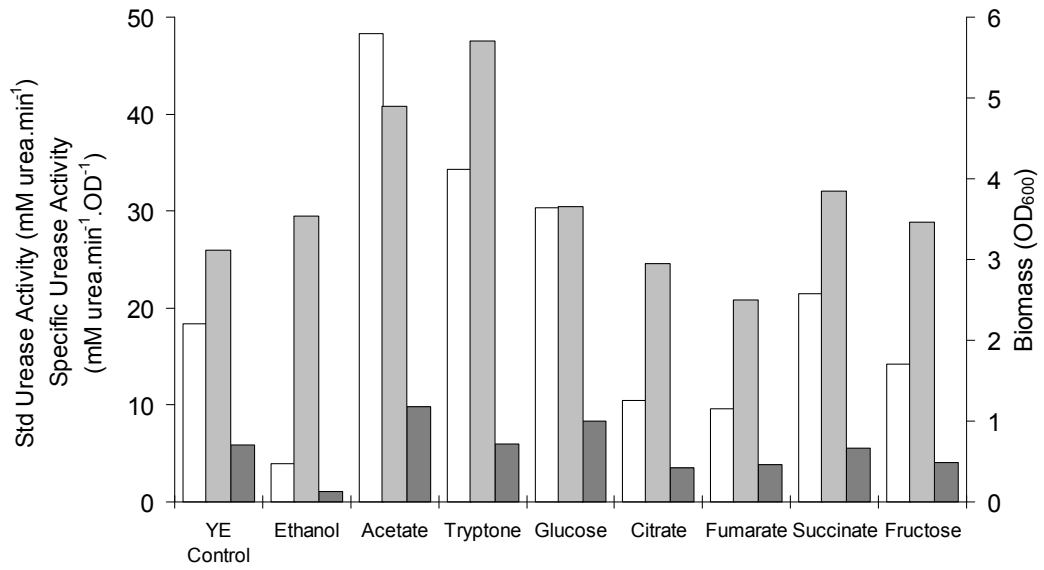


Figure 4.6: Effect of cultivating *S. pasteurii* with alternative substrates, on biomass (■), urease activity (□) and specific urease activity (■) for each media substitution. Test cultures contained 10 g.L⁻¹ yeast extract and 170 mM ammonium sulphate plus the alternative substrate. The YE control contained 10 g.L⁻¹ yeast extract and 170 mM ammonium sulphate only.

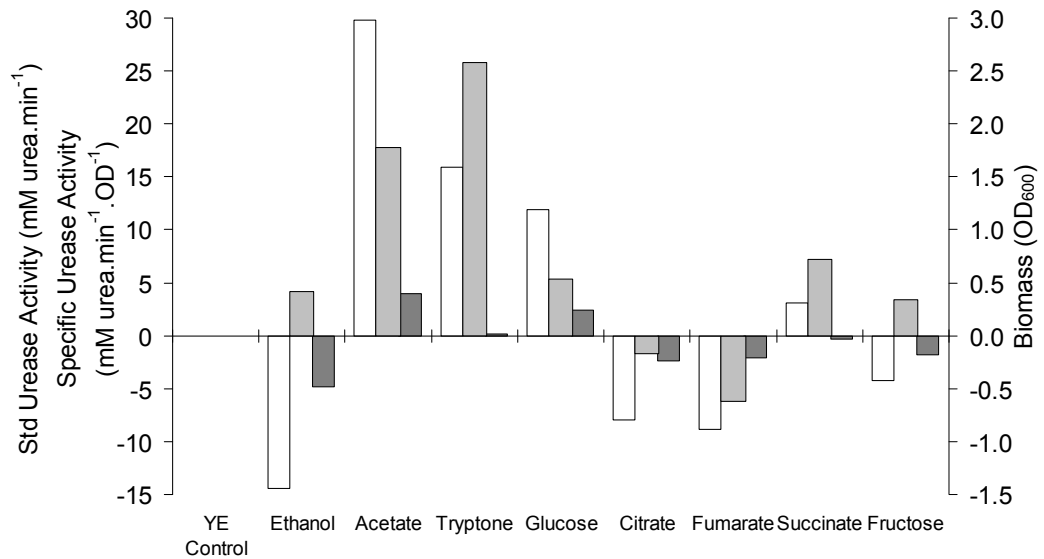


Figure 4.7: Change in biomass (■), urease activity (□) and specific urease activity (■) compared to the control culture.

4.3.1.4. Optimisation of acetate as an alternative substrate supplement

In order to optimise the concentration of acetate for maximum urease activity, several cultures were grown under acetate cultivation conditions (section 4.2.2.3) with 5 g.L⁻¹ yeast extract (containing 3 g.L⁻¹ total protein) and various concentrations of Na-acetate.

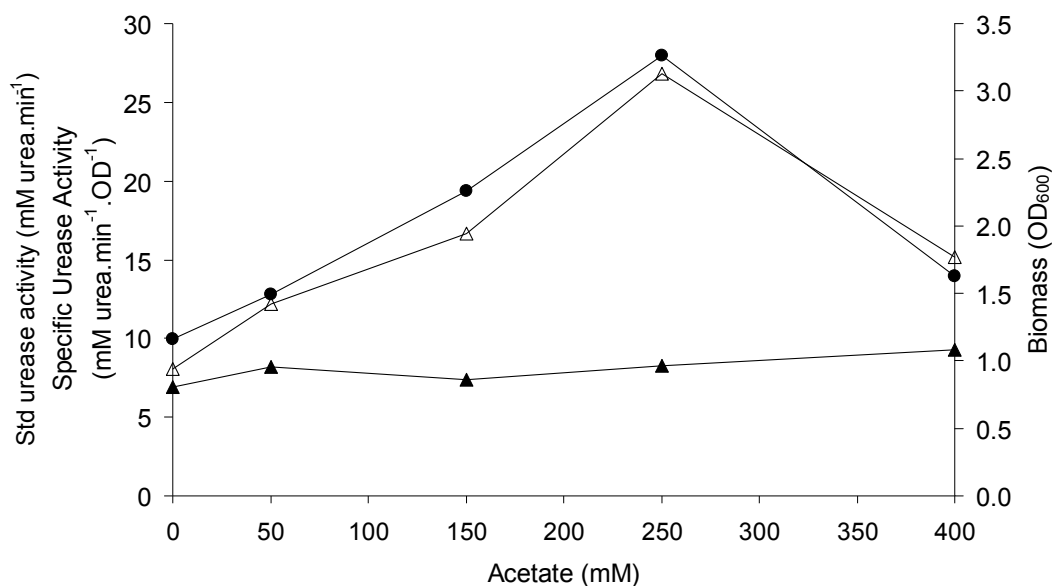


Figure 4.8: Effect of Na-Acetate concentration on urease activity (△), biomass (●) and specific urease activity (▲) when grown with 5 g.L⁻¹ yeast extract. Cultures were incubated for 48 hours at 28°C.

Maximum urease activity was produced in the 250 mM acetate medium (Fig 4.8). To compare this effect with the less expensive Vegemite medium, *S. pasteurii* was grown under the same conditions except for the substitution of 13.5 g.L⁻¹ Vegemite (containing 3 g.L⁻¹ protein) instead of the yeast extract component.

Under these conditions, maximum urease activity was produced in the 150 mM acetate medium which was 100 mM lower than the maximum produced with yeast extract (Fig 4.9), which may be due to the increase salinity of the medium. Urease activity in the culture containing no acetate was more than twice as high as the yeast extract parallel culture indicating that the organism could use other components available in the Vegemite, besides protein. The presence of more

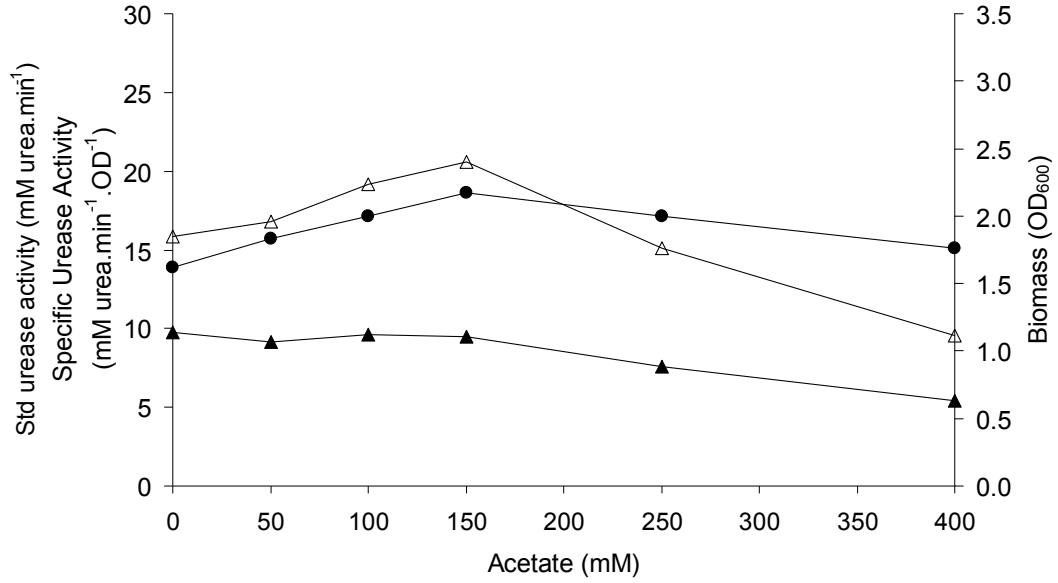


Figure 4.9: Effect of Na-Acetate concentration on urease activity (\triangle), biomass (\bullet) and specific urease activity (\blacktriangle), when grown with 13.5 g.L⁻¹ Vegemite. Cultures were incubated for 48 hours at 28°C.

complex components appears to have lessened the effect of acetate concentration in this experiment. The activity cost for the protein and acetate components was significantly cheaper when grown with Vegemite (Fig 4.10).

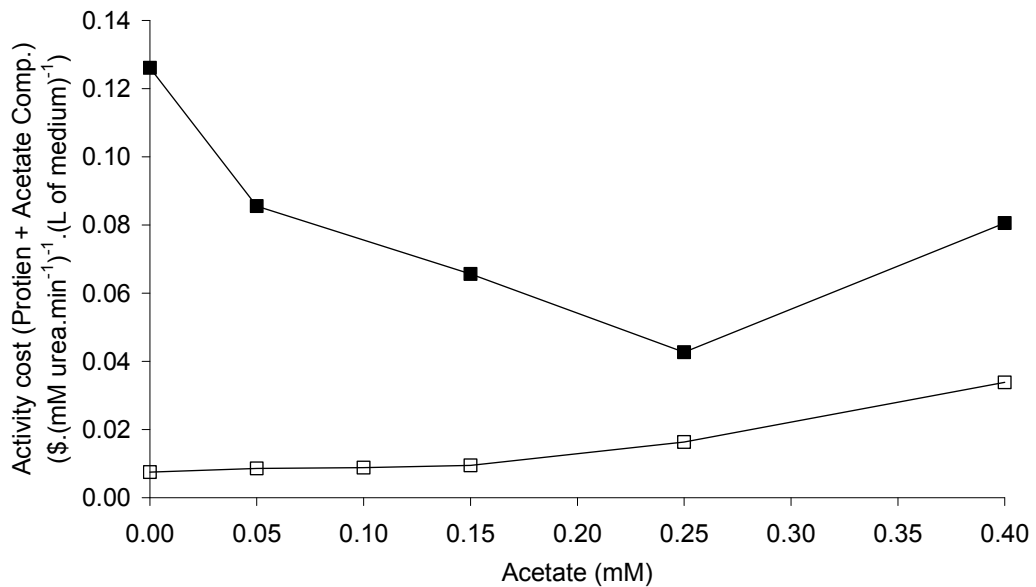


Figure 4.10: Activity cost for the protein and acetate components at various concentrations of acetate with either Vegemite (\square) or yeast extract (\blacksquare) as a protein source.

To determine how much of a cost saving could be achieved by cultivation of *S. pasteurii* on an acetate/Vegemite medium compared to a Vegemite medium without any acetate, the medium cost was plotted against urease activity produced (Fig 4.11). By using an acetate/Vegemite medium (150 mM/13.5 g.L⁻¹) activity was increased by 44% compared to the Vegemite medium (21.6 g.L⁻¹) for the same cost of \$0.20.

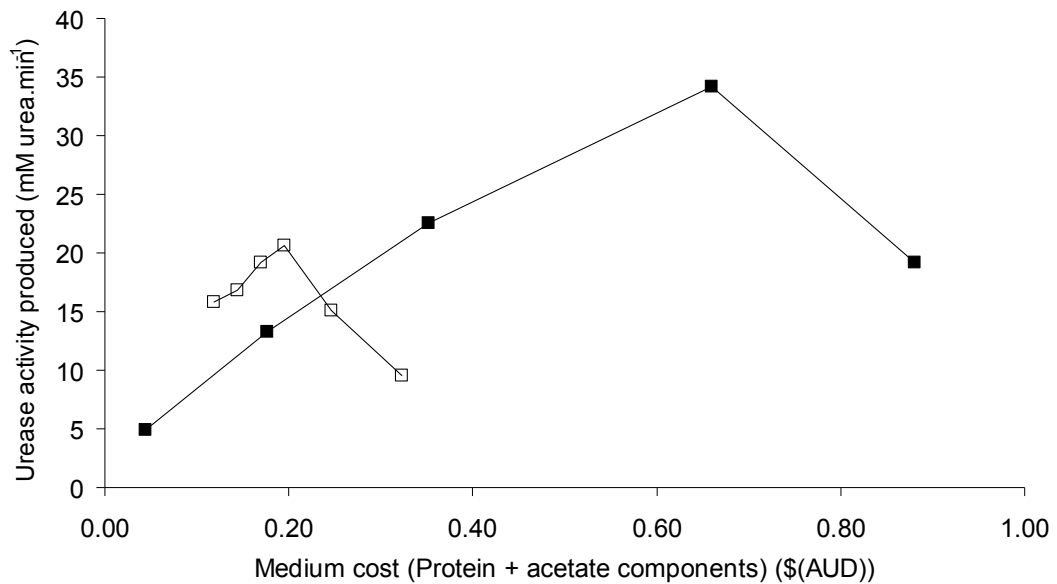


Figure 4.11: Medium cost (protein and acetate components) versus urease activity produced for Vegemite (5, 20, 40, 75 and 100 g.L⁻¹) (■) and Vegemite (13.5 g.L⁻¹) plus acetate (0, 50, 100, 150, 250 and 400 mM) (□) media.

4.3.2. Effect of oxygen limitation on specific urease activity

To investigate the effect of oxygen limitation on specific urease activity, two one-litre stirred tank reactors were set up with identical conditions except for the supply of oxygen. The high oxygen supply reactor had the capacity to supply oxygen at a rate that was 4.5 times faster than the low oxygen supply reactor. Both reactors were inoculated from the same inocula source.

The high oxygen supply reactor produced biomass 3.2 times faster than the low oxygen supply reactor, with a concomitant increase in urease activity (Table 4.2). This indicated that limited oxygen reduced growth but not the level of specific urease activity.

Table 4.2: Effect of high and low oxygen supply on specific urease activity.

	$k_L a$ (h^{-1})	Biomass (OD_{600})	Urease activity ($mM \text{ urea} \cdot \text{min}^{-1}$)	Specific urease activity ($mM \text{ urea} \cdot \text{min}^{-1} \cdot OD^{-1}$)
Low Oxygen	7.6	2.21	12.5	5.7
High Oxygen	34.2	7.17	36.4	5.1

Although similar specific urease activities were seen with high and low oxygen supply conditions, different cell morphology and motility were observed. Cells cultivated under high oxygen conditions appeared to have a normal physiology (rod shaped, non-motile), but under low oxygen conditions cells were shorter, wider, highly motile and demonstrated a chemotactic behaviour towards oxygen (Fig 4.12).

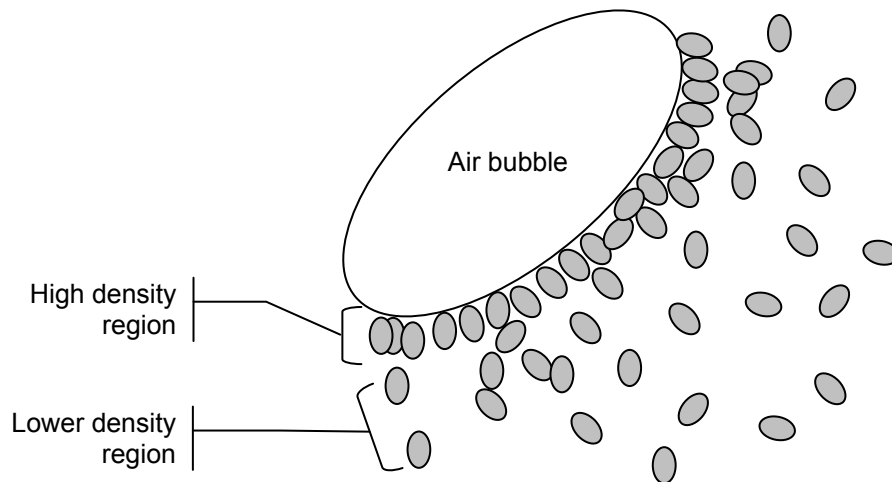


Figure 4.12: Behavioural difference observed with *S. pasteurii* cells under oxygen limitation. When placed on microscope slide cells actively form a dense region on the surface of an air bubble trapped between the slide and cover-slip.

4.3.3. Effect of oxygen limitation on urease activity and urease yield

To investigate the effect of oxygen limitation on urease activity and the efficiency of converting substrate to activity (urease yield), six shake-flask cultures were grown in $5 - 100 \text{ g} \cdot \text{L}^{-1}$ yeast extract. Urease activity was determined every 24 hours for four days.

As expected, the maximum urease activity in each culture was reached at different times depending on the concentration of substrate added; after 24 hours ($5 \text{ g} \cdot \text{L}^{-1}$),

48 hours (10, 25, 50 g.L⁻¹), 72 hours (75 g.L⁻¹) or 96 hours (100 g.L⁻¹), indicating that the cultures were increasingly oxygen limited. High levels of urease were produced under oxygen limited conditions. Up to 103 mM urea.min⁻¹ was produced in 100 g.L⁻¹ yeast extract after four days (Fig 4.13). The level of urease activity per biomass was high and generally proportional, ranging between 6.5 – 15.5 mM urea.min⁻¹.OD⁻¹ (Fig 4.14).

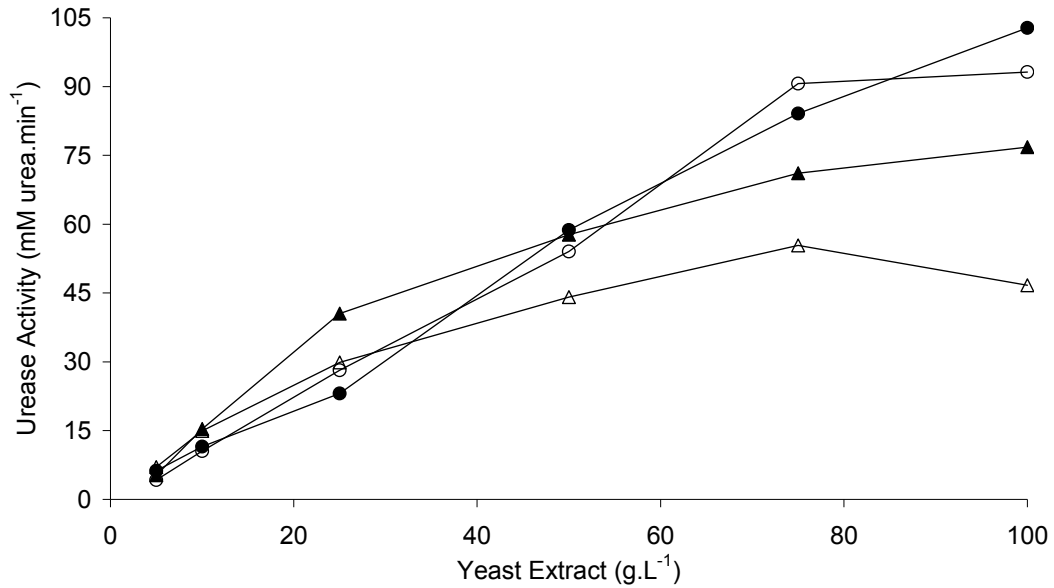


Figure 4.13: Urease activity of oxygen limited cultures over a four day incubation period (Day 1 (△), 2 (▲), 3 (○) and 4 (●)).

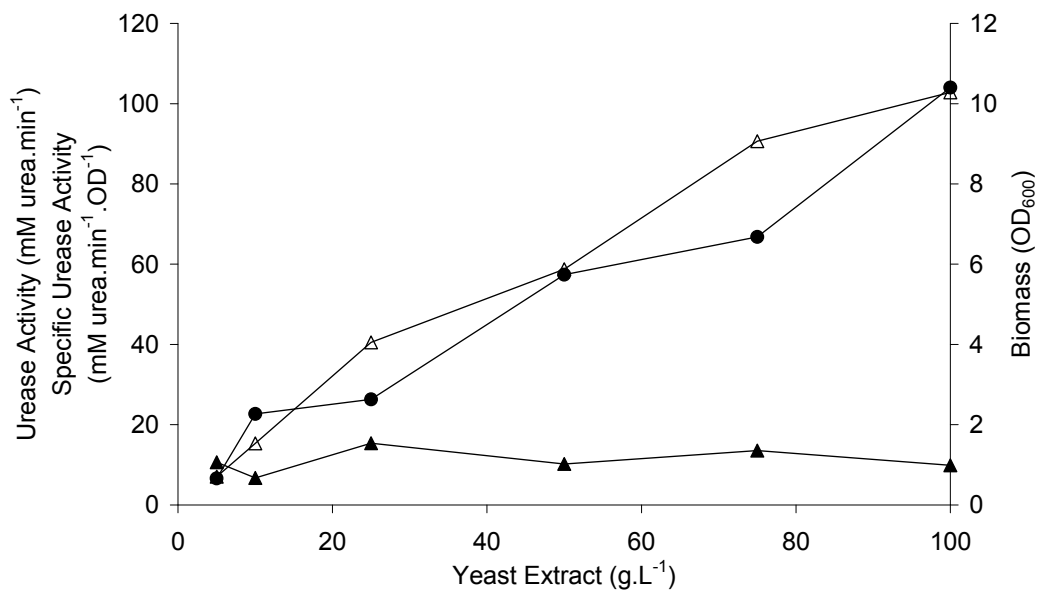


Figure 4.14: Maximum urease activity (△), specific urease activity (▲) and biomass (●) attained over the four day incubation period.

The yield of urease activity per gram of substrate added was reduced by approximately 25% in cultures with more than 50 g.L⁻¹ yeast extract (Fig 4.15), indicating that the substrate was used less efficiently over extended periods of oxygen limitation.

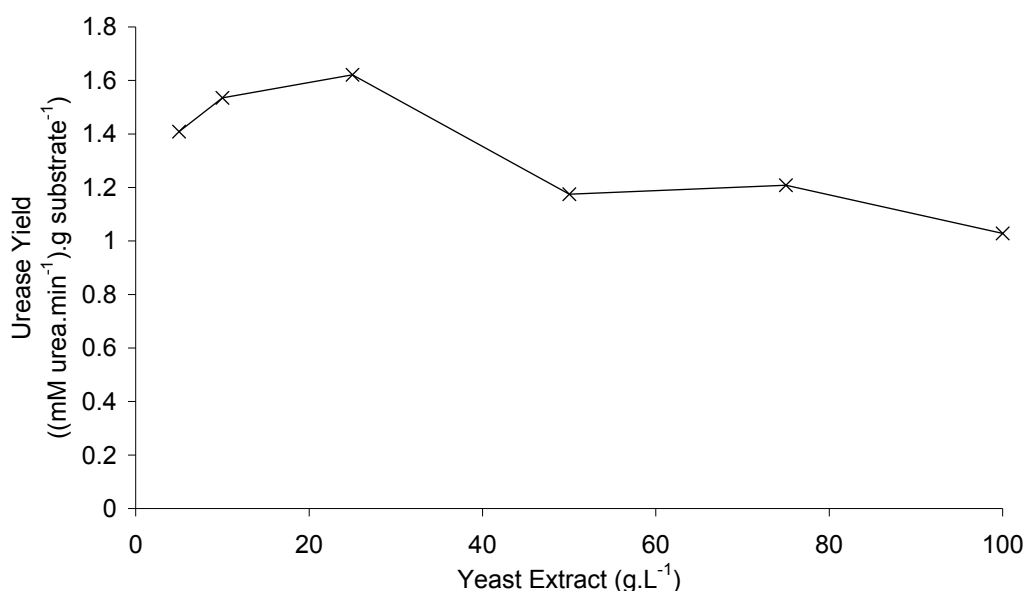


Figure 4.15: Maximum yield of activity per gram of yeast extract.

4.4. Discussion

A 20-fold reduction in medium costs without loss of urease activity was achieved by substitution of laboratory grade yeast extract with Vegemite (Fig 4.3). Further cost reduction was achieved when some of the Vegemite protein component was replaced with acetate (Fig 4.10; 4.11). Importantly, the final level of urease activity produced on the cheaper substrates was still sufficient for cementation with final activities of 21 and 34 mM urea hydrolysed.min⁻¹ on Vegemite/acetate and Vegemite media, respectively (Table 4.3).

Along with this cost saving, Vegemite also presented some additional processing needs, due to the presence of small insoluble particles when dissolved in water. The presence of these particles was not desired for cementation because of the potential for clogging permeable channels on injection into fine materials. The majority of the particles were sufficiently large to be gravity settled within 5

minutes for a one litre volume of medium and this could be done prior to sterilisation. Settling of the particles occurred at a faster rate when the water used to solubilise the paste, was cooled to 4°C, however solubility of the paste was slower at low temperature. The preferred method of preparation was to solubilise the paste in a small volume of warm water with stirring, then add additional cold water up to the required volume for settling purposes (Fig 4.16).

Table 4.3: Comparison of industrial media with laboratory medium.

Media	20 g.L ⁻¹ Yeast Extract 170 mM (NH ₄) ₂ SO ₄	13.5 g.L ⁻¹ Vegemite 150 mM acetate 170 mM (NH ₄) ₂ SO ₄	75 g.L ⁻¹ Vegemite 170 mM (NH ₄) ₂ SO ₄
Media cost per L (Protein component)	\$4.07	\$0.20	\$0.66
Activity produced	19 mM urea hydrolysed.min ⁻¹	21 mM urea hydrolysed.min ⁻¹	34 mM urea hydrolysed.min ⁻¹
Activity yield (\$/activity/L media)	\$0.21	\$0.01	\$0.02

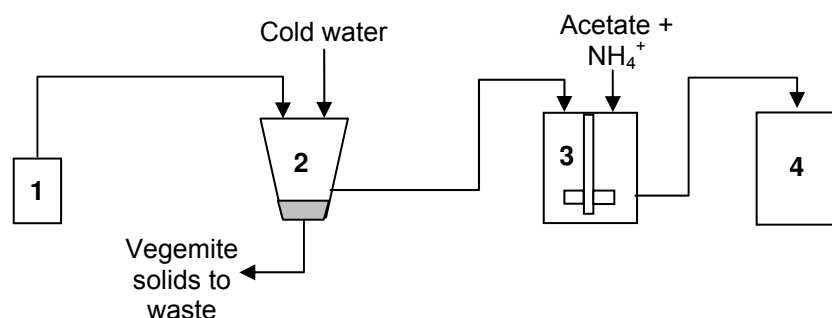


Figure 4.16: Enzyme production process for urease from *S. pasteurii*. Process involves (1) mixing of Vegemite with heated water, (2) transfer into settling tank, addition of cold water and gravity settling of solids, (3) transfer of clarified Vegemite into cultivation tank, addition of other medium components and cultivation of enzyme, followed by (4) harvesting of the enzyme into a holding tank at 4°C.

In a preliminary study, low oxygen supply was shown to limit the growth of *S. pasteurii* but it did not reduce the level of specific urease activity. This suggested that it may be possible to produce equivalent levels of urease under oxygen limited conditions, as produced under high oxygen conditions, providing that

sufficient time was allowed for growth (Table 4.2). This hypothesis was tested by growing *S. pasteurii* in oxygen limited shake-flask cultures with high concentrations of yeast extract. Given sufficient time for growth, it was shown that levels of urease that were proportional to biomass could be produced, and that high levels of urease activity could be attained (Fig 4.13, 4.14). Extended periods of oxygen limitation resulted in a reduction of 25% in urease activity yield per gram of substrate (Fig 4.15), which may have been due to the increased maintenance energy requirement to sustain cells over an extended period.

Evaluating the performance of urease under Biocementation conditions

5.1. Introduction

Thus far, a suitable microbial source of urease has been identified and the capacity of the microorganism to produce a high level of urease at an economical cost has been established. It was now necessary to investigate how urease performed under biocementation conditions and where the working boundaries of the process lay.

The biocementation process requires urease to function for an extended period under fairly severe environmental conditions. The enzyme must be functional at high concentrations of urea, ammonium, and calcium. It is also desirable for the enzyme to work across a range of temperatures. Due to economic considerations, it was preferable to use the organism intact, with minimal or no processing from the cultivation step, preventing the cost of enzyme extraction and maintaining maximum process simplicity. The objective of this chapter is to evaluate the performance of *S. pasteurii* urease under biocementation conditions.

5.2. Materials & Methods

5.2.1. Enzyme Form & Analytical Methods

All experiments were performed on native urease in whole unwashed *S. pasteurii* cells. Urease activity, specific urease activity and biomass were calculated as described previously (Chapter 2 - Section 2.2)

5.3. Results

5.3.1.1. Effect of temperature on urease activity

To determine the effective temperature range where biocementation is possible and to be able to predict rate changes with environmental temperatures, the activity of the enzyme was investigated between 15 and 80°C.

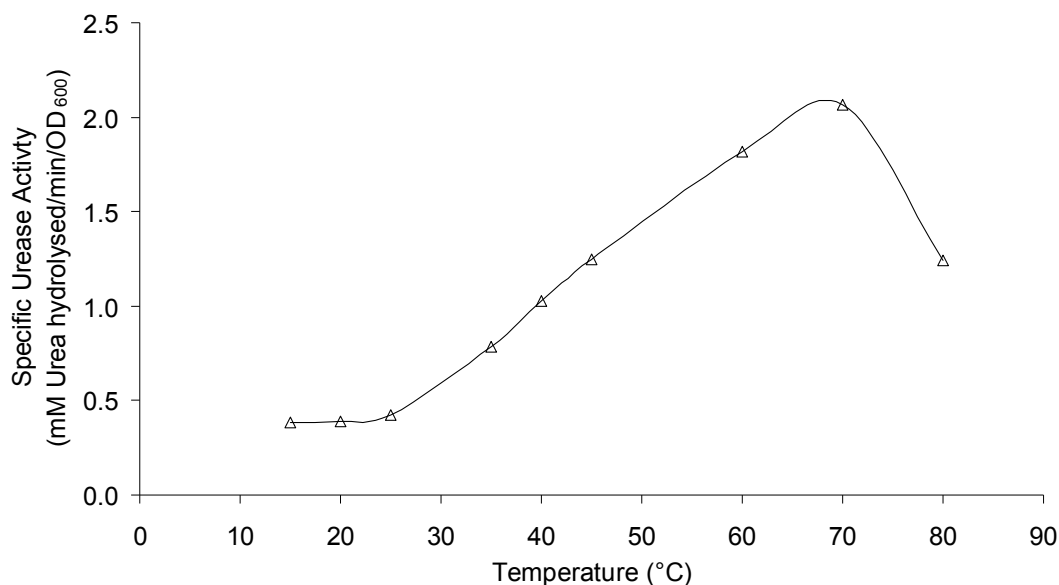


Figure 5.1: Effect of temperature on specific urease activity (\triangle) in *S. pasteurii*. The reaction mixture was incubated in a water bath at the desired temperature for 10 minutes before enzyme addition. Enzyme activity was determined over 5 minutes.

Enzyme activity was stable between 15 and 25°C and exhibited a linear increase of 0.04 mM urea hydrolysed.min⁻¹.OD⁻¹ per degree between 25 and 60°C. The enzyme had a temperature optimum at 70°C (Fig 5.1).

5.3.1.2. Maximum calcium/urea concentration per application

To obtain a desired strength increase, a particular amount of calcite is required to be delivered at a specific rate into the material. If all of the cement required for a desired strength can be delivered in one application of the enzyme and reactants, the process becomes less complex and more economical. The maximum amount of calcite that can be delivered in one application is dependent upon the sensitivity of the enzyme to high concentrations of reactants (urea/calcium) and products (ammonium/nitrate or chloride) of the biocementation process. To determine the maximum concentration of calcium and urea that can be applied in one application, several tests were conducted with the same concentration of enzyme and varying equimolar urea and calcium solutions.

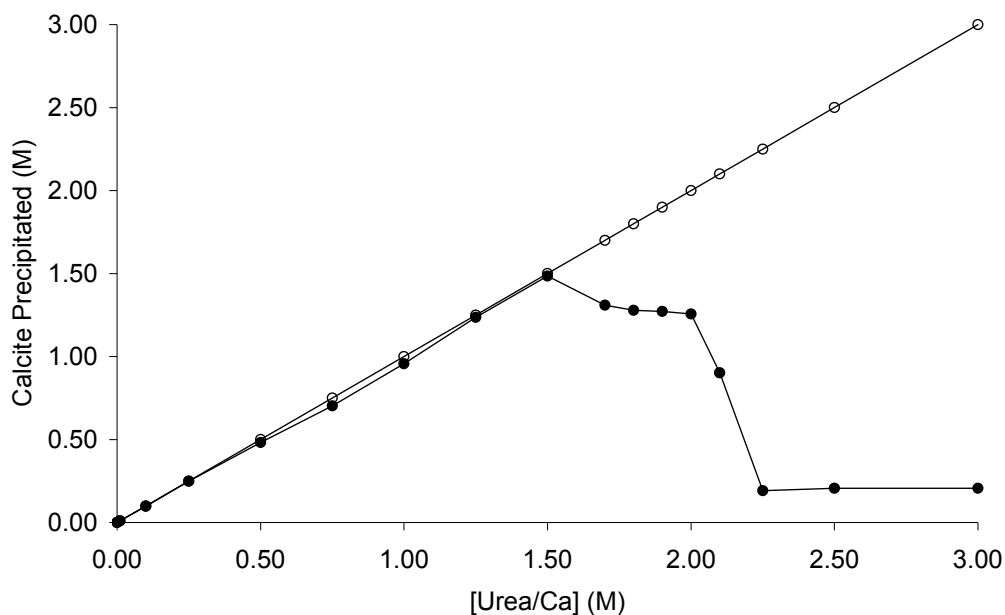


Figure 5.2: Actual (●) Vs Theoretical (○) amount of calcite precipitated at various concentrations of equimolar urea and calcium solutions after 18 hours incubation at 28°C.

All of the calcite was precipitated from reactions that had up to 1.5 M urea/calcium added, within the 18-hour incubation period. Less than the maximum possible precipitation occurred at concentrations between 1.7 and 2 M urea/calcium in the given time, and no useful precipitation was observed above 2.25 M urea/calcium. The lesser amount of precipitation observed between 1.7 - 2 M urea/calcium may be due to inhibiting substrate concentrations that reduce as the reaction progresses, which would result in a slowed reaction rate (i.e. insufficient time was allowed for the reaction to go to completion). It may be possible to increase the amount of urea/calcium per flush to more than 1.5 M if the separate effects of high urea and calcium concentrations on the enzyme are known.

5.3.1.3. Effect of urea concentration on activity

To determine the effect of high urea concentration on urease activity, the enzyme was tested between 0.01 and 3 M urea (Fig 5.3). 3 M was the maximum concentration tested because of solubility restrictions when mixed with calcium in

the biocementation reaction. *S. pasteurii* urease activity increased three-fold in the presence of 1.5 M urea, relative to the lowest concentration tested (Fig 5.3).

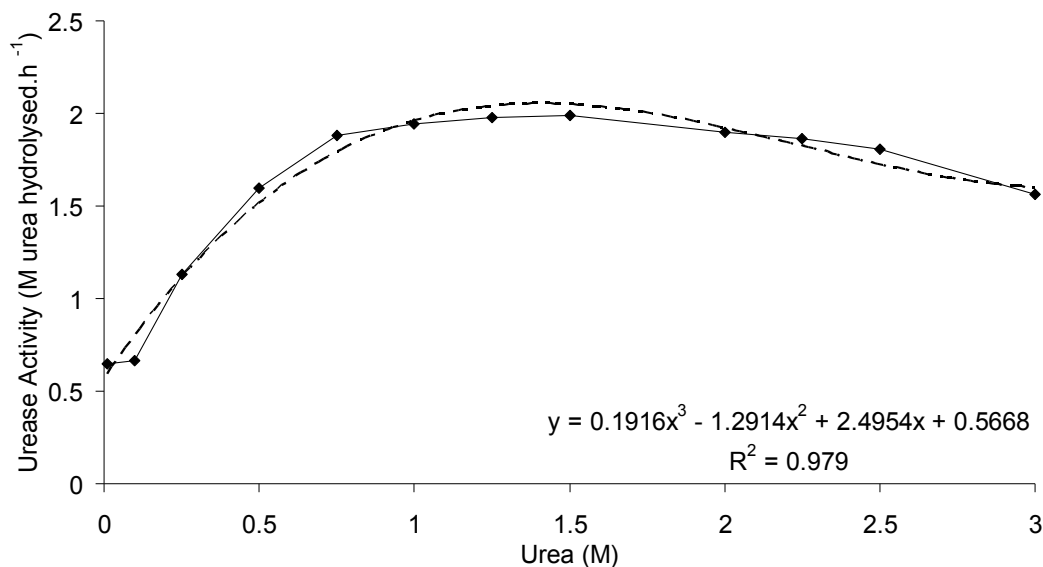


Figure 5.3: Effect of various concentrations of urea on *S. pasteurii* urease activity. Urease is maximally activated at 1.5 M urea to work three times faster than at 0.01 M urea.

High concentrations of urea were not responsible for a decrease in enzyme activity but instead resulted in an increase in enzyme activity at concentrations up to 1.5 M. Above this concentration the activation effect was increasingly reduced with 22% less activity at 3 M urea versus 1.5 M. This high tolerance to urea indicated that it was possible to form up to 3 M carbonate per flush.

5.3.1.4. Effect of calcium concentration on activity

To determine the effect of high calcium concentration on urease activity, the enzyme was tested between 0 and 3 M calcium nitrate. All tests contained 3 M urea. Urease activity was determined over the first 30 min (initial rate) and over an extended period between hours 1-5 (final rate). The initial rate of urea hydrolysis was inhibited by approximately 50% per mole of calcium nitrate present, with the enzyme completely inhibited at 2 M calcium nitrate (Fig 5.4).

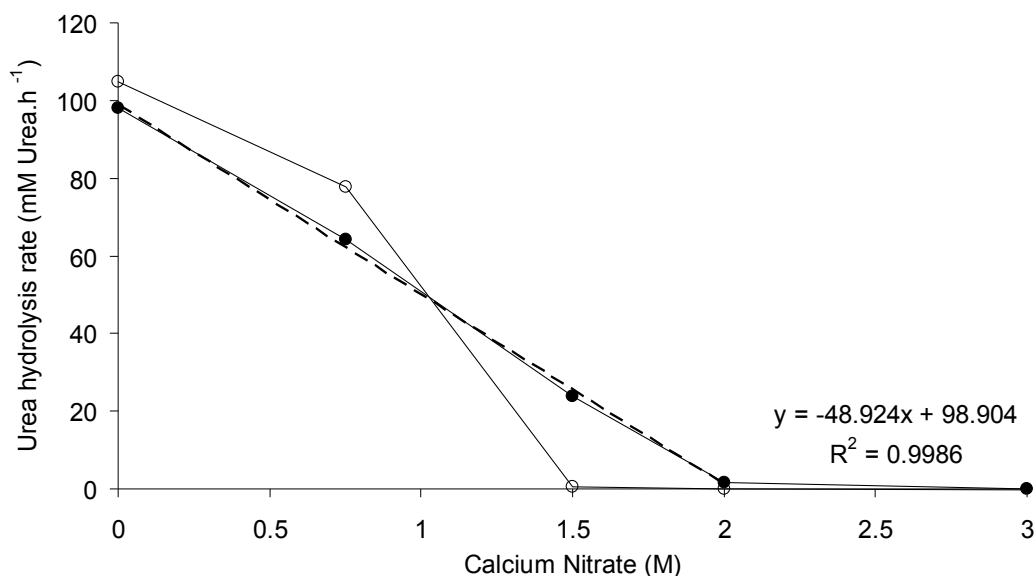


Figure 5.4: Effect of calcium nitrate concentration on urea hydrolysis rate. Urease activity was measured twice during the experiment - over the first 0.5 h (initial rate) (●) and from hours 1-5 (final rate) (○).

In this experiment, calcium was provided in the biocementation mix as calcium nitrate, which ionises in water to produce one mole of Ca^{2+} and two moles of NO_3^- ions. Calcium and nitrate concentrations may both contribute to the inhibition effect seen in Figure 5.4. Because of chemical solubility constraints, the only useful calcium sources for biocementation are $\text{Ca}(\text{NO}_3)_2$ and CaCl_2 . To take into account the effect of high concentrations of nitrate on the enzyme, two additional tests were conducted with:

- 1.5 M total calcium provided as 1.5 M $\text{Ca}(\text{NO}_3)_2$
- 1.5 M total calcium provided as 0.75 M $\text{Ca}(\text{NO}_3)_2$ and 0.75 M CaCl_2

Providing the calcium as a 50:50 mixture of nitrate and chloride salts, resulted in a 75% increase in initial activity compared to supplying the total amount of calcium as $\text{Ca}(\text{NO}_3)_2$ (Fig 5.5). The final rate was also significantly higher in the nitrate/chloride mixture compared to the nitrate only test. By using a 50:50 mixture of nitrate and chloride salts, the total amount of urea hydrolysed over the five-hour period was increased from 2 to 44% of the uninhibited reaction (Fig 5.6).

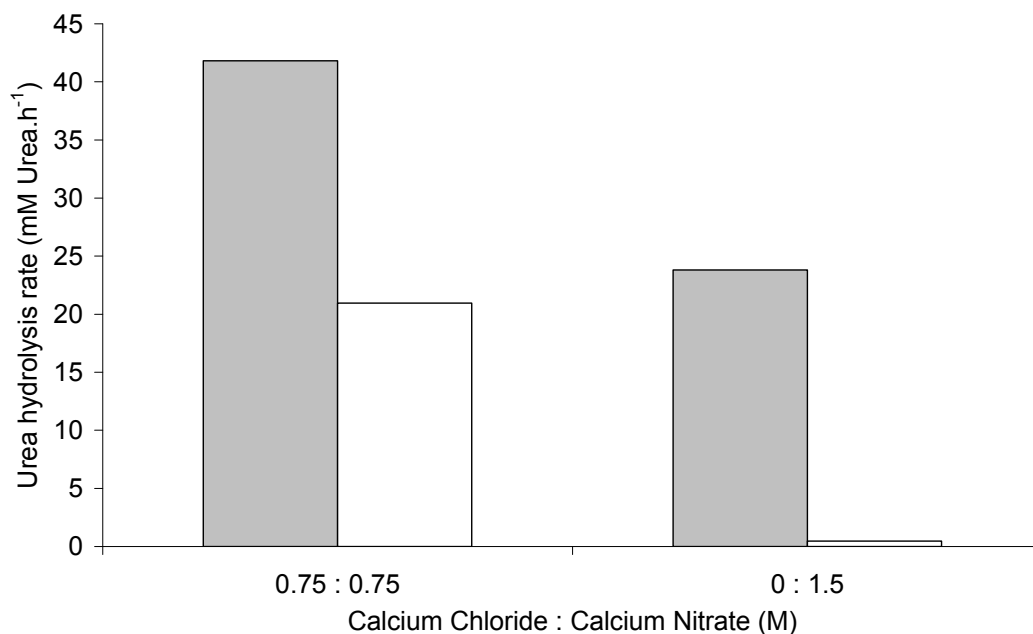


Figure 5.5: Effect of nitrate concentration on the urea hydrolysis rate. Urease activity was measured twice during the experiment - over the first 0.5 h (initial rate) (■) and from hours 1-5 (final rate) (□).

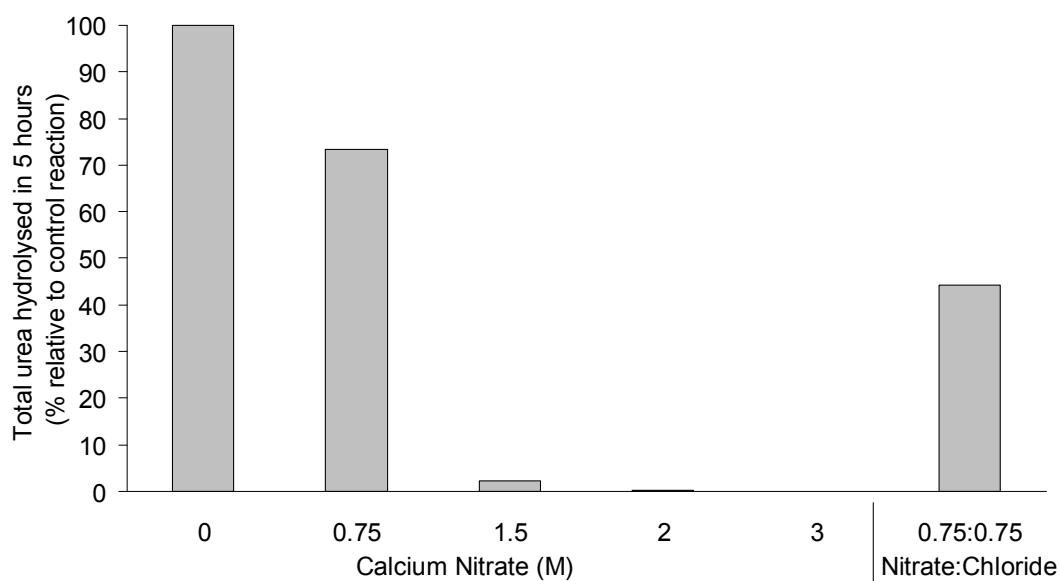


Figure 5.6: Extent of biocementation reaction after five hours at various calcium concentrations, relative to the uninhibited reaction (no $\text{Ca}(\text{NO}_3)_2$ added).

5.3.1.5. Effect of ammonium concentration on activity

High concentrations of ammonium are generated under cementation conditions. In order to determine the effect of high concentrations of ammonium on urease

activity, *S. pasteurii* cells were incubated with 1.5 M urea until the urea was completely hydrolysed. The initial pH of the system was 6.6, which quickly increased to 9.25 within 1 hour, where it remained for the rest of the experiment. The real rate of urea hydrolysis in the system decreased by 35% during the first hour of the reaction, however this decrease in activity could be completely accounted for, by the effect of pH on urease (Fig 5.7) (Appendix C). This indicated that even under high pH conditions, where the more toxic ammonia species represents approximately 50% of the total ammonium in the system, ammonium concentrations up to 3M had no effect on the rate of urea hydrolysis.

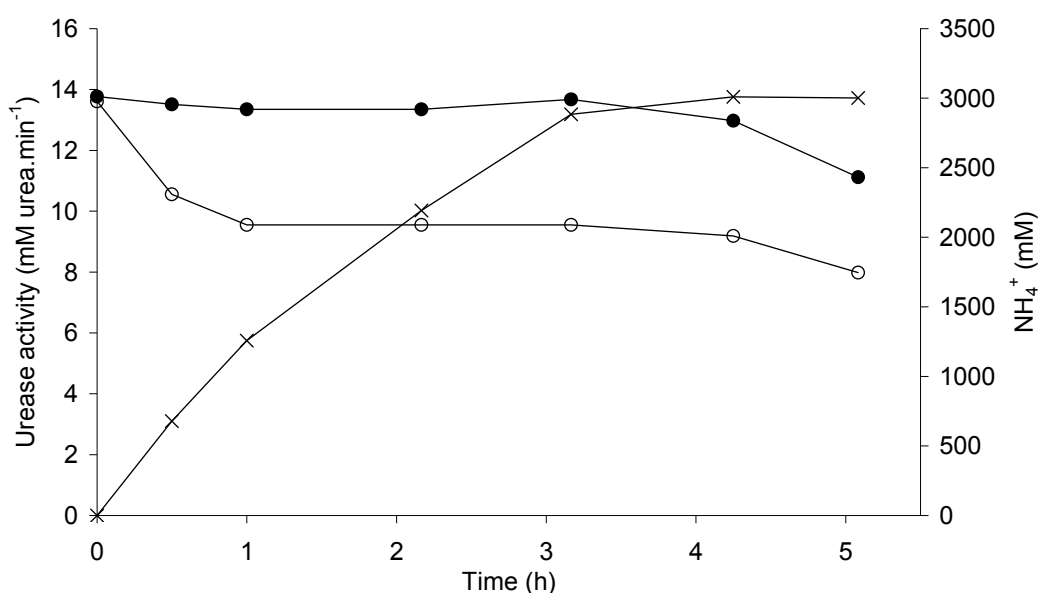


Figure 5.7: Effect of ammonium concentration on urease activity. Actual urease activity (○), urease activity adjusted for the effect of pH (Appendix C) (●) and ammonium concentration (×). Fully grown *S. pasteurii* cells were washed to remove all traces of ammonium from the growth medium prior to use. The initial pH of the solution was 6.6 which increased to 9.25 within 1 hour.

5.4. Discussion

Considering the effects of urea, calcium and nitrate concentrations on urease activity shown above (sections 5.3.1.2 - 5.3.1.4), the actual enzyme activity in the precipitating biocementation mixture must change substantially over the course of cementation, as the concentrations of the ions involved change. This means that the rate and hence the size and shape of calcite crystals that form, changes over

the course of cementation and either leads to desirable strength or non-desirable strength properties. The length of time that the enzyme works at the optimum rate for strength, will determine the overall strength properties of the cemented material.

To investigate the combined effect of these parameters on urease activity over the course of cementation, a simple model was generated based on the above data. The model assumptions were as follows:

- Urea activates urease according to Fig 5.3
- Calcium inhibits urease according to Fig 5.4
- Urease is reversibly inhibited by calcium (i.e. the inhibition is reversed as the concentration of calcium decreases) as indicated by the increase in urease activity between the initial and final rates at 0.75 M $\text{Ca}(\text{NO}_3)_2$ (Fig 5.4).
- Urease is not affected by ammonium as shown in Fig 5.7
- Urease is stable over the time course
- Urease is available to substrate at all times (i.e. not concealed by calcite precipitation)
- The reaction is conducted at 25°C

Under these assumptions, urease activity was determined by the following equations at each calculation interval:

$$\text{Urease activity}_{\text{NEW}} = \text{Urease activity}_{\text{OLD}} \times [(\text{UA}) + (\text{CI} \times \text{current Ca conc.})]$$

Urea activation (UA)

$$\begin{aligned} &= (\text{Urease activity}_{\text{OLD}} \text{ at } x \text{ M urea}) / (\text{urease activity}_{\text{OLD}} \text{ at } 0 \text{ M urea}) \\ &= (0.1916x^3 - 1.2914x^2 + 2.4954x + 0.5668) / 0.5668 \\ &(\text{where } x = \text{current urea concentration; } R^2 = 0.98; \text{ Fig 5.3}) \end{aligned}$$

Ca Inhibition (CI)

$$\begin{aligned} &= (\text{Urease activity at } 1 \text{ M Ca}) / (\text{Urease activity at } 0 \text{ M Ca}) \\ &= -48.924 / 98 \\ &= -0.489 \text{ (} R^2 = 0.98; \text{ Fig 5.4)} \end{aligned}$$

$$\text{Ca or Urea conc.}_{\text{NEW}} = \text{Ca or Urea conc.}_{\text{OLD}} - (\text{Time interval} \times \text{Urease activity}_{\text{OLD}})$$

The simulated rate of urea hydrolysis was not constant over the course of cementation and changed significantly according to the activation effect of urea and the inhibition effect of calcium. As the concentration of these two components changed over the course of cementation so did the rate of urea hydrolysis. Although the simulation indicated that the starting urease activity was relatively similar ($1.4 - 1.7 \text{ mM urea.min}^{-1}$) for all the urea/calcium concentrations tested, development of activity over the course of cementation was clearly different. Reactions with starting urea/calcium concentrations between $0.5 - 1 \text{ M}$ resulted in an immediate gradual decline in level of urease activity and were essentially complete within 20 hours. Sustained high levels of urease activity were seen for 7 and 11 hours, at 1.5 and 2 M urea/calcium respectively, before activity declined (Fig 5.8). It should be noted that the model is limited to consider only the inhibition effect of calcium, which decreases over the cementation, and not the effect of nitrate ions, which remain constant. It has been shown that nitrate also has an inhibitory effect on urease activity (Fig 5.6), however the effect was not quantified and this is a limitation of the model.

Cementation was deemed to be complete once the urea/calcium concentration decreased below 0.01 M . The modelled simulation indicated that the time required for the reaction to go to completion, was proportional to the concentration of urea/calcium added and that the average urease activity over the course of cementation was higher at urea/calcium concentration up to 1.5 M . Between 1.5 and 2 M no change in average urease activity was evident, suggesting that the enzyme is maximally activated under these conditions (Fig 5.9).

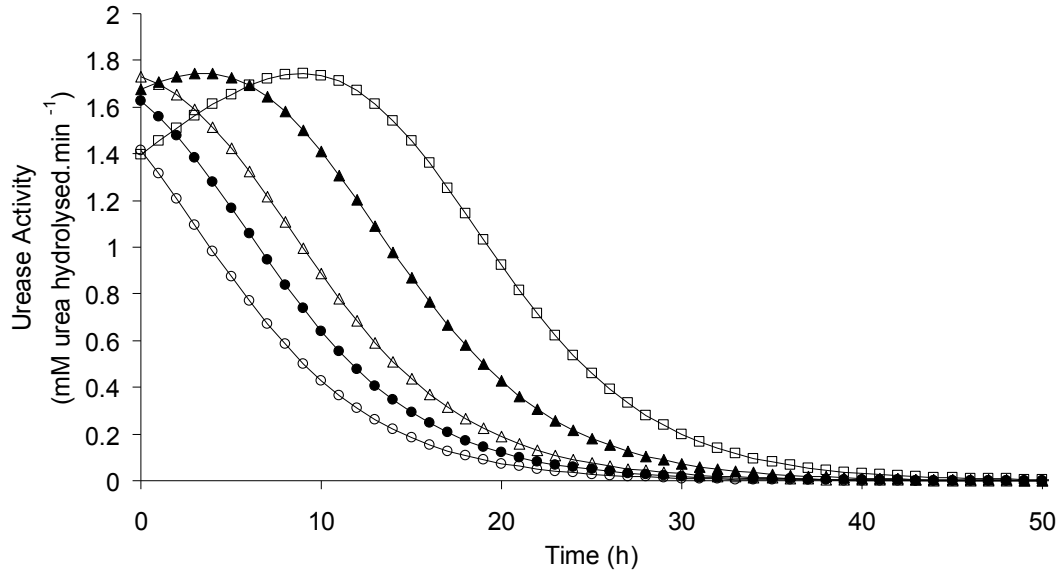


Figure 5.8: Modelled effect of different urea/calcium concentrations on the same applied urease activity during the course of cementation, taking into account activation and inhibition effects of the changing concentrations of urea and calcium as they are precipitated as calcite. Urea/calcium concentrations tested were 0.5 (○), 0.75 (●), 1.0 (△), 1.5 (▲) and 2.0 (□) M.

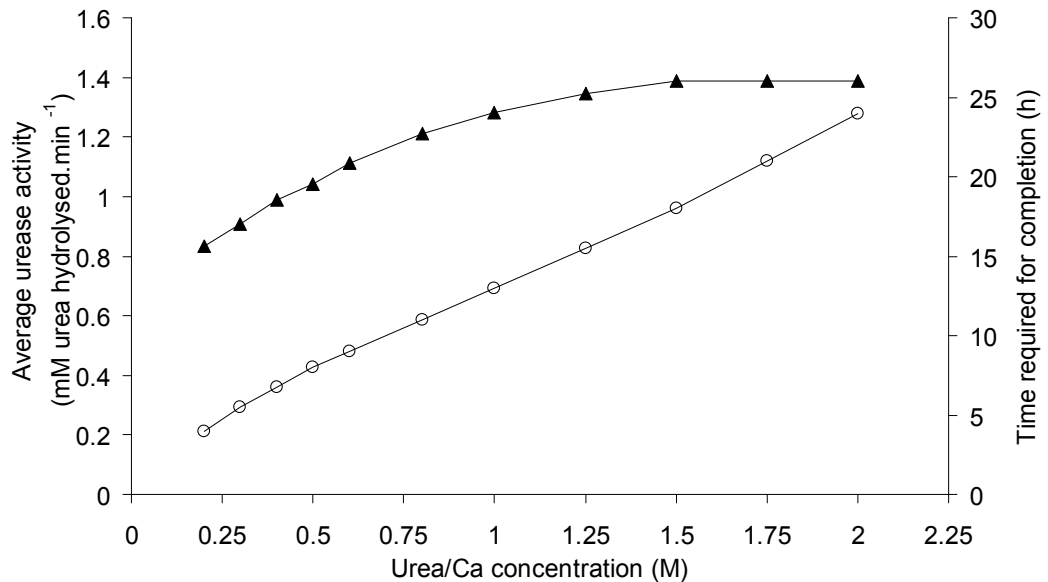


Figure 5.9: Simulated time required for the cementation reaction to go to completion (○) and simulated average urease activity (▲) over the entire cementation reaction. Completion was deemed to have occurred when urea/calcium concentrations had decreased to below 10 mM.

The large effects of urea and calcium concentration on *S. pasteurii* urease activity present a suitable means of controlling the level of urease activity during cementation. By altering the starting conditions of these two species, it is theoretically possible to generate a known effect on urease activity. For example, a low urease activity culture may still be useful for cementation, if the urea and calcium concentrations can be adjusted to provide a suitable activity. To investigate the controlling potential of both urea and calcium on urease activity, two further simulations were conducted. The first simulation was conducted with 1.5 M calcium and various concentrations of urea (Fig 5.10) and the second with 1.5 M urea and various concentrations of calcium (Fig 5.11).

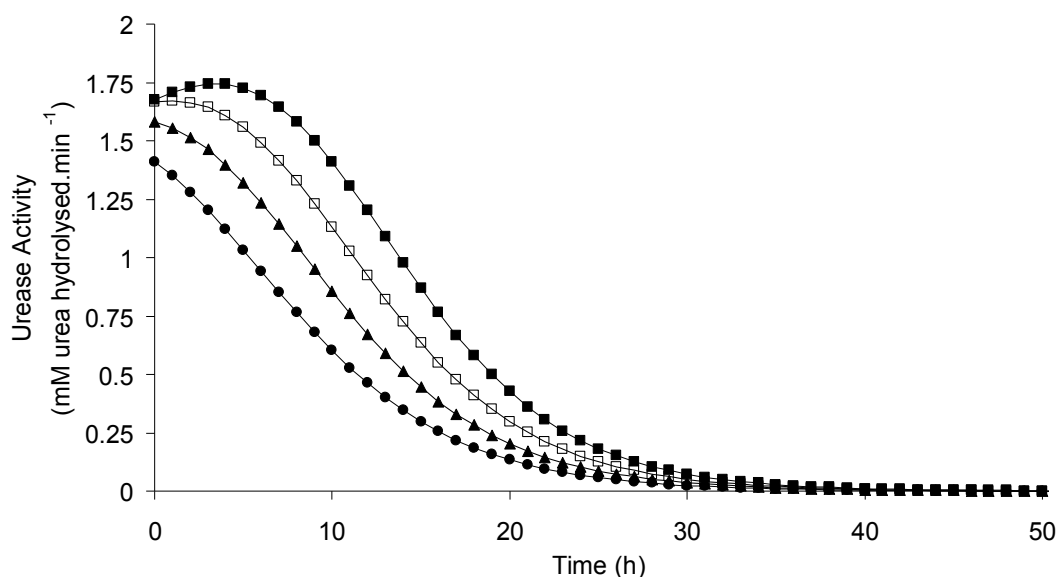


Figure 5.10: Simulated urease activities over the course of cementation with 1.5 M calcium and various concentrations of urea (0.75 (●), 1.0 (▲), 1.25 (□) and 1.5 (■) M).

Results from the model indicated that varying urea concentration produced a larger range and thus greater control over urease activity compared to varying calcium concentration. A two-fold increase in urea concentration resulted in approximately doubled urease activity for most of the cementation time (Fig 5.10). A two-fold increase in calcium concentration lowered urease activity by approximately 10% over the first 8 hours of cementation, with no effect observed after this (Fig 5.11). It must be acknowledged that providing disproportionate

concentrations of urea and calcium will of course only produce the lower concentration of calcite cement, but this method may provide a means to convert low enzyme activity preparations into useable enzyme for cementation.

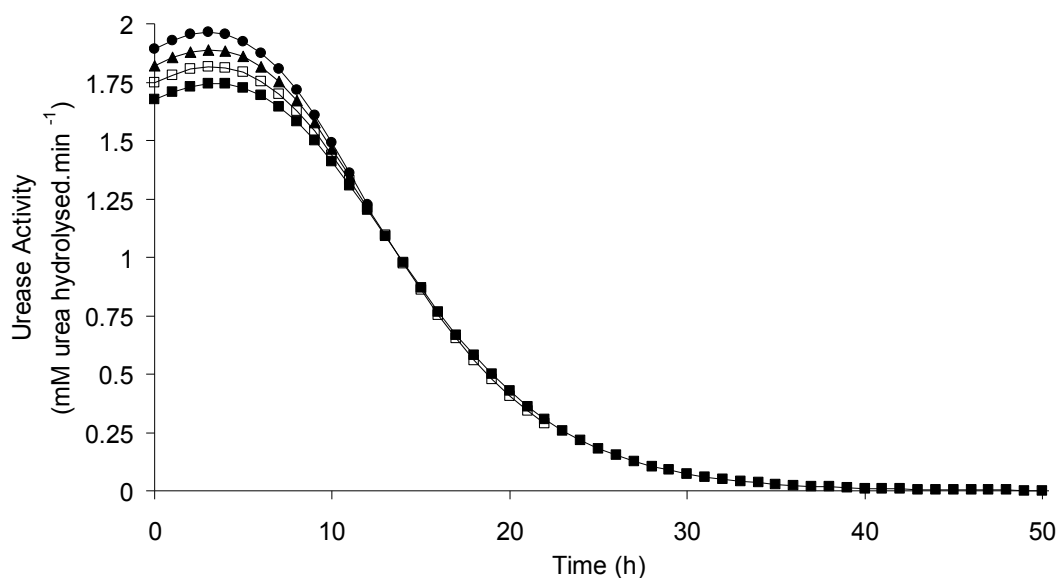


Figure 5.11: Simulated urease activities over the course of cementation with 1.5 M urea and various concentrations of calcium (0.75 (●), 1.0 (▲), 1.25 (□) and 1.5 (■) M).

The activation of urease at high concentrations of urea may be attributed to a higher diffusivity of urea into the cell, due to an increased concentration gradient across the cell membrane. Urea is a small, uncharged molecule that can move easily across the cell membrane. Unlike some other urease positive bacteria, *S. pasteurii* does not have an active uptake system for urea (Morsdorf and Kaltwasser, 1989), which indicates that its uptake of urea is dependent on diffusion kinetics. If the concentration gradient across the cell membrane is increased, and the enzyme is not saturated, it would be expected that the urease activity inside the cell would increase with an increase in external concentration of urea. This could be tested by lysis of the cells and measuring the rate of urease hydrolysis in the lysed urease extract compared to whole cells exposed to the same external concentration of urea.

It is clear that the rate of urea hydrolysis is not constant over the course of cementation, but largely controlled by the concentrations of urea and calcium present. If good mechanical strength is achieved at an optimum precipitation rate (or within an optimum rate range) then it should be possible to control the mechanical strength properties of the cemented material by applying the concentration of urea/calcium that can maintain the desired urea hydrolysis rate (or range rate) for the longest time. An investigation into which urea hydrolysis rate gives which strength property to the cemented material, is presented in the following chapter.

In summary, urease from *S. pasteurii* is a suitable enzyme for use under biocementation conditions. It is functional in temperatures up to at least 80°C and can tolerate concentrations of up to 3 M urea, 2 M calcium when supplied as $\text{Ca}(\text{NO}_3)_2$ and even higher concentrations when calcium is supplied as a 50:50 nitrate/chloride mixture. Ideally and if time had permitted, it would have been desirable to fully investigate the effects of supplying different concentrations of calcium as a 50:50 nitrate/chloride mixture to establish the maximum tolerance of total calcium.

Biocementation trials

6.1. Introduction

The previous chapter indicated that urease from *Sporosarcina pasteurii* is functional under the general conditions needed for biocementation. It has been established that the rate of urea hydrolysis during the cementation reaction is not constant during cementation, but largely controlled by the concentrations of soluble urea and calcium present, which are constantly changing due to precipitation of solid calcium carbonate from the system. It was desirable to know the minimum concentration of urease necessary to still produce good mechanical strength and to investigate the effect of different concentrations of enzyme on the binding strength of the cement.

Calcite crystal shapes and sizes are extremely varied in nature and are often highly complex structures, with over 300 different forms previously described (Klein and Hurlbut, 1999). Because the solubility product (K_{sp}) of calcite is extremely low ($3.3 \times 10^{-9} \text{ mol.L}^{-1}$ at 25°C (Sawada, 1998)) when compared to its constituent ions, it is straightforward to achieve the supersaturation required to precipitate calcium carbonate crystals from solution. Precipitation can be achieved by simply mixing together moderate concentrations of soluble Ca^{2+} and CO_3^{2-} ions. As soon as the concentrations of both Ca^{2+} and CO_3^{2-} ions exceed 3.3 nmol.L^{-1} at 25°C , calcite will precipitate. The types of crystals that form when the reaction happens very quickly are soft, powder-like crystals which starkly contrast against very hard, naturally occurring limestone and cave stalactites/stalagmites which form very slowly. If the rate is too fast, little strength is achieved, but with a slower rate of formation, the binding strength of calcite can be significantly improved.

Crystals form from solution when the solute concentration in a solvent exceeds its solubility, which is termed supersaturation (Randolph and Larson, 1988). The formation of crystals proceeds in two phases, firstly the formation of nuclei (nucleation) where new crystals are formed, and secondly a phase where very few new nuclei are formed and the growth of existing crystals dominates (Mullin,

1993; Sawada, 1998). Nucleation is a complex phenomenon which is not yet well understood outside of pure species systems. Nucleation is affected by temperature, the degree of over-saturation, and the presence of other surfaces (e.g. dust, vessel walls, colloids) (Nyvlt *et al.*, 1985). It has been suggested that bacterial cells themselves can act as nucleation sites for the formation of crystals (Ferris *et al.*, 1987). It is clear that the activation energy required for a critical nucleus to form decreases with an increase in saturation and that the presence of other particles increases the ease with which nuclei form (Nyvlt *et al.*, 1985). The biocementation system is very far from the pure systems that have been studied, and contains many other factors which can influence crystal growth (e.g. sand grains, dust, waste products from the culture broth, presence of high concentrations of NH_4^+ , Cl^- and NO_3^- and the presence of bacterial cells), thus the type, shape and size of crystals produced could not be predicted and measured with any degree of certainty in this study.

Rather than determining the type of crystals that are precipitated during biocementation, a more functional parameter of the overall effect of a biocementation treatment is the improvement in mechanical strength. Ideally, this would be determined by directly measuring the strength before and after treatment, by increasing the stress on the sample under confining pressure and determining the shear failure (breaking) point. The nature of this type of direct strength testing is destructive and sample dimensions required for accurate determination were outside the realm of laboratory scale experiments. Instead, ultrasonic velocity was used to determine strength. The velocity of ultrasonic waves through matter is a non-destructive and widely used technique to determine the properties of many different materials including stainless steel (Vasudevan and Palanichamy, 2003), metal alloys (Schneider, 1997), concrete (Ramyar and Kol, 1996), crude oil (Fukushima and Ichimura, 1959), red blood cells (Hianik *et al.*, 2000), bone (Turner and Eich, 1991) and kidney stones (Singh and Agarwal, 1990). Ultrasound velocity can also be used as a direct estimation of geomechanical properties including mechanical strength of rock formations (Schön, 1996).

The strength properties of rocks mainly depend on the bonding type and quality of the solid particles and the internal structure of the rock skeleton. For unconsolidated sediments such as sand, the strength is dependent upon the degree of cementation between the sediment particles (sand grains). The uniaxial compressive strength for some naturally occurring rocks are given in table 6.1 (Schön, 1996).

Table 6.1: Uniaxial compressive strength of natural rocks (Adapted from Schön (1996) and Klein & Hurlbut (1999)).

Rock Type	Calcite content (%)	Uniaxial compressive strength (MPa)
Limestone	100	90 – 120
Sandstone	40 - 80	35 – 150
Unconsolidated sand	0	0.004 – 0.012

A correlation exists between uniaxial compressive strength and ultrasound velocity for fine-medium grained natural sandstone (McNally, 1987). The sandstone was sourced from the German Creek formation (Queensland, Australia) and the following mathematical relationship was derived from 142 samples ($R^2 = 0.91$) (Fig 6.1).

$$\sigma = 1277 \times \exp\left(\frac{-11200}{v}\right)$$

Where:
 σ = uniaxial compressive strength (MPa)
 v = ultrasonic velocity (m.s^{-1})

Such an exponential relationship between strength and ultrasonic velocity exists for all materials (Schön, 1996). It must be stressed however, that the absolute values of the relationship given in Figure 6.1 are only valid for this particular rock formation. For this reason, results using biocemented rocks are given as ultrasonic velocities in m.s^{-1} . Where it has been necessary to interpret the data with respect to strength, the relationship derived by McNally (1987) has been used. All interpretations based on calculated strength have been made considering the relative changes only. The baseline value (where the x-axis intercepts the y-axis) is given as the velocity of ultrasonic waves through water, which is 1500 m.s^{-1} .

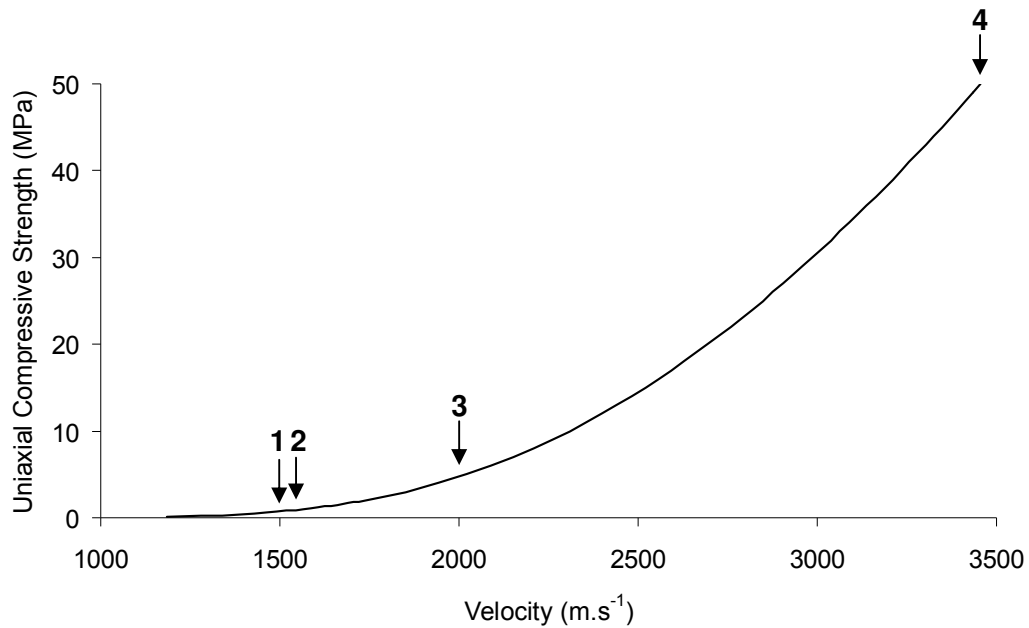


Figure 6.1: Relationship between uniaxial compressive strength and velocity for fine-grained sandstone from the German Creek formation, Queensland, Australia (McNally, 1987; Schön, 1996). The numbered points indicated typical ultrasonic velocities for water (1), unconsolidated sand (2), soft rock (3) and hard rock (4).

Urease is the most expensive consumable component of the biocementation process. In order to efficiently use urease, it was necessary to determine the relative strength generated at different urea hydrolysis rates. For example, can more strength be achieved with two treatments at a low urease activity or one treatment with double the urease activity? In addition, it was also important to know how strength was imparted to the material during cementation, to determine when any additional treatments should be applied. Finally, alternative methods of applying urease were considered for potential ways to reuse the enzyme in subsequent treatments.

In summary, the objectives of this chapter were to determine:

- the effect of urea hydrolysis rate on strength
- the nature of strength development, during the cementation reaction
- potential methods for reusing urease in subsequent treatments.

6.2. Materials & Methods

6.2.1. Experimental Set-up

6.2.1.1. Application of cementation reactants into the core

Cores for cementation consisted of 50 ml plastic syringes that were dry packed with $-300\ \mu\text{m}$ Si-sand under continuous vibration to give an even density. Cores were then up-flushed with water and tapped to remove air pockets. After flushing with water, the volume occupied by the sand decreased due to the lubrication effect between the sand particles and the stopper was adjusted to maintain a confining pressure. Ca/urea solution and cells were premixed immediately before injection into the core by pouring both liquids into a single vessel and twice drawing and expelling the liquid with a second 50 ml syringe, to ensure proper mixing. One and a half times the pore volume was flushed in to ensure full displacement of water.

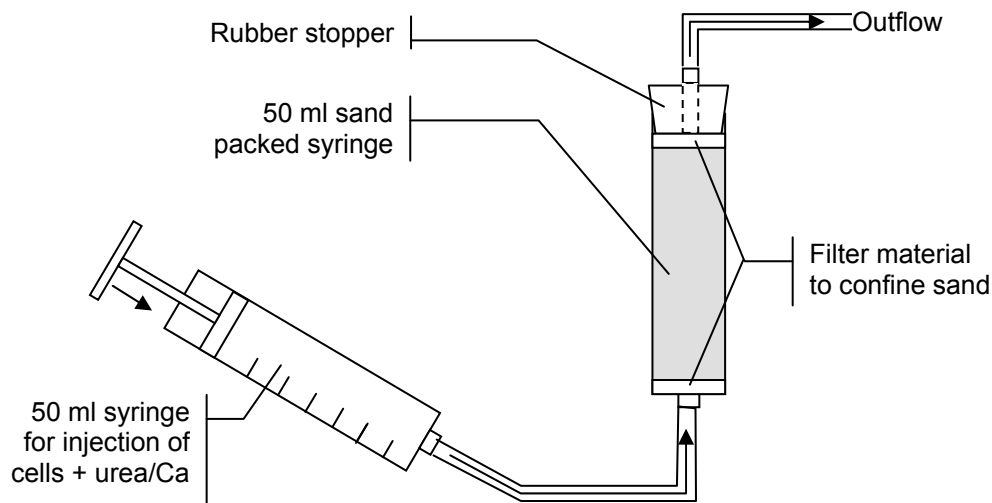


Figure 6.2: Method for applying cementation reactants into a sand core for cementation.

6.2.1.2. *In-situ* cementation measurements

Ultrasound waves were sent through the diameter of each core at three positions along the length (15, 45 and 75 mm from the injection end) (Fig 6.3). Measurements were taken repeatedly over the course of the experiment. Simultaneously, 1 ml samples were withdrawn from the cores through a capillary tube inserted down the side of the core to avoid ultrasound signal disruption (see

Fig 6.3 insert). The samples were centrifuged at 13 500 rpm to remove any suspended particles (sand or bacteria). The supernatant was then transferred to a clean tube and stored at -20°C awaiting ammonium analysis.

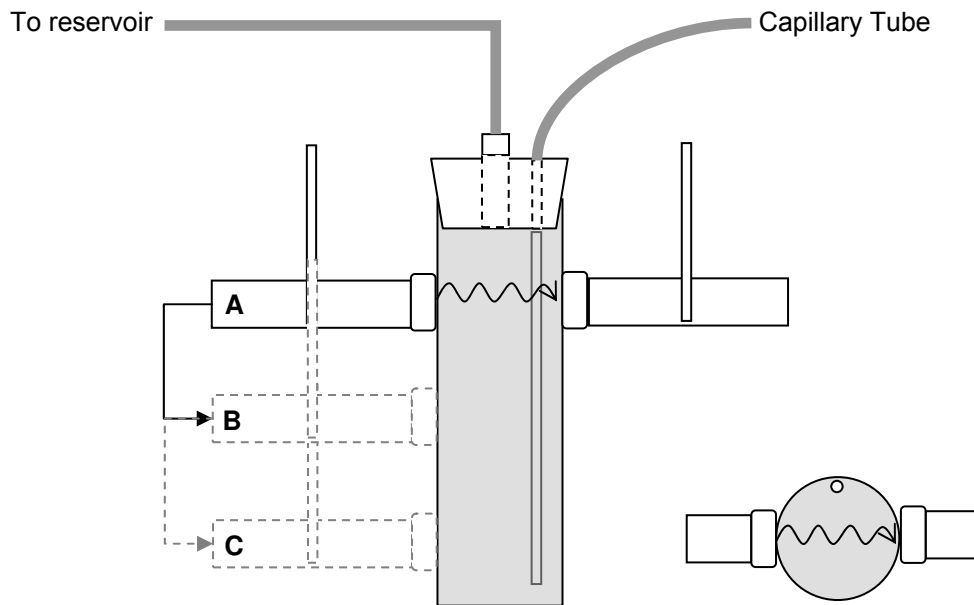


Figure 6.3: Schematic of core set-up for measuring ultrasound velocity during the rate change over the course of cementation experiment. One transducer sends the ultrasonic pulse and the other receives it. Transducers recorded measurements at three positions along the length (15 mm (A), 45 mm (B) and 75 mm (C) from the injection point). Inset: Top view of core indicating position of capillary tube and path of sound wave.

6.2.2. Analytical Methods

6.2.2.1. Velocity Measurements

Velocity was determined using ultrasonic p-wave piezoelectric transducers, which produced a series of sound signals on one side of the core, which were received by the opposite transducer. The time taken for the ultrasonic signal to travel through the sample from the source to the receiver was recorded. The transducers were Panametrics type V303S with a dominant frequency of 1 MHz. The signal was then digitised using a Nicolet 430 digital oscilloscope, which is a two-channel 12-bit system with a sample interval of 100 nanoseconds (Sherlock, 1999). Five repeated signals were recorded at each position (Fig 6.4). The average arrival time for the maximum amplitude peak was measured and used to determine the

ultrasonic velocity through the width of the core, according to the following equation:

$$\text{Velocity (m.s}^{-1}\text{)} = \frac{\text{Internal diameter of Core}}{[(\text{Average arrival time} - \text{Delay}) \times 1000000]}$$

Where: the units of internal core diameter are (m), the units of velocity are (μs) and delay is the time (in μs) taken for the signal to travel within the source and receiver transducers and through the syringe walls (i.e. Average velocity minus delay gives the time taken to travel through the internal diameter of the core only). The delay was $18.3 \mu\text{s}$.

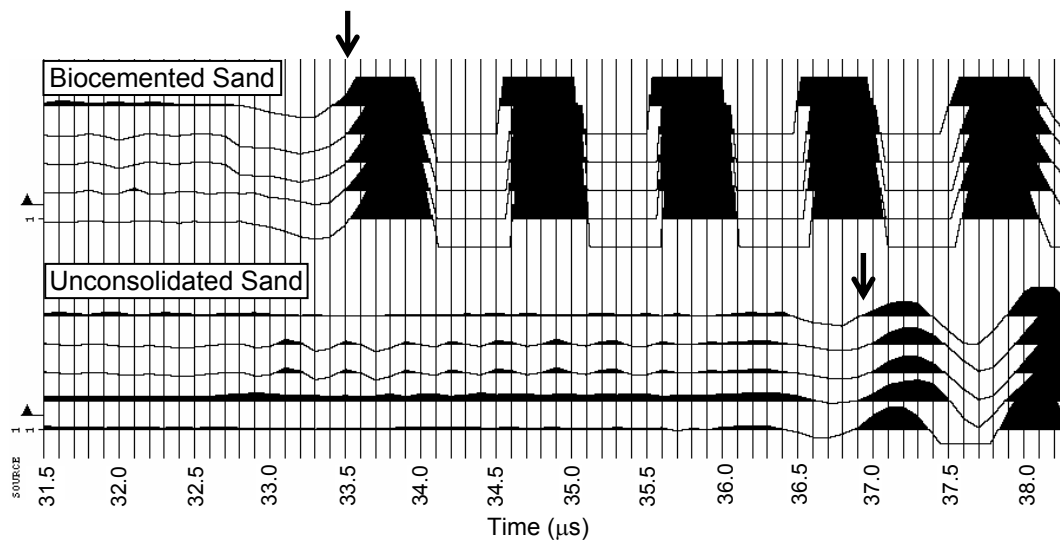


Figure 6.4: Example of ultrasonic data used to calculate velocity in unconsolidated and biocemented sand samples. Time is recorded along the x-axis. Five replicate signals at each position were averaged and used to calculate velocity. Note that the arrival time for the sound wave to reach the opposite transducer was significantly longer in unconsolidated sand compared to biocemented sand (indicated by vertical arrows).

6.2.2.2. $\text{NH}_4\text{-N}$ Analysis

Ammonium concentration was determined as described previously (Chapter 2 – Section 2.2).

6.3. Results

6.3.1. Cementation Trials

6.3.1.1. Effect of Enzyme Rate on Strength

The effect of enzyme rate on strength was determined by measuring the velocity of ultrasound waves across the diameter of the cores. A higher degree of cohesion

(cementation) will allow the sound wave to travel at a faster velocity (i.e. the faster the velocity - the greater the degree of cementation). Eight cores were treated with 1.5 M equimolar urea/calcium solutions (calcium supplied as 0.75M $\text{Ca}(\text{NO}_3)_2$ and 0.75 M CaCl_2) and cemented at various rates with different concentrations of urease. Two flushes were performed and ultrasound measurements were taken at three positions (A, B and C) after 24 hours (Fig 6.3).

The velocity measurements at the three positions (A, B and C) on each core were within $\pm 10\%$, indicating that the degree of cementation along the core length was uniform (Fig 6.5). To obtain an overall core result, the velocities for the three positions were averaged (Fig 6.6A).

After one biocementation treatment, strength was observed to increase with increasing enzyme activities between 2.2 – 13.3 mM urea.min⁻¹ (Fig 6.6B). Treatments with urea hydrolysis rates above 13.3 mM urea.min⁻¹ did not exhibit any difference in strength. A different response was seen after a second treatment had been applied. All cores that were treated with enzyme activities between 4.4 – 13.3 mM urea.min⁻¹ had attained similar strengths, with less strength observed in cores with higher urease activities (Fig 6.6B).

It is desirable to obtain maximum cementation strength for the cost of the process. In order to assess the improvement in strength relative to the amount of enzyme applied, the amount of strength was divided by the amount of enzyme input into the system. Enzyme input was calculated over both flushes (e.g. if two applications of 4.4 mM urea.min⁻¹ produced a strength of 18 MPa, the strength improvement relative to enzyme input would be $18 \div 8.8 = 2.05 \text{ MPa} \cdot (\text{mM urea} \cdot \text{min}^{-1})^{-1}$ (Fig 6.7). Similar amounts of strength were achieved per enzyme input after one treatment but more strength was achieved at low compared to high enzyme input after two treatments. Six-times more strength was achieved per enzyme input from two applications at 4.4 mM urea.min⁻¹ compared to two applications at 17.8 mM urea.min⁻¹ (Fig 6.7).

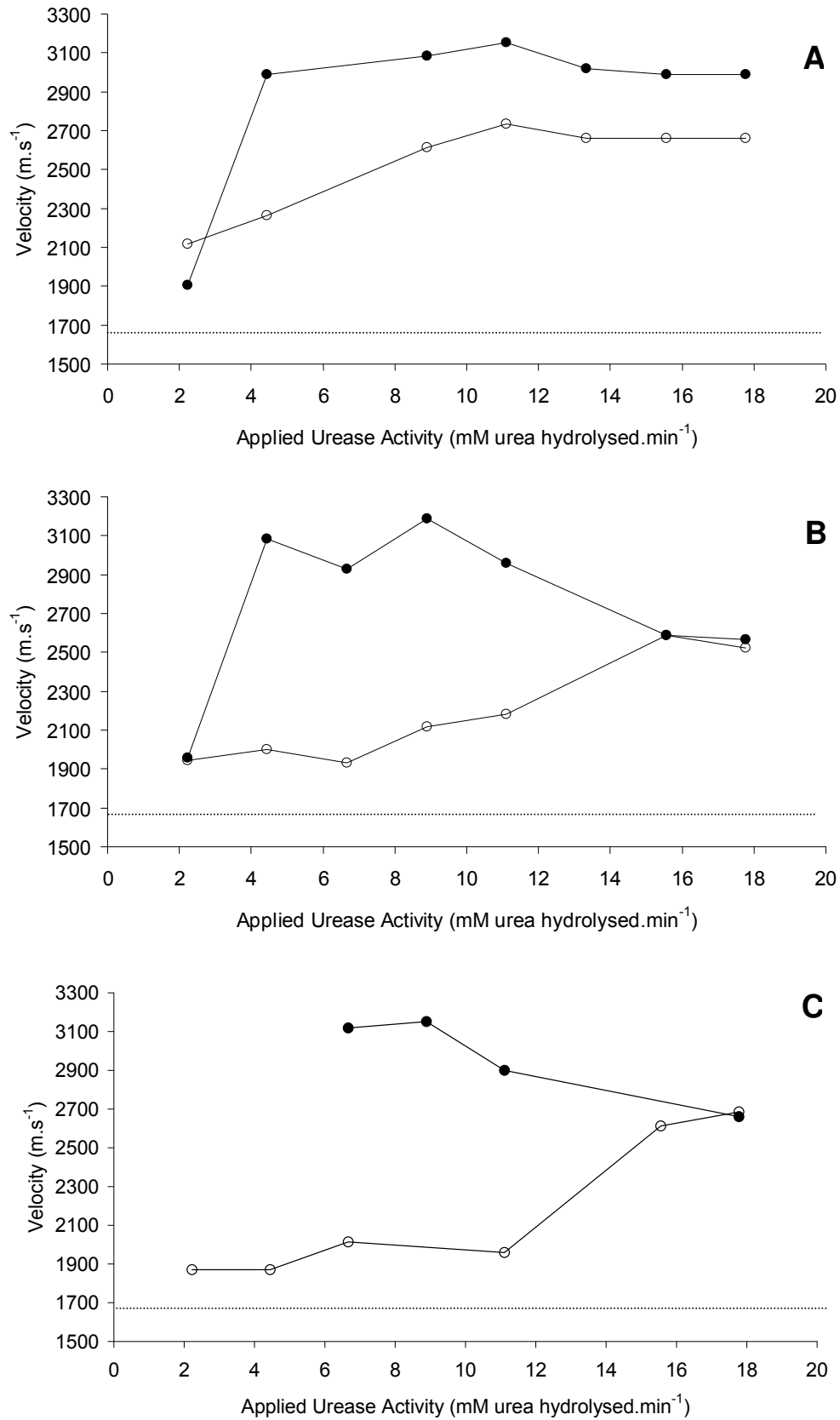


Figure 6.5: Velocity versus urease activity at three positions along the 90 mm core length (A=15; B=45; C=75 mm) from injection point for first (○) and second (●) biocementation treatments with 1.5 M equimolar urea and calcium solution, after 24 hours. The dotted line at 1675 m.s⁻¹ indicates the velocity through urea/Ca and sand only (without bacteria).

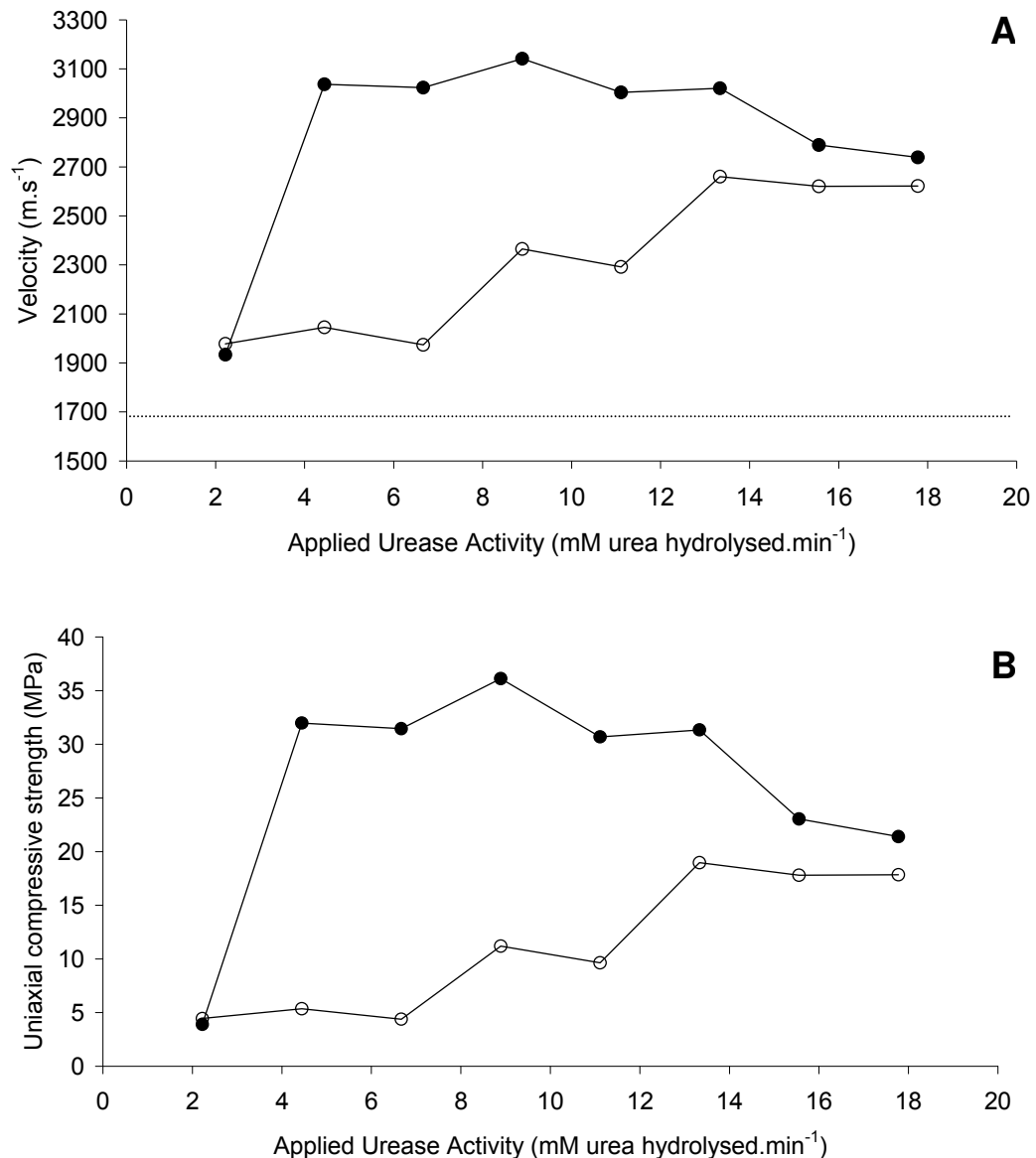


Figure 6.6: Average velocity (A) and uniaxial compressive strength (B) recorded over the entire 90 mm core length for first (O) and second (●) treatments with 1.5 M equimolar urea and calcium solution, after 24 hours. The dotted line on the velocity figure at 1675 m.s⁻¹ indicates the velocity through urea/Ca and sand only (without bacteria).

6.3.1.2. Strength Development During the Course of Cementation

To investigate the strength development during the course of cementation, the velocities of four cores were continuously monitored over a 42 hour period, during which two applications of bacteria and reactants were applied. This allowed observation of strength changes as the reaction was proceeding.

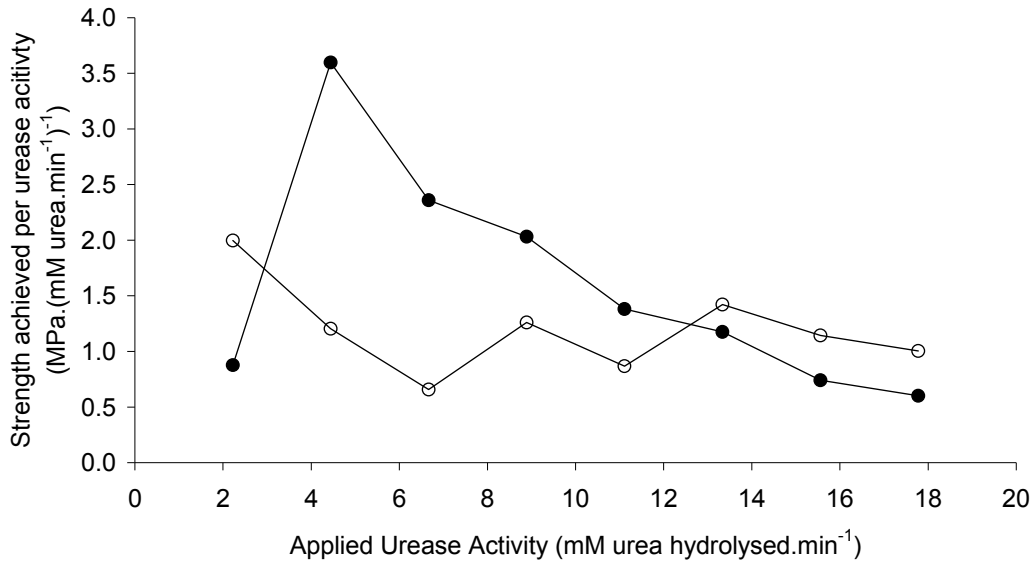


Figure 6.7: Strength achieved per urease activity input for first (○) and second (●) biocementation treatments.

The first biocementation treatment only weakly reacted, indicating a problem with the bacterial enzyme and this treatment was considered unsuccessful (Appendix E, Fig E.2). The cores were flushed with water to remove reactants and re-treated with a second application (Fig 6.8). The starting velocity at time zero was not consistent (2100 – 2600 m.s⁻¹) between each core, due to a small amount of cementation during the first application (Appendix E).

An increase in ultrasound velocity was evident in all locations tested, except for 15 mm and 45 mm positions on the core that was treated with the lowest enzyme activity (6 mM urea.min⁻¹) (Fig 6.8). The degree of strength development was different at each position along an individual core, suggesting that the deposition of cement was not uniform (Fig 6.9).

The production of ammonium substantially slowed down or stopped completely in all cores after 4 hours (Fig 6.9). The cessation of ammonium production in the 12 mM urea.min⁻¹ activity core, was explained by depletion of urea at this time (3000 mM NH₄⁺ = 1.5 M urea) however the other cores stopped before the reaction had gone to completion.

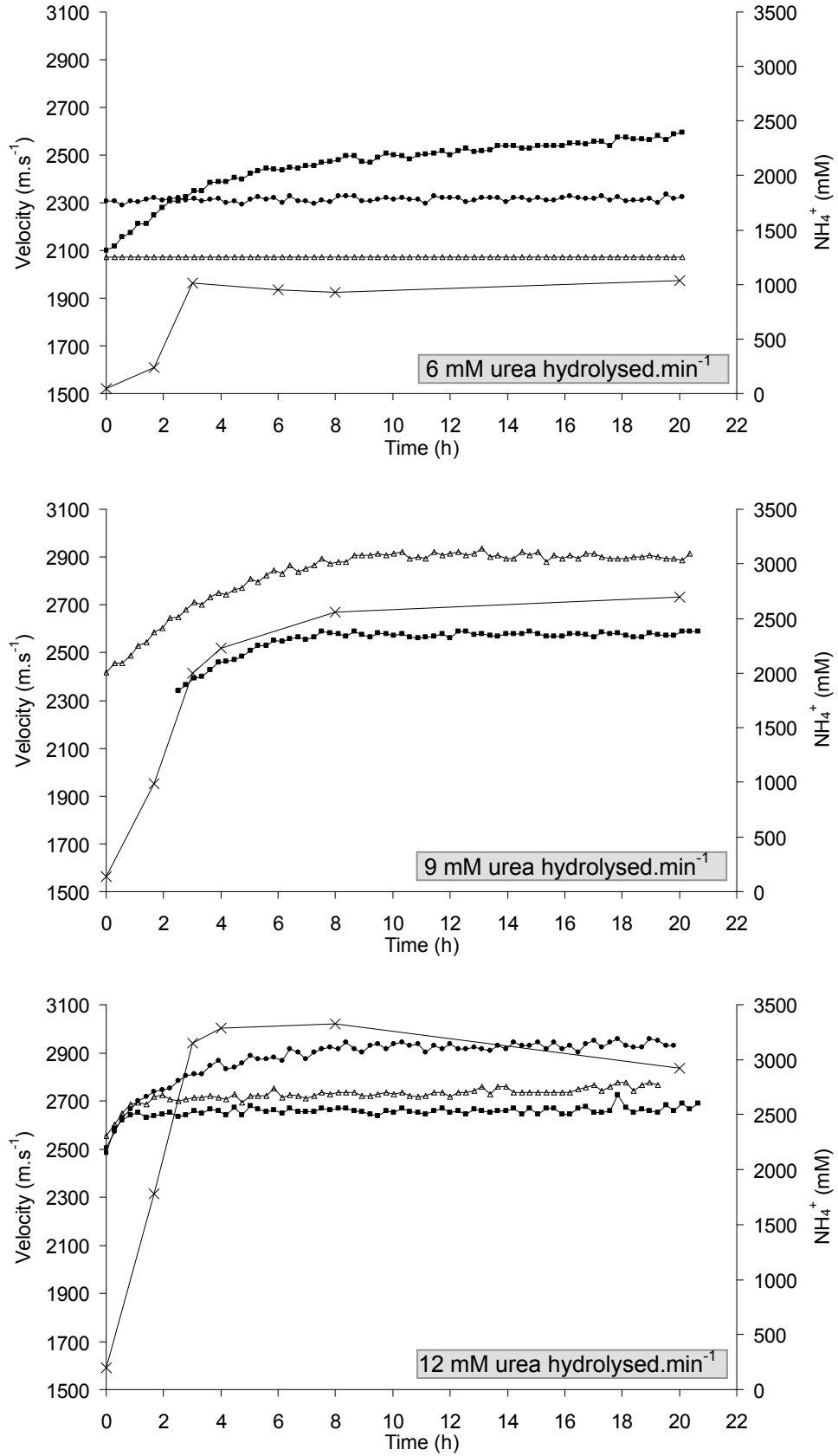


Figure 6.8: Effect of different bacterial enzyme concentration on velocity development during cementation at three positions along core (15 mm (●); 45 mm (△) and 75 mm (■)) and concomitant production of ammonium from urea hydrolysis (×). Urease was applied as whole bacterial cells at various activities as stated on each chart.

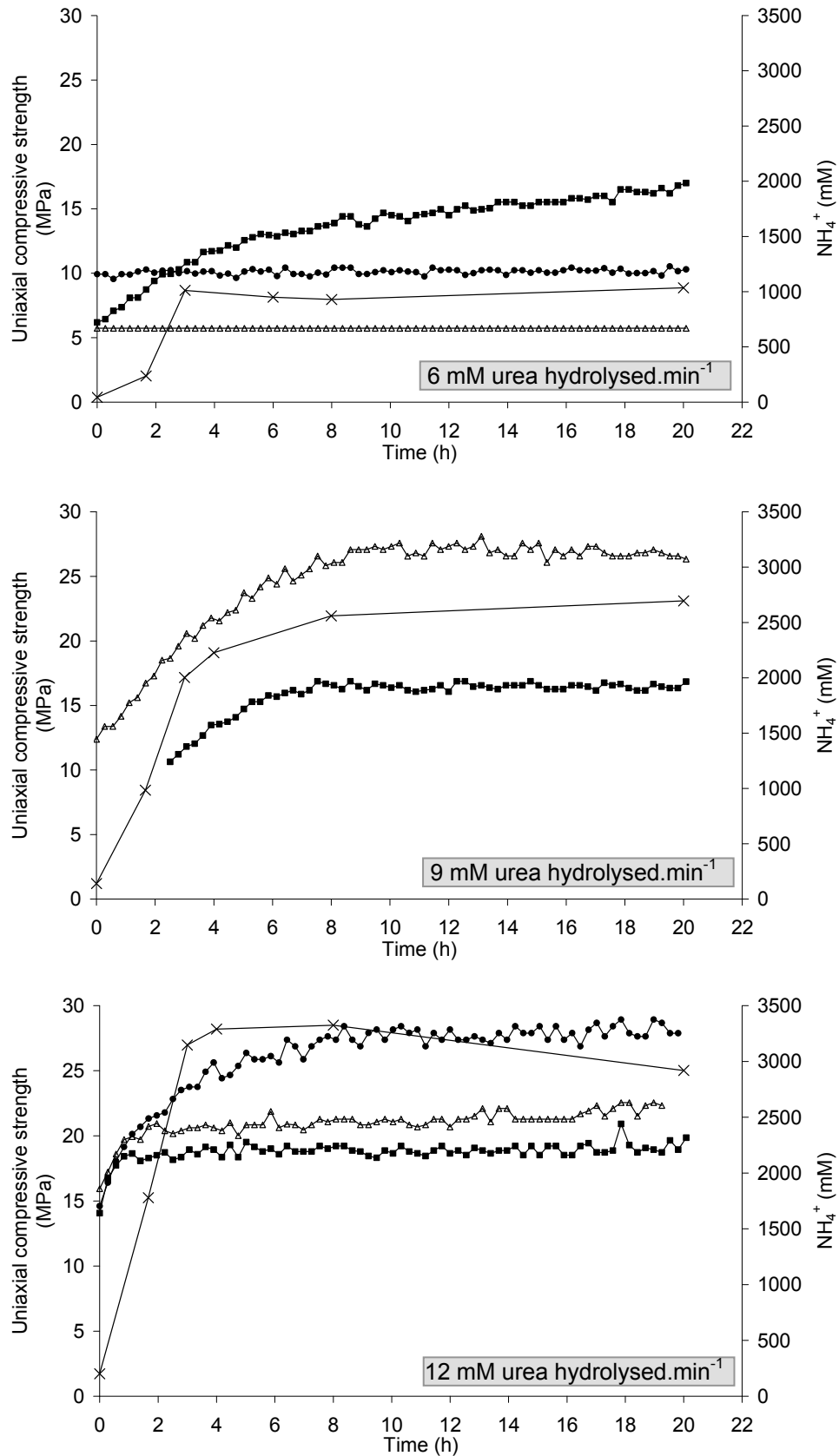


Figure 6.9: Effect of different bacterial enzyme concentrations on strength development during cementation at three positions along core (15 mm (●); 45 mm (△) and 75 mm (■)) and concomitant production of ammonium from urea hydrolysis (×). Urease was applied as whole bacterial cells at various activities as stated on each chart.

Comparison of the bacterial enzyme system to the soluble plant enzyme system showed a marked difference in the nature of velocity and strength improvement. Soluble plant enzyme produced a linear and constant improvement in all positions along the core length, which was also reflected in the ammonium production rate (Fig 6.10), suggesting that the non-uniform distribution in the bacterial system was due to the particulate bacterial cells. The in-situ rate of ammonium production was significantly less in the soluble plant system compared to the same applied activity in the bacterial system (Fig 6.8, 6.9), indicating that the bacterial system was more tolerant under cementation conditions. Note that the extent of

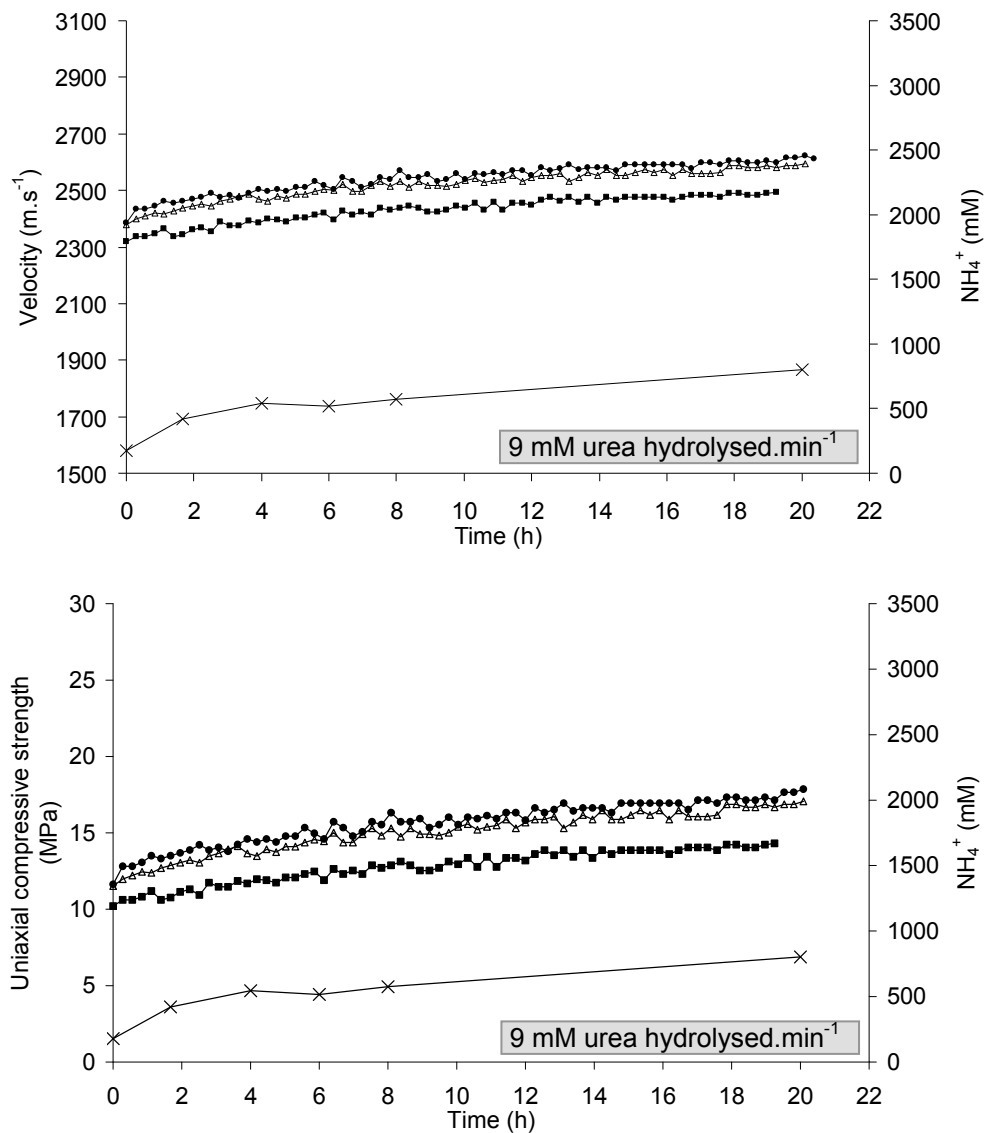


Figure 6.10: Velocity and strength improvement over time using soluble plant enzyme at three positions along the core length from the injection point (15 mm (●); 45 mm (△) and 75 mm (■)) and ammonium concentration (×) during the cementation reaction. Urease was applied as soluble enzyme at 9 mM urea hydrolysed.min⁻¹.

the cementation strength improvement in the plant system was still sufficient for the core to become hard enough to be easily handled, with a final velocity in the range of soft rock (Fig 6.1).

Ammonium concentration was monitored during the experiment which allowed the in-situ urea hydrolysis rate to be calculated. The rate inside the core, under cementation conditions was lower than the applied activity and was variable over the course of the cementation, which gave the same trend as the modelled rate of urea hydrolysis (Fig 6.11, 5.8). Initial activities were lower than the applied activity, which was to be expected due to the effect of high Ca concentrations in the cementation mixture, however the activity in all cores became close to zero at approximately 6 hours. The urea hydrolysis rate inside the core was much higher for the bacterial enzyme system compared to the same applied activity of soluble plant enzyme (Fig 6.11).

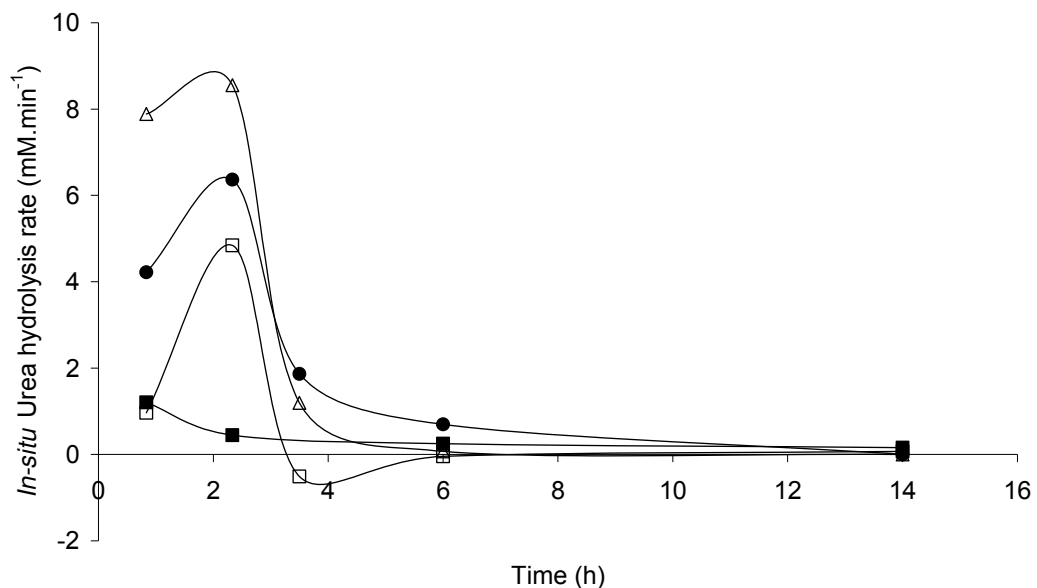


Figure 6.11: *In-situ* urea hydrolysis rate inside the core at different whole-cell bacterial urease activities: 6 mM (□), 9 mM (●) and 12 mM (△) urea hydrolysed.min⁻¹ and soluble plant enzyme: 9 mM urea hydrolysed.min⁻¹ (■).

In order to elucidate the urea hydrolysis rate that produced the highest cementation strength, the change in strength per mM of urea hydrolysed was calculated and compared against the urea hydrolysis rate for each interval. Data

was calculated for the medium ($9 \text{ mM urea.min}^{-1}$) and high ($12 \text{ mM urea.min}^{-1}$) activity cores (Fig 6.12, 6.13).

In all three cases, the highest gain in strength per urea hydrolysed occurred at low urea hydrolysis rates. Despite similar urea hydrolysis rates observed in both cores over the 3 – 4 and 4 – 8 h intervals, the same urea hydrolysis rates did not produce the same strength improvement over these intervals (Fig 6.12, 6.13). This suggested that cementation factors other than urea hydrolysis rate also played a role in the degree of strength developed.

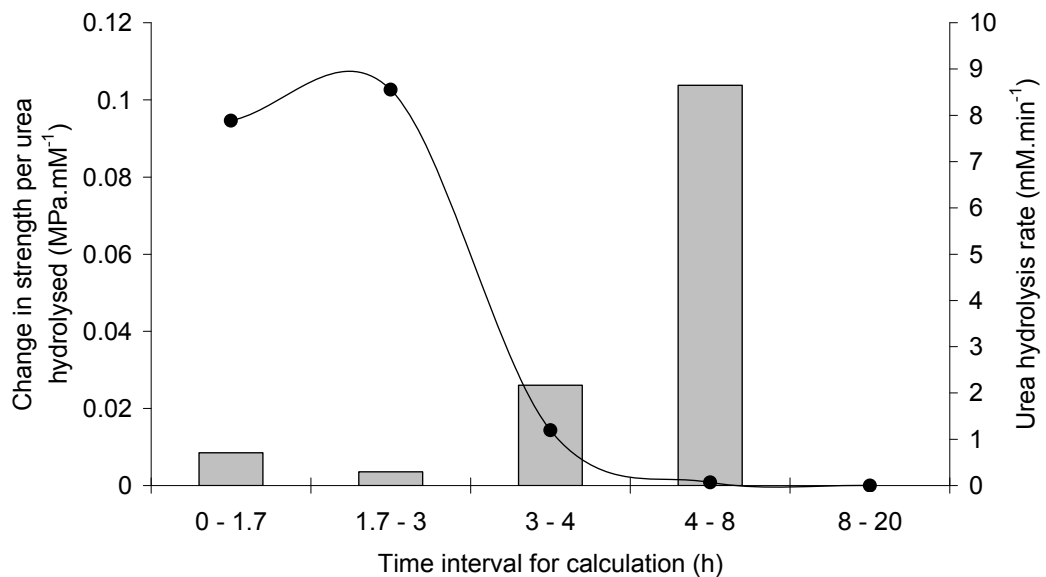


Figure 6.12: Change in strength per urea hydrolysed (■) versus urea hydrolysis rate for each interval (●) for high activity core, 12 mm from the injection point.

The ammonium concentrations measured from cores in the in-situ experiment were then compared to the predicted ammonium concentrations under the same conditions, using the model developed in chapter 5. At all three concentrations, the modelled rate of ammonium production was faster than the actual measured rate (Fig 6.14). The model takes into account the separate effects of urea, calcium (when applied as 100% $\text{Ca}(\text{NO}_3)_2$), and ammonium on the rate of urea hydrolysis (section 5.4). The effect of nitrate was shown to be inhibitory to urease (Fig 5.5, 5.6) but was not considered by the model because the effect was not

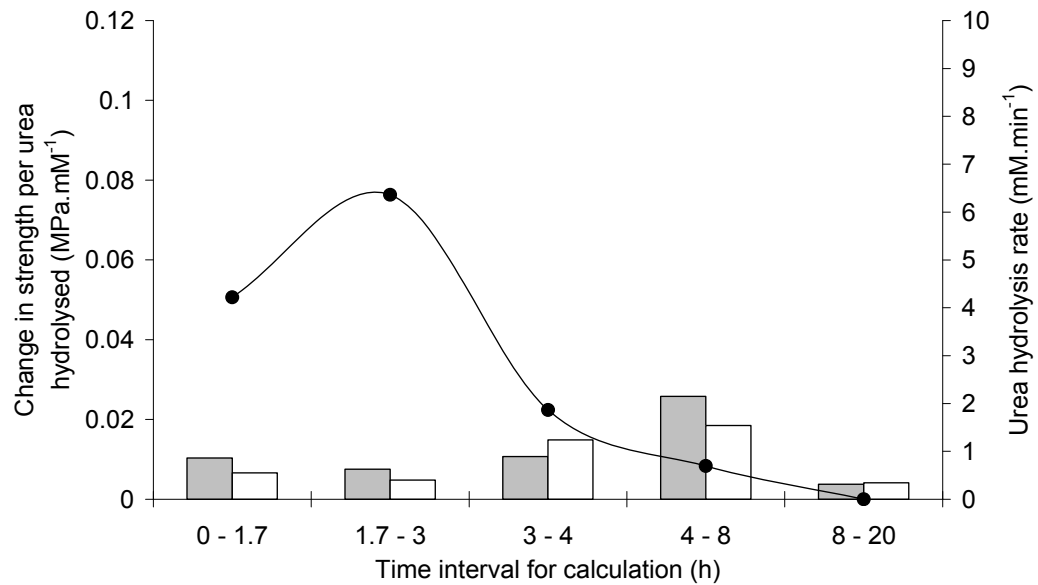


Figure 6.13: Change in strength per urea hydrolysed at 45 mm (■) and 75 mm (□) from the injection point, versus urea hydrolysis rate for each interval (●) for medium activity core.

fully quantified. In the real system calcium was supplied as a 50:50 mixture of nitrate and chloride salts, which would have lessened the inhibition effect of nitrate, resulting in a lower modelled rate compared to the real rate (Fig 5.5). This was not the case and thus does not explain the lower real rate, indicating that another parameter exists in the real system that decreases the rate of urea hydrolysis, which is not considered by the model. Possible parameters that have not been considered which could negatively affect urease activity are:

- Decreased availability of the substrate to the cell, due to a slower rate of diffusion. This may occur if there was significant precipitation of calcite of the bacterial surface (Hammes *et al.*, 2003a) or from the formation of calcareous flocs (Fig 7.4)).
- A decrease in the number of active cells able to contribute to urea hydrolysis, as one might expect of cells trapped the middle of calcareous flocs (Fig 7.4).

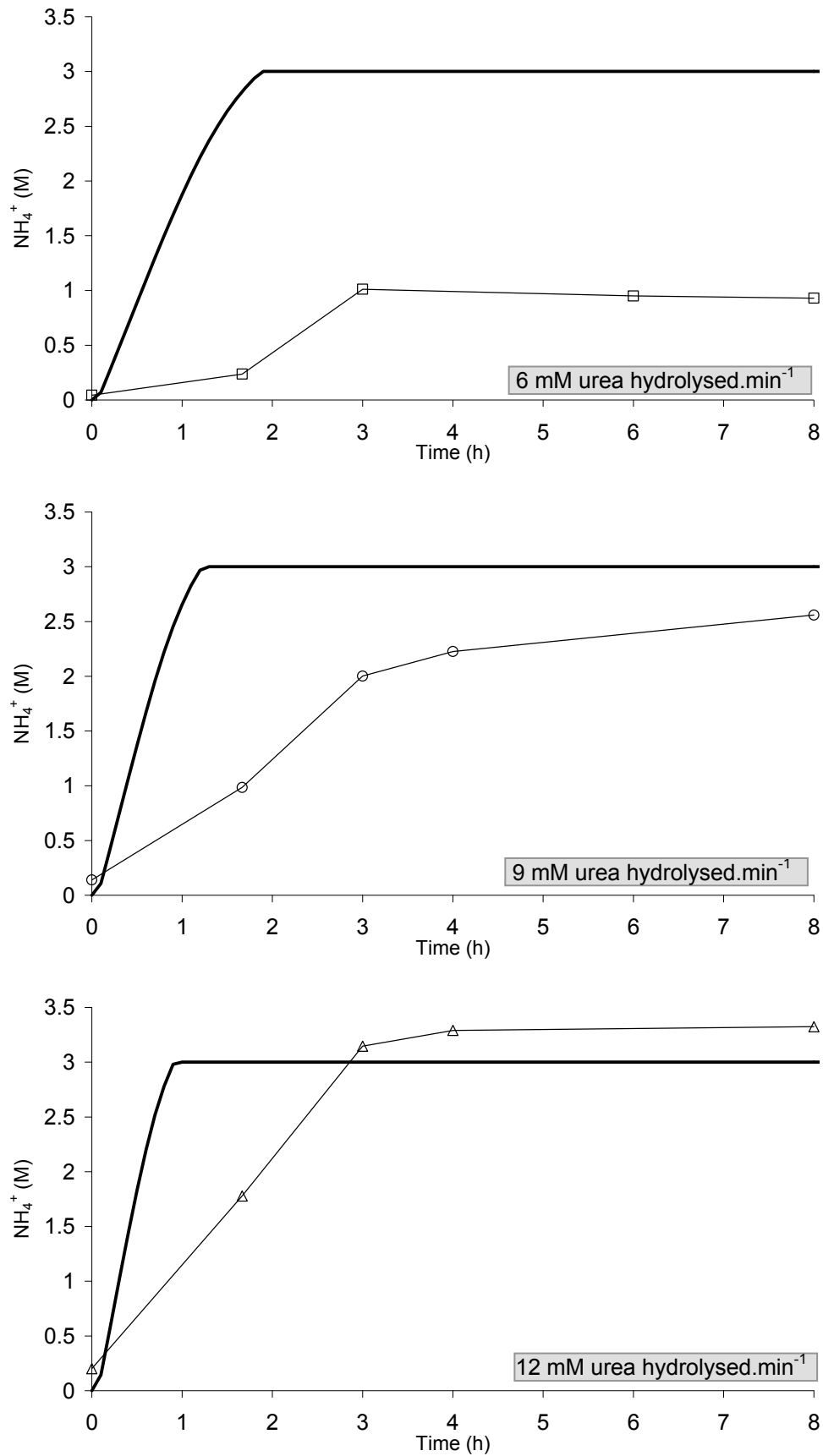


Figure 6.14: Model predicted ammonium concentrations (solid line) and actual ammonium production (open symbols) from urea hydrolysis during biocementation treatment for continuously monitored cores.

6.3.2. Alternative application methods

6.3.2.1. Reapplication of enzyme every treatment

During repeated urease applications, it was observed that the rate of urea hydrolysis became faster in subsequent applications even though the same amount of bacterial enzyme was applied. To quantify this effect the same amount of bacterial enzyme and reactants were applied in two consecutive treatments. The production of ammonium was determined by the periodic removal of samples via a capillary tube inserted into the core. Between treatments, water was flushed through the core to remove any spent liquid. The second treatment was applied 24 hours after the first treatment and each treatment contained an applied enzyme activity of $11 \text{ mM urea} \cdot \text{min}^{-1}$ and 1.5 M equimolar urea/Ca solution.

During the initial hours of cementation, the first treatment maintained an ammonium production rate of $7.2 \text{ mM NH}_4^+ \cdot \text{min}^{-1}$, which relates to $3.6 \text{ mM urea hydrolysed} \cdot \text{min}^{-1}$. The initial urea hydrolysis rate in the second application was double this rate ($7.8 \text{ mM urea} \cdot \text{min}^{-1}$), indicating that bacterial urease activity from the first treatment was still active in the second (Fig 6.15). The higher level of activity in the second treatment resulted in earlier completion of the reaction.

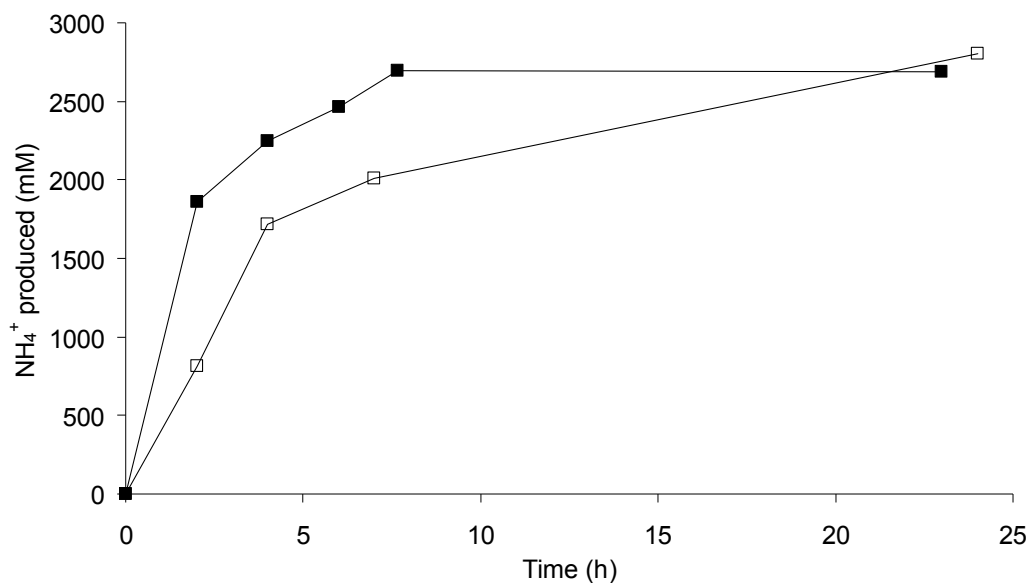


Figure 6.15: *In-situ* production of ammonium versus time for the first (□) and second (■) applications of enzyme and reactants inside a sand core. Both applications contained an applied enzyme activity of $11 \text{ mM urea hydrolysed} \cdot \text{min}^{-1}$ under standard conditions.

6.3.2.2. Immobilisation of enzyme and re-treatment with reactants

If urease activity from previous applications is still viable after the reaction is completed, it may be possible to apply less or even no enzyme in subsequent applications and still generate strength-building cement. As urease is the most expensive consumable component of the cementation reaction, the ability to reuse the catalyst in subsequent treatments represented a significant economic improvement to the process. To determine if residual urease activity present in the core after two cementation treatments could be reused, a third application was applied containing only calcium and urea reactants (without any additional enzyme). Even though no additional enzyme was added, approximately 1 M of ammonium was produced in 4 hours (4.5 mM ammonium produced.min⁻¹), which related to an average urease activity of 2.25 mM urea hydrolysed.min⁻¹ (Fig 6.16). The retention of residual activity in the core that could be reused by application of further urea and calcium, presents the potential to achieve additional strength at a very limited additional cost. The residual enzyme in the core was only active for the first few hours after application with no further ammonium production beyond 4 hours.

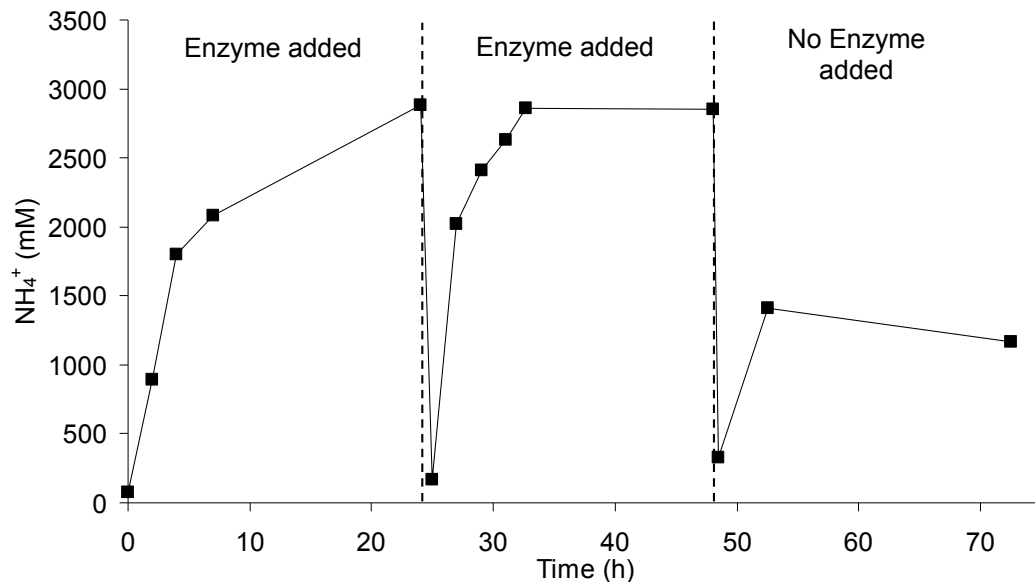


Figure 6.16: Residual urease activity immobilised in core after two previous applications of enzyme and reactants.

6.4. Discussion

The initial actual rate of urea hydrolysis *in-situ* was approximately 30% lower than the applied activity and varied significantly over the course of cementation (Fig 6.11). During all cementations with whole cell bacterial enzyme, the rate of urea hydrolysis was slowed dramatically after approximately 4 – 6 hours (Fig 6.9, 6.15). In one case this was explained by depleted substrate (Fig 6.9 – 12 mM urea.min⁻¹ core), but in all other cases the reaction was not yet complete, suggesting that the cementation conditions adversely affected urease activity beyond this time. Contrastingly, it was observed that residual urease activity that had been retained on the column after 24 hours could be reused by addition of fresh urea/calcium solution (Fig 6.16). The early cessation in activity before reaction completion and the reactivation of activity after draining and application of new solution, could be explained by removal of an inhibitory product that accumulated during the reaction. The soluble products of the biocementation reaction are ammonium, nitrate and chloride. Ammonium has been shown to have no effect on *S. pasteurii* urease activity at concentrations of up to 3M (Fig 5.7). This suggested that nitrate and/or chloride built up to inhibitory concentrations, which resulted in suppression of urease activity. If this were the case, one would have expected to see a concentration dependent effect on the time of inhibition; i.e. one would expect to observe an earlier inhibition at higher rates of urea hydrolysis, which was not evident (Fig 6.11).

Another possible hypothesis for reduced enzyme activity could be the precipitation of cement on the bacterial cell surface, which would slow diffusion of substrate to the organism, thus resulting in reduced activity. Bacterial cells have been shown to act as nucleation sites for precipitating minerals, which results in deposition of crystals on their cell surface (Ferris *et al.*, 1986; Ferris *et al.*, 1987). Hammes *et al.* (2003) produced calcareous cell flocs by a similar process to biocementation (urea hydrolysis in a calcium-rich environment) and used a cell staining technique to determine membrane integrity after calcite had been formed. This technique indicated the presence of live cells on the outside of the floc and dead cells on the inside of the floc (Hammes *et al.*, 2003a), suggesting that cells may eventually entomb themselves in calcite. Mineral precipitation is known to reduce the cell surface area available for nutrient uptake, which eventually leads

to loss of ATP generating capacity via dissipation of the PMF and results in cell death (Southam, 2000). If this were the case during biocementation, again one would have expected to see a calcite dependent effect on the time of inhibition; i.e. one would expect to observe an earlier inhibition at higher rates of urea hydrolysis. This was observed in figure 6.15 but not in figure 6.9, and requires further investigation.

Strength development during cementation was investigated with whole-cell bacterial enzyme and compared to soluble plant enzyme (Fig 6.9, 6.10). The development of strength in the bacterial system was non-uniform in different sections of the core, which contrasted against the soluble plant system, suggesting that an interaction between the sand and the bacterial cells resulted in a filtering effect of the enzyme when injected into sand. This effect is further investigated in Chapter 7. Both systems were applied with an activity of 9 mM urea.min⁻¹, however under the same cementation conditions, the initial rate of urea hydrolysis in the plant system was 25% of the rate in bacterial system, indicating that the soluble plant enzyme was more sensitive to the cementation conditions (Fig 6.9, 6.10, 6.11). Improved bacterial enzyme tolerance may be attributed to the protective effect of the cell membrane, which acts as a selectively-permeable barrier between the enzyme and the external environment (Prescott *et al.*, 1993).

In order to understand the effect of enzyme activity on strength, it was necessary to consider the first biocement treatment. Increasing enzyme concentrations between 2.2 – 13.3 mM urea.min⁻¹ produced increasing degrees of strength, after 24 hours of incubation (Fig 6.6B). With knowledge of the reduced durability of urease under cementation conditions in hindsight, it is not clear if this effect truly reflects the degree of strength produced at the various urea hydrolysis rates or if the reduced strengths are due to reduced total amounts of precipitated calcite. The actual urea hydrolysis rate was not monitored during this experiment. Based on the assumption that urease activity decreased by 30% in the cementation environment and that it was durable for 6 hours before reducing activity, full conversion of 1.5 M urea to carbonate would require an applied activity of 6 mM urea.min⁻¹. This would mean that the first two activities tested did not have sufficient opportunity to fully precipitate the available reactants.

During the second application, the real urea hydrolysis rate was further complicated by retention of residual activity from the first flush (Fig 6.16). Similar strengths were obtained in cores cemented at activities between 4.4 – 13.3 mM urea.min⁻¹, with approximately 30% less strength achieved at higher urease activities (Fig 6.6). These results suggested that fast urea hydrolysis rates, produced less cementation strength than slow urea hydrolysis rates. Larger strength improvements per urea hydrolysed were also shown at slower urea hydrolysis rates for cores that were continuously monitored during cementation (Fig 6.12, 6.13). The most efficient biocementation treatment which resulted in high strength production was with two treatments of 4.4 mM urea.min⁻¹, which yielded six-times more strength per enzyme input into the system compared to two treatments at 17.8 mM urea.min⁻¹ (Fig 6.7).

In order to assess the economic impact and quantify the cost of biocementation with regard to strength, the enzyme cost to treat 1 m³ of sand was determined (Table 4.3, Appendix F, Fig 6.17). The highest strength per cost was achieved with two treatments of 4.4 mM urea.min⁻¹ at a cost of \$41.90 per m³. A 10%

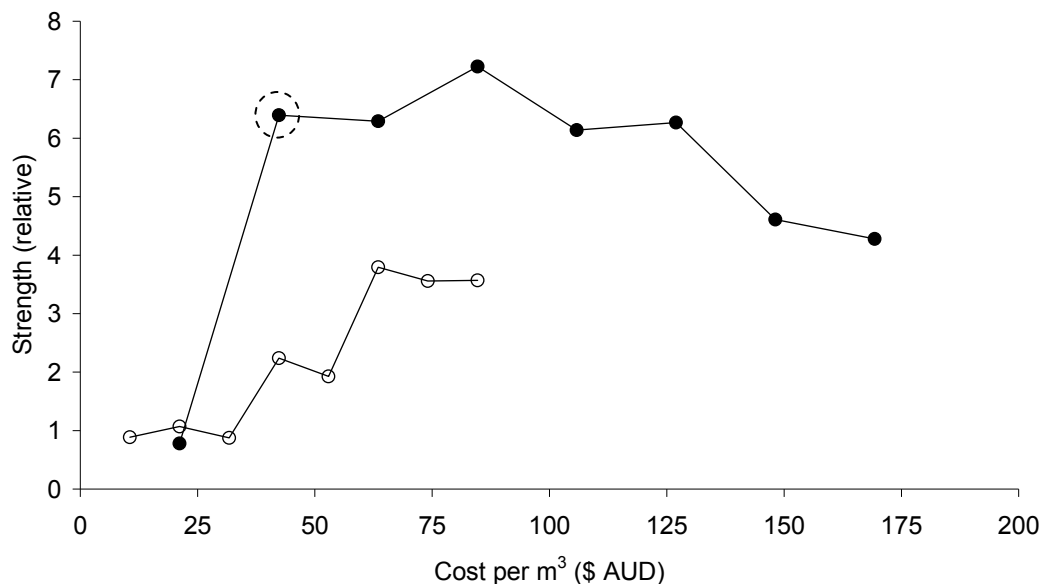


Figure 6.17: Economic evaluation of cost to treat 1 m³ of sand versus relative strength achieved for either one (○) or two (●) treatments of (in order) 2.2, 4.4, 6.7, 8.8, 11.1, 13.3, 15.6 and 17.8 mM urea hydrolysed.min⁻¹. The highest strength achieved per cost was with two treatments of 4.4 mM urea.min⁻¹, indicated by dashed circle. One cubic metre of sand was assumed to have a 50% pore volume (i.e. a pore capacity of 500 L).

improvement in strength could be achieved with two treatments of 8.8 mM urea.min⁻¹, however this small strength improvement doubled the enzyme cost to \$83.81 per m³ (Fig 6.17, Appendix F).

In summary, low urea hydrolysis rates were conclusively shown to produce higher strengths per carbonate produced, compared to high urea hydrolysis rates (Fig 6.12, 6.13). The most effective biocementation treatment for high strength was two applications of 4.4 mM urea.min⁻¹, for a cost of \$41.90 per m³ (Fig 6.17). In contrast to the soluble plant enzyme system, the nature of strength development during cementation in the bacterial system was not linear, but was initially fast followed by a dramatic reduction in rate after 4 – 6 hours of cementation (Fig 6.11). The dramatic reduction in rate may have been contributed to slower diffusion of urea to the cell due to the formation of calcareous flocs (Fig 7.4) or to precipitation of calcite on the bacterial surface. Residual activity was detected in two cores, 24 hours after a biocementation treatment, indicating that a proportion of urease activity was still active up to 24 hours after cementation. This suggested that a product of the cementation reaction, other than ammonium, may have been inhibitory to urease activity, which when removed allowed the bacterial enzyme to again become active. This also suggested that there was potential to reuse the enzyme in subsequent treatments, which represents a significant cost-saving for the process (Fig 6.16).

Industrial application of the Biocementation technology

7.1. Introduction

Having established the fundamental basis for biocementation and an understanding of how the parameters of the reaction affect its rate, it then became appropriate to test the technology in an industrial application. Interest was received from a Dutch geotechnical company regarding the application of Biocement for the stabilisation and strengthening of sand used for railroad embankments and dikes. Biocement offered significant advantages over existing technologies in that it could provide a significant strength improvement whilst still maintaining the permeability of the strengthened material, and the *in-situ* application alleviated the need for removal of existing material, mixing with a binding agent and replacing it.

The industrial trial provided an excellent opportunity to upscale the economic urease production system developed in Chapter 4 to pilot-scale and identify any differences compared with laboratory scale production. The trial also required that larger sized cores (38 mm ID x 170 mm) were produced to allow the direct measurement of shear strength, stiffness and permeability after treatment. Pending the successful outcome of this preliminary trial, a Dutch field site had been identified as a future site to field-test the biocementation treatment. This site contained a peat/sand soil mixture, and thus it was desirable to determine if there were any differences in cementation of a peat/sand mix, compared to clean sand.

In summary, the objectives of this chapter were:

- To culture *S. pasteurii* at pilot-scale, and determine any differences in urease production compared to laboratory-scale cultivation.
- To compare shear strength and stiffness before and after treatment
- To determine the degree of reduction in permeability after treatment

- To determine the effect of biocementation in clean sand compared to a clean sand/peat mix (9 parts sand to 1 part peat)

7.2. Materials & Methods

7.2.1. Cultivation

6.2.1.1. Vegemite Acetate medium

An industrial economic medium has previously been defined for *S. pasteurii* (Table 4.3). Vegemite acetate medium consisted of 13.5 g.L⁻¹ Vegemite, from which the solids were removed by gravity settling followed by decanting the upper fraction (Fig 4.16), and 150 mM acetate added as glacial acetic acid. The pH was adjusted to 7 with 6 M NaOH.

For the 10 L inoculum cultivation, the medium was sterilised and 10 g.L⁻¹ urea was added by sterile filtration, post-sterilisation. For the pilot-scale cultivation, the medium was not sterilised and urea was added without sterile filtration.

6.2.1.2. 10 L Pilot-scale inoculum cultivation

A pilot-scale inoculum was grown under sterile conditions in a 10 L stirred tank reactor (Chemap, Germany), at 30°C with a starting pH of 8.25.

6.2.1.3. 100 L Pilot-scale cultivation

The pilot-scale cultivation was conducted under non-sterile conditions in a 120 L custom-built fibreglass airlift reactor (courtesy of Andrew Brown & Co., Perth) (Fig 7.1), with a working volume of 100 L.

The reactor was specifically designed for economic cultivation of *S. pasteurii*. The vessel was constructed to a simple fibreglass design, which was low cost and easily cleaned. Economical mixing and aeration was achieved by air-lift using a wastewater treatment plant air diffuser (courtesy of ESI, Perth) and a removable internal tube with recirculation holes. The vessel was located outside to simulate industrial conditions, and was temperature controlled to 30°C, with a starting pH of 8.15. Before cultivation the interior of the reactor was washed with a 5% hypochlorite solution and rinsed well with clean water. The trial was conducted

in summer and the average minimum and maximum outside temperatures during the trial period were 20-35°C.

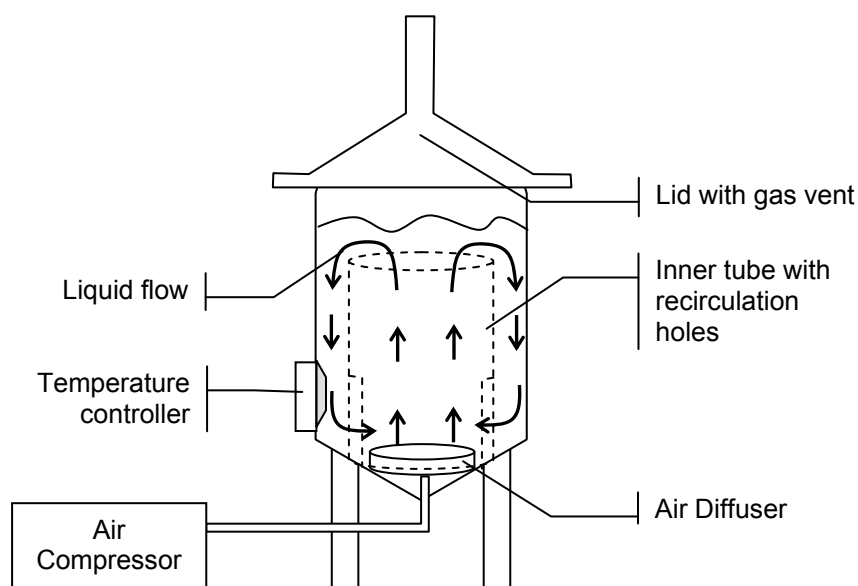


Figure 7.1: Custom-built fibreglass airlift reactor (courtesy of Andrew Brown & Co., Perth). The reactor was fitted with a wastewater treatment plant membrane air diffuser (courtesy of Environmental Solutions International (ESI), Perth) and with an inner tube with recirculation holes to aid mixing, aeration and temperature control of the culture.

7.2.2. Analytical Methods

Urease activity, specific urease activity and biomass were calculated as described previously (Chapter 2 - Section 2.2)

7.2.3. Cementation Trials

Cementation trials were conducted in either <300 μm silica sand (i.e. all sand particles less than 300 μm), a commercial Dutch construction sand containing some shale (Koolschijn) or a 9 parts Koolschijn sand to 1 part Peat mix as indicated for each experiment (Koolschijn and peat supplied by GeoDelft, The Netherlands). The sand was dry packed with continuous vibration, to give an even density of approximately 1.75 g.cm^3 for sand cores or 1.65 g.cm^3 for sand/peat cores, into 38 (internal diameter) by 170 mm PVC pipe columns. Cores were then up-flushed with water and tapped to remove air pockets. Ca/urea solution and enzyme were premixed immediately before injection into the core via a pressurised vessel, based on a method developed by Calcite Technology Pty. Ltd

(Fig 7.2). One and a half times the pore volume (void volume) was flushed in to ensure full displacement of water. Between cementation flushes, water was flushed through the cores to remove any spent liquid and the number of cementation flushes for each core is indicated for each experiment. Post-cementation, cores were flushed with a bleach solution and oven-dried at 60°C.

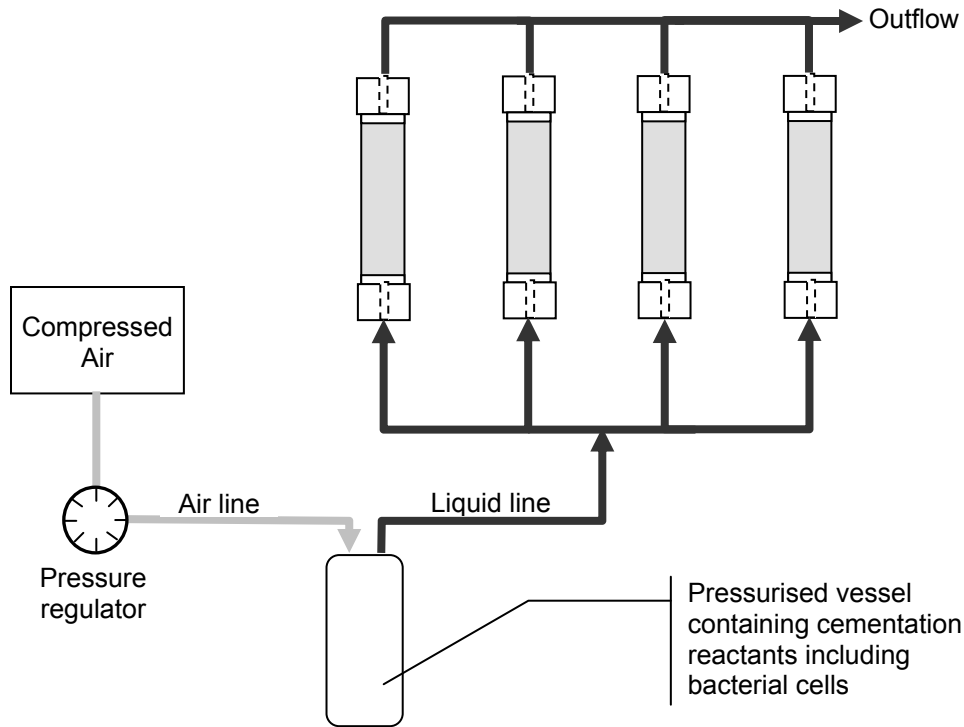


Figure 7.2: Method for injecting cementation liquid into sand cores, based on the method developed by Calcite Technology Pty. Ltd. The cementation reactants (calcium/urea solutions and bacterial cells) were immediately mixed and put into the vessel which was closed and pressurised using compressed air. The liquid line was then opened to allow the cementation solution to up-flush through the sand cores. After the cores were fully saturated, the liquid line was closed and cementation solution remained in cores for 24 hours. Up to four cores were treated at once.

7.3. Results

7.3.1. Urease preparation

6.3.1.1. Inoculum cultivation

To provide the amount of enzyme required for the larger sized cores required, it was necessary to scale-up urease production from laboratory to pilot-scale. A 10 L stirred tank reactor was used to produce a suitable inoculum for the 100 L pilot-scale reactor. *S. pasteurii* was cultivated under sterile conditions on Vegemite acetate medium. A normal level of activity and biomass (22 mM urea.min⁻¹ and

OD₆₀₀ of 6, respectively) was produced within 10 hours (Fig 7.3, Table 4.3), indicating that there was no difference in scaling the cultivation from 1 L to 10 L under the same conditions. At this time, a fed-batch feed addition was attempted to further boost activity. 4 L of the culture was harvested from the vessel and replaced with 4 L of concentrated feed, which contained the equivalent amount of nutrients in 10 L of new feed. Despite providing the same amount of nutrients as at the beginning of cultivation, the yield of urease activity per gram of substrate was only 30% of that produced in the first 10 hours (Fig 7.3). This confirmed earlier results (Fig 3.6) that a component in the culture broth produced during normal growth was responsible for suppression of urease activity, and as a result fed-batch growth was not a suitable cultivation method for yielding high urease activity from *S. pasteurii*.

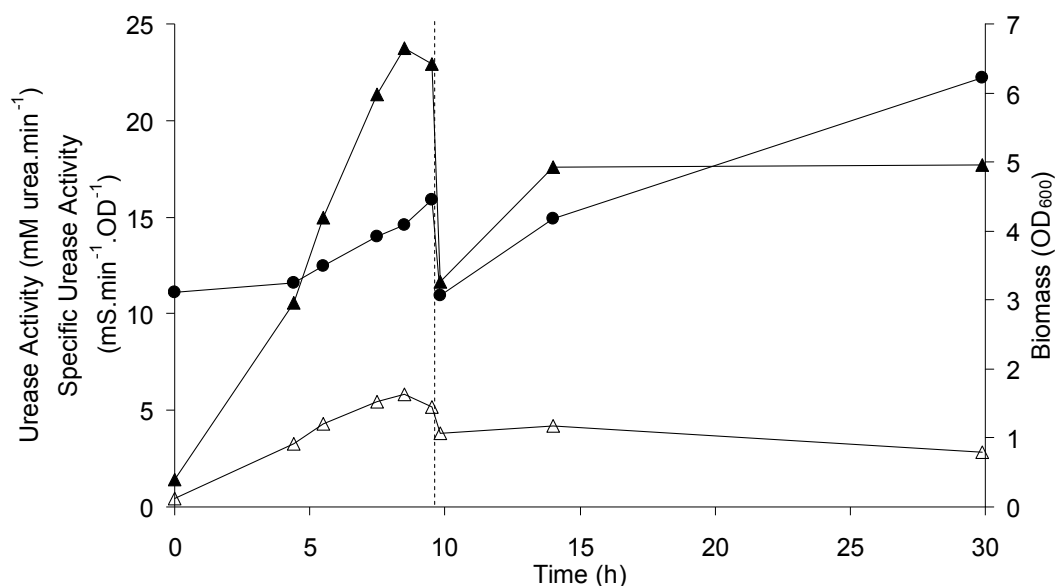


Figure 7.3: Production of a pilot-scale inoculum in a 10 L stirred tank reactor. Urease activity (\triangle), specific urease activity (\blacktriangle) and biomass (\bullet). *S. pasteurii* was cultivated on Vegemite urea medium under sterile conditions. At 9 hours a fed-batch was attempted to further boost activity (indicated by dashed line).

6.3.1.2. Pilot-scale cultivation

Five litres of the inoculum were transferred from the inoculum cultivation (above) to the pilot-scale airlift reactor (Fig 7.1) (5% inoculum) and cultivated non-sterilely on Vegemite acetate medium. Previous experiments had indicated that *S. pasteurii* could be cultivated under non-sterile conditions with up to 50% contamination, without affecting the level of urease activity (Fig 2.12). The pilot-scale cultivation was conducted under non-sterile ‘clean’ conditions, which involved washing the interior of the reactor immediately prior to inoculation, with a 5% hypochlorite solution and rinsing well with water. The production of urease activity was approximately 20-times slower in the airlift reactor compared to the stirred tank reactor (Fig 7.3, 7.4). Slower biomass and urease production were expected in the airlift reactor due to a lower k_{La} , however it had been previously shown that proportional levels of biomass and urease activity could be attained under oxygen limited conditions, if given sufficient time for growth (Fig 4.13, 4.14). Even though a higher biomass concentration was attained in the pilot-scale cultivation compared to the (pre-fed batch) inoculum cultivation, the maximum urease activity was approximately 4-times lower, at 6 mM urea hydrolysed.min⁻¹ (Fig 7.3, Fig 7.4).

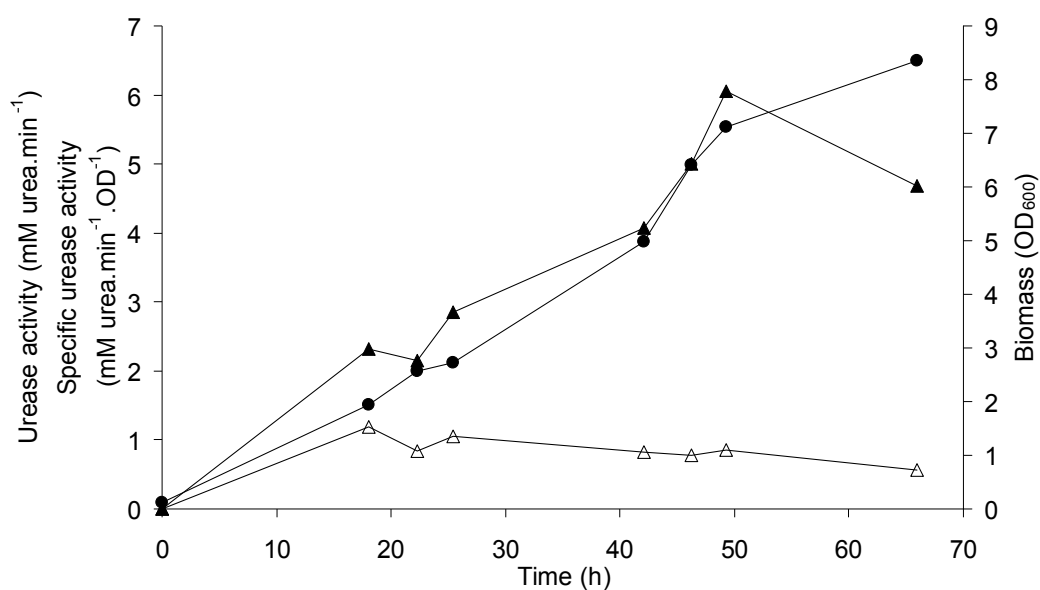


Figure 7.4: Pilot-scale cultivation of *S. pasteurii* for the production of urease. Urease activity (▲), specific urease activity (△) and biomass (●). Cultivation was conducted under non-sterile conditions, on Vegemite urea medium at 30°C.

7.3.2. Cementation of Koolschijn sand

6.3.2.1. Optimum enzyme activity for Koolschijn sand

It had previously been determined that the most efficient biocementation treatment with regard to strength in <300 μm silica sand, was two treatments of 4.4 $\text{mM urea}\cdot\text{min}^{-1}$ (Fig 6.7, 6.17). In order to determine the optimum enzyme activity range for Dutch Koolschijn sand, four cores were flushed with various amounts of urease activity and the degree of cementation was compared. The cementation depth was estimated by lightly squirting water from a wash bottle on the side of the PVC core mould and removing any sand that was washed out. A “soft” rating indicated that the remaining cemented sand could be penetrated with light pressure on a wooden skewer, and a “hard” rating indicated that it could not be penetrated with the skewer (Table 7.1).

Table 7.1: Effect of different urease activities on cementation depth in Koolschijn sand.

Core #	Sand Type	Applied Urease Activity Growth Medium	Cemented Depth (mm)	Top	Bottom	Injection Pressure (psi)
1	Kool	5 $\text{mM urea}\cdot\text{min}^{-1}$ Medium: Vegemite/acetate/urea	99	Soft	Hard	10
2	Kool	7.5 $\text{mM urea}\cdot\text{min}^{-1}$ Medium: Vegemite/acetate/urea	125	Soft	Hard	10
3	Kool	10 $\text{mM urea}\cdot\text{min}^{-1}$ Medium: Vegemite/acetate/urea	108	Soft	Hard	10
4	Kool	12.5 $\text{mM urea}\cdot\text{min}^{-1}$ Medium: Vegemite/acetate/urea	107	Soft	Hard	10

The cores were up-flushed from the bottom to top (Fig 7.2), and the top sections of all four cores could be washed out, which indicated very weak cementation in the upper section. This was confirmed by activity measurements, which were taken 24 hours after injection into the core. Approximately three times more enzyme activity was recovered at the injection end of the column, compared to the outlet end, indicating that the bacterial cells were filtered out as they were injected through the core. This was an undesirable phenomenon as it did not allow the depth of cementation to be controlled. The greatest cementation depth was evident in the cores treated with 7.5 and 10 $\text{mM urea}\cdot\text{min}^{-1}$, thus the optimum

activity for treatment of Koolschijn sand was determined as the median value between these activities at $8.75 \text{ mM urea} \cdot \text{min}^{-1}$ (Table 7.1).

Non-uniform cementation had been observed previously (Fig 6.9) but not to the same extent as was seen in this experiment. The major difference between the application method used in pilot-scale cementation (Fig 7.2) and the application method used previously (Fig 6.2) was the time required for the cementation liquid to be injected into the core. Using the current method, extra time was required to pressurise the vessel and several cores are treated at once, requiring longer times before the cementation components were fully injected into the sand cores.

On closer inspection of the cementation mixture, the precipitation of white crystals were evident immediately after mixing the reaction components together. The white precipitate was filtered and on contact with acid, a fizzing gas was released confirming that it was carbonate-based and most likely CaCO_3 (Klein and Hurlbut, 1999). The initial stages of cementation were then observed under the microscope, by initiating the reaction on a glass slide. Immediately after the addition of calcium to the bacterial suspension, very small calcite crystals began to form. Cells that came into contact the crystal appeared to become bound to it. Within 3 – 4 minutes, 70 – 80 % of the cells were captured in the crystal matrix (Fig 7.5).

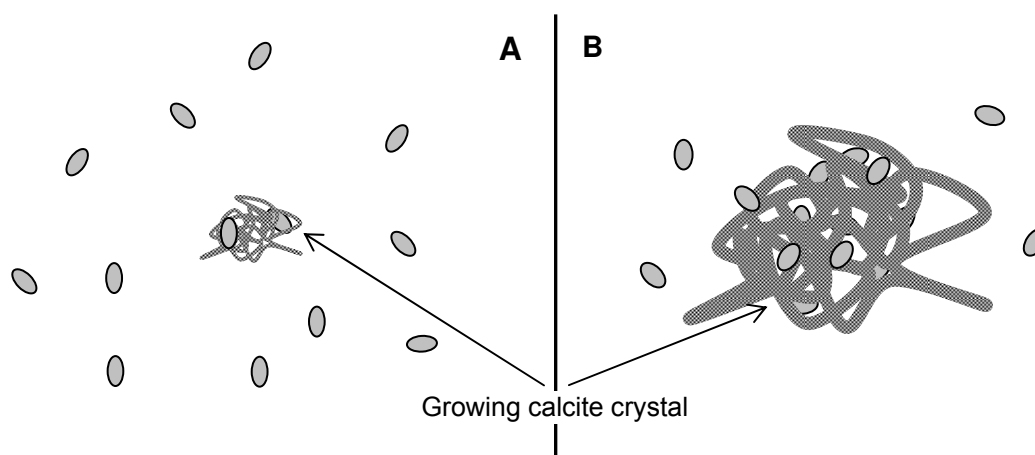


Figure 7.5: Schematic of trapped microbial cells in the growing calcite crystal. (A) Immediately after addition of calcium to the bacterial cell suspension, very small calcite crystals began to form. (B) Within 3-4 minutes, 70-80% of cells were attached to the precipitating crystal and bound into a concentrated mass.

The formation of calcite prior to injection into the sand core and the coagulation of bacteria into the crystal matrix were not desirable for cementation and a modification to the cementation protocol was required. During cultivation of *S. pasteurii*, urea in the growth medium was hydrolysed and the resultant culture broth contained a high concentration of carbonate ions. When the enzyme was mixed with calcium prior to flushing into the sand core, calcite was immediately precipitated and cells became bound to the crystal (Fig 7.5). These results suggested the following hypothesis: Solid crystals were flushed into the core and became lodged in the lower pore spaces, resulting in a lower concentration of bacterial cells reaching the upper sections of the core and thus a lower depth of cementation. Possible ways to avoid this problem are investigated in the next section.

6.3.2.2. Trouble-shooting non-uniform cementation

A second cementation trial was conducted to test possible ways to overcome the non-uniform cementation. Several hypotheses were tested to elucidate why the enzyme was being filtered out:

- The pressure applied was not sufficient to push the enzyme into the sand quickly enough before the reaction had started.
- The Koolschijn sand has a high capacity to retain bacteria.
- The presence of up to 167 mM carbonate in the medium (generated from urea hydrolysis during growth) resulted in the early precipitation of CaCO_3 , which produced solid particles that clogged the pores and retained bacteria.

Urea was initially included in the industrial economic medium because it was an economical and readily available source of ammonium, and it is inhibitory to many contaminant organisms (Madigan *et al.*, 2000). Providing an alternative source of ammonium was available, urea was not required for urease production by *S. pasteurii* (Fig 3.2) and a new batch of urease active bacteria were cultivated in a urea-free medium containing 10 g.L⁻¹ yeast extract, 100 mM CH_3COONa and

10 g.L⁻¹ (NH₄)₂SO₄. These cells were used to test the above hypotheses (Table 7.2).

Table 7.2: Effect of sand type, increased injection pressure and soluble enzyme on the cementation depth of cores. Sand type: Si = Silica sand; Kool = Koolschijn sand. Medium: YE/Ace/NH₄⁺ = 10 g.L⁻¹ Yeast ext, 100 mM CH₃COONa and 10 g.L⁻¹ (NH₄)₂SO₄. A soft rating indicated that the cemented sand could be penetrated with light pressure on a wooden skewer and a hard rating indicated that the sand could not be penetrated.

Core #	Sand Type	Applied Urease Activity Growth Medium	Cemented Depth (mm)	Top	Bottom	Injection Pressure (psi)
5	Kool	8.75 mM urea.min ⁻¹ Medium: Yeast Extract/acetate/NH ₄ ⁺	136	Soft	Hard	10
6	Si	8.75 mM urea.min ⁻¹ Medium: Yeast Extract/acetate/NH ₄ ⁺	170	Hard	Hard	10
7	Kool	8.75 mM urea.min ⁻¹ Medium: Yeast Extract/acetate/NH ₄ ⁺	167	Hard	Hard	15
8	Kool	8.75 mM urea.min ⁻¹ Medium: Soluble plant derived enzyme	170	Hard	Hard	10

The use of a carbonate-free enzyme solution slightly improved the depth of cementation in Koolschijn sand by cementing approximately 6% more of the core (core 2 versus 5). Full cementation of the core was achieved in silica sand versus Koolschijn sand under the same conditions, suggesting that the nature of Koolschijn sand played a role in filtration of the cells, even in the absence of prior precipitation of calcite (core 5 versus 6). Full cementation in Koolschijn sand was achieved by increasing the injection pressure from 10 to 15 psi (core 5 versus 7). When treated with soluble enzyme instead of a particulate bacterial suspension, non-homogeneous cementation was not evident in Koolschijn sand (core 5 versus 8), suggesting that an interaction between the sand and the bacterial surface was responsible in part, for non-uniform cementation (Table 7.2).

6.3.2.3. Investigation of possible reasons for non-uniform cement distribution

The cementation process involves pre-mixing enzyme and cementation reactants before injection into a prepacked sand column (section 7.2.3). In order to

determine if the particulate nature of bacterial cells caused a filtration effect when injected into sand, washed *S. pasteurii* cells were up-flushed through a 1.6 m $<300\ \mu\text{m}$ silica sand column at a rate of 15 ml per minute and the distribution of urease activity was determined from samples withdrawn at various points along the column. Samples were withdrawn sequentially from top to bottom to avoid disturbing the liquid below the sample point. The distribution of urease was relatively uniform along the full length of the column, indicating that the bacteria were not filtered out due to their physical size properties (Fig 7.6).

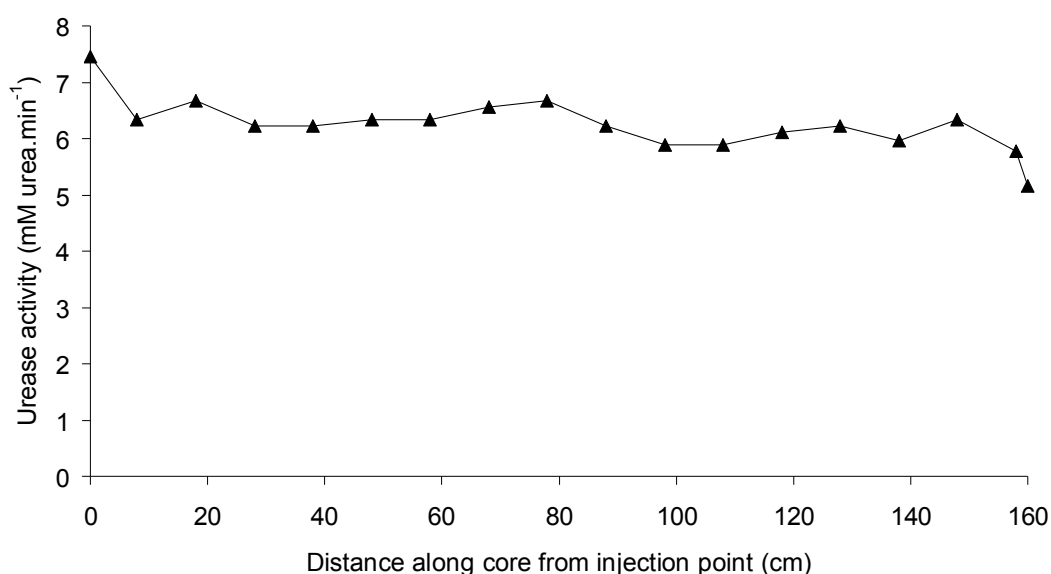


Figure 7.6: Distribution of urease activity in washed *S. pasteurii* cells after injection into a 1.6 m -300 μm Si-sand packed column.

To investigate whether or not different sand types play a role in the retention of *S. pasteurii* in the column, washed bacteria were trickled through several different sands by recycling through the core via a reservoir. Urease activity was determined in the drained column after recycling for five minutes. Neither the silica sand nor the industrial construction sand retained any urease activity however swimming pool filter (SPF) sand did retain a significant proportion of the added activity (Fig 7.7). Full retention of activity was possible in SPF sand within 1 minute, when the washed bacteria were supplied to the column immediately after mixing with 0.1 M $\text{Ca}(\text{NO}_3)_2$. This indicated that the type of sand used for

cementation played a role in the retention of *S. pasteurii* cells, and that the retention of cells could be enhanced by pre-mixing with calcium ions before injection.

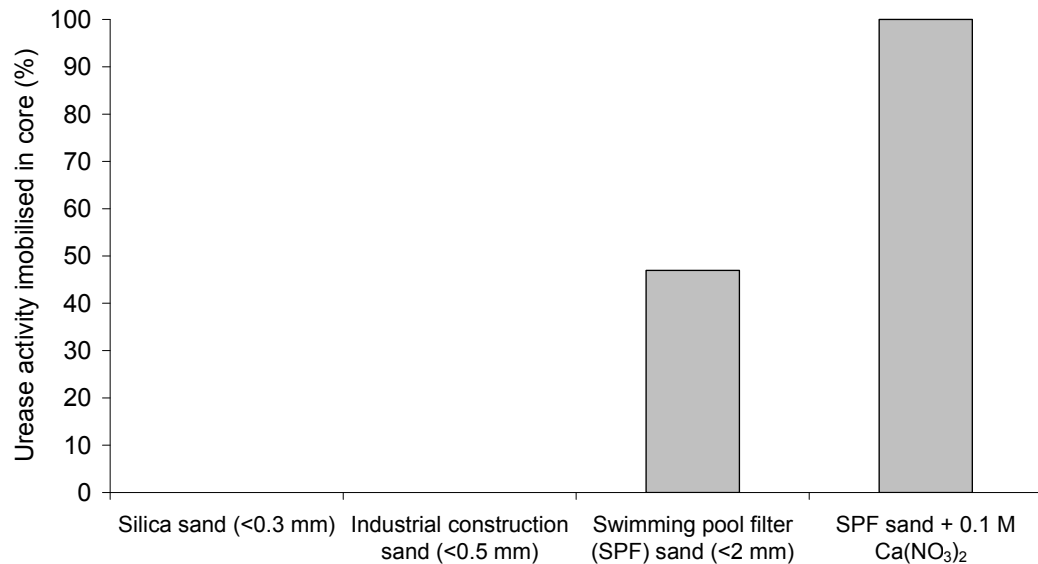


Figure 7.7: Proportion of *S. pasteurii* urease activity retained in the sand core after five minutes of recycling through 10 cm packed sand columns of different sand types, via a reservoir.

6.3.2.4. Determination of Strength Properties after Biocementation

Having established a suitable cementation method to achieve uniform cementation in Koolschijn sand (using a urease activity of $8.75 \text{ mM urea} \cdot \text{min}^{-1}$ from cells cultivated in Vegemite acetate and ammonium medium and injected into the core at 15 psi), samples were then prepared for the purpose of determining the physical strength properties imparted after biocementation. Samples were prepared using either Koolschijn sand (K) or 90% Koolschijn plus 10% peat mix (w/w) (KP) and treated with 2, 3 or 4 biocementation treatments as per section 7.2.3. After cementation, the strength properties of the cores were investigated by triaxial testing, and shear strength and stiffness were determined. Triaxial testing is a compression test of a cylindrical rock sample under confining pressure where the loading path is followed by a computer. This test aimed to simulate the

conditions that may occur to *in-situ* rock material where it is subjected to a confining pressure and deviatoric stress. Shear strength is a measure of how much stress (force ÷ area) can be applied before the material undergoes shear failure (the situation whereby the soil can no longer sustain increases in the applied load without excessive deformations and stress re-distribution resulting). Stiffness is the ratio between stress and strain at 50% shear strength, and gives an estimate of the force required to give a certain degree of displacement.

Biocementation in Koolschijn sand had a clear improvement on strength and stiffness, with an average increase in shear strength by a factor of 8, and increase in stiffness by a factor of 3 (Fig 7.8). The Koolschijn/peat samples (KP) were clearly different, showing less strength and less stiffness compared to K sand. Two biocementation treatments in KP sand showed no improvement compared to uncemented K sand however it should be noted that the strength properties of uncemented KP sand was not determined and may have been lower. Increasing the number of applications in KP sand improved shear strength (Fig 7.8).

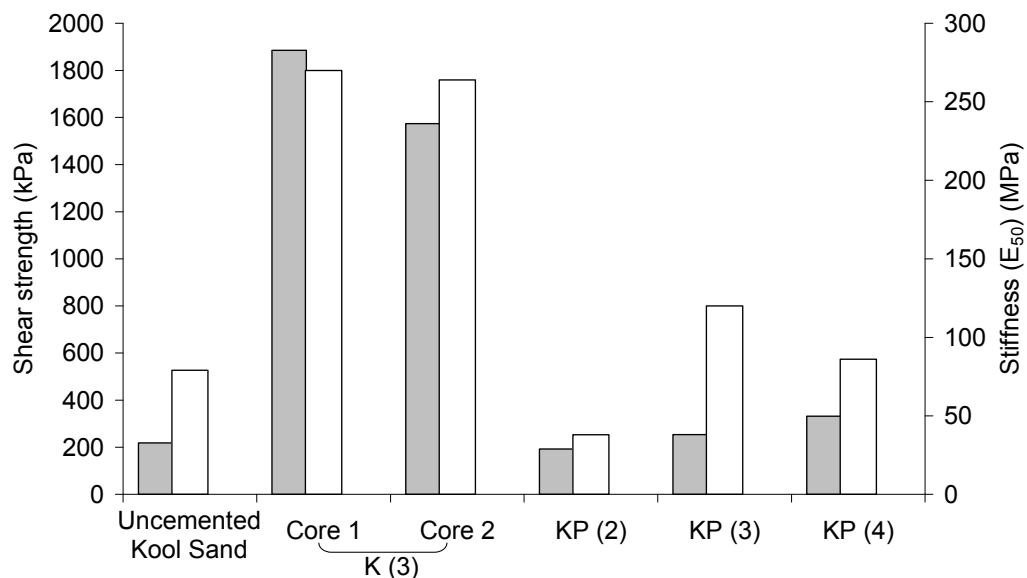


Figure 7.8: Shear strength (■) and stiffness (□) in Koolschijn sand (K) and Koolschijn sand mixed with 10% peat (KP) after biocementation treatments (Number of treatments are indicated in brackets). Before the triaxial shearing phase samples were flushed with CO₂ to remove any non-water soluble gases and saturated with water. Stiffness was determined using Young's modulus at 50% of peak stress and shear strength was determined at 50% of the maximum deviator stress.

One of the advantages of biocementation over other traditional stabilisation treatments is the conservation of permeability after treatment. During biocementation calcite is precipitated within the pore spaces to provide strength but not as to completely block them. To determine the degree of pore volume reduction after cementation, four Koolschijn cores were cemented (three treatments) and the pore volume was compared to that of unconsolidated sand. Pore volume was decreased between 2 and 14%, representing a minor reduction after cementation, thus leaving the permeability of the core largely unchanged (Fig 7.9).

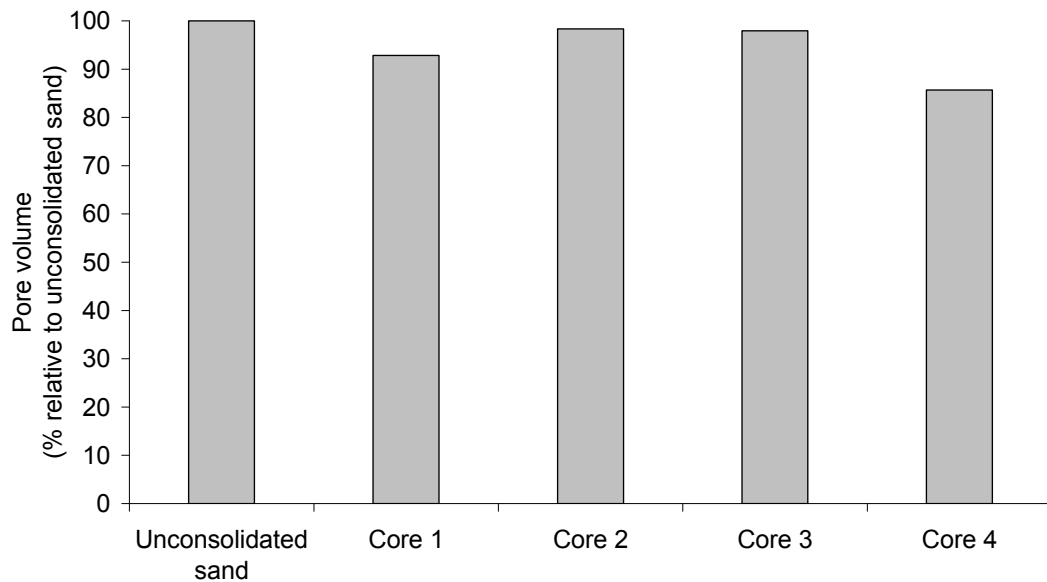


Figure 7.9: Difference in pore volume before and after treatment in Koolschijn sand with three biocementation treatments. Permeability tests were conducted using a 30 cm head difference, 300 kPa back pressure and a consolidation pressure of 100 kPa.

7.4. Discussion

For the purposes of comparison, the inoculum culture (Fig 7.2) was conducted under laboratory-scale conditions and the 100 L cultivation (Fig 7.3) was conducted under pilot-scale conditions. The production of urease under pilot-scale conditions was significantly slower compared to that seen at laboratory-scale. The pilot-scale system reached its maximum urease activity at 6 mM urea hydrolysed.min⁻¹ and OD₆₀₀ 7 after 50 hours (Fig 7.3). The laboratory-scale system produced its maximum urease activity at 23 mM urea hydrolysed.min⁻¹

and OD₆₀₀ 5 after 9 hours (Fig 7.2). Pilot-scale versus laboratory-scale cultivation presented two major differences for *S. pasteurii*:

1. Oxygen was supplied by airlift versus sparging and stirring

Supplying oxygen by the airlift method is less efficient than supply by sparging and stirring, thus it would expected that biomass growth was slower in the airlift reactor. This was confirmed by the production of similar final levels of biomass in both reactors, and growth was approximately 4.5 times slower in the airlift reactor (Fig 7.2, 7.3). Despite having similar levels of biomass, the maximum level of urease activity in the laboratory reactor was almost four-fold higher than the pilot-scale reactor. The reason as to why slower growth would result in lower specific activities is not clear. The mechanism for controlling urease activity in *S. pasteurii* has been investigated (chapter 3), but it has not been fully elucidated in this study and requires further investigation.

2. Non-sterile cultivation versus sterile cultivation

The other major difference between pilot- and laboratory-scales was the sterility of the cultivation. Laboratory scale was conducted under sterile conditions (autoclaved medium, sterile inoculation, 0.2 µm filtered airflow), whereas pilot scale was conducted under clean but non-sterile conditions (reactor surface was hypochlorite-washed and rinsed before inoculation, medium was not autoclaved, air was supplied by compressor directly from atmosphere). The pilot-scale cultivation was continuously monitored by microscopy for contamination, and no contaminating species were observed, even after more than 60 hours of cultivation. This was likely due to the high concentrations of urea and ammonium present and the high pH conditions. It was considered that the process of autoclaving the medium may have solubilised additional nutrients but as similar levels of biomass were obtained in both cultivations, this was not considered significant.

The pre-precipitation of calcite prior to injection into Koolschijn sand was encountered with using bacteria that had been cultivated with urea (Fig 7.4). This had not been considered previously and has dual implications for biocementation.

Firstly, a significant proportion of calcium was precipitated wastefully (up to 167 mM or 10%) outside of the core and secondly, the production of solid particles resulted in a filtration effect which affected the depth of cementation. This effect was more significant in Koolschijn sand than in silica sand, but did not fully account for the reduction in cementation depth as carbonate free enzyme produced only a slightly improved result (Table 7.2). Full cementation of the entire 170 mm core depth was not achieved with carbonate-free enzyme, unless the injection pressure was increased from 10 to 15 psi. As soluble enzyme did not reduce the cementation depth, it can be assumed that there was an interaction between the Koolschijn sand and the bacterial surface which promoted attachment.

The retention of cells in the core was tested in several other types of sands and it was found that different sands had different capacities for the retention of *S. pasteurii* (Fig 7.5, 7.6). In addition, flushing cells through a core immediately after mixing with calcium ions resulted in a greater degree of retention. The retention of active enzyme in the core has been previously observed (Fig 6.11) and this is a significant finding in that it may be possible to control the retention, so as to selectively immobilise the catalyst in the core and reuse the activity over several flushes, thus making the process significantly cheaper.

Clear improvements on shear strength and stiffness were evident in Koolschijn treated sand (Fig 7.7). Biocemented Koolschijn/peat sand produced a clearly different response with only marginal improvements in strength, even after four biocementation treatments. The presence of peat in the sand had a negative effect of the strength of cementation and it would be desirable to conduct further trials with lesser concentrations of peat (e.g. 0.25, 0.5, 1%) to determine if peat-containing soils are suitable for cementation.

The difference in pore volume was determined before and after biocementation. The application of three biocementation treatments in Koolschijn sand resulted in a minor decrease in pore volume between 2 – 14%, which still enabled a high degree of permeability to the rock after cementation (Fig 7.8). The 12% variation in pore volume reduction within the same treatment was possibly due to variation in column packing.

General Discussion

8.1. Features of Biocement

Biocementation via microbial carbonate precipitation (MCP) by way of urea hydrolysis, presents a promising novel biotechnology for the consolidation of loose materials. The treatment can produce significant strength improvements in sandy materials with an 8 – 9-fold increase in shear strength (1.8 MPa) and a 3-fold increase in stiffness (275 MPa) after three applications (Fig 7.8) indicating that the deposition of calcite strongly adhered to the pore surfaces. This is the first published study to achieve effective consolidation of loose sand via MCP by way of urea hydrolysis.

Biocement has several advantages over existing technologies. The conservation of permeability is an important parameter for consolidated materials, because it allows the movement of moisture through the stone, thus preventing early deterioration due to water logging, which is one of the main problems of other microbial consolidation technologies (Rodriguez-Navarro *et al.*, 2003; Tiano *et al.*, 1999). To a large extent, the treated material retained its original porosity after treatment, with only minor decreases in pore volume evident (Fig 7.9). Unlike injectable grouts and synthetic polymers which can displace the existing material, the biocementation reactants are initially aqueous and infiltrate into the pore spaces of material, thus requiring much lower injection pressures for application.

An additional advantage may be achieved with the bacterial system by reuse of the enzyme in subsequent applications. It was shown that a proportion of residual bacterial urease was immobilised in the core after an application, and that the enzyme activity could be reused by application of cementation reactants only (no additional enzyme) (Fig 6.16). Because urease is the most expensive consumable component in the system, the reuse of urease activity represents an additional and significant cost-saving for the process.

8.2. Depth of cementation

Compared to other microbial consolidation technologies, the depth of cementation was improved from 0.5 mm to 170 mm, being the maximum cementation depth attempted in this study (Table 7.2) (Castanier *et al.*, 2000a; Rodriguez-Navarro *et al.*, 2003). This improvement was attributed to by use of a carbonate-free growth medium (i.e. no urea), which prevented the precipitation of particulate calcite before injection, and by increasing the injection pressure (Fig 7.5). It has been shown in this study that different sand types have different capacities for retention of bacteria (Fig 7.6, 7.7), and further that the presence of calcium can significantly enhance the retention of bacteria (Fig 7.7). Both of the previous studies with low cementation depth were applied to existing limestone where calcium is present, which may have resulted in decreased penetration. The method of injection is considered to be the main factor in increasing cementation depth, which may be further improved by an interdisciplinary approach considering the geotechnical and engineering as well as microbiological aspects. The injection method could be improved by applying the enzyme under cold conditions (e.g. 4°C) with cooled solutions to minimise the precipitation reaction before injection into the material. Alternative injection devices may also improve cementation depth. For example, pumping the cementation liquid through a lance with holes in it, that is inserted into the material for cementation and slowly withdrawn so as to cement from the inside out. The most suitable injection method will vary depending on the application and can be tailored to achieve a desired result.

8.3. Consistency of urease activity

Although biocementation offers some significant advantages over other technologies, some problems were encountered with the consistency of urease activity that has not yet been fully resolved. *P. vulgaris* and *S. pasteurii* were evaluated in this study for their suitability to produce urease for biocementation (Chapter 2). Although *S. pasteurii* produced clearly more urease per cell than *P. vulgaris*, urease production in this organism was less consistent and varied with respect to biomass (Fig 2.8). High specific urease activity could be achieved by harvesting cells from the culture medium and reinoculating into fresh medium (Fig 3.6), however the same improvement could not be achieved by fed-batching nutrients into the culture medium (Fig 7.3), suggesting that a factor in the medium

which increased during growth was responsible for repression of high specific activities. It was further shown that the lack of high specific urease activity was not due to the depletion of urea (Fig 3.4, 3.5) and was not due to high concentrations of ammonium (Fig 3.9). pH was shown to have a regulatory effect with higher specific urease activity exhibited under sub-optimum growth conditions (Fig 3.9) however the effect was not clearly switchable (Fig 3.7, 3.8), which indicated that another regulatory mechanism(s) existed, in addition to pH, to control the level of urease activity in *S. pasteurii*.

As the growth optimum pH was shown to be repressive to urease activity, a two-stage growth system, where the organism is grown and then subject to optimum conditions for producing high levels of urease activity, seems preferred for maximum urease production (Fig 3.9). However, lowering pH did not result in an increased specific urease activity (Fig 3.7, 3.8) and the capacity for restoration of urease activity after loss was low (Fig 3.12, 3.13, 3.14). Chemostat and fed-batch cultivation were shown to produce poor urease activities (Fig 3.11, 7.3). Contrastingly, the reasonably high levels of urease activity were achieved (28 mM urea.min⁻¹, Fig 3.6 hours 0 – 10) in batch cultivation of *S. pasteurii* on industrial medium, and thus this method is recommended until a better understanding of urease regulation is acquired. The actual repressive mechanism was not fully elucidated in this study and requires further investigation. Despite not fully exploiting the urease capability of *S. pasteurii*, sufficient urease activity for biocementation could still be achieved to levels suitable for biocementation.

8.4. Tolerance of *S. pasteurii* under cementation conditions

At high concentrations, urea significantly enhanced urease activity in *S. pasteurii*, probably because of an increased rate of urea diffusion due to the higher concentration gradient across the membrane (Fig 5.3). High concentrations of ammonium were thought to be the most-likely inhibitory product of urea hydrolysis, but ammonium did not affect urease activity in *S. pasteurii* (Fig 5.7). The organism was exposed to concentrations of up to 3 M ammonium at pH 9.25, where ammonium existed as 1.5 M NH₄⁺ and 1.5 M of the usually more toxic NH₃ (Fig 1.5). This high level of tolerance for cementation conditions further confirmed that this organism was suitable for biocementation.

8.5. Optimisation of cementation conditions

The cementation conditions required for biocementation include high concentrations of calcium, urea, ammonium and nitrate and/or chloride. The tolerance of cementation conditions by *S. pasteurii* was shown by full precipitation of 1.5 M calcite within 18 hours (Fig 5.2). Normal intracellular Ca levels are in the order of 0.1 – 1 μM which are approximately 1000-times less than the normal external environment conditions (Norris *et al.*, 1996). High calcium concentrations of up to 1.5 M were tolerated by the cell representing an external concentration that was 10^6 -times higher outside compared to inside the cell (Fig 5.4). It has been suggested that this calcium gradient allows microorganisms an additional role in calcite precipitation by active extrusion of calcium via the $\text{Ca}^{2+}/2\text{H}^{+}$ pump, which results in localised areas of alkalinisation near the cell surface (Hammes and Verstraete, 2002). The urease activity of *S. pasteurii* was completely inhibited by 2 M $\text{Ca}(\text{NO}_3)_2$ and showed approximately 80% inhibition at 1.5 M (Fig 5.4). This inhibition could be reduced to 60% of the uninhibited rate by providing calcium as a 50:50 mixture of nitrate and chloride ions, indicating that nitrate was also inhibitory to urease activity (Fig 5.5, 5.6).

8.6. Cost of urease production

An economic medium was developed for *S. pasteurii* which reduced the medium cost to \$0.20 per L with an activity yield of 21 mM urea.min⁻¹ (Table 4.3). *S. pasteurii* has a high preference for protein based-media (Mörsdorf and Kaltwasser, 1989; Wiley and Stokes, 1962) and it may be possible to grow the bacteria on a waste product such as liquid protein waste from dairy industries. The problem with waste sources is that they are often dilute and would therefore require concentration of the bacteria after growth to achieve sufficient urease activities for biocementation. Further economisation of the medium could be attained, if a suitable method for the concentration of bacteria could be found.

8.7. Optimisation of application for strength

The most cost effective treatment that generated high strength cementation was two applications of 4.4 mM urea.min⁻¹, given 24 hours apart (Fig 6.17) at a total cost of \$41.90 per m³ (Appendix F). This value represents the cost of the growth medium consumables to produce the required amount of enzyme. In general, low

urea hydrolysis rates were conclusively shown to produce higher strengths per carbonate produced compared to high urea hydrolysis rates (Fig 6.6B 6.12, 6.13). This is in agreement with the suggestion of Ramachandran *et al.*, (2001), who investigated the effect of bacterial concentration (and hence urease activity) on the compressive strength improvement in porous Portland cement mortar cubes. The authors found that low concentrations of bacteria produced a 24% increase in compressive strength, whereas high concentrations of bacteria produced no improvement. In their study it was not possible to conclusively establish this finding because no difference in total calcite could be detected in the bacterially treated cubes versus the control cubes (which were treated with urea/Ca only) (Ramachandran *et al.*, 2001). The lowest enzyme concentration tested in this study was 2.2 mM urea.min⁻¹ (Fig 6.5). It would be desirable to test whether lower concentrations of enzyme with a higher number of applications or with longer incubations times, could produce significant strength. This was not evaluated in this study and requires further investigation.

A trend of apparent limited 4 – 6 hour life-span for the enzyme was observed under cementation conditions, independent of cementation rate (Fig 6.11). This has a significant impact on the amount of cementation that can be achieved in one application. The immobilisation of most enzymes, including urease, have been shown to stabilise enzyme activity for longer periods, under adverse conditions (Bachmeir *et al.*, 2002). The immobilisation potential for *S. pasteurii* urease inside the cementation material has been shown (Fig 6.16) and further investigation of this is considered worthwhile.

8.8. Remaining problems to be addressed

Several problems exist in the system, that have not yet been fully addressed:

- Variability of urease activity in *S. pasteurii* – Although *S. pasteurii* has the capability to generate very high concentrations of urease and a high tolerance towards the chemicals (Ca²⁺, urea, NH₃/NH₄⁺, NO₃⁻ and Cl⁻) involved in cementation, the mechanism controlling urease activity has not been clearly elucidated in this study. This lack of knowledge represents a risk for low activity production, which should be

addressed before commencing large-scale production. It would also be desirable to enrich for wild-strains that may be suitable for biocementation.

- Production of NH_4^+ – The hydrolysis of urea generates 2 moles of ammonium for every mole of urea, which represents up to 3 M of ammonium per application. In some cases three applications were applied, and in large scale application this would generate 1500 L of 3 M ammonium chloride or nitrate solution as waste per m^3 of treated material. High concentrations of ammonium are highly toxic to most organisms and this waste should be treated by nitrification/denitrification processes (requiring the addition of a carbon source) before disposal.

8.9. Application potential of the technology

Two options exist for the application of bacterial urease in cementation; (i) use of whole bacterial cells and (ii) use of a soluble enzyme extract made by cell lysis via a homogeniser or other large scale industrially-suitable method.

The whole cell system is likely to provide less uniform cementation (gradient from the injection point) and the potential to immobilise the enzyme in the cementation material. This whole cell application method is well suited to the consolidation of sandy soils, particularly for stabilisation and strengthening of landforms. Potential applications for this technology include the stabilisation of dunes and embankments, where the retention of porosity is important to enable water movement through the material whilst preventing erosion of the structure. The technology was considered suitable for strengthening dikes in the Netherlands, where the ability for water to move through the material is essential (personal communication: Mr Gert Grew, GeoDelft). There is also scope for this technology in the agricultural industry, for the temporary seasonal cementation of pineapple and strawberry plantation rows, where significant problems exist in the removal of soil by tropical rains (personal communication: Mr Bruce Moore, Agronomist for Golden Circle, QLD).

A soluble urease extract could be produced for an additional cost, with easy injection properties and no capacity for immobilisation. This type of soluble enzyme extract would be more suitable for fine-scale homogeneous cementation, such as would be required for homogenous geological rock models or fine restoration of heritage structures, such as the preservation of sand forms from buried chariot remnants in China (personal communication: Dr Geoff Hewitt, Archaeologist at La Trobe University, Melbourne).

8.10. Conclusion

This study has conclusively established that high strength economical cementation can be achieved using MCP via urea hydrolysis to “proof-of-concept” stage. This is the first published study to achieve effective consolidation of loose sand via the application of bacterial cultures. The biocementation technology has many advantages over existing consolidation technologies, including retention of porosity after treatment, in-situ application and the potential for reusing the enzyme in subsequent applications. Cementation can be readily achieved without any additional processing of the bacterial culture liquid (e.g. concentration, lysis, removal of medium), by directly mixing the enzyme with the cementation components (Ca/urea) and injection into the cementation material. A cost-efficient cementation procedure has been developed, however the potential for further cost savings is apparent in the areas of medium development (e.g. dairy waste) and enzyme immobilisation and reuse, which require further investigation. There are still currently two problems to be addressed before large-scale cementation is attempted; (i) consistency of *S. pasteurii* urease and (ii) a suitable method for dealing with high ammonium concentration waste.

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Appendices

Appendix A	Conductivity as a measure of urease activity
Appendix B	Conversion of conductivity rate to urea hydrolysis rate
Appendix C	Standardisation of urease activity measured under different pH conditions
Appendix D	Ammonium determination by a modified Nessler method
Appendix E	Data from ultrasound velocity monitoring during cementation (Chapter 6 – Section 6.3.2.1)
Appendix F	Cost calculation for biocementation of 1 m ³ of material with various treatments

Appendix A

Conductivity as a measure of urease activity

Urease activities were determined by calculating the slope of conductivity changes versus time, measured under standard conditions of 1.5 M urea at 25°C.

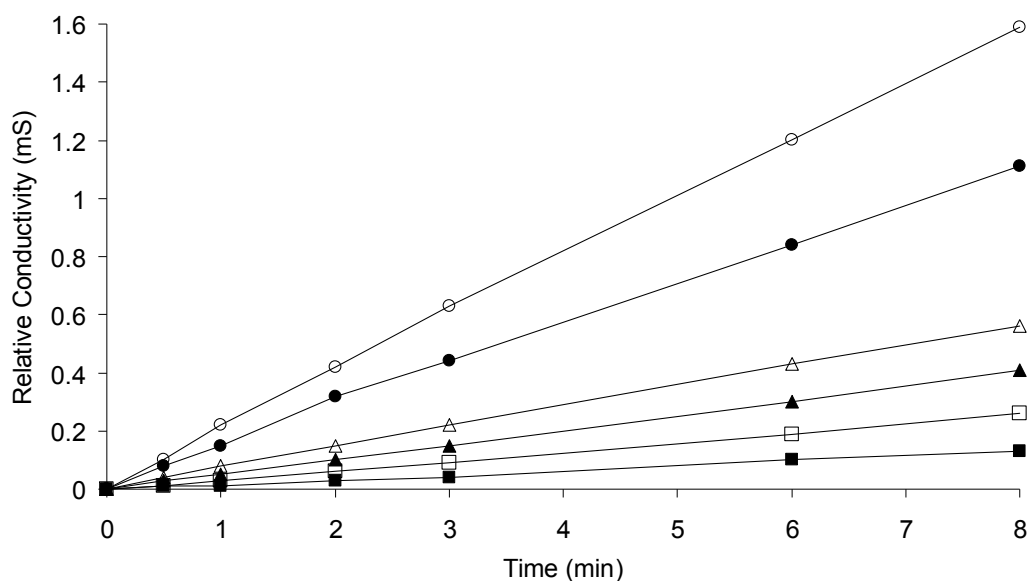


Figure A.1: Relative conductivity change for different dilutions of whole cells of *Proteus vulgaris*, measured at 1.5 M urea at 25°C. Relative concentrations of cells were 1x (■), 2x (□), 3x (▲), 4x (△), 8x (●) and 12x (○).

Providing urea was in excess, conductivity changed linearly with the production of ionic NH_4^+ and CO_3^{2+} over the eight minutes tested. The slope of conductivity change was directly proportional to the concentration of active urease present (Table A.1).

Table A.1: Calculated slopes of each line in Figure A.1.

Relative urease concentration	Slope (mS.min ⁻¹)	Slope/Relative Concentration	Relative urease concentration	Slope (mS.min ⁻¹)	Slope/Relative Concentration
1	0.0166	0.0166	4	0.0698	0.0175
2	0.0327	0.0163	8	0.1376	0.0172
3	0.0507	0.0169	12	0.1981	0.0165

Appendix B

Conversion of conductivity (mS) to urea hydrolysed (mM)

Urease activities were determined by measuring the relative change in conductivity when exposed to urea under standard conditions of 1.5 M urea at 25°C. A standard curve was generated by determining the conductivity change resulting from complete hydrolysis of several concentrations of urea by purified urease (Sigma Cat. No. U-7127). The amount of ammonium present at the end of hydrolysis was also determined to ensure that the reaction had gone to completion (Fig B.1).

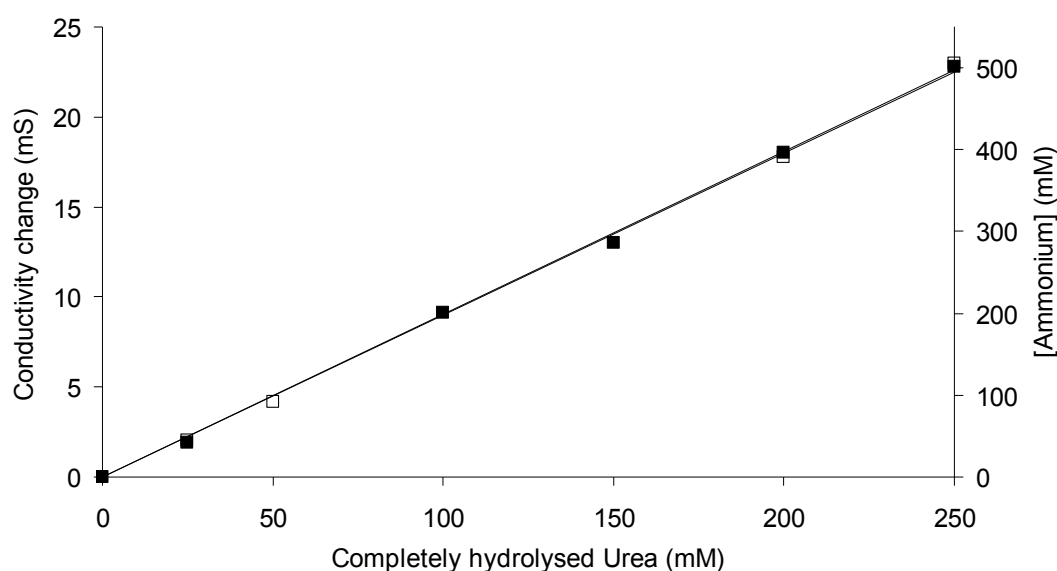


Figure B.1: Standard curve of the conductivity change (■) and ammonium concentration (□) present after complete hydrolysis of urea.

From this figure, the following relationships were determined:

$$\text{Urea hydrolysed (mM)} = \text{Conductivity (mS)} \times 11.11 \quad (R^2 = 0.9988)$$

$$\text{Urea hydrolysed (mM)} = \text{Ammonium (mM)} \times 0.50 \quad (R^2 = 0.9991)$$

Appendix C

Standardisation of urease activity measured under different pH conditions

To ensure that all measured changes in urease activity were real changes and not the pH effects on the enzyme, all activities were standardised to pH 7.

The effect of pH on urease activity was determined by measuring the slope of conductivity change over five minutes, by an aliquot of *S. pasteurii* suspension under different buffered pH conditions. Conductivities were determined under standard conditions of 1.5 M urea and 25°C. Conductivity measurements (mS.min⁻¹) were converted to urea hydrolysis rate (mM urea hydrolysed.min⁻¹) according to Appendix B.

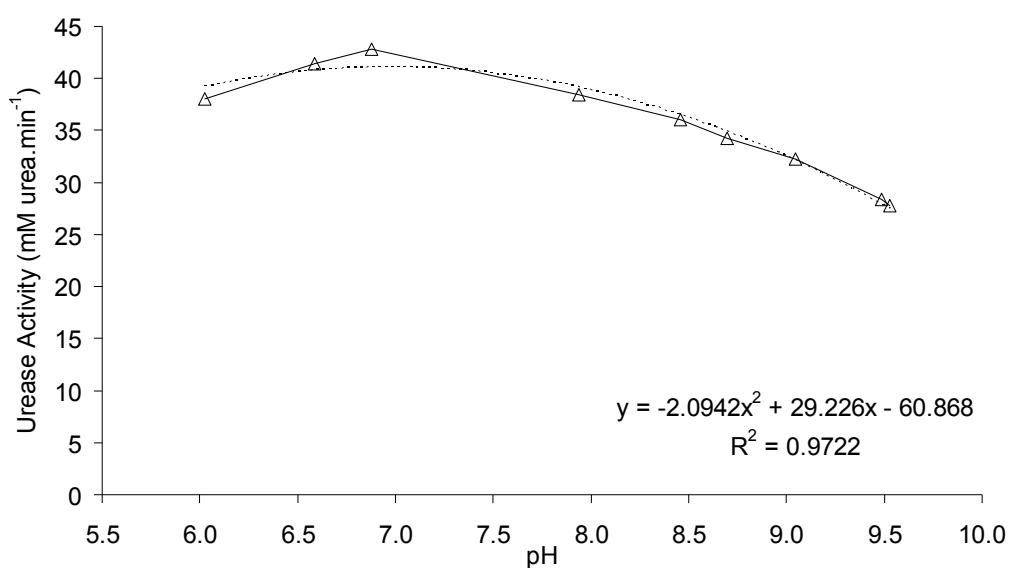


Figure C.1: Effect of pH on urease activity between pH 6 and 9.5.

A polynomial equation ($y = -2.0942x^2 + 29.226x - 60.868$) was fitted to the line and standardised activities were determined according to the following formulae:

$$\text{Activity at pH 7} = \text{Activity at pH } x \times \frac{\text{Std Activity at pH 7}}{\text{Std Activity at pH } x}$$

For example:

Standardisation of an activity of 25 mM urea hydrolysed.min⁻¹, measured at pH 9.25.

$$\begin{aligned}\text{Standard activity at pH 7} &= [-2.0942*(7^2)] + [29.226*(7)] - 60.868 \\ &= 41.10 \text{ mM urea hydrolysed.min}^{-1}\end{aligned}$$

$$\begin{aligned}\text{Standard activity at pH 9.25} &= [-2.0942*(9.25^2)] + [29.226*(9.25)] - 60.868 \\ &= 30.29 \text{ mM urea hydrolysed.min}^{-1}\end{aligned}$$

$$\begin{aligned}\text{Activity at pH 7} &= 25 \times \frac{41.10}{30.29} \\ &= 33.92 \text{ mM urea hydrolysed.min}^{-1}\end{aligned}$$

Appendix D

Ammonium determination by a modified Nessler Method

Ammonium concentration was determined by a modified Nessler method as described in the Materials and methods section of Chapter 2 (Greenburg *et al.*, 1992). Standards were prepared from analytical grade NH_4Cl .

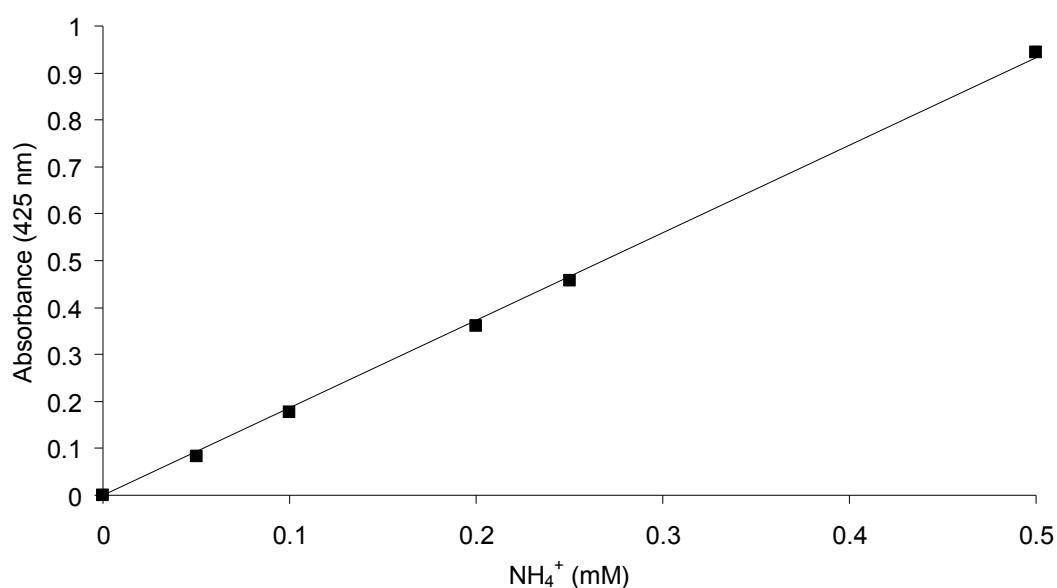


Figure D.1: Standard curve of ammonium concentration versus absorbance for the modified Nessler method.

A linear relationship exists between absorbance and ammonium concentration in the range of 0 – 0.5 mM NH_4^+ , which is described by:

$$y = 1.865 x \quad (R^2 = 0.9992)$$

Appendix E

Data from ultrasound velocity monitoring during cementation

(Chapter 6 – Section 6.3.2.1.)

This appendix includes data for the first unsuccessful biocementation treatment of cores that were continuously monitored for ultrasound velocity improvement during the cementation reaction.

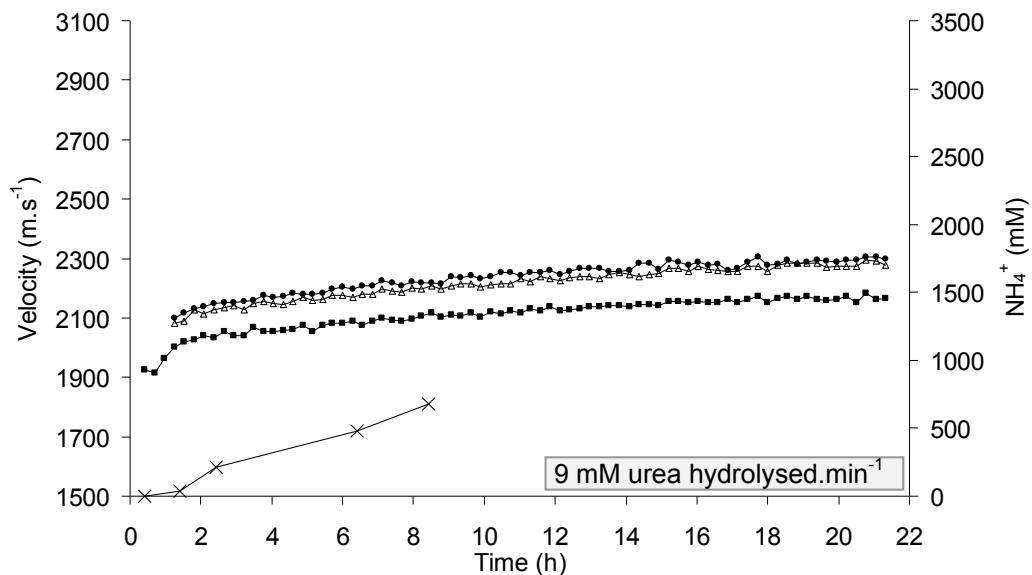


Figure E.1: Continuous monitoring of ultrasound velocity at three different positions along the core length from the injection point (15 mm (●); 45 mm (△) and 75 mm (■)) and ammonium concentration (×) during the biocementation reaction. Urease was applied as soluble enzyme at a concentration of 9 mM urea hydrolysed.min⁻¹.

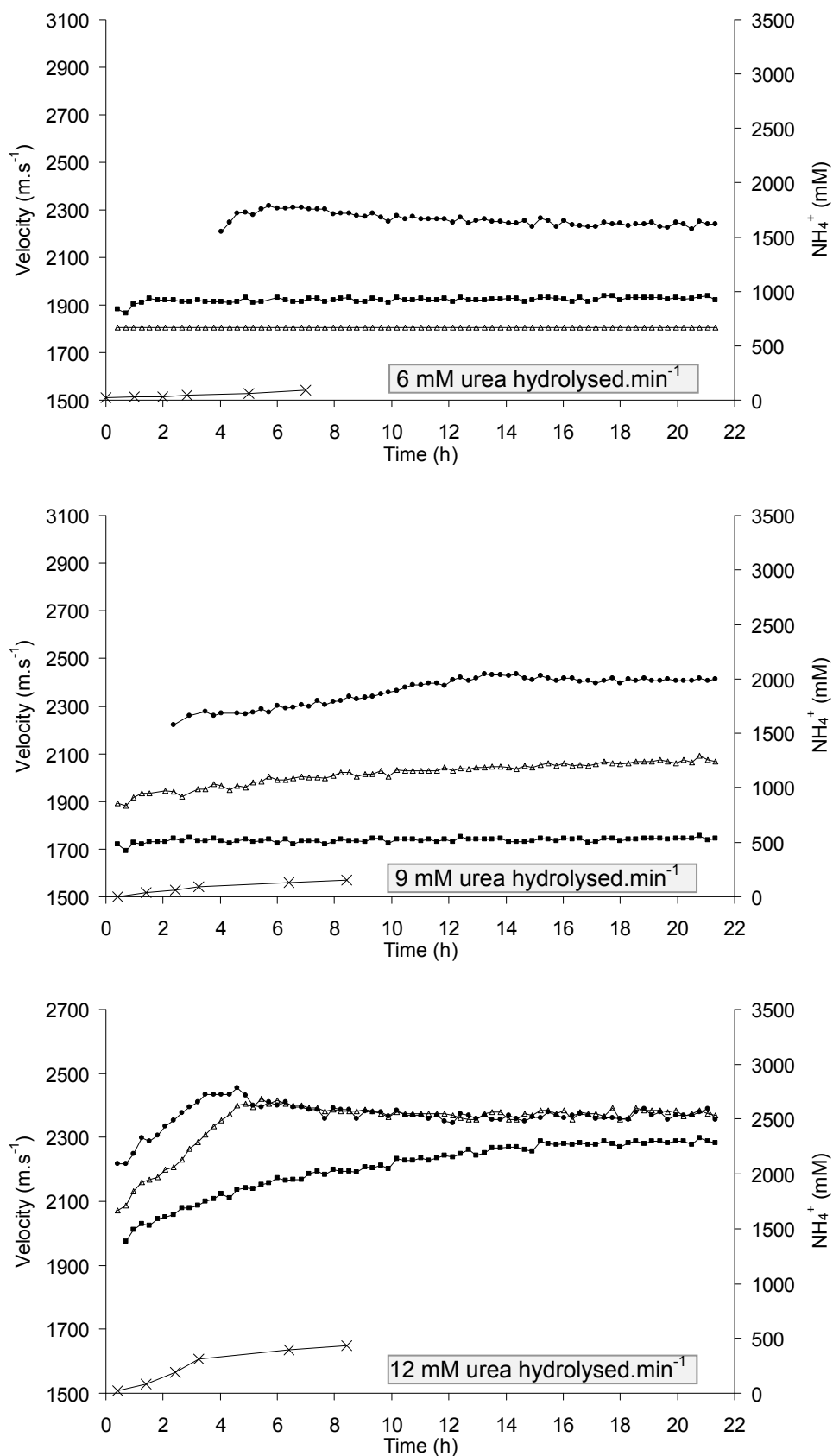


Figure E.2: Continuous monitoring of ultrasound velocity at three different positions along the core length from the injection point (15 mm (●); 45 mm (△) and 75 mm (■)) and ammonium concentration (×) during the biocementation reaction. Urease was applied as whole bacterial cells at various concentrations as stated on each chart.

Appendix F

Cost calculation for biocementation of 1 m³ of material with various treatments

All costs have been calculated using urease grown on the industrial medium containing 13.5 g.L⁻¹ Vegemite (Kraft), 150 mM acetate and 170 mM (NH₄)₂SO₄, which yields 21 mM urea.min⁻¹ for a cost of \$0.20 per L (Table 4.3).

1 m³ of sand is assumed to have a pore volume 50%; i.e. a pore capacity of 500 L.

$$\text{Treatment Cost} = \left[\frac{\text{Enzyme activity required}}{\text{Enzyme activity in culture}} \times L \text{ per treatment} \right] \times \text{Cost per L}$$

Table F1: Cost and enzyme requirement of various biocementation treatments.

Treatment (mM urea.min ⁻¹)	Enzyme required (mM urea.min ⁻¹)	Enzyme required (L)	Cost (\$AUD)
One application			
1 x 2.2	2.2	52.4	10.48
1 x 4.4	4.4	104.8	20.96
1 x 6.7	6.7	159.5	31.90
1 x 8.8	8.8	209.5	41.90
1 x 11.1	11.1	264.5	52.90
1 x 13.3	13.3	316.7	63.34
1 x 15.6	15.6	371.4	74.28
1 x 17.8	17.8	423.8	84.76
Two applications			
2 x 2.2	4.4	104.8	20.96
2 x 4.4	8.8	209.5	41.90
2 x 6.7	13.3	316.7	63.34
2 x 8.8	17.6	419.	83.81
2 x 11.1	22.2	528.6	105.72
2 x 13.3	26.6	633.3	126.66
2 x 15.6	31.2	742.9	148.58
2 x 17.8	35.6	847.6	169.52

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