High Strength *In-Situ* Biocementation of Soil by Calcite Precipitating Locally Isolated Ureolytic Bacteria

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Declaration

I declare that, except where specific reference is made in the text to the work conducted by other authors, 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.

Salwa M. Al-Thawadi
Abstract

This study has contributed to the patented technology of biocement (Microbial Biocementation, WO/2006/066326). Biocementation or biogrout is a sand consolidation technology, in which the carbonate released from microbial urea hydrolysis precipitates with an excess of calcium ions to form \textit{in-situ} calcite (CaCO$_3$) precipitation. Under the right conditions this can result in soil solidification and has found significant commercial interest.

This study has enriched and isolated highly urease active bacteria, particularly suitable for the fermentation process. Six strains with different properties relevant for biocementation were isolated. The most urease active strain (strain MCP11) produced sufficient urease to allow the use of the non-concentrated cell suspension for biocementation experiments. Activities and specific activities were 11-28 mM urea hydrolysed.min$^{-1}$ and 2.2-5.6 mM urea hydrolysed.min$^{-1}$.OD$^{-1}$ respectively. A separate strain (strain MCP4) showed spontaneous flocculation at the end of the batch growth, showing its increased tendency to attach to surfaces. This can be useful for effective cell concentration and for improved attachment during the cementation process.

The possibility of causing cementation by using enrichments rather than pure strains has been documented. This may allow a cheaper production of the urease than by traditional pure culture processes.

Urease production was optimised by increasing the concentration of yeast extract and the addition of Ni$^{2+}$ ions to the growth media, resulting in increasing urease activity as the reproducible urease yield. This was accomplished by the addition of 10 $\mu$M Ni$^{2+}$ ions and increasing the level of yeast extract to 20 g.L$^{-1}$.
Some of the isolated strains were suitable for biocementation process producing mechanical strength ($\geq 0.6$ MPa) within several hours depending on the rate of urea conversion. This mechanical strength enhancement of the cemented columns was performed without a large decrease in the permeability.

The formation of CaCO$_3$ crystals in the presence of high concentration of calcium and urea was monitored. This crystal growth was monitored over time by video recording the ureolytic reaction on a microscopic slide. The crystals also were examined through SEM. It was found that two types of CaCO$_3$ precipitates were formed; these precipitates were calcite rhombohedral crystals and spheroids. Video clips showed that the rhombohedral crystals originated from the spheroids. These spheroids were fragile, not stable and were considered to be vaterite.

This study suggested that the strength of the cemented column was caused mostly due to the point-to-point contacts of rhombohedral CaCO$_3$ crystals and adjacent sand grains.

A method of producing high strength cemented samples from sand was developed. This method first attaches the cells into the sand-column by growing them in the presence of calcium ions as little as 6 mM. Then, the cells were incubated in-situ for about 48 hours to enable attachment to the surface of the sand granules. Then the cells were reused over 20-times by continuous supply of cementation solution (equi-molar concentration of calcium and urea). This method produced a mechanical strength of up to 30 MPa, which is equivalent to construction cement.

The mechanical strength could be increased by supplying the bacteria in-situ with a food source and 10 $\mu$M Ni$^{2+}$ ions, allowing some measures of reaction rate control in-situ. To our knowledge, this study was the first study to use biological cementation to produce strength comparable to that
of traditional cemented construction materials such as sandstone and concrete.

The key factors for the optimal CaCO$_3$ precipitation (strength production) \textit{in-situ} were examined. It was found that \textit{in-situ} urease activity was the key factor for strength production. The maximum \textit{in-situ} urease activity was achieved by supplementing the cementation solution with growth media, and the use of 0.5 M urea and Ca$^{2+}$ as cementation solution. The \textit{in-situ} urease activity differed according to the different bacterial strains which tolerated the cementation conditions differently.

One of the advantages of the present study was that cementation of porous media could be achieved without clogging the injection end. The injection end could be clogged by CaCO$_3$ precipitation due to cementation reaction (cells, calcium and urea). By sequentially flushing the cells and cementation solution, clogging of the injection end could be avoided and high penetration depth was achieved as long as there was sufficient passage of cementation solution. Uniform cementation along 1 m packed sand-column was obtained. This uniformity was confirmed by the urease activity measurement, calcite precipitation and mechanical strength production. For finer sand, homogenous cementation proved more difficult.
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1.1. Background

Calcium carbonate (CaCO₃) is one of the most common minerals on earth. CaCO₃ precipitation is a common phenomenon, forms natural rock and exists in environments such as marine water, fresh water, and soils (Castanier et al., 1999; Ehrlich, 1998). The increase in the concentration or decrease in the solubility of the calcium or carbonate in solution causes the natural precipitation of CaCO₃. Abiotic change (e.g. evaporation or changes in temperature or pressure) or biotic action (microbial action) participates in the natural precipitation of CaCO₃. The biotic contribution in CaCO₃ precipitation exceeds the abiotic in most environments on earth (Castanier et al., 2000a). The rate of microbiological CaCO₃ precipitation correlated with cell growth. Stocks-Fischer et al (1999) have found that this microbial rate of precipitation was significantly faster than that of chemical precipitation.

Hammes and Verstraete (2002) suggested that the chemical CaCO₃ precipitation is controlled by four factors:

1. The calcium ions concentration;
2. The dissolved inorganic carbon concentration (carbonate);
3. The pH; and
4. The presence of nucleation sites.

In microbial CaCO₃ precipitation, the first three factors are the key for CaCO₃ precipitation, while the forth factor is not a key factor because the bacteria themselves behave as nucleation sites.

1.2. The Role of Urease Activity in Calcium Carbonate Precipitation

1.2.1. Urease

Urease was the first enzyme to be isolated in its crystalline form from Canavalia ensiformis (jack bean) (Summer, 1926). The first three-dimensional structural model of urease was observed by X-ray of a bacterial
source, *Klebsiella aerogenes* (Jabri et al., 1995; Karplus et al., 1997). *Klebsiella* urease consists of three subunits. Each subunit has an active site with a bimetallic Nickel center (Mulrooney and Hausinger, 1990; Mobley et al., 1995; Benini et al., 1999). These primary structures of *K. aerogenes* and *Sporosarcina pasteurii* urease (Mulrooney and Hausinger, 1990) are highly homologous.

Urease is synthesised under condition of nitrogen starvation (Mobley et al., 1995). Mobley and his colleagues (1995) found that urease level is increased 20 to 25-fold when *Bacillus subtilis* cells were grown in nitrogen poor medium. There are contradictory statements regarding the location of urease in the bacterial cells. Reithel (1971) stated urease is a cytoplasmic protein whereas Mclean *et al* (1985: 1986) found that the urease is located in the membrane and periplasm of *Staphylococcus sp.* and *Protus mirabilis*.

### 1.2.2. The Urease Reaction Mechanism

Urea is released into the environment as a result of biological action. For example all mammals excrete urea as a detoxification product. Urease (urea amidohydrolase; EC 3.5.1.5) is widely distributed in soil and aquatic environments. Biotic urease activity is widespread in the environment and includes the action of bacteria, yeasts, filamentous fungi (Mobley and Hausinger, 1989), algae (Yates, 1999), and a number of higher plants including jack beans (*Canvalia ensiformis*), soybean leaf and seed (*Glycine max*), pigweed (*Chenopodium album*) and mulberry leaf (*Morus alba*) (Sirko, 2000).

Urease hydrolyses the substrate urea generating ammonia and carbamate (Equation 1.1). Carbamate spontaneously decomposes to produce another molecule of ammonia and carbonic acid (Equation 1.2) (Mobley and Hausinger, 1989). The two ammonia molecules and carbonic acid subsequently equilibrate in water with their deprotonated and protonated forms, resulting in an increase in the pH (Equation 1.3 and 1.4) (Mobley and Hausinger, 1989).
In general, the bacterial urease is regulated through five regulation modes (Sissons et al., 1990; Sissons et al., 1992; Mobley and Hausinger, 1989 and Mobley et al., 1995). These modes are:

1. Constitutive—where the enzyme is fundamentally produced regardless of the environmental conditions (e.g. B. pasteurii and S. uresa).

2. Repression—where the presence of NH₃, NH₄⁺, urea and other ammonia precursors inhibit the urease production. Micro-organisms in this group include P. aeruginosa, A. eutrophus, B. megaterium (Kaltwasser et al., 1972) and K. aerogenes (Friedrich and Magasanik, 1977). Urease is re-activated (de-repressed) in the presence of a low concentration of nitrogen source (urea or ammonium) (Mobley and Hausinger, 1989; Mulrooney et al., 1989).

3. Induction — where the urease expression is induced in the presence of the substrate urea or other inducers. Urease activity was induced five to 25 times compared to the non-induced enzyme in the presence of urea (Mobley et al., 1991). Proteus and Providencia species have been reported to induce urease with urea (Rosentein et al., 1981; Mörsdorf and Kaltwasser, 1989).
4. Developmental regulation — where the expression of urease varies with the developmental stages. For example, the level of urease and urease transcript increases during swarming stage of bacterial cells (*P. mirabilis* and *P. vulgaris*) (Falkinham III and Hoffman, 1984).

5. Regulation by pH — where the urease level is regulated by pH through controlling the rate of urease synthesis (Sissons *et al.*, 1990; Sissons *et al.*, 1992). In *Streptococcus salivarus*, there was a six-fold increase in the urease activity within one hour due to lowering the pH (Li *et al.*, 2000).

### 1.2.3. Effect of Minerals on Urease Activity

There are different effects of minerals on urease activity have been proposed (Smith *et al.*, 1993; Bachmeier *et al.*, 2002; Hammes *et al.*, 2003a). Some of the studies showed contradictory effect of these minerals on urease activity. The presence of Ni$^{2+}$ ions in the active site of the urease is essential for the functional activity as well as the structural integrity of the enzyme. Bachmeier and her colleagues (2002) have shown an increase of CaCO$_3$ precipitation by the addition of Ni$^{2+}$ ions in the growth medium of *E. coli* (pBU11). They claimed that the CaCO$_3$ precipitation rate was increased dramatically by the addition of Ni$^{2+}$ ions (5–100 µM), showing the highest rate in the presence of 5 µM. This importance of Ni$^{2+}$ ions to urease activity was confirmed by Mobley *et al.* (1995) who claimed all forms of purified urease required Ni$^{2+}$ ions. Contradictorily, the presence of additional Ni$^{2+}$ ions proved not to increase the CaCO$_3$ precipitation by *S. pasteurii* in another study (Bachmeier *et al.*, 2002).

In addition to Ni$^{2+}$ ions, activity of urease was enhanced by, but not dependent on — the presence of Na$_2$EDTA, DL-dithiothreitol (0.1-5 mM), Ca$^{2+}$, Ba$^{2+}$ and citrate (2-20 mM) (Smith *et al.*, 1993). The effect of Ca$^{2+}$ on urease activity was confirmed by another study which showed that the urease activity increased up to 10-folds in the presence of 30 mM Ca$^{2+}$ ions compared to the activity in the absence of Ca$^{2+}$ for certain isolates (Hammes *et al.*, 2003b). Contradictorily, the presence of Na$^+$, K$^+$, Cl$^-$, Br$^-$, acetate or
nitrate (2-20 mM) did not affect the urease activity; however the presence of 
Li⁺, Mg²⁺, Zn²⁺ or I⁻ caused the decrease in urease activity (Smith et al., 1993).

**1.3. Energy of Bacteria that Degrade Urea (Ureolytic Bacteria)**

Several microorganisms produced ATP through urea hydrolysis (Mobley and Hausinger, 1989). In *S. pasteurii* for example, the generation of ATP is coupled with urea hydrolysis, and specifically NH₄⁺ production (Figure 1.1). The proton motive force (or electrochemical potential) (Δp) controls the generation of ATP, according to Equation 1.5.

\[
\Delta p = \Delta p_{HI} + \Delta \psi
\]  

(1.5)

The pH gradient (the difference between the pH inside and outside the cells) is symbolised by ΔpH while Δψ is the membrane potential or charge gradient.

The generated ATP in neutrophilic organisms (organisms that prefer to grow in a neutral medium) depends on proton concentration gradient. The cells expel outside the protons from the electron transport chain, causing an increase in the concentration of protons (low inside/high outside). The protons then will be moved back into the cell according to the concentration gradient through the ATP-synthase; resulting in ATP production (Prescott et al., 1993).

For alkalophiles (organisms that grow optimally in high pH conditions), the condition is different. The pH outside the cell is high (low protons), so the protons will diffuse from inside the cell according to the concentration gradient (high inside/low outside) (Figure 1.1). The alkalophiles need the protons to generate ATP; therefore, they develop two mechanisms to drive the protons back into the cells. These mechanisms are increasing the pH inside the cells causing alkalinity of the cytoplasm (i.e. reducing ΔpH) and increasing the Δψ by the efflux of a cation (NH₄⁺) via ATP synthesase rather
than $H^+$. Due to the increase in the charge separation across the membrane ($\Delta \psi$) the proton motive force drives back the protons into the cell against the concentration gradient.

### 1.4. Ureolytic Bacteria and Cementation Reaction

Many organisms can use urea as a source of nitrogen by importing urea into the cell cytoplasm. One of the most robust ureolytic bacteria is *Sporosarcina pasteurii* (formerly known as *Bacillus pasteurii*). *S. pasteurii* is an aerobic, spore forming, rod shaped bacterium. It uses urea as an energy source and produces ammonia which increases the pH in the environment and generates carbonate, causing $Ca^{2+}$ and $CO_3^{2-}$ to be precipitated as $CaCO_3$ (Equation 1.6-1.8) (Kroll, 1990; Stocks-Fischer *et al*., 1999). Alkaline pH is the primary means by which microbes promote calcite precipitation (Ehrlich, 1998; Castanier *et al*., 2000a; Fujita *et al*., 2000). Based on various studies (Kroll, 1990; Stocks-Fischer *et al*., 1999; Castanier *et al*., 2000b; Van Lith 2003), a schematic model describing the role of ureolytic bacteria on calcium carbonate precipitation is illustrated in Figure 1.2.

\[
Ca^{2+} + \text{Cell} \rightarrow \text{Cell-} Ca^{2+} \text{...........................................(1.6)}
\]
\[
Cl^- + HCO_3^- + NH_3 \leftrightarrow NH_4Cl + CO_3^{2-} \text{...........................................(1.7)}
\]
\[
\text{Cell-} Ca^{2+} + CO_3^{2-} \rightarrow \text{Cell-CaCO}_3 \downarrow \text{.................................(1.8)}
\]
There are two metabolic pathways for bacterial carbonate formation. These are autotrophic and heterotrophic pathways (Castanier et al., 2000b). Regarding autotrophic pathway, CO$_2$ is used as a carbon source causing its depletion in the bacterial environment. In the presence of Ca$^{2+}$ ions, such depletion enhances the production of CaCO$_3$ (Equation 1.9).

$$\text{Ca}^{2+} + 2\text{HCO}_3^- \leftrightarrow \text{CaCO}_3 + \text{CO}_2 + \text{H}_2\text{O} \quad \text{..........................(1.9)}$$
Regarding heterotrophic pathways, bacteria can precipitate CaCO₃ through active or passive precipitation. In active precipitation, the production of CO₃²⁻ is due to ionic exchange through the cell membrane by calcium and/or magnesium ionic pump. During passive precipitation, the production of CO₃²⁻ is due to ammonification of amino acids, dissimilatory reduction of nitrate, or degradation of urea or uric acid. In all cases, ammonia as a metabolic end product is produced which induces a pH increase.

**Figure 1.2:** Schematic model based on the literature (Kroll, 1990; Stocks-Fischer et al., 1999; Castanier et al., 2000b; Van Lith 2003) summarizing the role of ureolytic bacteria in CaCO₃ precipitation in the presence of a high concentration of Ca²⁺ ions. The processes involved in CaCO₃ precipitation are: (1) hydrolysis of urea (Equations 1.1-1.3), (2) increasing the alkalinity (Equation 1.4), (3) surface adsorption of Ca²⁺ ions (Equation 1.7) and (4) nucleation and crystal growth (Equation 1.8-1.9). EPS stands for extra-polysaccharide in the case of the presence of EPS surrounding the ureolytic cells.

1.5. **Molecular Basis of Bacterial Calcium Carbonate Precipitation**

The mechanism of CaCO₃ formation is poorly understood at both the molecular and genetic level. Barabesi and his colleagues (2007) carried out the first study that suggested that the CaCO₃ biomineralisation process was genetically controlled. They identified the genes responsible for CaCO₃ formation in *B. subtilis*. The operon *lcfA* consists of five genes (Figure 1.3);
icfA, ysiA, ysiB, effB, and etfA. Previously, Wipat et al (1996) has described the transcriptional unit (icfA, ysiA, ysiB, effB, and etfA) as a cluster of genes encoding proteins used in fatty acid metabolism. Mutant strains containing each of the five genes were formed by insertional mutagenesis (Barabesi et al., 2007). Those mutant strains were unable to precipitate CaCO$_3$. Thus, Barabesi and his colleagues (2007) have suggested that all the genes in the icfA operon could be participating in CaCO$_3$ precipitation. Moreover, Barabesi and his colleagues (2007) have suggested that there is a link between the CaCO$_3$ and fatty acid formation confirming the previously mentioned findings of Wipat et al (1996).

![Gene map of the B. subtilis chromosome region containing urease gene cluster (from icfA to etfA) and results of RT-PCR as described by Barabesi et al (2007). Arrows show the direction of the transcription. At both ends of the region, there are two transcription terminators. Major RT-PCR cotranscripts (A4-C1, B6-D1 and C6-E3) are shown as bars with their sizes in base pairs (bp).](image)

**Figure 1.3:** Gene map of the *B. subtilis* chromosome region containing urease gene cluster (from icfA to etfA) and results of RT-PCR as described by Barabesi et al (2007). Arrows show the direction of the transcription. At both ends of the region, there are two transcription terminators. Major RT-PCR cotranscripts (A4-C1, B6-D1 and C6-E3) are shown as bars with their sizes in base pairs (bp).

1.6. Applications of Calcium Carbonate Precipitation via Bacterial Urea Hydrolysis

Bacterial CaCO$_3$ formation as a result of urea hydrolysis is known as bacterial calcite precipitation (BCP). BCP is highly desirable because it is natural and pollutant free. There are several applications for BCP, most of which considered for purposes other than strength development. Some of these applications are: (1) Removal of contaminants (e.g. radio-active pollutants); (2) Removal of calcium ions from wastewaters; (3) Protection and restoration of limestone monuments and statuary; (4) Creation of sacrificial patinas on limestone and production of biological mortars or cements; and (5) Plugging the pores of the oil-recover reservoir rock. Below are more details of these applications.
1.6.1. Removal of Contaminants
The capturing of divalent radionucleotide Strontium$^{90}$ ($^{90}\text{Sr}^{2+}$) in the groundwater, was investigated after the addition of high concentration of urea and very low concentration of Ca$^{2+}$ ions (Fujita et al., 2000; Warren et al., 2001). Strontium carbonate ($^{90}\text{SrCO}_3$) was precipitated; in such a way that ($^{90}\text{Sr}^{2+}$) replaces Ca$^{2+}$ ions in the calcite crystal preventing the spread of radio-nucleotide contamination.

1.6.2. Removal of Calcium Ions from Wastewaters
The potential of removing Ca$^{2+}$ ions from industrial wastewaters facilitated by BCP instead of chemical precipitation was studied (Hamme et al., 2003c). The presence of a high concentration of calcium ions (500-1500 mg.L$^{-1}$) in the wastewater causes severe scaling in the pipelines and reactors due to calcium formation as carbonate, phosphate and/or gypsum. By the addition of a low concentration of urea (0-16 g.L$^{-1}$), up to 90% of the calcium ions were removed from the examined wastewater.

1.6.3. Protection and Restoration of Limestone Monuments and Statuary
Physical, biological and chemical factors may cause the weathering of monumental stones. Consequently, a loss of cohesion of stone material and progressive mineral matrix dissolution will be enhanced. In the case of calcareous stones, the porosity will increase due to CaCO$_3$ leaching and weakening of the superficial structure of the stone (Tiano, 1999). The attempt which was done by Tiano (1999) in stopping or slowing down the deterioration of monumental statuary by ureolytic bacteria was unsuccessful, as no significant difference in strength was shown after CaCO$_3$ precipitation. In a recent study, microbial sealant at which a new additional layer on the surface of an old concrete layer was achieved (Figure 1.4) (Ramakrishnan, 2005). It was concluded that cracks remediation by $S$. pasteurii may enhance the strength and the durability of the structure.
Figure 1.4: Remediation of micro-cracks found in the concrete by *S. Pasteurii*. A new layer of CaCO$_3$ (surface 2) was precipitated over the surface of the cement mortar. The remediated concrete regained 82% of its original strength (Ramakrishnan, 2005).

1.6.4. Creation of Sacrificial Patinas and Biological Mortars

Le Metayer *et al.* (1999) have successfully studied bacterial cementation aiming at surface coating, biological mortars and creation of patinas on limestone. Their method depends on spraying the entire surface of limestone with bacteria followed by a nutritional medium containing calcium and urea. A protective surface coating of CaCO$_3$ (several micrometers deep) was formed. They have claimed that calcite particles were precipitated within the bacterial cells and then expelled afterwards. A high penetration depth of 500 $\mu$m was reported by immersing the limestone sample in the cementation media (Rodriguez-Navaro *et al.*, 2003). The use of *Myxococcus xanthus* (a slow growing bacterium) resulted in CaCO$_3$ precipitation at the wall of the porous materials without plugging them.
1.6.5. Plugging the Pores of the Oil-Recover Reservoir Rock

Bacterial cells were used to plug the highly permeable rocks of the oil-reservoir. Between 8 and 30% of the total oil present in oil-reservoir was recovered from ordinary oil production method (Leonard, 1986). Oil-recovery depends on primary and secondary treatments (water flood) to recover the crude oil in the pores of the reservoir rock (Bryant, 1987). To improve the recovery method after primary and secondary treatments, conventional methods depend on chemical or thermal energy is used. These conventional methods are considered inefficient as they led to 67% retention of the total oil within the pores of the reservoir rock (Bryant, 1987). Therefore, there was an interest in the use of microbes to enhance the oil-recovery, which can be through:

- Microbial production of bio-surfactants and biopolymers at the surface;
- Microbial growth in the pores of the oil-reservoir rocks producing gasses, surfactants and other chemicals; or
- Microbial plugging of the pores in the oil-reservoir channels, which may resulted in increasing the sweeping effectiveness of the recovery process.

The rocks of the oil-reservoir contain high permeability zones. When the water is injected to displace oil, it will move through the pores of the highest permeable zone, bypassing much of the oil. Because of the small size of the bacteria, they will move to high permeable areas, plugging the pores, and as a consequence the sweep efficiency and oil recovery will be enhanced up to 100% (Bryant, 1987; Behlülgil and Mehmetoğlu, 2002).

In the ordinary method of bacterial enhancement of oil-recovery, plugging of the pores was due to bacterial multiplication (Knapp et al., 1983; Macleod et al., 1988), production of gasses that increases the pressure (Jack et al., 1982) production of organic acids, surfactants (Zobell, 1946) and polymer production (Macleod et al., 1988). Much attention is devoted to the plugging of highly permeable zone via bacterial urea hydrolysis. This type of plugging probably offers a feasible alternative to block the rock pores; improving the
residual oil recovery. Complete plugging within days was achieved by mixing bacteria with sand before packing into cores followed by application of calcium, urea and carbonate (Gollapudi et al., 1995). Moreover, it was found that the bacteria plug the sand granules by providing a nucleation site at which CaCO₃ was precipitated through alkaline environment (Stocks-Fischer et al., 1999).

1.7. Stone Formation by Bacterial Calcite Precipitation (Biogrun)

Microbial plugging could be greatly enhanced by using microorganisms with high urease enzyme activities indirectly involved in CaCO₃ consolidation (Stocks-Fischer et al., 1999). Besides the high urease activity, a high tolerance to urea, calcium, ammonium and either nitrate or chloride (depending on the calcium salt used) enhance the microbial plugging (Whiffin, 2004). There is a lack of knowledge regarding the high strength production of the biocemented products (sand stone formation). Most of the CaCO₃ precipitation studies achieved a consolidation or patching treatments for existing material as described previously (section 1.6). Whereas there are few studies aimed at the production of sand stone (Table 1.1). Sand stone production depends on how strong is the binding between the particles, which affects the cementation quality of the precipitated calcite.

The growth of CaCO₃ crystals for the purposes of artificially cementing sediments proved difficult because of the low yields obtained from a number of different reactions at room temperature. However, the successful bonding of calcareous sediments with derivatives of aluminium alkoide indicates that CaCO₃ is a promising route to stabilise loose particles (Koplick, 1989). Superior to this sediment cementation attempt, the loose particles were well cemented by chemical precipitation of CaCO₃ through Calcite In-situ Precipitation System (CIPS), producing high degrees of calcite cementation similar to the natural sediments within less than 24 hours. This CIPS technology which is (a non-microbial cementation process) was similar to the natural process that forms the rocks (Ismail et al., 2002). This successful
rock formation by chemical CaCO₃ precipitation indicates a great deal of scope for further work on the strength of microbiological CaCO₃ precipitation in porous media.

Table 1.1: Biocementation conditions which are reported in the literature for the production of CaCO₃. The purpose of this CaCO₃ precipitation was to form stone via CIPS (Calcite In-situ Precipitation System), microbial or plant urease. The loose materials (sand granules) were packed and then injected by combining calcium/urea with the bacterial or plant enzyme. According to the CIPS, the components of the solutions were not mentioned.

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th>Urea (mM)</th>
<th>Ca (mM)</th>
<th>Urease activity</th>
<th>Maximum Strength (MPa)</th>
<th>Depth of Penetration (mm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial and plant urease</td>
<td>1500</td>
<td>1500</td>
<td>4-18 mM urea.min⁻¹</td>
<td>1.8 (3 applications)</td>
<td>170</td>
<td>(Whiffin, 2004)</td>
</tr>
<tr>
<td>plant urease</td>
<td>200</td>
<td>600</td>
<td>0.3 g.L⁻¹</td>
<td>NM</td>
<td>300</td>
<td>(Nemati &amp; Voordouw, 2003)</td>
</tr>
<tr>
<td>CIPS</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.679-3.8 (1 application)</td>
<td>179</td>
<td>(Ismail, 2002)</td>
</tr>
</tbody>
</table>

ND: Not Defined, Property to Calcite Technology Pty Ltd (Perth, Australia)
NM: Not Measured

Nemati and Voordouw (2003) have described the use of urease to cement a porous medium. In this study, reducing the permeability of a porous medium by enzymatic CaCO₃ precipitation through Canvalia ensiformis was achieved. Nemati and Voordouw used between 0.1 and 1.0 M (>33 g.L⁻¹) calcite together with high urease activity for a successful plugging of the sand core. Unfortunately, the strength build-up was not monitored.

The study of Whiffin (2004) was the first published study in bacterial plugging of loose sandy material through urea hydrolysis (Biogrout) for the purpose of strength production. This study was successful in producing strength of 1.8 MPa. This relatively high strength as compared to the previous studies was achieved through three applications of bacterial cells and cementation solution. Whiffin (2004) has established a biocementation method which depends on a fast flow rate to inject the biocementation mix (bacteria, calcium and urea). The fast flow rate is not recommended to consolidate fine sands. The used cementation solution (calcium/urea) ranged between 0.75
and 1.5 M. In some cases Whiffin (2004) has recorded a full precipitation of calcite within 18 hours. Due to the calcite precipitation, which blocks the pores, a low penetration depth (maximum of 170 mm) was achieved. Beside this low penetration depth, the inconsistency of urease production by \textit{P. vulgaris} and \textit{S. pasteurii} are considered to be another problem which has arisen throughout this study.

1.8. Advantages of Sand Stone Formation by Ureolytic Bacteria

The biocementation process (microbial consolidation) is advantageous over the ordinary cementation processes by:

- Retention of the permeability was evident by the absorbance of water recorded in the biocemented surfaces (Tiano \textit{et al.}, 1999). For consolidation of loose material, it is vital to conserve the permeability so that the water moves through the voids in the stone hindering the deterioration due to water logging.

- Cost-saving process as the bacterial enzyme was reused in subsequent applications (2-3) of calcium and urea only (Whiffin, 2004). The extent of reusing the cells \textit{in-situ} was not determined by Whiffin (2004).

- Consolidation of porous media can be achieved by the direct use of bacterial culture without the need to concentrate the cells or extract urease from the bacteria. Thus, there is no need for additional processing for the bacterial culture to produce sand-stone.

- Its economical effective process more than, for example, the CIPS technology. One of the components of CIPS cementation solution is originated from a plant source (Property to Calcite Technology Pty Ltd., Perth, Australia). Thus CIPS is a challenging process since it is subjected to seasonal variation, extraction cost and transport.
Its dependence on bacteria which are more tolerant to the cementation condition than the plant source (Whiffin, 2004).

1.9. Derivation of Thesis Objectives
The process of biocementation by the use of components derived from a plant source —CIPS technology— and ureolytic bacteria was well established for the consolidation of short packed sand-columns (Ismail, 2000; Ismail et al., 2002; Namati and Voordouw, 2003; Whiffin, 2004). The injection in all of these attempts depends on combining the cementation solution and the enzyme/or bacteria immediately prior to the injection, avoiding the ex-situ precipitation of CaCO$_3$. This type of injection required very fast flow rate. No published work has discussed the possibility of sequential injection of bacteria followed by calcium/urea solution with and/or without immobilizing the cells in-situ. In addition, the mechanism by which the bacteria can form CaCO$_3$ crystals and their types are not known.

To achieve high strength production of a packed sand-column via ureolytic bacteria, it is feasible to enrich and isolate robust ureolytic bacteria that tolerate high concentrations of urea, NH$_4^+$, Ca$^{2+}$ and Cl$^-$ (produced from CaCl$_2$ salt) and produce urease constitutively.

To avoid the clogging of CaCO$_3$ at injection end due to cementation reaction; a mechanism of attaching ureolytic bacteria first to sand granules followed by up-flushing cementation solution is of a great interest. By this mechanism, high penetration depth (cementation of long packed-sand column) is expected. Thus, the objectives of this thesis are to produce different strength of sand cores "Commercially acceptable" and high penetration depth using robust bacterial cells isolated locally.

1.10. Thesis Objectives
Naturally, the biomineralisation process occurs at a very slow rate over geological times (Stocks-Fischer et al., 1999). For effective biomineralisation, bacterial mediated precipitation must be enhanced by
using microorganisms of relatively high urease activity (Stocks-Fischer et al., 1999; Whiffin, 2004). The precise objectives of this study are:

1) To enrich, isolate and identify bacterial strains that are suitable for calcite precipitation;

2) To examine the process by which the bacterial cells can form the CaCO$_3$ crystals and type of the produced crystals;

3) To determine the key factor(s) that control the biocementation process \textit{in-situ};

4) To determine the effect of feeding the cells during biocementation process \textit{in-situ}; and

5) To establish a method of achieving a different range of strengths from soft to high-strength formation using urease producing bacteria and continuous supply of cementation solution (calcium/urea).
Chapter 2
Enrichment and Isolation of Highly Urease Active Aerobic Bacteria from Soil and Sludge

2.1. Introduction

*S. pasteurii* is widely used as a source of urease. It produces intracellular urease which is close to 1% of the cell dry weight (Benini et al., 1999). A study done on clogging sand column by using *S. pasteurii* showed inconsistency in urease production by the desired microbe (Whiffin, 2004). Accordingly, it was highly desirable to obtain new strains of urease positive bacteria that offer specific advantages over the existing strain (*S. pasteurii*). The advantages could be: higher urease specific activity, higher stability (robust), higher tolerance to high concentration of ammonium, cheaper medium used, less need for sterile conditions, and more consistent in urease production. Hence, one of the aims of this chapter is to design methods to enrich and isolate highly urease active bacteria.

Before proceeding to biocementation attempts with the new isolates, it was necessary to optimize their urease production. Hence, this chapter aims at studying the conditions that affect the urease activity such as:

- The minimum requirement of Ni²⁺ ions;
- The effect of yeast extract (YE) concentration in the growth medium;
- The addition of Urea (substrate) at late Log-phase; and
- The presence of very low concentration of calcium/urea or calcium only in the bacterial culture.

2.2. Materials and Methods

2.2.1. Enrichment of Highly Active Ureolytic Bacteria

To screen for new strains with high level of urease activity, high specific activity (the activity showed by each cell) and high stability, soil and sludge samples were collected from different locations that are likely to contain ureolytic bacteria (Table 2.1). One g of soil or sludge was placed in 50 ml of growth media (250 ml shaking flasks, at 28°C, for 36 hours). The enrichment media consisted of 10 g.L⁻¹ Yeast extract (YE), 1-5 M urea, 152 mM
ammonium sulphate and 100 mM sodium acetate. For some enrichment cultures, YE was replaced with 20 g.L\(^{-1}\) corn steep liquor or 20 g.L\(^{-1}\) Vegemite, a food quality yeast extract used in Australia.

2.2.2. Isolation of Highly Urease Active Bacteria from Enrichment Cultures

To screen for pure colonies, the enrichment cultures were diluted and streaked on Nutrient Agar plates (NA, pH 8). NA contained 8% of filter sterilized urea which was added after the autoclaving. The plates were incubated at 28°C for 48 hours. Sterile phenolphthalein drops (indicator for the increase in pH caused by urease activity) were placed on the surface of the colonies and the change in the color was recorded. The single colonies that showed a pinkish color were individually transferred to a liquid media similar to the enrichment medium, and were treated under the same conditions of enrichment cultivation.

2.2.3. Conditions that Affect Urease Activity, Specific Urease Activity and/or Bacterial Biomass

2.2.3.1. Examining the Effect of the Addition of Urea at Late-Log Phase

For this test, bacterial isolates (50 ml, MCP6) were grown in the absence of ammonia and urea at 28°C. When the optical density of the culture reached 4 (late log phase), and urease activity reached 2.8 mM urea hydrolysed.min\(^{-1}\) (0.25 mS.min\(^{-1}\)), YE-medium (to a final concentration of 5%) and urea (to a final concentration of 0.25 M) were added to the culture. Then, this culture was incubated in the orbital shaker for several hours.
Table 2.1: Ureolytic bacterial enrichment conditions with different urea concentrations and C-sources. 1 g of soil or sludge was added to 50 ml of the growth media at pH 9.0. It was incubated in 250 ml shaking flask at 28°C with shaking for 36-48 hours.

<table>
<thead>
<tr>
<th>Enrichment #</th>
<th>Media</th>
<th>Inoculums sample</th>
<th>Urea concentration (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>*</td>
<td>Waste water sludge</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>*</td>
<td>Soil from Murdoch University agricultural field</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>**</td>
<td>Waste water sludge</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>**</td>
<td>1g Soil from Murdoch University agricultural field.</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>***</td>
<td>Waste water sludge</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>***</td>
<td>Soil from Murdoch University agricultural field.</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>*</td>
<td>Soil from garden bed from the suburb of Murdoch</td>
<td>1.6</td>
</tr>
<tr>
<td>8</td>
<td>*</td>
<td>Waste water sludge</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>*</td>
<td>Waste water sludge</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>*</td>
<td>Soil from Murdoch University agricultural field.</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>*</td>
<td>Soil from garden bed from the suburb of Murdoch</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>*</td>
<td>Soil from sheepcote</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>*</td>
<td>Soil from stable</td>
<td>5</td>
</tr>
</tbody>
</table>

* 10 g.L⁻¹ YE, 8.2 g.L⁻¹ NaCH₃COO, 10 g.L⁻¹ (NH₄)₂SO₄
** 20 g.L⁻¹ Corn steeper, 8.2 g.L⁻¹ NaCH₃COO, 10 g.L⁻¹ (NH₄)₂SO₄
*** 20 g.L⁻¹ Vegemite, 8.2 g.L⁻¹ NaCH₃COO, 10 g.L⁻¹ (NH₄)₂SO₄

2.2.3.2. Concentration of Urease Activity by the Presence of Cementation Solution

A bacterial isolate (1L, MCP6) was grown in the absence of urea. After the optical density (OD) reached 1.25, cementation solution (equimolar concentration of calcium chloride and urea) was added to a total concentration of 30 mM. This cementation solution was added gradually to the culture, with soft stirrings to avoid damaging the cells. White precipitates
were formed after a short period of soft stirring. These flocks were easily re-suspended.

2.2.3.3. Minimum inhibition of Ni\textsuperscript{2+} Ions Concentration

Bacterial culture (MCP11) was grown in the presence of different concentrations of Ni\textsuperscript{2+} (0-7290 µM) for 24 hours. Then samples (1 ml) were collected for mass (OD at 600 nm) and urease activity (by conductivity) measurements.

2.2.3.4. Effect of Calcium Concentration on Urease Activity and Coagulation

Bacterial culture (MCP11) was grown in the presence of different concentrations of Ca\textsuperscript{2+} (0-2000 mM) for 24 hours. This experiment was carried out in 50 ml test tube. Then samples (1 ml) were collected for mass (OD at 600 nm) and urease activity (by conductivity) measurements.

2.2.3.5. Effect of Different Concentrations of Yeast Extract (YE) on Urease Activity

The bacterial growth medium and conditions were similar to the enrichment experiment (current chapter, section 2.2.2); except that:

- Concentration of urea was 1 M
- Concentrations of yeast extract were 10, 20 or 40 g.L\textsuperscript{-1}.

2.2.4. Identifying the Best Ureolytic Strain for Biocementation Process

Total genomic DNA from pure cultures (5 ml of MCP1, MCP4 or MCP11) was extracted using ultra clean™ soil DNA kit (Mo-Bio Laboratories Inc., CA). DNA was extracted using the manufactures directions with the following modifications: the amount of buffer S3 was doubled followed by an extra wash of the column with buffer S4. The extracted DNA was used in PCR amplifications to target the bacterial 16S rRNA gene using eubacteria-specific primers designed by Muyzer et al (1993). The forward primer Eub341F (5’-CCTACGCGGAGGCAGCAG-3’) and the reverse primer Eub 907R (5’-CCGTCATATGTTAGTCTACG-3’) [numbered according to E. coli]
were included in a final volume of 25 ml, and contained 20 mM Tris HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 mM of each primer and 0.5 U of Taq Ti DNA polymerase (Fisher Biotech, Australia). PCR amplification was initiated by denaturation at 95°C for 10 min and was followed by 40 cycles of denaturation (95°C, 30 s), annealing (55°C, 15 s) and then by extension (72°C, 30 s). A final extension was carried out at 72°C for 3 min. PCR products were separated by electrophoresis in 1.2% agarose gels, stained with ethidium bromide and visualized on a UV trans-illuminator. PCR products were purified using a Qiagen PCR purification kit (Qiagen, Australia). Nucleotide sequences of the PCR products were determined using the same primers, and the ABI-PRISM BigDye terminator V 3.0 cycle sequencing kit (Perkin Elmer) using an ABI-Capillary DNA sequencer (Perkin Elmer) at the State Agricultural Biotechnology Centre (SABC) at Murdoch University. Sequencing was carried out in duplicate. Putative identification of each sequence was carried out using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

2.2.5. Biomass Determination

The biomass was determined by measurement of optical density (OD) spectrophotometrically at 600 nm (Pharmacia Biotech, Novaspec II).

2.2.6. Urease Activity

Ionic products from non-ionic substrates are liberated when urea is hydrolyzed according to the following equation:

\[
\text{H}_2\text{N-CO-NH}_2 + 2 \text{H}_2\text{O} \xrightarrow{\text{Urease}} 2 \text{NH}_4^+ + \text{CO}_3^{2-} \quad \text{..... (2.1)}
\]

The ionic products generate an increase in overall conductivity of the solution, and the rate at which conductivity increases is proportional to the concentration of active urease present. The conductivity was recorded over 3-6 minutes under standardized conditions of 1.5 M urea at 25°C (Whiffin, 2004).
2.2.6.1. The Conductivity Measurement as an Indicator of Urease Activity

Conductivity was used to determine the enzymatic rate of reaction. The method has a number of advantages; the device used is a robust, easy, and inexpensive assay system. The sensitivity of the method depends on the concentration of ion in the solution. Practically, conductivity has a mid-range sensitivity but the events occurring within 10 s may be accurately recorded (Lawrence and Moores, 1972). The most sensitive of all conductimetric assays is the urea hydrolysis (Lawrence and Moores, 1972; Equation 2.1).

The conductivity (mS) of each strain was measured and converted to urease activity by multiplying the conductivity with the dilution factor. When exposed to different concentrations of urea; the relative change in conductivity and amount of \( \text{NH}_4^+ \) (at the end of reaction) were measured (Whiffin, 2004). From the generated standard curve, Equation 2.2 was derived.

\[
\text{urea hydrolysis (mM) = conductivity (mS) } \times 11.11 \quad (R^2 = 0.9988; \text{Whiffin, 2004})
\]

(2.2)

2.2.6.2. Specific Urease Activity

Specific urease activity is the amount of urease activity per unit biomass. It was calculated as follows:

\[
\text{Specific urease activity (mM urea hydrolysed.min}^{-1}.OD^{-1}) = \frac{\text{Urease activity (mM urea hydrolysed.min}^{-1})}{\text{Biomass (OD}_{600})}
\]

2.2.6.3. \( \text{NH}_3 \)-N Analysis (Nesslers)

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 \( \text{NH}_4^+ \) analysis, the sample was thawed and then diluted to be in the range of 0-0.5 mM. This diluted sample (2 ml) was mixed with 100 \( \mu \text{l} \) 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 derived from ammonium chloride standards which were treated under the same conditions (Appendix A).

2.3. Results

2.3.1. Experimental Setup: To screen for ureolytic bacteria, conditions of high urea concentration and pH value were selected. Increasing urease production by the addition of $\text{Ni}^{2+}$ ions and yeast extract concentrations were tested.

2.3.2. Enrichment Culture for Screening Ureolytic Bacteria
To screen for ureolytic bacteria, it is necessary to select the conditions at which these types of microbes grow and produce urease. For selective enrichment media for ureolytic bacteria, the presence of urea as a substrate and an alkaline pH ($\geq 9.0$) was necessary. After initial incubation of the selective enrichment, different levels of urease activity were obtained (Figure 2.1), ranging from 2-42.2 mM urea hydrolysed.min$^{-1}$.

Derived from the high level of urease activity which was obtained from some enrichment cultures, it was expected that also unpurified enrichment cultures could give successful biocementation in the presence of cementation solution.

2.3.3. Isolation of Pure Isolates with High Urease Activity
The primary enrichments described above were transferred repeatedly to a fresh medium and then plated out on urease agar plates as described. Different ureolytic colonies were obtained. The most ureolytic active colonies could be spotted from pH increases in the agar media indicated by a colour change to pink by Phenolphthalein. In the presence of $\text{NH}_3/\text{NH}_4^+$, the solution becomes alkaline. Thus it turns pink. This pH increase was used as an indicator of urease activity (Equation 2.1).
Figure 2.1: Urease activity of the enrichments (see table 2.1) incubated for 36-48 hours, at pH 9.0 and 28°C. Yeast extract medium was used (10 g.L⁻¹) in the presence of filter sterilized urea (1-5 M) and 1 g soil or sludge.
A number of different pure bacterial isolates were obtained (Figure 2.2) according to the previously described method (current chapter, section 2.2.3). This method would allow the production of similar strains from suitable soil environments.

![Urease activity measurement by conductivity of the isolated ureolytic strains (1-13) from 13 enrichment cultures in Figure 2.1.](image)

**Figure 2.2:** Urease activity measurement by conductivity of the isolated ureolytic strains (1-13) from 13 enrichment cultures in Figure 2.1.

The common features of the three most suitable strains for biocementation process were as follows: Rod shaped cells that were characterized by the following features:

- Between 1-5 µm long, endogenous of urease production (i.e. after precipitating the cells by centrifugation, it was found that urease activity was only in the precipitate and not supernatant), fast growing (0.5-2.0 hours), highly tolerant to 1 M urea and 2 M ammonia, spore forming, relatively high urease activity (> 3.3 mM urea hydrolysed.min⁻¹). Online monitoring of urease activity, specific urease activity and bacterial growth for some isolated strains were carried out (Figure 2.3).

The three most promising isolates as can be seen in figures (2.2 and 2.2) were:

- **MCP6:** Without further optimisation, urease activity of 4.4-7.8 mM urea hydrolysed.min⁻¹ (0.4-0.7 mS.min⁻¹) was produced by
MCP6 (Figure 2.3). In contrast to other isolated strains, MCP6 produced the enzyme mostly during the mid phase (after 5-8 hours from being inoculated by fresh cells, Figure 2.3). This means that production of urease by this strain could be induced. Thus, MCP6 could be useful for certain application if the urease of this strain can be controlled.

- **MCP4 (Flocculating strain):** The flocculation behavior of this strain facilitates its separation from the growth medium and is expected to assist in the immobilisation into sand-columns. Its urease activity ranged from 5.6 to 11.1 mM urea hydrolysed min⁻¹ (0.5-1 mS min⁻¹) (Figure 2.3).

- **MCP11:** Without further optimisation, urease activity of 9-11 mM urea hydrolysed min⁻¹ (0.8-1 mS min⁻¹) was achieved by MCP11 (Figure 2.3); no need for urea as inducing agent. This last point could be significant for biocementation trials as it avoids the production of carbonate and ammonia in the medium which avoids rapid formation of CaCO₃ when contact with the cementation solution. Also MCP11 strain was observed to maintain its urease activity upon storage over several weeks at 4°C.

To assess the ability of one of the isolates to tolerate biocementation conditions, MCP4 (flocculant strain) was used for large scale biocementation process (Figure 2.4). It was used to plug compacted sand (sand distribution, appendix B) in a glass tank according to the method described by Whiffin (2004). The cementation solution and bacterial culture was mixed and flushed through the sand instantly (in less than 15 min). Unequal cementation was achieved; the stone formation was only around the injection tubes, presumably due to the unequal distribution of cells. This unequal distribution of cells may have occurred because the bacteria had flocculated in the injection tube and clogged the sand. However, in principal, the success of some sand
stone formation suggested that the newly enriched strains may be suitable for biocementation process. In addition, the cementation which was performed close to the injection point supports the idea that the use of flocculated cells may be suitable for cementation of more porous material (i.e. coarse sand, Appendix B).
Figure 2.3: Online monitoring of biomass (●), urease activity (●) and specific urease activity (▲) of some of the isolated ureolytic strains (MCP1, MCP2, MCP4, MCP5, MCP6 and MCP11). These isolates were incubated for 24-42 hours at 28°C in YE-based medium with 0.3 M urea, in the absence of ammonium acetate.
Figure 2.4: Initial biocementation of porous media (sand, particle size 300-400 µl) attempt for one of the isolated strains (MCP4). Plugging was performed at the injection ends. The flow of solution was from left to right and took about 15 min.

2.3.4. Effect of Increasing the Concentration of Yeast Extract on the Bacterial Urease Activity and Specific Activity

In an attempt to increase the urease activity of the isolated bacteria, the cells were grown on different concentrations of Yeast extract (YE). Because the flocculated MCP4 was proven to be suitable only for surface coating, while this study aims at stone formation then, the non-flocculating strain (MCP6) was used for this test. As expected, there was an increase in urease activity as the YE was increased allowing the cells to grow (Figure 2.5). By increasing the YE 2 and 4-folds, the OD increased by 38% and 50% respectively (Figure 2.5). This increase in OD caused an increase in urease activity, maintaining the specific urease activity about constant. The percent increase in OD from using 20 g.L⁻¹ to 40 g.L⁻¹ YE was 12%. This low increase in growth does not encourage the use of more than 20 g.L⁻¹ YE in the cultivation media.
The increase in YE concentration in the growth media serves an important factor to enhance the bacterial growth and consequently the urease activity.

2.3.5. Effect of Urea Addition on Urease Activity

To test for any stimulating effect of urea on the urease activity/specific urease activity, the substrate urea was supplied to the bacterial culture at early stationary phase. There was no increase in urease activity (Figure 2.6) indicating, that urease was not induced by the presence of its substrate at the stationary phase.
Figure 2.5: The effect of increasing the YE in the growth media on biomass (●), urease activity (●) and specific urease activity (▲) of MCP6. The bacteria were incubated for 42 hours at 28°C in a medium with (A) 10 g.L⁻¹, (B) 20 g.L⁻¹ and (C) 40 g.L⁻¹ YE.
Figure 2.6: The effect of urea (substrate) supplementation at the early stationary phase of MCP6 growth. The bacteria had an optical density of 3.7 (●) and were grown on media with 10 g.L\(^{-1}\) YE, in the absence of ammonium acetate and urea. The addition of urea had no significant effect on urease activity (■) and urease specific activity (▲).

2.3.6. Concentrating Bacterial Cells by the Addition of Cementation solution (Calcium Ions and Urea)

Concentrating urease activity by mixing the cells with calcium and urea may be necessary to obtain sufficiently strong urease activity for biocementation. It was found that the urease activity was concentrated approximately 20-times (Table 2.2) by adding calcium and urea (cementation solution) to the solution. By cementation solution addition bacterial cells are precipitated while urease activity stays viable, resulting in higher urease readings of the precipitate. This co-precipitation of bacterial cells with CaCO\(_3\) could be used as a cost effective and perhaps more cost effective method than using commercial flocculants or concentrating the cells by centrifugation. To calculate the cost effective of using the calcium ions to precipitate the cells instead of using commercial flocculants or concentrating the cells by centrifugation needs further experiments. It is logic that the use of low concentrations of calcium ions is cheaper than ordering commercial flocculants which are needed in high concentrations. At the same time centrifugation of cells need energy which will be added to the cost of the process.
Table 2.2: Bacterial cell concentrations by co-precipitation with CaCO$_3$ formed from urease activity (of 20 mM of calcium and urea was added). Urease activity of the bacterial culture before the addition of ca$^{2+}$ ions and urea was 0.2 mS.min$^{-1}$.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Urease activity (mS.min$^{-1}$)</th>
<th>Volume (ml)</th>
<th>Urease activity (mS.ml.min$^{-1}$)</th>
<th>Total urease activity (mS.min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>0.08</td>
<td>950</td>
<td>58.9</td>
<td>* 0.14</td>
</tr>
<tr>
<td>Pellet</td>
<td>1.55</td>
<td>38</td>
<td>76</td>
<td></td>
</tr>
</tbody>
</table>

* Total urease activity (mS.min$^{-1}$) = (urease activity of supernatant + pellet (mS.ml.min$^{-1}$))/(the total volume of supernatant + pellet (ml))

2.3.7. Minimum Requirement of Ni Ion Concentrations

To determine a suitable concentration of Ni$^{2+}$ ions which enhances urease activity without decreasing the biomass, bacterial cells were grown in the presence of different concentrations of Ni$^{2+}$ ions. The minimum concentration of Ni$^{2+}$ that did not inhibit the growth but accelerated the urease activity was 10 µM (Figure 2.7A). The addition of as little as 10 µM Ni$^{2+}$ ions increased urease activity 3-folds. At Ni$^{2+}$ concentrations higher than 270 µM a dramatic drop in urease activity and biomass was observed (Figure 2.7B) indicating Ni toxicity. Although there was a drop in urease activity and biomass, the specific urease activity (urease activity of each cell) increased 1-2.0 folds. This increase indicates that the presence of Ni$^{2+}$ ions in the growth medium is important in enhancing the specific urease activity.

The above results have raised the following question: Could the Ni$^{2+}$ ions accelerate the specific urease activity of an already grown bacterial culture, leading to an increase in overall urease activity? To answer this question, different concentrations of Ni$^{2+}$ ions were added to an already grown bacterial culture and incubated in an orbital shaker for several hours in the presence of 10% medium added as a source for urease synthesis. The addition of low concentration of Ni$^{2+}$ (1 mM) could double the urease activity of this grown culture (Figure 2.8) while higher concentration caused a slight inhibition.
Providing the culture with sufficient Ni$^{2+}$ is a simple way of maintaining an increased activity. Hence, all future experiments well be using 10 µM Ni$^{2+}$ in the growth medium.

Figure 2.7: The effect of different concentrations of Ni$^{2+}$ ions (µM) on biomass (●), urease activity (●) and specific urease activity (▲) of MCP11. The bacteria were incubated for 24 hours at 28°C in a growth medium containing 20 g.L$^{-1}$ YE and pH 9.0.
Figure 2.8: The effect of the addition of different concentrations of Ni\(^{2+}\) ions to a grown bacterial culture (after being grown to the desired OD) on biomass (●), urease activity (★) and specific urease activity (▲) of MCP11. The bacteria were grown in 50 ml test tubes in a shaker at 28°C for 24 hours. Yeast extract to a final concentration of 4 g.L\(^{-1}\) and Ni\(^{2+}\) ions were added and left in the shaker for 17 hours.

2.3.8. Effect of Calcium Concentration on Urease Activity and Coagulation

The effect of calcium concentration on urease activity was examined. It was found that urease activity was stable at concentration up to 1 M (Figure 2.9). At 2 M calcium, there was a dramatic decrease in urease activity. Accordingly, urease activity was inhibited by the presence of a concentration above 1 M calcium ions. Determining the inhibitory amount of calcium chloride on urease activity enables the proper determination of calcium chloride concentration to be used in the cementation solution (calcium/urea). This concentration of Ca\(^{2+}\) ions suppose not to exceed 1 M.
Figure 2.9: Effect of calcium concentration on urease activity. Inhibitory effect of calcium on urease activity at concentration of 2 M was evident.

The effect of calcium concentration on coagulating the cells was examined in a test tube type experiment (as described in section 2.2.3.4). As the concentration of calcium increased, the volume of the coagulated cells increased until a steady state was performed (Figure 2.10). This coagulation caused by the presence of calcium only did not significantly lower the urease activity. In the presence of urea and calcium, the CaCO$_3$ will be precipitated at the surface of the bacteria, causing the cells to precipitate (as mentioned in section 2.3.6). The more calcium/urea solution was added, the more cells were precipitated. Moreover, there was a chemical precipitation of CaCO$_3$ crystals in the solution due to the presence of Ca$^{2+}$ and CO$_3^{2-}$, high pH and supersaturation. Thus, the presence of sufficient amount of calcium/urea in the bacterial culture prior to the injection; could cause penetration difficulty through the packed sand-column. This penetration of cells will be worse if fine sand was used. The penetration difficulty could be attributed to:

- The precipitation of CaCO$_3$ at the surface of the bacterial cells which increases the diameter of the cells; and
- The chemical precipitation of CaCO₃ crystals might block the pores of the packed sand-column.

Based on above, the settlement of cells by the presence of calcium only was superior to the settlement by the presence of urea and calcium in terms of penetration depth.

![Graph showing the amount of flocculated cells from bacterial suspension (measured by a graduated cylinder) for given concentrations of calcium added. The optical density in each test at 600 nm was 0.3.](image)

**Figure 2.10:** Amount of flocculated cells from bacterial suspension (measured by a graduated cylinder) for given concentrations of calcium added. The optical density in each test at 600 nm was 0.3.

### 2.3.9. Identification of Isolated Ureolytic Bacteria by 16S rRNA Sequence

To examine the molecular differences that control the ureolytic behavior of the ureolytic isolates, three isolates (MCP1, MCP4 and MCP11) from different environmental locations (as mentioned previously in Table 2.1) were genetically examined. PCR amplification of 16S rRNA was performed (Appendix C). By blasting this sequence at NCBI (GeneBank), it was found that the isolates were all closely related to one another. They were mostly closely related to a *B. species* which was identified by a previous study conducted by Hammes *et al* (2003a). All of the isolates were 100% related to *B. sphaericus* group, 97% related to *B. pasteurii* and 96% related to a *S. species*. Although the
isolated bacterial strains behaved differently regarding the urease production, ability to coagulate and growth rate; they have belonged to the same genera. The observed differences in the close related strains might be due to the different environments from which they were isolated. In addition, the enrichment media and conditions may be enriched for *Bacillus* related species.

Knowing that ureolytic strains were closely related to one another is of a great interest to the investigators in the field of bacterial fossils.
2.4. Discussion

A method to specifically enrich bacteria from most soil within a short cultivation period (36-48 hours), ideally suitable for biocementation process was designed. Selection conditions (high pH, presence of urea up to 5 M) have enriched for a superior Bacillus type bacteria that can degrade urea, is highly tolerant to urea and ammonia at pH 9.0 and hence ideally suited the biocementation process. It may not be necessary to isolate strains but merely to use selective enrichment conditions which contain urea as a substrate and setting the pH to be alkaline (around 9.0).

From the enrichment cultures, different ureolytic bacterial strains were isolated with high urease activity (up to 42 mM urea hydrolysed.min\(^{-1}\)). This urease activity was higher than the 10 mM urea hydrolysed.min\(^{-1}\) which is required for biocementation process as was suggested by whiffin (2004). The ureolytic cells could be concentrated through the presence of 20% cementation solution, conserving their ability to degrade urea. As respect to the extent and speed of concentration, better cellular concentration (coagulation) could be achieved by the presence of calcium ions only, which inhibits the urease activity when exceeds 1 M.

Although the differences in some bacterial behaviors between the isolates were evident, the most effective isolates were closely related to one another. Similar finding was shown in a previous study (Hammes et al., 2003a) on identification of ureolytic strains isolated from various environmental locations. This close relationship between the isolates might be due to the dominance presence of Bacillus species as was confirmed by Fleske et al (1998) who revealed that Bacillus species represented at least 40% of Dutch soil. Moreover, Stock-Fischer and her colleagues (1999) have stated that Bacillus species are selected by the isolation and cultivation methods.
The conditions that enhance urease activity were examined. It was found that enhancing the activity by the presence of Ni$^{2+}$ ions and yeast extract (YE) was successfully achieved. As expected, urease activity increases in the presence of YE as the bacterial cells will grow. The specific urease activity was accelerated almost Three-times by the presence of Ni$^{2+}$ ions in the growth medium (10 µM). The presence of Ni$^{2+}$ ions in the active site of urease is essential for the functional activity as well as the structural integrity of the enzyme. Bachmeier and his colleagues (2002) have shown an increase in calcite precipitation by the addition of Ni$^{2+}$ ions during their work on *E. coli* (pBU11). They claimed that the calcite precipitation rate increased dramatically by the addition of nickel (5-100 µM), showing the highest rate was in the presence of 5 µM Ni$^{2+}$ ions. In contradiction, another study showed that there was no increase in the calcite precipitation by *S. pasteurii* in the presence of additional Ni$^{2+}$ ions (Bachmeier *et al.*., 2002).

### 2.5. Conclusions

- Even before isolation attempts were performed, the enrichments produced were of high levels of activity. Thus it may not be necessary to isolate strains but merely to use selective enrichment conditions.
- The urease activity and/or specific urease activity can be increased by increasing the concentration of yeast extract (YE) and Ni$^{2+}$ ions (10 µM) in the growth medium.
- Derived from the coagulation effect of the cells followed by their settlement in the presence of calcium ions, it can be concluded that the activity could be easily concentrated by the presence of calcium ions in the bacterial culture.
Chapter 3
Calcium Carbonate Crystals Produced by Ureolytic Bacteria

3.1. Introduction

Biomineralization is a biologically mediated process leading to mineral nucleation and growth of mineral products (Tang, 1997). Lowenstam and Weiner (1989); Mann et al (1989) have defined two modes of biomineralization: biologically induced biomineralization (BIB) and boundary organized biomineralization (BOB). In BIB, an organism changes its local microenvironment providing suitable conditions for the chemical precipitation of minerals thus biomenerals are not directly associated with cellular structures. Whereas in BOB, an organism produces organic matrix within or on which inorganic particles are grown, thus a nucleation intracellularly or on the cell wall is observed during BOB.

3.1.1. Nucleation and Growth of Crystals

The transformation from solution to solid occurs when the free energy (free potential of Gibbs) of the initial solution phase is greater than the sum of the free energies of the crystalline phase plus the final solution phase (Gibbs 1876; 1878). It is known that all thermodynamics processes attempt to minimize the free energy. Accordingly, the free energy of the single crystalline state is minimal.

The process that produces a new phase with low free energy from an old phase with high free energy is called nucleation (new crystal formation) (Hohenberg and Halperin 1977, Chaikin and Lubensky, 1995). Nucleation occurs either as primary nucleation or secondary nucleation. Primary nucleation is the production of nuclei independent of the presence of suspended crystals while the secondary nucleation requires the presence of suspended solute crystals (Randolph and Lorson, 1988).

A high degree of supersaturation of lattice molecules is required for the CaCO$_3$ crystals to nucleate and grow (Sawada, 1998). There are three different mechanisms for crystal growth (Figure 3.1) (Randolph and Larson 1988; Ohtaki, 1998) which are summarized as follows:

- **Kossel mechanism:** This mechanism depends on the stepwise of a mononuclear layer which is supposed to occur on a surface of an ideal crystal (Figure 3.1A1). A mononuclear layer ($P'$) is joined to a smooth surface of crystal ($P$). The end of the layer ($P'$) is an incomplete line and has one or more kinks ($K$, important sites at which molecules that attach there build more bonds to neighboring ones attached to other regions). This kink region has the highest binding energy and is the most appropriate region for the integration of a unit molecule from the solution phase.

- **Two-dimensional nucleation:** Subsequent spread of crystals around the nucleus forming a new layer (Figure 3.1B1). Once propagation step (growth of crystals) reaches the edge of the face, the surface is covered with another smooth surface without steps or kink sites. The two-dimensional nucleation is favorable at a high degree of supersaturation.

- **Screw dislocation:** The dislocation of crystals occurs in a spiral form in the propagation step (Figure 3.1C1). Crystals are not ideal structures; certain types of defect in the crystal structure could allow the incessant formation of steps (ledges or raised partial layers) on the surface. A new crystal arriving at the surface would then always find a step on which to adhere. The particular defect which provides this structure is called a screw dislocation. This mechanism of crystal growth occurs at a low degree of supersaturation.
Figure 3.1: Three models of crystal growth (Sawada, 1998) (A1): Kossel model, (B1): Two-dimensional model and (C1): Screw dislocation.

3.1.2. Types and Morphology of Calcium Carbonate Crystals

Calcite, aragonite and vaterite are three crystal forms of CaCO$_3$ nucleation (same chemical formula, different structure). Calcite is considered as the most stable form of CaCO$_3$, with simple rhombohedral shape (De Yoreo and Vekilov, 2003) (Figure 3.2). Its formation is favoured by the presence of magnesium, manganese ions and orthophosphate. Furthermore, crystal aging supports calcite precipitation (Wray and Daniela, 1957).
Figure 3.2: A pure calcite crystal (De Yoreo and Dove, 2004)

Aragonite is a metastable CaCO$_3$ form and changes into calcite over geologic time. It is an orthorhombic crystal and obtained at high temperature (Wray and Daniela, 1957; Tai and Chen, 1998) or low temperature in the presence of magnesium ions (Tai and Chen 1998) and pH less than 11 (Tai and Chen 1998). Its formation is favoured by the presence of magnesium and manganese ions but not orthophosphate (Tai and Chen, 1998).

Vaterite is rarely found in nature (Sanchez-Moral, 2003). It is produced in the pH range from 8.5 to 10 with the initial relative supersaturation between 6.5 and 8.5 (Kralj et al., 1990), low Ca$^{2+}$ concentration (Yagi et al., 1984), or low temperature and high Ca$^{2+}$ concentration (Roques and Girou, 1974). The vaterite morphology is influenced highly by the pH and temperature. Three vaterite crystal types are known: Spherulite (at pH < 9.3 and below room temp), hexagonal-plate (at pH 9.6), and lettuce (at pH 8.5 and temperature of 40°C) (Tai and Chen, 1998). Generally speaking, the crystal morphology is one of the parameters affecting the applications of CaCO$_3$ precipitates for standard purposes. For example, the plate shape precipitates are desirable for paper making and paint making (Ota et al., 1989).

3.1.3. Microbial Calcium Carbonate Precipitation

Bacterial CaCO$_3$ precipitation under appropriate conditions is a general phenomenon (Bouquet et al., 1973). There are a number of species of
CaCO$_3$ minerals associated with bacteria, for example vaterite formation by *Acinobacter sp.* (Sanchez-Moral *et al.*, 2003), aragonitic sherulites by *Deleyahlophila* (Rivadeneyra *et al.*, 1996), calcite by *E. coli* (Bachmeier *et al.*, 2002) and magnesium calcite spherulites and dumbbells by the slime-producing bacteria, *Myxococcus xanthus* (Gonzàlez-Muñoz *et al.*, 2000; Holt *et al.*, 1993).

The CaCO$_3$ precipitation rate in general is a linear function of the ion concentration product of [Ca$^{2+}$] and [CO$_3^{2-}$] (Longdon *et al.*, 2000) hence obeying 2$^{nd}$ order kinetics or 1$^{st}$ order kinetics if one of the reactants (e.g. calcium) is in excess. Different rate constants have been obtained for bacterial CaCO$_3$ precipitation (Table 3.1). The microorganisms can influence the attainable saturation and the rate of CaCO$_3$ precipitation, controlling the polymorph of the produced CaCO$_3$ crystals (Bosak *et al.*, 2004; Bosak and Newman, 2005). When the concentration of Ca$^{2+}$ and CO$_3^{2-}$ exceeds the solubility product ($K_{sp}$), supersaturation of solution is reached. The higher the supersaturation (S) is the more likely precipitation of CaCO$_3$ is to take place.

**Table 3.1:** Different rate constant of bacterial urea hydrolysis.

<table>
<thead>
<tr>
<th>Source</th>
<th>Rate constant (d$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>In agriculture Topsoil (mixed micro-organisms)</td>
<td>0.01 - 0.11</td>
<td>Nielsen <em>et al</em> (1998)</td>
</tr>
<tr>
<td>In Vadese Zone subsoils (mixed micro-organisms)</td>
<td>0.09 - 1.68</td>
<td>Swensen and Bakken (1998)</td>
</tr>
<tr>
<td><em>Sporosarcina pasteurii</em></td>
<td>0.09 - 0.91</td>
<td>Ferris <em>et al</em> (2004)</td>
</tr>
</tbody>
</table>

The saturation level (S) of a solution with respect to CaCO$_3$ is defined by:

$$S = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}}$$

.................................(3.1)
Where \([\text{Ca}^{2+}]\) and \([\text{CO}_3^{2-}]\) represent the concentration of the dissolved \(\text{Ca}^{2+}\) and \(\text{CO}_3^{2-}\) respectively and \(K_{sp}\) is the calcite solubility product (Table 3.2).

**Table 3.2:** The solubility product \((K_{sp})\) of CaCO\(_3\) at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>(K_{sp})</th>
<th>Data Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.99×10(^{-8})</td>
<td>(Lange, 1967)</td>
</tr>
<tr>
<td>25</td>
<td>0.87×10(^{-8})</td>
<td>(Lange, 1967)</td>
</tr>
<tr>
<td>18-25</td>
<td>4.8×10(^{-9})</td>
<td>(Pauling, 1970)</td>
</tr>
</tbody>
</table>

Ferris *et al* (2004) revealed that the ureolytic bacteria (*S. pasteurii*) precipitate CaCO\(_3\) crystals through three different stages: (1) The development of supersaturated solution, (2) nucleation at the point of critical saturation (i.e. the supersaturation at which CaCO\(_3\) actually initiates), (3) spontaneous crystal growth on the stable nuclei.

It seemed worthwhile to study microbiologically formed CaCO\(_3\) crystals (type, shape, size and growth) at the micro or nano-scale. Such a micro-scale study will clarify some aspects related to the cementation process in the crystallographic level. The aims of this chapter are:

- To determine the morphology and types of CaCO\(_3\) crystals which were precipitated in the presence of high concentrations of cementation solution (calcium/urea);
- To determine the type of CaCO\(_3\) crystals formed by different ureolytic bacteria;
- To examine the size of CaCO\(_3\) crystals formed at different concentrations of cells and cementation solution;
- To investigate the role of ureolytic bacterial strain used in the production of CaCO\(_3\) crystals; and
- To monitor the growth of CaCO\(_3\) crystals (time-lapse).
3.2. Materials and Methods

3.2.1. Calcium Carbonate Precipitation by Ureolytic Bacteria

Bacterial culture (MCP11, urease activity of 1–2.8 mM urea hydrolysed.min\(^{-1}\)) was mixed with cementation solution to a final molarity of 1 M (equimolar of calcium/urea solution, calcium supplied as CaCl\(_2\).2H\(_2\)O) in a microcentrifuge tube. The final urease activity on the microscopic slide was 0.6–1.6 mM urea hydrolysed.min\(^{-1}\). Immediately, two drops (20 µl) of this mix were placed on microscopic slide and then covered with coverslip.

In the case of testing the ability of CaCO\(_3\) to precipitate on sand granules, 200 mg silica sand granules (9 µm diameter) were placed on a microscopic slide. Then, two drops of the cementation mix (bacterial culture and cementation solution) were placed at the surface of the sand granules. To avoid the drying of the sample on the microscopic slide, the edges of the coverslip were sealed with nail polish. CaCO\(_3\) crystal formation was examined (immediately and after 24 hours) by an Olympus compound microscope (BX51) fitted with a DP70 Digital Camera. One week later, the samples were subjected to Scanning Electron Microscope (SEM) examination (SEM- Philp XL20). Energy Dispersive x-ray Microanalysis of CaCO\(_3\) crystals was carried out using Oxford ISIS-5175 micro-analyser.

3.2.2. SEM Sample Preparation

Subsequent to growing CaCO\(_3\) crystals on a microscopic slide covered with coverslip, parts of the coverslip were placed on aluminium stubs using “Carbon Tabs” (supplied by ProSciTech, Queensland, Australia). The stubs were then placed in a dust proof container and allowed to dry completely at room temperature, overnight before being coated with a 20 nm layer of Gold in a Balzers Union Ltd. “sputter coater”. SEM samples for X-ray analysis were not coated with gold but otherwise treated the same way.
3.2.3. Preparation of Sample: Spherical Crystal Growth (Time-Lapse)

Bacterial culture (20 µl, 3.4 mM urea hydrolysed.min⁻¹, 25°C) was placed on a microscopic slide, on which a coverslip was placed. Two opposite edges of the coverslip were sealed with nail polish, and then the slide was examined under the microscope. After selecting an appropriated field of focus, a 1 M cementation solution (equimolar of calcium/urea solution) was placed close to one of the unsealed edges, opposite to which a piece of tissue was placed to ease the diffusion of the cementation solution. After most of the solution had diffused under the coverslip, the remaining two unsealed edges were sealed with nail polish to reduce the evaporation of the liquid. Then, time-lapse images of the spherical crystal formation and growth were taken for 22 hours, with an interval of 2 hours.

3.2.4. Calcium Carbonate Precipitation by Pure Urease

The above CaCO₃ precipitation procedures (as shown in section 3.2.1) were followed except that the bacterial culture was replaced with plant urease enzyme (the final urease activity on the microscopic slide was 3.3 mM urea hydrolysed.min⁻¹).

3.2.5. The Effect of Different Concentrations of Bacterial Cells on the Size of the Calcium Carbonate Crystals Formation

Biocementation experiments on microscopic slides were carried out for the non-diluted bacterial culture (urease activity of 3.3 mM urea hydrolysed.min⁻¹, specific urease activity of 1.3 mM urea hydrolysed.min⁻¹.OD⁻¹, dry weight of 30 g.L⁻¹) and for three different dilutions (1.0×10⁻¹ (3 g.L⁻¹), 1.0×10⁻² (0.3 g.L⁻¹), and 1.0×10⁻³ (0.03 g.L⁻¹)). The culture was diluted by a sterile saline solution to reach the desired dilution factor. During the biocementation experiment as mentioned previously in section 3.2.1, all the samples were further diluted almost two times. After 24 hours of CaCO₃ precipitation, the crystals were examined under the Olympus light microscope and the
averages of different spheres sizes (4-6 spheres) were measured in 6-9 individual fields of focus.

3.2.6. The Effect of Different Concentrations of Cementation Solution on the Size of the Calcium Carbonate Crystal Formation

Biocementation experiments on microscopic slides were carried out using different concentrations of cementation solution (10-1250 mM equivalent concentration of calcium/urea). The size of the calcite spheres were measured by Image-Pro Express software from Media Cybernetics.

3.2.7. Calcite Precipitation by Different Ureolytic Strains

CaCO₃ crystals precipitation by S. pasteurii (urease activity of 3 mM urea hydrolysed.min⁻¹), MCP4 (urease activity of 2 mM urea hydrolysed.min⁻¹) and MCP6 (urease activity of 3.5 mM urea hydrolysed.min⁻¹) were examined under the Olympus microscope according to the method previously mentioned for MCP11 (section 3.2.1).

3.3. Results

3.3.1. Experimental Setup: In order to characterise (type, size, shape and growth) the CaCO₃ crystals which were precipitated due to bacterial activity in the presence of high concentration of cementation solution, the bacterial cells were mixed with cementation solution and immediately placed on a microscopic slide. Then the precipitated CaCO₃ crystals were examined under Olympus light microscope and Scanning Electron Microscope (SEM).

3.3.2. Microscopic Examination of Calcium Carbonate Precipitation (Light Microscope)

3.3.2.1. The Formation of Spheres

Spherical CaCO₃ deposits started to precipitate within minutes from the initiation of the biocementation process (Figure 3.3A). These spheres
were mostly clustered together in aggregates of two or more (Figure 3.3B, 3.4A, B), while some were compacted forming non-spherical aggregates (Figure 3.4C). The spheres appeared to be similar in size (Figure 3.4C) in one field of focus (around 22 µm in diameter), while different (100-400 µm in diameter) in another field (Figure 3.4D).

3.3.2.2. The Formation of Spheres and Rhombohedral Crystals Together

It was found that rhombohedral crystals appeared at a later stage of biocementation process (9 hours to 1 week) (Figure 3.5; Figure 3.6). Observations indicated that the rhombohedral crystals could originate from the spherical crystals. In Figure 3.6, three stages of CaCO$_3$ precipitation were obvious (an intact sphere, a slightly open sphere with part of the rhombohedral crystals protrusion, and free rhombohedral crystals without shell).
Figure 3.3: Light microscopic images of the (A) initiated (some are surrounded with dashed circle for clarification), and (B) the intact spherical CaCO$_3$ crystals produced by MCP11 (final urease activity of 1.6 mM urea hydrolysed min$^{-1}$) after 2-3 min, and 24 hours respectively from the addition of 1 M cementation solution.
Figure 3.4: Light microscopic images of similarly sized (around 22 µm) CaCO$_3$ spheres at (A) 10X (overview), (B) 40x, (C) 100x (enlarged image) and (D) different sized (100-400 µm) at 100x (in another field of focus). Calcium carbonate produced by strain MCP11 (final urease activity of 0.6 mM urea hydrolysed min$^{-1}$) after 48 hours from the addition of 1 M cementation solution.
Figure 3.5: Light microscopic images of the degenerated spherical CaCO$_3$ crystals produced by MCP11 (final urease activity of 1.6 mM urea hydrolysed min$^{-1}$) after 1 week from the addition of 1 M cementation solution.

Figure 3.6: Light microscopic image showing three steps of producing the rhombohedral crystals from the spheres. The image was taken after 9 hours from the addition of 1 M of cementation solution to the isolate MCP11 (final urease activity of 2 mM urea hydrolysed min$^{-1}$ and specific urease activity of 2 mM urea hydrolysed min$^{-1}$ OD$^{-1}$). Where (1) illustrates the intact sphere, (2) illustrates part of the rhombohedral crystals appears from the spheres and (3) illustrates the rhombohedral crystals in a spherical arrangement were free without shell. Note the clear zone around the spherical and rhombohedral crystals.
3.3.3. Calcium Carbonate Crystals Examined by Scanning Electron Micrographs (SEM)

3.3.3.1. The Nature of Calcium Carbonate Precipitation

Spherical and rhombohedral crystals in spherical arrangements were observed through SEM (Figure 3.7). It was established that these crystals were composed of CaCO$_3$ as shown by the Energy Dispersive X-ray (EDS) (Figure 3.8).

The Non-stable nature of the spheres (Figure 3.9; Figure 3.10), and their spherical shape (Braissant et al., 2003) suggests that the spherical CaCO$_3$ precipitate was vaterite. This suggestion was supported by XRD analysis which was carried out by Andreassen (2004). Andreassen has shown that CaCO$_3$ crystals (spheroids) at the beginning of the reaction between concentrated sodium carbonate and calcium nitrate were vaterite.

According to literature, the rhombohedral crystals with cubic faces are typical of calcite (De Yoreo and Dove, 2004; Söllner et al., 2003; Guo et al., 2003). This calcite formation was supported by XRD analysis (Söllner et al.; 2003, Guo et al., 2003).
Figure 3.7: SEM image of (a) spherical and (b) rhombohedral CaCO$_3$ crystals. This image was taken after 1 week from the progress of biocementation reaction using bacterial cells with urease activity of 1.6 mM urea hydrolysed.min$^{-1}$ and 1 M cementation solution (final urease activity on the microscopic slide was 1.6 mM urea hydrolysed.min$^{-1}$).
Figure 3.8: Energy dispersive x-ray spectroscopy (EDS) of bacterially induced CaCO$_3$ crystals using SEM-SiLi detector. Those spectra indicate Ca peaks associated with the spherical (A) and the rhombohedral (B) calcite crystals. Peaks for Si, Cl and Na originated from the glass, cementation solution and culture media respectively.
Figure 3.9: SEM micrographs of decomposed spherical calcite produced by bacteria (final urease activity of 1.6 mM urea hydrolysed min⁻¹). It seems that the spherical shell after certain time from being intact, dissolve gradually (arrows in A and B). (C) A schematic diagram of the degenerated spherical crystals that failed to produce rhombohedral crystals pointed with arrows in (A).
Figure 3.10: SEM micrographs of collapsed spherical calcite crystals (A). (B) Is enlarged image of the area surrounded by a box in (A). The bacterial strain was grown in the presence of 3.4 mM CaCl$_2$·2H$_2$O, with urease activity of 1.6 mM urea hydrolysed min$^{-1}$.

3.3.3.2. Surface Texture of Calcium Carbonate Precipitation

The outside surface texture of the spheres was smooth with regular openings (Figure 3.11). They appeared to consist of small acicular (needle-shaped) deposits (Figure 3.12). The thickness of the spherical
wall varied. Some were thick (4 µm) (Figure 3.13A) and others were thin (0.08 µm) (Figure 3.13B). Some appeared to be hollow (Figure 3.12; Figure 3.13) and others with internal protrudings (Figure 3.11; Figure 3.14). The differences may be due to different stages of the sphere development and possible transformation into the rhombohedral crystals.

Figure 3.11: SEM image of the structured openings at the upper surface of the spherical calcite (see arrows) produced by MCP11 (final urea activity 1.6 mM urea hydrolysed.min⁻¹).
Figure 3.12: SEM micrographs of collapsed spherical crystals illustrate the acicular deposits of the CaCO$_3$ spheres. (A) An overview image. (B) An enlarged image of the area surrounded by box in (A).
Figure 3.13: SEM micrographs of collapsed spherical crystals (a broken or decomposed spherical shell) with different thickness of shell (A) 4 µm (channel-like internal structure) and (B) 0.08 µm. The small oval crystals (~3 µm) in (A) were repeatedly seen when the biocementation reaction was maintained for several days.
3.3.4. Possible Mechanisms for Strength Formation

The spherical crystals were able to cover the surface of the sand granules, connecting them together (Figure 3.15) and providing one mechanism for strength formation (i.e. bridging between the sand granules by CaCO$_3$ crystals was the mechanism which led to strength formation).

There were imprints of the bacterial cells shape at the surface of the spherical crystals (Figure 3.15D) in contrast to the absence of similar imprints at the surface of the rhombohedral crystals (Figure 3.16). The absence of these imprints at the rhombohedral surface supports the assumption that a chemical reaction has occurred to precipitate this
type of crystals. This allows the possibility that entrapping and/or diffusion of Ca\(^{2+}\) and CO\(_3^{2-}\) ions through the spherical shell was the cause of the precipitation of rhombohedral crystals after the critical supersaturation was achieved.

Figure 3.15: SEM micrographs of calcite crystals embedded on the coverslip and attached to 100-200 µm silica sand grains (A-D). The sand grains were fully covered by spherical CaCO\(_3\). The space between the two sand grains was full with rhombohedral crystals (arrow in C). Those images were taken after 1 week from the initiation of biocementation reaction between bacterial cells (final urease activity of 1.6 mM urea hydrolysed.min\(^{-1}\)) and 1 M cementation solution. The dashed circles in D show the signs of bacterial cells (~1 µm) surface at the surface of the spherical calcite.
3.3.5. Calcium Carbonate Precipitation by Pure Plant Urease

To determine whether the cause of the formation of spherical deposits was dependent on the presence of bacterial cells; pure soluble *Canavalia ensiformis* (jack bean) urease with final activity of 3.3 mM urea hydrolysed min⁻¹ in the absence of bacterial cells was used to precipitate CaCO₃ crystals. Aggregations of poorly arranged rhombohedral crystals were formed (Figure 3.17). By comparison in the presence of bacteria (Figure 3.7; Figure 3.9), rhombohedral calcite crystals were well organized in spherical arrangement form; suggesting that the presence of bacterial cells or the localised rather than uniform
production of carbonate plays a role in spherical arrangement of the rhombohedral crystals

Figure 3.17: Light microscope image of spread out CaCO₃ rhombohedral crystals (≤1 µm) produced by pure urease, with a final urease activity of 3.3 mM urea hydrolysed min⁻¹.

3.3.6. Time-Lapse of the Spherical Calcite Growth

To understand the growth pattern of the spherical CaCO₃ deposits, the crystal formation by ureolytic bacteria in the presence of high concentration of calcium and urea (1 M, equimolar concentration) was monitored during 22 hours. It was found that the size of the spherical CaCO₃ increased (Figure 3.18; Figure 3.19), until a point was reached (at around 14-24 hours) beyond which no further crystal growth occurred. Similarly, after 12-15 hours, there was no further initiation of new spheres. Their growth in size seemed to stop when one sphere touched another (note the compressed crystals previously shown in Figure 3.4C). Different sizes of CaCO₃ spheres were precipitated and classified as large (15 µm), medium (10 µm) and small (3 µm) spheres.
(Figure 3.18; Figure 3.19). This classification applied to this field of focus (at which the time-lapse images were taken) while in another field of focus there were different sizes of spheres, the largest being 98 µm in diameter.

During the phase of sphere formation, above half of the spheres (56%) were associated with a single cell. The rest of the spheres were associated with a single cell only at the beginning of the spherical formation, and then 2-5 cells appeared around the spheres (Figure 3.18, spheres growth.ppt file provided as a soft copy).

3.3.7. Location of Crystal Formation

From time lapse studies and from frequent microscopical investigations it appeared that the spherical crystals were formed close to the bacterial cells and at times using the cell itself as the nucleation site. However, at other times crystals were formed away from bacterial cells on the glass of the slide, such that a uniform claim cannot be made on the question whether the local supersaturation of calcite caused by the bacterial carbonate release was the location of crystal formation.
**Figure 3.18:** Different light microscopic images of the spherical calcite crystals development (Time-lapse for 22 hours). The bacterial culture (3.4 mM urea hydrolysed.min⁻¹) was subjected sequentially to 1 M cementation solution (equivalent concentration of calcium/urea) on a microscopic slide. The dashed circle represents the growth of two spheres (a and c). (a) Large, (b) Medium and (c) Small spheres. These images are shown as a power point presentation as well to show the cells which were associated with the spheres (provided as a soft copy).
**Figure 3.19:** The growth of three different spherical calcite crystals and their numbers during 22 hours of biocementation reaction (Based on the time-lapse images in Figure 3.18).

### 3.3.8. Time-Lapse of the Appearance of Rhombohedral Crystals

To be certain of the origin of the rhombohedral crystals, the spherical deposits were monitored online. It was found that the rhombohedral calcite crystals originated from the spherical ones (Figure 3.20). The spherical crystals first increased in size, followed by the appearance of the rhombohedral crystals subsequent to the dissolution of the spherical shell. This claim was supported by Kralj et al (1997) who stated that vaterite and calcite were precipitated spontaneously; with initial precipitation of vaterite followed by calcite. The transformation of vaterite to calcite was supported by recording the pH as a functional of time and a mathematical model which predicted the changes of the solution and the solid phase during the CaCO$_3$ formation process.

The process of the nucleation and growth of CaCO$_3$ crystal is complicated. It is well known that high degree of supersaturation forms precipitates spontaneously. The precipitation of metastable vaterite is kinetically favourable over the stable calcite at high degree of
supersaturation (Kralj et al., 1990). The metastable phase grows continuously faster than the stable phase. When the supersaturation drops, the transformation of vaterite (solubility product: log $k_{sp}$ = -7.91 at 25°C) to calcite (solubility product: log $k_{sp}$ = -8.45 at 25°C) will occur. By using the ions in the solution, the calcite might precipitate lowering the ionic activity product (IAP, the total activity of the ions that include a specified solid phase) below the solubility product ($k_{sp}$, ionic product when the system is in equilibrium) of the vaterite. This decrease in the IAP below $k_{sp}$ causes the dissolution of vaterite (Sawada, 1998; Kralj et al., 1990; Kralj et al., 1997). After reaching the supersaturation, the calcite crystal will precipitate in a particular shape depending on pH, ionic strength (Cl$^-$, Na$^+$) and additives. Accordingly, any sphere enclosing the rhombohedral crystal provides a more defined condition of supersaturation.

In conclusion these experiments provide interesting insights into possibilities of strength formation of the biocementation, however, the conditions that control the formation of the rhombohedral crystal will need to be studied more extensively.

3.3.9. Calcium Carbonate Transformation from Spheres to Rhombohedral Crystals Precipitation (Video-Clip)

At the early stage of biocementation process, as soon as the contact between the cells and cementation solution was made, the cells become usually immobilized. This was microscopically visible by the flagella attaching to the slide surface causing a circular movement of the cells on the spot. In some instances it appeared as if small spheres where formed around a single bacterial cell, but there were also instances where spheres were formed more than 10 μm away from the nearest cell. A small white nucleus appeared at one end of the cell. This small nucleus might indicate that calcium ion can bind to the external surface of the bacterial cell. The cells appeared to be in the centre of the sphere during their precipitation. Small spheres were formed from a
single cell, gradual increase in size, followed by the appearance of rhombohedral crystals (Video clip saved as spheres to rhombohedral crystals). Although in literature—as discussed above in section 3.3.8—the spherical vaterite transformation to rhombohedral calcite crystals were recorded in the absence of bacterial cells, the role of bacteria in producing the spheres cannot be denied. Initial precipitation of sphere around a single bacterial cell was observed repeatedly under the light microscope.

Figure 3.20: Olympus light microscope images showed the origin of rhombohedral crystals after (A) 0.5 (B) 2.5 and (C) 3.5 hours from the progress of biocementation reaction. The urease activity of the bacteria used for CaCO₃ precipitation was 14 mM urea hydrolysed·min⁻¹ and specific urease activity was 4 mM urea hydrolysed·min⁻¹·OD⁻¹.
3.3.10. Calcium Carbonate Precipitation by Different Ureolytic Bacteria

To examine the specificity of the formation of spherical deposits to one strain, CaCO₃ precipitation by different strains were examined. The light microscopic images revealed that spheres were produced by several ureolytic strains isolated from soil (MCP4 and MCP6) and also by S. pasteurii (Figure 3.21: Figure 3.23). Thus spherical deposits were a general phenomenon for CaCO₃ precipitation and not specific to MCP11.

Figure 3.21: Light microscopic images of CaCO₃ crystals precipitation on the microscopic slide for S. pasteurii (the final urease activity of the microbe was 2 mM urea hydrolysed.min⁻¹) after 21 hours from the progress of 1 M cementation reaction (equimolar molar of calcium/urea).
Figure 3.22: Light microscopic images of CaCO$_3$ crystals embedded on the microscopic slide for MCP4 (urease activity of the microbe was 1.1 mM urea hydrolysed.min$^{-1}$) after 2 hours from the addition of 1 M cementation solution (equimolar of calcium/urea). The budding which is shown at the spheres surface (dashed circles) were actually small spheres stoped when attached to another spheres surface during crystal growth.

Figure 3.23: Light microscopic images of CaCO$_3$ crystals embedded on the microscopic slide for MCP6 (urease activity of the microbe was 1.8 mM urea hydrolysed.min$^{-1}$) after 40 min from the addition of cementation solution (1M equivalent concentration of calcium/urea).
3.3.11. The Effect of Different Concentrations of Bacterial Cells on the Size of the Calcium Carbonate Crystals

Based on the hypothesis that each spherulite is formed by one bacterial cell, it was expected that by using lower bacterial concentrations, fewer but bigger spheres would be produced. However, results showed that the sphere sizes increased with the increase in bacterial cells concentration (Figure 3.24). This correlation between bacterial cell numbers and sphere sizes produced does not support the above hypothesis.

3.3.12. The Effect of Different Concentrations of Cementation Solution on the Spherical Calcium Carbonate Crystals

The size of the spheres increased as the concentration of the urea and calcium ions increased (Figure 3.25). There was a sharp increase in the spherical size at concentrations of 10-250 mM above which the increase in the size was limited.

![Figure 3.24: Different sizes of CaCO₃ crystals (spheres) produced by different concentrations of cells.](image-url)
Figure 3.25: Different sizes of CaCO₃ crystals (spheres, average of 6-9 spheres) produced by different concentrations of cementation solution (equimolar concentration of calcium/urea). Lowest concentration of calcium/urea tested was 10 mM.
3.4. Discussion

As a general phenomenon, ureolytic bacteria in the presence of high concentration of calcium and urea produce two types of CaCO$_3$ precipitations; spherical deposits (Figure 3.3B; Figure 3.4) and rhombohedral crystals (Figure 3.6; Figure 3.7; Figure 3.14).

Both spherical deposits and rhombohedral crystals were observed in one study addressing the removal of waste cations e.g. Sr$^{2+}$ (Warren et al., 2001). Rhombohedral calcite crystals due to ureolytic bacterial activity in the absence of the spherical deposits were observed in other studies (Bang et al., 2001; Bang et al., 2002; Mitchell and Ferris, 2006). The chemical precipitation of rhombohedral crystals was described by Warren and his colleagues (2001). They did not observe embedded cells (S. pasteurii). In contrast, other authors noted that S. pasteurii imbedded in the rhombohedral crystals indicated a direct involvement of the cells in the formation of the rhombohedral crystal (Figure 3.25) (Bang et al., 2001).

![Figure 3.25: SEM micrographs rhombohedral (A) and undefined CaCO$_3$ crystals with bacterial signs at the surface (B) (from Bang et al., 2001).](image-url)
Rhombohedral calcite crystals nucleate through self-assembly process which requires the combination of calcium cation and carbonate anion (Guo et al., 2003). The actual mechanism of the formation of those rhombohedral crystals inside the spheres remains still unclear, However; in literature, it was found that agglomerates of rhombohedral calcite appeared at pH of 8.5 (Tai and Chen, 1998) and supersaturation index of 30-40 (Dittrich et al., 2003).

The decomposable behaviour of the microbial spherical CaCO\(_3\) crystals supports the assumption that they may be suitable to be used as a template to encapsulate certain micro and macro-molecules. Suckhorukov and his colleagues (2004) have successfully chemically prepared decomposable, spherical, porous calcite crystals with an average size of 5 µm to be used for the encapsulation of biomolecules mainly proteins. Those spheres were suitable to enclose biomolecules due to their high surface area, the presence of nano-pores and channels in the spherical shell.

A complete picture of the development of rhombohedral calcite crystals inside the spherical calcite was hypothesized according to what was observed under SEM (Figure 3.26). This Figure could lead to the idea that the ureolytic bacteria in the presence of high concentration of calcium and urea were able to control the shape of the grown crystals and nucleation (orientation and location) as other organism do such as molluscs (Calvert, 1992), corals (Martin and Le Tissier, 1991), coccolith (Young, 1999) and stone like otolith in Zebrafish (Söllner, 2003). However evidence to prove this idea could not be shown in this thesis.

The current work on the CaCO\(_3\) crystals formation by ureolytic bacteria has provoked the following question: Which type of the calcite crystals is responsible for strength production? Is it the spherical, rhombohedral or both crystals? This question will be addressed next chapter.
Figure 3.26: A schematic diagram of the sequence development of rhombohedral calcite crystals within the spherical CaCO$_3$ crystals during ureolytic bacterial activity. This diagram was hypothesised according to the time lapse which was taken for the spheres development and dissolution (Figure 3.12; Figure 3.15). During the biocementation reaction, the cells start by forming a small sphere (A) which grew gradually and enclosing so oriented small rhombohedral crystals that they will eventually join to form a single crystal (B) (note the remaining parts of the spheres at the surface of the rhombohedral crystals) (B).
3.5. Conclusions

- The spherical crystal formation is a general phenomenon of ureolytic microbes in the presence of urea and calcium ions while the microenvironment around the cells is not disturbed.
- The rhombohedral calcite crystals originated from the spherical CaCO$_3$ deposits and appeared obvious at a later stage of biocementation reaction. However, not all spheres transformed into rhombohedral calcites.
- Derived from the time lapse and video clip of the bacterial precipitation of CaCO$_3$ crystals, it was concluded that microenvironment around the bacterial cells is important for rhombohedral calcite precipitation.
Chapter 4
Strength Production by Concentrating the Cells

In-Situ

4.1. Introduction

Coating particles and partial infilling of void spaces between the particles are considered as two phenomena occurring during the precipitation of CaCO$_3$. What enhances the strength of the cemented particles is the point-to-point contact between the particles and not the filled spaces (Sharma and Fahey, 2003a). According to Ismail (2002); the strength of the consolidated sand increases with strength of the individual particles, sand density, and decreases with particle size, particles pre-coating with CaCO$_3$, and roundness of particles.

The strength of a cemented sand-column is measured by destructive and non-destructive methods, as follows:

- **Direct strength testing (destructive):** 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).

- **Indirect strength testing (non-destructive):** The velocity of ultrasonic waves through matter is a widely used technique to determine the properties of many different materials including concrete (Ohdaira and Masuzawa, 2001), bone (Fumio et al., 2003), tungsten cobalt hard alloys (Belotskii et al., 1984) and soil (Khazin et al., 1974). Ultrasound velocity can also be used as a direct estimation of geomechanical properties including mechanical strength of rock formations (Schön, 1996; Lee et al., 2004).

One of the most important strength measurements in geotechnical engineering is shear wave velocity. It is a robust and reproducible technique for monitoring the strength development during cementation. However, remoulding (Kong et al., 2004), density (Bell, 1993) and
chloride content (Kèzdi, 1979) affect the shear wave velocity. The shear wave velocity test, measures the vertical stress acting on the sand granules at a specific frequency (Blewett and Woodward, 2002). Civil engineers are highly recommending this technique, especially because it represents the actual behavior of the sample precisely and it is a promising measurement technique for the field (Blewett and Woodward, 2002). Samples for shear wave velocity testing are held in a split mould, in such a way that the sample can be extracted without any damage (personal communication, Mostafa Ismail, 2006, COFS, UWA, Australia).

In recent studies, ureolytic bacteria have been used to consolidate lose sand by combining the cells with calcium/urea solution (cementation mix), followed by fast injection into packed sand-columns under high pressure. The cells were allowed to react with calcium/urea solution under batch mode conditions. To increase strength, several applications of the cementation mix were applied (Whiffin, 2004). This way of consolidation is not suitable for application in the field when the consolidation of several meters is required (details will be given in chapter 6). A different way of sand consolidation which depends on concentrating the cells into the packed sand-column followed by up-flushing the cementation solution (urea and calcium) will be examined.

Using large quantities of cells for biocementation jeopardizes the future of biocementation technology as the process will be expensive. To lower the cost of the process, the possibility of reusing the cells should be examined. If the strength develops to a satisfactory level after addition of cementation solution, then there is no need for further application of cells. Thus, the aims of this chapter are:

- To produce strength by concentrating the cells in-situ through three applications of cells followed by three applications of cementation solution.
- To monitor the strength development in-situ by shear wave velocity in two attempts:
After up-flushing one application of cells and cementation solution;

- After up-flushing a second flush of cementation solution (calcium/urea), a second application of bacterial cells and a third application of cementation solution.

- To determine the relationship between the development of CaCO$_3$ crystals and strength.

### 4.2. Materials and Methods

#### 4.2.1. Concentrating the Cells in a 10 cm Packed Sand-Column

#### 4.2.1.1. Sand Compaction

SiO$_2$ sand (90-400 µm) was dry packed in a 60 ml plastic syringe (10 cm long, diameter of 3 cm). The packed sand-columns were tapped for about 3 min with a rubber hammer to give an even bulk density. They were then up-flushed with 3-void volumes of deionized water for 2 hours and the stoppers were inserted to maintain a confining pressure.

#### 4.2.1.2. Experimental Running

The packed sand-columns were up-flushed with microbial culture (MCP11), urease activity of 3.9 mM urea hydrolysed.min$^{-1}$ and a specific urease activity of 1.7 mM urea hydrolysed.min$^{-1}$,OD$^{-1}$. Four-void volumes of the bacterial culture were up-flushed at a flow rate of 833 ml.h$^{-1}$. Cementation solution (1 M, equivalent concentration of 1M calcium/urea solution) was up-flushed into the columns (1.1-void volume) directly after up-flushing bacterial cells. Four sand-columns were prepared for testing different applications (from one to 4 flushes). Then, the columns were kept for 24 hours at room temperature for the reaction to complete (Figure 4.1).
Figure 4.1: Schematic diagram of the experimental set-up used for the biocementation of packed sand-columns by concentrating the cells in-situ. Between 1 and 4 applications of cementation mix (cells and cementation solutions were sequentially loaded) were applied. P: peristaltic pump.

4.2.1.3. ESEM
Thin sliced section of the cemented column (4 flushes) was prepared and examined by Environmental Scanning Electron microscope (ESEM, examined by offshore centre, UWA, Australia). It was dried in an oven overnight at 40°C, and then placed in a hand made Aluminium boat where Epotech 301 epoxy resin is poured over it to impregnate the sample and set it in a plastic block; this was allowed to set at 40°C in the oven overnight. The Aluminium was peeled off and the sample was cut at the desired spot with a 20 cm diameter, 0.3 mm thick diamond saw blade. The surface was then ground flat using 220, 400, and 600 grit Carborundum powders, successively, on a cast iron grinding lapidary wheel. The sample was then surface impregnated by placing on a hotplate at 40°C allowed to dry for 2-3 hours before having Epotech 301 spread over the surface. After a half an hour the excess was wiped of with a tissue and allowed to dry overnight. The surface was then ground smooth with 600 grit Carborundum, before surface impregnating again overnight. This was repeated as often as necessary till the surface was perfect. Thick glass slides which are 75x25 mm in size are ground flat (+/-1 micron) to 1300 micron thickness on a precision Logitech LP30 grinding machine. The sample was glued to the slide with the Epotech 301 resin and allowed to set overnight on a
hotplate at 40°C. The excess was cut off with the diamond saw and then ground down to 70 microns thick using 600 grit Carborundum then finished with 1200 grit Aluminium Oxide grinding powder on the Logitech. The glass slide was then cut down to 50 mm length to fit the polishing machines. The sample was then polished with 6 micron diamond paste on a Kemet KFA polishing cloth with Planopol 5 polishing machine for 1 hour. It was finally polished using first 1 micron diamond paste then ¼ micron diamond paste on a Planopol 2 machine with Kemet MSA polishing cloths for ½ hour each.

4.2.2. Split-Mould Setup for Shear Wave Velocity Test

For measuring the shear wave velocity, the sample preparation technique described by Ismail (2000) was used. This technique (Figure 4.2; Figure 4.3) is summarized as follows:

- A rubber membrane (to avoid destroying the sample when dismantling the split mould) was placed on the base and held tightly by stretching two O-rings (Figure 4.2) on the pedestal recess (Figure 4.3).
- An aluminium split mould with two outlets for applying the vacuum was used. The two halves of the split mould were assembled and clamped, after greasing the edges of each half with anti-vacuum lubricant to enable the vacuum application.
- The membrane was stretched over the machined top edge of the mould, and the O–rings were rolled gently over it.
- Napkin tissue was placed on the base (emitter) and top (receiver) around the sensors (piezoelectric transducers).
- The membrane was stretched firmly to the mould wall when a vacuum was applied through the mould outlets.
- A known weight dry soil was poured into the split mould with one napkin tissue at the bottom.
- To achieve a certain soil density, an adaptor (stainless steel, T-shape) was placed on the top of the poured soil, and the mould was vibrated gently by tapping with a rubber hummer.
- The density adaptor was removed after reaching a density of 1.6 g.cm\(^{-3}\).
- The top cap with filter was inserted into the mould; the wrinkles of the filter were avoided to prevent errors of sensors readings.
- The upper (receiver) and lower (emitter) sensors were placed in the same directions.
- To obtain the stability of the sample during the flushing process, a seating pressure was applied by using a dead weight of 80 kg.

![Figure 4.2: Mould and ancillary items (Ismail, 2000).](image-url)
4.2.3. Production of Bacteria for the Split-Mould

The bacterial culture (MCP11) was grown in a shake flask at 28°C with urease activity of 4.3 mM.urea hydrolysed.min⁻¹ and specific urease activity of 2 mM.urea hydrolysed.min⁻¹.OD⁻¹).

4.2.4. Preparing the Split Mould for Cementation Process (Washing of Sand and Up-Flushing Cells)

SiO₂ sand (90-400 μm) was dry packed in the split mould, according to the procedures mentioned previously in the sample preparation (section 4.2.2). After setting up the device as in Figure 4.3, the mould was up-
flushed with 1.2-void volumes of deionized water (to remove, any solute from the sand that may affect the biocementation process), followed by about 4-void volumes of bacterial culture. Subjecting the sand to gentle vibration decreased the volume by at least 8% (West and Menke, 2004) enhancing the contact between the sand granules and allowing an increase in the shear strength. Then 1.2-void volume of 1 M cementation solution was up-flushed. The flow rate of all the solutions which were up flushed was 850 ml.h⁻¹.

4.2.5. Shear Wave Measurements after one Application of Cementation Reactants (Cells and Cementation Solution)

The shear wave velocities were measured immediately after up-flushing the cementation solution. The strength development was followed over a 20 hours period.

The shear wave velocity was determined by producing 10 khz signals from the transducer at the base of the split mould and received by the opposite transducer. The transducers depend on converting the electrical signals (produced by piezoelectric element) into mechanical vibration (transmit mode) and from mechanical vibration into electrical signal (receive mode). The time taken for the wave to reach the receiver was recorded as arrival signals. The shear wave velocities were continuously monitored and calculated according to Equation 4.1. Those velocities (m.s⁻¹) were plotted versus curing time (h) to monitor the strength development during the course of biocementation.

\[
Velocity \ (m \cdot s^{-1}) = \frac{Sample \ height \ (mm)}{Arrival \ time \ (\mu s) \times 1000} \quad \ldots \ldots (4.1)
\]

By using the shear wave velocity setup (described above), it is difficult to relate the strength development in the sand-column with the urea hydrolysis rate and the type and size of crystals precipitation. Any attempt to open the mould for sampling would disturb the transducers
and consequently the shear wave velocity readings. As a result, two procedures were done:

1. In the split-mould, three samples (1 ml each, over a period of 11 hours from the addition of cementation solution) of the outflow solution were taken for ammonium analysis.
2. One parallel sand-column was prepared (dummy column) in a 60 ml syringe for testing the crystal shape, assuming that identical conditions to the split mould had been maintained. After up-flushing the bacterial culture followed by the cementation solution using the same flow rate which was used for the shear wave velocity experiment, several samples were collected for SEM.

4.2.6. Retention of Cells within the Split-Mould According to the Optical Density and Conductivity Measurements

To estimate the fraction of cells and urease activity trapped among sand granules, the cell density (OD) and urease activity in out-flow fractions were subtracted from the in-flow values. Thus, Fractions of 100 ml were collected during up-flushing bacterial cells into the split mould.

4.2.7. Urea Degradation in the Split-Mould

One ml samples were collected from the outlet tube at the top base of the split mould, by pushing very little solution from the inlet (in the base). It was not possible to take the samples directly from the cemented column within the split mould during the biocementation process to avoid disturbing the shear wave velocity measurements.

4.2.8. SEM of Calcium Carbonate Precipitation during Biocementation Process

SEM samples were collected during the first six hours from the beginning of the biocementation process in the dummy column, to relate the development of CaCO₃ precipitation to strength which was measured by shear wave velocity. SEM stubs with sticky tape were
placed at the surface of the cemented column and pushed downward so that the crystals stuck to the tape. The stubs with the sand granules were washed with deionized water gently to remove off excess cells and reactant (Ca$^{2+}$ and CO$_3^{2-}$) avoiding any increase in the crystal size. Then, they were kept upward (the surface exposed to air) at room temperature to dry.

4.2.9. Monitoring Strength Development Using Shear Wave Velocity (Second Attempt)

The strength development was monitored over a 75 hours period, by using the shear wave velocity measurement. During this period, different flushes of cells and/or cementation solution were applied. These flushes can be divided into three stages (Table 4.1). In stage one, the packed sand-column within the split mould was up-flushed with water followed by the bacterial cells (almost 4-void volumes) and then cementation solution (1.2-void volume). After around 4 hours from the progress of the first biocementation reaction, further amount of cementation solution (1.2-void volume) was up-flushed with a flow rate of 850 ml.h$^{-1}$, initiating a second stage of biocementation process (second cementation solution application). Then, a second flush of bacterial cells (almost 4-void volumes) was applied followed by up-flushing a third flush of 1 M cementation solution.

4.2.10. Urea Degradation at the End of Each Application

To measure the urease activity in the cemented column after stage 1 and 2 (Table 4.1), 1 ml samples from the washed out solution (well mixed) were analysed for ammonium. After the third stage (Table 4.1), the cemented sand-column was extracted from the split mould before being sent for Unconfined Compression Strength (UCS) test. One ml solution was removed from the centre of the cemented column by syringe for ammonium analysis. The samples were centrifuged to separate the cells preventing further degradation of urea and kept in the freezer until the time of analysis.
Table 4.1: Categorizing the different applications of cells and cementation solution in the second attempt of shear wave velocity (Vs) experiment.

<table>
<thead>
<tr>
<th>Stages</th>
<th>Time (h) of cementation reaction monitored by Vs</th>
<th>Action</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>Flushing bacteria</td>
<td>First application of cells and cementation solution</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Equivalent concentration of calcium/urea solution (1 M)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>Equivalent concentration of calcium/urea solution (1 M)</td>
<td>Second application of cementation solution</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>Flushing bacteria</td>
<td>Second application of cells and third application of cementation solution</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Equivalent concentration of calcium/urea solution (1 M)</td>
<td></td>
</tr>
</tbody>
</table>

4.3. Results

4.3.1. Experimental Setup: To determine the possibility of obtaining strength from concentrating the bacterial cells in-situ, and the type of CaCO$_3$ crystals formation; four packed sand-columns (in plastic syringe) were up-flushed with bacterial cells first followed by up-flushing the cementation solution. After one day from the initiation of CaCO$_3$ precipitation, further three applications of cementation mix (cells and cementation solution) were applied.

To monitor the strength development in-situ, indirect strength measurement by shear wave velocity was applied. While the strength was measured, in-situ urease activity was determined (by NH$_4^+$ analysis). In addition, the shape of the CaCO$_3$ crystals during the cementation reaction was determined (by SEM) in a dummy column.

4.3.2. Concentrating the Microbial Cells within a 10 cm Packed Sand-Column

Continuous up-flow of the ureolytic cells through the packed sand-column, concentrate the cells in-situ. Due to this concentration of cells,
a soft rock (1200 kpa, tested by Offshore Centre, UWA, Australia) was obtained through three applications of the cells and cementation solution (Figure 4.4). The cemented sand-column was permeable, uniform with a smooth surface. With one or two applications of cells and cementation solution, the consolidation of sand was very weak (Figure 4.5). Thus by concentrating the cells \textit{in-situ}, consolidation of sand was achieved without clogging the injection point as the direct contact between the cementation solution and cells was \textit{in-situ}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.4}
\caption{Unconfined compression strength (UCS) of the cemented sandy material after three applications of cells and cementation solution, sequentially loaded. (A) Row data (to calculate B, examined by Offshore Centre, UWA, Australia), (B) actual strength (C) biocemented sand-column (D) biocemented sand-column after performing UCS.}
\end{figure}
Figure 4.5: The biocemented sand-column which was treated with a second application of cementation mix. The column was poorly consolidated. This column was too weak to the extent that it was damaged during mould extraction (plastic syringe). Thus, the UCS test was not performed for this sample.

4.3.3. ESEM Examination

To examine the arrangement of CaCO₃ crystals between the sand granules, thin slices of the cemented sands with four applications of cementation mix (bacterial cells and cementation solution) were examined under ESEM. It was shown that point-to-point contacts binding the granules together (Figure 4.6; Figure 4.7). These contacts were composed of rhombohedral CaCO₃ crystals in spherical arrangements, which were found in the pore spaces between the sand granules (Figure 4.6, Appendix D). The rhombohedral crystals in spherical arrangement were solid (Figure 4.6D) and some were coating the surface of the sand granules (Figure 4.6C: D; Figure 4.7). The presence of voids between the sand granules (Figure 4.6) indicated that improved packing of the sand was advisable in future experiments.

4.3.4. Comparison between Bacterial Calcium Carbonate and CIPS Precipitation

Artificial cemented samples were prepared by chemical cementation process using CIPS (Calcite In-situ Precipitation System) which was developed by CSIRO (Commonwealth Scientific and Industrial Research Organization, Australia). CIPS cementation technique is achieved by flushing two solutions (plant source liquid and calcium/urea) (Whiffin, 2004) through sand leading to CaCO₃ precipitation at the surface of the sand granules. The CaCO₃ bonds in
the contact points among the granules provide cohesive strength (Ismail, 2000; Ismail et al., 2002).

The precipitation of CaCO$_3$ by CIPS differs from the bacterial precipitation, in that the crystals which coat the sand granules are without the spherical rhombohedral appearance (Figure 4.8). They have granular appearance but did not arrange in spherical spot. A comparison between the two types of CaCO$_3$ precipitation is shown in Table 4.2.
Figure 4.6: ESEM micrograph of silica sand cemented by bacterial CaCO₃ after 2 weeks from the completion of the cementation reaction (Appendix D). The CaCO₃ was precipitated as rhombohedral crystals in a spherical arrangement forming point-to-point contacts between the sand granules (dash circles in A). A, B: overview of point-to-point contact of CaCO₃, C: A close view of the rhombohedral crystals in spherical arrangement; inset circle: magnification of CaCO₃ crystals showing solid crystals (not hollow) from inside. D-F: closer images to the rhombohedral crystals in the point-to-point contacts (different field of focus).
Figure 4.7: Thin section of the cemented packed sand-column which was treated with four applications of cells and cementation solution (cementation mix) sequentially uploaded, examined by Olympus light microscope. A: an overview showing point-to-point contact of CaCO$_3$. B-D: magnification of the biocemented sand granules in (A) clarifying the “spot-welding” effect of CaCO$_3$ (calcite precipitation filling the gap between the adjacent sand granules (appears as grey crystals or spots)).
Figure 4.8: Sand granules cemented by CIPS at (A) one flush, (B) 10 flushes (Ismail, 2000; Ismail et al., 2002).
Table 4.2: A comparison between bacterial and chemical (CIPS) CaCO$_3$ precipitation.

<table>
<thead>
<tr>
<th>Bacterial calcification (Current study)</th>
<th>CIPS calcification (Ismail et al., 2002)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Rhombohedral crystals in spherical arrangement.</td>
<td>• Crystals are different in shape (not in spherical arrangement).</td>
</tr>
<tr>
<td>• Coating mechanisms in addition to the distribution of point-to-point contact (big spot, 40-80 µm).</td>
<td>• Concentration between particles, appears as layers of CaCO$_3$ for high concentration, and small spot (10-20 µm) for low concentration</td>
</tr>
<tr>
<td>• Smooth (spheres) and/or granular (rhombohedral) in spherical arrangement.</td>
<td>• Granular in texture.</td>
</tr>
<tr>
<td>• Sequential flush, slow or fast flush.</td>
<td>• Combined, only fast flow rate to prevent clogging.</td>
</tr>
</tbody>
</table>

4.3.5. Shear Wave Velocity Measurement (Indirect Strength Measurement) during One Application of Bacterial Cells and Cementation Solution

4.3.5.1. Retention of the Bacterial Cells In-Situ

According to OD measurements, low mass of cells (1.1 g) were retained in a 300 cm$^3$ sand-column. The calculation was based on the estimate that 1 OD of cells is equivalent to 1 g.L$^{-1}$; accordingly, the retained OD in-situ was converted into mass (g) after knowing the OD of each 100-ml out-flow fraction (i.e. during up-flushing 4-void volumes of bacterial cells into the sand-column, the out-flow samples were collected as 100-ml fractions and then the OD was measured). Then the total mass was calculated by adding the retained mass of cells in each out-flow fraction together.

According to the above mentioned calculation, it was found that 50% of cells were retained within the sand-column of the split mould. This
percent is equivalent to urease activity of 0.022 mmol urea hydrolysed cm\(^{-3}\). min\(^{-1}\).

4.3.5.2. Urease Activity during the Biocementation Process in the Split-Mould

Urease activity within the split mould increased for the first 4-5 hours of biocementation reaction (Figure 4.9), after which the activity remained constant. This pattern of urea degradation almost synchronised with that of the shear wave velocities development (Figure 4.11), which increased sharply with time for the first 3-5 hours.

4.3.5.3. Monitoring Shear Wave Velocity during Biocementation Process

There were two types of waves that appeared in the signal spectra, compression and shear waves (Figure 4.10). Compression waves (referred as mechanical longitudinal, pressure or density waves) are transmitted by the compression of a fluid (e.g. a sound wave in air). Its vibration occurs in longitudinal direction, and can be generated in liquids, gasses and solid. Shear waves (referred as S-waves, rotational or transverse waves) generate vibrations which are transverse to the propagation direction within the material being examined. The shear waves are transmitted in solids, however, transmitted inefficiently in liquids and gasses. Hence shear waves are a good measure of stiffness.
Figure 4.9: Urea degradation in the outlet of the split mould during the shear wave measurement. (A) \( \text{NH}_4^+ \) production from the degradation of 1 M urea, (B) Percentage of urea conversion calculated from (A).
Figure 4.10: Example of shear wave data used to calculate velocity in (A) unconsolidated sample (after the bacteria were up-flushed completely), (B) after 50 min and (C) after 20 hours from the start of the biocementation reaction. The vertical lines indicate the arrival time (signal received) of the wave at the opposite transducer. Note that the waves before the vertical line are compression waves (more obvious in B).

The strength of the biocemented column was successfully monitored over a 20 hours period using shear wave velocity measurement. As the strength increased, the transmitted signal of the consolidated sample reached the upper-transducer faster than in the unconsolidated column. Therefore the arrival time will decrease, leading to an increase in the shear wave velocity according to Equation 4.1 (shown previously).
The velocity of the consolidated column increased around 3-fold (Figure 4.11). The velocity of the unconsolidated sand after up-flushing water or bacterial culture was 200 m.s\(^{-1}\). In theory, fluids do not contribute to shear strength (West and Menke, 2004). The shear wave velocity increased very fast after up-flushing 1 M cementation solution (Figure 4.11), to the extent that, most of the strength was gained within the first 3-4 hours of the biocementation reaction. Therefore, it would be adequate to allow only about 4-5 hours before applying a subsequent flush of cells and/or cementation solution. The consolidation step completed over a short period of time, almost 6 hours from the start of cementation reaction (Figure 4.11). The rate of strength development decreased after approximately 2 hours from CaCO\(_3\) precipitation (Figure 4.11) at which 50% of urea was converted (Figure 4.9). This gradual decrease in the rate of strength development might be attributed to one or more of the following:

- The decrease in urea concentration \textit{in-situ};
- The inhibition of the cementation reaction due to the high concentration of end products (e.g. NH\(_4^+\) ions, CO\(_2\)); and/or
- The decay of the urease.

The rate of strength development was calculated from the velocity change versus the change in experimental time (elapsed time). The rate at which biocementation reaction changes its velocity is known as acceleration (Equation 4.2).

\[
\text{Acceleration (m} \cdot \text{s}^{-2}) = \frac{dvs}{dt} \tag{4.2}
\]
Figure 4.11: Development of shear wave velocity (m.s\(^{-1}\)) over curing time of 20 hours (○). A: After completing flushing water, B: After completing up-flushing bacterial culture (above which the cementation reaction started) and C: After completing up-flushing cementation solution (calcium/urea) solution. (■) is for the rate of shear wave velocities developed during the biocementation process after one application which affects the acceleration.

The shear stiffness (\(G_{\text{max}}\)) of the consolidated sand increased 11-folds after the completion of the cementation reaction according to Equation (4.3). The obtained shear velocities for the unconsolidated and consolidated packed sand-columns within the split mould were 200 and 675 m.s\(^{-1}\) respectively (Figure 4.11). By knowing that the density of the sand-column was 1.6 g.cm\(^{-3}\), then the shear stiffness according to Equation (4.3) was 64,000 and 729,000 (kg.m\(^{-1}\).s\(^{-2}\) or N.m\(^{-2}\)) for the unconsolidated sand and consolidated sand columns respectively.

\[
G_{\text{max}} = \rho \cdot V_s^2 \quad \text{.................................................................(4.3)}
\]

Where, \(G_{\text{max}}\) is the shear module or shear stiffness, \(\rho\) is the total density and \(V_s\) is the shear wave velocity.
Consolidation of sand was observed subsequent to dismantling the split mould (Figure 4.12) with softer upper part than bottom. The non-uniformed pattern of the cementation was probably attributed to the unequal distribution of cells throughout the packed sand-column in which the number of cells were higher at the bottom than the top enhancing the formation of more point-to-point contacts. However, no other known mechanism could allow the cells to be higher at the top than the bottom.

4.3.5.4. SEM Images from the Dummy Column that was Running in Parallel with the Split-Mould

To examine the crystals formation in the split mould that was subjected to shear wave velocity, a dummy column subjected to cementation conditions similar to the split mould was setup. During the biocementation process, both rhombohedral and spherical crystals were formed at the surface of the sand granules. After 2-3 min from the biocementation progress, a sample from the top surface of the sand column was examined under SEM; resulting in the formation of a reasonable size of spheres (1-10 µm) (Figure 4.13, Table 4.3). However, after a period of 2 hours; the rhombohedral crystals of spherical arrangement appeared besides the intact spheres (Figure 4.14). The number of spheres was low (around 14-33%) as compared to the rhombohedral crystals (Table 4.3). The number of the rhombohedral crystals increased with time to reach almost 100% after 4 hours from the initiation of cementation reaction (Figure 4.15B, Table 4.3). Thus, the spheres developed to reach the maturation (i.e. the formation of rhombohedral crystals) confirming what was shown repeatedly in chapter 3.
Figure 4.12: The steps of opening the split mould after the completion of biocementation process.
Figure 4.13: SEM images of the spherical CaCO$_3$ crystals formed after 2-3 min from the cementation reaction. See the different sizes of the sphere: (A) an overview, (B) a close view.
Table 4.3: The Types of CaCO$_3$ crystals found at the surface of the sand granules in the dummy column. The crystals development was monitored during 4 hours from the start of the cementation reaction.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Size (µm)</th>
<th>Type</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.067</td>
<td>1 - 10</td>
<td>Spheres</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>≤18</td>
<td>Spheres</td>
<td>—</td>
</tr>
<tr>
<td>2.7</td>
<td>11-18</td>
<td>Rhombohedral crystals of spherical arrangement (67-86%). Spheres (14-33%)</td>
<td>Few of the spherical crystal, almost most spheres were open. Rhombohedral crystals of spherical arrangement are dominant. Bacteria are obvious at the surface of spheres and rhombohedral crystals.</td>
</tr>
<tr>
<td>3.5</td>
<td>10-25</td>
<td>Rhombohedral crystals of spherical arrangement (60-80%). Spheres (10-20%). ~1 µm oval crystals appeared (0-30%).</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>10-25</td>
<td>Rhombohedral crystals of spherical arrangement (100%).</td>
<td>The rhombohedral crystals were in two different spherical arrangements (Figure 4.15B).</td>
</tr>
</tbody>
</table>
**Figure 4.14:** SEM images for the spheres which were produced after 2.7 hours from the biocementation process (A-C: different field of view). Note the rhombohedral crystals in a spherical arrangement are more numerous than the intact spheres (B, C).
4.3.5.5. Shear Wave Velocity Measurement for Several Applications of Cells and Cementation Solution

To determine the retention of the bacterial cells within the split mould, fractions of the out-flow liquid (i.e. cells not reacted with cementation solution) was collected for OD and urease activity measurement. According to OD and urease activity measurements, low mass of cells (1 g) and urease concentration (0.016 mmol urea hydrolysed.cm$^{-3}$.min$^{-1}$) were retained within the sand-column of the split mould. The retention of cells and urease In-situ was calculated as mentioned previously (section 4.3.3.1).
To determine the urease activity at the end of each stage (Table 4.1), out-flow liquid fraction were examined for NH\textsubscript{4}\textsuperscript{+} analysis. It was found that 85%, 94% and 100% of urea was hydrolysed when the first (up-flushing cells followed by 1 M cementation solution), second (up-flushing 1M cementation solution) and third (up-flushing cells followed by 1 M cementation solution) cementation reaction had proceeded respectively (Figure 4.16).

Figure 4.16: Urea hydrolysis in the split mould during the shear wave velocity measurement in three different stages. Stage 1: Up-flushing of cells followed by 1 M cementation solution (equivalent concentration of calcium/urea). Stage 2: Up-flushing of 1 M cementation solution only. Stage 3: Up-flushing of new application of cells followed by 1 M cementation solution).

By monitoring shear wave velocity online, 11% increase in the strength was recorded after up-flushing a second flush of cementation solution without the addition of new cells (stage 2, Table 4.1) (Figure 4.17); confirming the possibility of reusing the cells in-situ. This increase in velocity was recorded within 2.5 hours. The strength which was obtained after a second application of cementation mix (cells and cementation solution) was around 3-folds less than the one which was obtained by the first application (Figure 4.17). Furthermore, the strength
development after the second application of cementation solution was stopped after 2 hours from the start of biocementation reaction. This cementation period is considered to be half the period which was taken to stop the strength development in the case of the first application of the cementation solution.

Figure 4.17: Development of shear wave velocity (\( V_s \)) over curing time of 75 hours (○). A: Water up-flushed was completed, B: Bacterial culture was completed, C: First flush of 1 M cementation solution (calcium/urea) was completed (first stage), D: Second flush of cementation solution was completed (second stage), E: Second flush bacteria was completed, F: Third flush of cementation solution was completed and G: End of biocementation process. (●) is for the rate of shear wave velocities developed during the biocementation process after one application which affects acceleration.

When a second application of bacteria was up-flushed followed by a third application of cementation solution; it was expected that the increase in shear wave velocity will be at least similar to what was recorded for the first application as further CaCO\(_3\) was precipitated. In addition, further addition of cells would increase the point-to-point contact and consequently the strength. Therefore, the maximum velocity should be around 1600 m.s\(^{-1}\) added to the increase which was recorded after the addition of a second flush of biocementation solution (202.5 m.s\(^{-1}\)). Unfortunately, after the second application of the bacterial cells and cementation solution, the obtained velocity was 3-times less
than what was recorded for the first application of bacterial cells and cementation solution. This low obtained velocity was either due to the low in-situ urease conversion or low point-to-point contact. The urea was completely converted. Therefore, the low velocity was due to the low point-to-point contact. This means that the second application of cells did not increase the point-to-point contact to the same extent of the first application. Thus, the additional of bacteria did not give a great boost to the strength development, since the pattern of its strength development resemble the strength pattern when a second application of only cementation solution was performed (Figure 4.17).

### 4.3.5.6. Unconfined Compression Strength of the Cemented Column (UCS)

The unconfined compression strength of the cemented column was 600 Kpa (0.6 Mpa, Figure 4.18). This strength is 50% lower than the previously recorded one (section 4.3.2.) due to lower concentration of cells in-situ. The cells were 1.2-void volume instead of 3-void volumes in the former experiment. According to the ISRM (International Society for Rock Mechanics) classification, this cemented column is considered as a very soft rock (Figure 4.19). The maximum velocity which was recorded after a full urea conversion (1156 m.s\(^{-1}\)) suggests that a rock was formed. Engineers have postulated that the shear wave velocity in the range of (1000-4000 m.s\(^{-1}\)) is an evidence of a rock formation (personal communication, Mostafa Ismail, 2005, COFS, UWA, Australia). Thus, while ultrasound waves provide a method for monitoring the relative strength development (Figure 4.11; Figure 4.17) it did not accurately measure the absolute strength production.
Figure 4.18: The unconfined compression strength (UCS) of the cemented column within the split mould after monitoring the shear wave velocity during two flushes of bacterial cells and three flushes of cementation solution (calcium/urea). (A) Raw data (to calculate B, tested by Centre of Offshore Foundation Systems (COFS), UWA, Australia), and (B) Actual strength (kpa). On the right of the Figure the biocemented sand-column prior (C) and subsequent (D) to performing the UCS test was shown.
Figure 4.19: The ISRM classification of geomaterials (Ismail, 2000).

Figure 4.20: Urea hydrolysis in the split mould during the shear wave velocity measurement in three different stages. Stage 1: Up-flushing of cells followed by 1M cementation solution (equivalent concentration of calcium/urea). Stage 2: Up-flushing of cementation solution only. Stage 3: Up-flushing of new application of cells followed by cementation solution.
4.4. Discussion

4.4.1. Strength Production by Concentrating the Cells *In-situ*

A soft rock was produced by concentrating the cells *in-situ* through 3-4 applications of sequential loading of cells followed by cementation solution (calcium/urea). Interestingly, the obtained strength was attributed to the point-to-point contact of CaCO₃ crystals, which formed bridges between the adjacent granules (Figure 4.6). The production of strength due to this type of contact was confirmed by Sharma and Fahey (2003b). Moreover, Ismail and his colleagues (2002) have stated that the strength of the cemented matrix —by using CIPS— will increase as the contact points increase; enhancing the potential cementation sites for CaCO₃ precipitation. The void spaces in ESEM images (Figure 4.6) indicated that the sand granules in the biocemented column should be compressed more to increase the number of the effective point-to-point contacts which is a critical factor for improving strength (Ismail, 2000; Ismail *et al*., 2002).

A rapid strength build-up through biocementation reaction was successfully monitored using shear wave velocity after concentrating the cells *in-situ.* According to this velocity measurement, most of the strength was developed within the first 3-4 hours of the initiation cementation reaction (Figure 4.11) at which greater than 60% of urea conversion was achieved (Figure 4.9B). This is comparable with the study done by Bang *et al* (2001) which showed that calcite precipitation by both free and immobilized *S. pasteurii* was completed within 4 hours. Moreover Ismail *et al* (2002) —in his study on the artificial sand granules cementation by CIPS— has stated that most of the developed strength and the precipitated CaCO₃ were obtained within a period of 5 hours.

On their study regarding the modification of clay soils using *S. pasteurii,* Kong *et al* (2004) have failed to raise the shear strength of the clay soil. Their goal was to precipitate CaCO₃ by mixing *S. pasteurii* with 1.68 M
or 0.8 M urea with clay soil or clay soil and a stabilizer (cement or lime). A reduction in the shear strength of all samples modified with bacteria was recorded in addition to the absence of CaCO$_3$ precipitation. The researchers attributed the unsuccessfulness in increasing the shear strength to the deleterious material in the clay and pH which was in the range of 7.0-9.0. Their conclusion was not convincing because in the presence of urease active microbe, pH below 9.0 will not be detected since the microbe will automatically degrade urea rising up the pH to be around 9.0. pH 9.0 is suitable for the biocementation process (Mobley et al., 1989). Accordingly, it seems from the low pH measurement and the absence of CaCO$_3$ crystals in the tested sample that the cells were probably inactive prior and/or during the cementation reaction. Although they have based their study on bacterial urea hydrolysis, unfortunately the study did not show any measurement for the urease activity of the desired microbe.

The success in the strength production by concentrating the cells *in-situ* has provoked the following question: Is it possible to attach the bacterial cells *in-situ* and by how much? This question will be addressed next chapter.

**4.4.2. Strength Development during Different Applications of Cells and Cementation Solution Sequentially Loaded**

The bacterial cells were reused to the extent that almost a complete urea conversion was obtained. This should result in 2-times more CaCO$_3$ build-up. However, the shear wave velocity only showed a minor increases (12%). As the strength and shear wave velocity are positively correlated (Figure 4.21), then the minor increase in velocity was probably due to strength and CaCO$_3$ ratio.
The shear wave did not significantly measure the actual strength production. In agreement, some researchers —during monitoring cementation process by ultrasound— have found that 1 M cementation solution (equivalent concentration of calcium chloride and urea) and 2 M NH₄Cl (produced from biocementation reaction) caused an increase in the ultrasound velocity in sand (Geodeflt, 2006a).

### 4.4.3. Relating the Crystal Formation to Strength Development

As soon as the contact between the cells and cementation solution had occurred, the spherical crystals formed, and consequently increased in size with time (Figure 4.13 and Table 4.3) leading to the precipitation of rhombohedral crystals after 2-3 hours from the biocementation progress. In parallel, the strength of the cemented column —as was measured by shear wave velocity— increased with the increase in crystal size, until it reached the maximum when the crystals were in the rhombohedral form. After 2 hours from the initiation of biocementation reaction, the rhombohedral crystals were dominant and their
appearance in the dummy column was assured by the consolidation of the column (i.e. the consolidation of the dummy column was assessed by tapping the surface of the column). This consolidation supports the claim that the obtained strength seemed to be caused by the rhombohedral crystals. After 3 hours from the initiation of the cementation process (Figure 4.11), the strength development as measured by shear wave velocity was limited at the time that small oval crystals were formed in the dummy column (Table 4.3; Figure 4.15A). Those small crystals which were formed at the end of the biocementation process and repeatedly found at low urease activity probably did not add much to the strength.

4.5. Conclusions

- The final strength of the biocemented sand could be attributed to the rhombohedral crystals and not to the spheroids as they no longer exist at the end of cementation process.
- Low urease activity (0.02 mmol.cm\(^{-3}\).min\(^{-1}\)) could increase the stiffness of the packed sand-column 11-16 times.
- Shear wave velocity is a useful measurement for monitoring the development of strength which was produced by bacterial action; however, it did not estimate the actual strength of the biocemented column.
- The strength is produced within the first 3-4 hours of the biocementation process.
Chapter 5
Attachment of Bacterial Cells to the Sand Granules

5.1. Introduction

Microbial interaction with interfaces (a common border between two different objects, devices or systems through which information is passed) is usually described as attachment, retention, adhesion, adsorption or deposition. This interaction could be reversible or irreversible. The reversible bacterial attachment is a condition in which bacterial cells remain very close to a surface such as sand granules, glass and stainless steel for several minutes (Vigeant, 2002). These cells are reversibly attached to surfaces due to physicochemical forces (e.g. hydrophobicity, surface energy and electrostatic interaction). This attachment can be interrupted by washing (Vigeant, 2002), changes of chemistry of the solution, and cell motility (Marshall and Bitton, 1980). The reversible attachment is followed by an irreversible firm attachment which is a time dependant process, and mediated by extracellular material like EPS (Fletcher, 1973).

Microbial cells are attached to different surfaces. Bacteria *E. coli*, *Bradyrhizobium japonicum*, *Rhizobium leguminosarum*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, for example, have been found to be attached to surfaces such as glass and quartz (McClaine and Ford, 2002; Fowle, 2004), sand (Ozawa, 1986) pea root hair tips (Smit *et al.*, 1987), silica beads (Deshpande and Shonnard, 2000) and steel (Herald, 1989) respectively.

5.1.1. Importance and Significance of Microbial Attachment

The attached cells might benefit from the attachment to surfaces via:

- Nutrients which are found at surfaces such as simple, soluble carbon sources and organic macromolecules which may make
the surface nutritionally rich environment for the bacteria to obtain these nutrients (Deflaun et al., 1990); and
- Protection from predators by the formation of biofilm.

5.1.2. Factors that Affect Microbial Attachment

Attachment of bacteria to solid surfaces is affected by biological and chemical factors. Biological factors include: the presence of specific surface protein (Schie and Fletcher, 1999); extracellular polymers formation, for example, polysaccharide materials which connect cells to autoclaved sand (Balkwill and Casida, 1979); the presence of appendages (flagella and fimbrie) (Schie and Fletcher, 1999) which are responsible for reversible adhesion (El-Hamdaoui, 2003); the degree of cell surface hydrophobicity (Schie and Fletcher 1999) in which hydrophobic bacteria attach more than hydrophilic cells (Van Loosdrecht et al., 1987); the degree of cell electrostatic charge (Schie and Fletcher 1999), growth phase at which bacterial cells attach to surfaces at log-phase (Deflaun et al., 1990); cell concentration since the attachment decreases with an increase in cell concentration (Johnson and Logan, 1996); and cellular size since large cells (1.8 µm) retained more than the small ones (Fontes et al., 1991).

Chemical factors that affect the attachment include: the availability of nutrients. For example, the presence of glucose increases the bacterial attachment (Balkwill and Casida, 1979); and high ionic strength increases the bacterial attachment to porous media (Deshpande and Shonnar, 2000).

Deshpande and Shonnard (2000); Vandervivere and Kirchman (1993) have studied the effect of nutrient (citrate) and ionic strength (0.5M \( \text{Na}_2\text{HPO}_4 \) and 0.5M \( \text{K}_2\text{HPO}_4 \)) on the bacterial attachment. Their results revealed that high ionic strength (up to 1 M phosphates) and the presence of citrate in bacterial suspension enhanced the bacterial attachment. Another study revealed that the presence of \( \text{Ca}^{2+} \) ions in
the culture media enhanced rhizobial attachment (Morris et al., 1989). Based on these studies, the aim of this study was to determine the effect of citrate salts and calcium ions on bacterial retention in-situ. Additionally, the incubation period of bacterial cells in-situ prior to the injection of cementation solution was examined.

5.2. Materials and Methods

5.2.1. The Effect of Premixing Bacterial Cells in the Presence of Calcium ions and Citrate Salt on Attachment

5.2.1.1. Bacterial Conditions

The bacterial isolate (MCP11) was grown in MA (20 g.L⁻¹ YE, 8.2 g.L⁻¹ sodium acetate and 10 g.L⁻¹ sodium sulphate) at pH 9.0 and 28°C. It was grown in the absence of urea to avoid the formation of CaCO₃. The absence of urea in the growth medium therefore, reduces the aggregation of the cells before being up-flushed into the packed sand-column. The urease activity of the bacteria was 19 mM urea hydrolysed.min⁻¹ (1.7 mS.min⁻¹), with specific urease activity of 3.3 mM urea hydrolysed.min⁻¹.OD⁻¹ (0.3 mS.min⁻¹) and OD of 5.9.

5.2.1.2. Packing Sand-Column and Cell Placement

Plastic syringes (60 ml) were dry packed with 90-400 µm Si-sand (100 g) under continuous vibration to give an even density. The packed sand-columns were up-flushed with water (4-void volumes) and tapped to remove air pockets. After up-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. The columns were up-flushed with cells (1.3-void volume, flow rate of 0.4 L.h⁻¹) in the absence of calcium ions, mixed with 18 mM calcium chloride, 18 mM Na-citrate or equimolar CaCl₂/Na-citrate (18 mM). The optical densities at 600 nm of the inlet and outlet were recorded at each 10 ml fractions.
5.2.1.3. Cementation Reaction

Cementation solution (0.75 M equimolar of calcium/urea) was up-flushed into the four packed sand-columns directly after upflushing the bacterial cells was complete (zero incubation). To ensure full displacement of the liquid, 1.3-void volume of the cementation solution was up-flushed.

5.2.2. The Effect of Incubating the Cells *in-situ* after being Pre-mixed in the Presence of Calcium and Citrate Salts on Attachment and Strength Production

The bacterial condition, packing columns and cementation conditions were similar to the previously mentioned procedures (section 5.2.1.1 to 5.2.1.3) except that:

- Six sand-columns were prepared;
- The concentrations of the chemicals which were tested to enhance the attachment was (6 mM CaCl$_2$, 6 mM Tri-sodium citrate); and
- The concentration of the cementation solution was 1 M.

5.2.3. Effect of Growing Bacterial Cells in the Presence of Calcium Ions and Citrate Salt on the Attachment to the Sand Granules

- Four packed sand-columns were left to dry after draining the water from the core for more than 72 hours at room temperature.
- The concentration of urea in the growth media was 0.3 M, at pH 9.0 and 28$^\circ$C.
- Tri-sodium citrate dihydrate (3.4 mM), calcium chloride (3.4 mM), and a mix of calcium chloride and tri-sodium citrate dihydrate (final concentration of 3.4 mM, equivalent concentration) were added to the media separately depending on the examined column (Table 5.1).
- The urease activity of the bacteria in the three different treatments is shown in Table 5.1.
To assure the equality of the inflow of bacterial cells, the bacterial culture was mixed softly by stirrer during bacterial up-flow.

**Table 5.1:** The urease activity and optical density (OD) at 600 nm (average of four readings) of the bacterial culture at the moment of the in-flow through the packed sand-columns.

<table>
<thead>
<tr>
<th>Column</th>
<th>Urease activity (mS.min(^{-1}))</th>
<th>Urease activity (mM urea hydrolysed.min(^{-1}))</th>
<th>OD at 600 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.44</td>
<td>4.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Grown in the presence of Na-citrate</td>
<td>0.35</td>
<td>3.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Grown in the presence of CaCl(_2)</td>
<td>0.35</td>
<td>3.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Grown in the presence of CaCl(_2) and Na-citrate</td>
<td>0.30</td>
<td>3.3</td>
<td>2.1</td>
</tr>
</tbody>
</table>

### 5.2.4. Extending the Incubation Period of Cells *In-Situ* after being Grown in the Presence of Calcium Ions

#### 5.2.4.1. Bacterial Growth and Placement

- Three packed sand-columns were prepared.
- The cells were grown in the presence of 0.3 mM urea.
- Bacterial culture (3-void volumes) was up-flushed into the column.
- OD at 600 nm was 2.5.
- Urease activity was 5.6 mM urea hydrolysed.min\(^{-1}\) (0.5 mS.min\(^{-1}\)) and specific urease activity was 2.24 mM urea hydrolysed.min\(^{-1}\).OD\(^{-1}\) (0.2 mS.min\(^{-1}\).OD\(^{-1}\)).
- The cells were incubated for 0, 24 and 48 hours and then the cementation solution (1.3-void volumes) was up-flushed.
- Flow rate of cementation solution was 827.6 ml.\(^{-1}\).
- Two applications of cementation solution were injected.
5.2.4.2. Strength Measurements of the Cemented Columns (Pocket Penetrometer)

The unconfined compression strength was measured by a modified pocket penetrometer. A pocket penetrometer is usually used to measure the strength of extremely soft cohesive sand. The pocket penetrometer which was used to measure the strength of the cemented sand had a fixed tip (diameter of 0.6 cm, and surface area of 0.2826 cm²). It is possible to measure the strength of a well cemented sand-column (low strength) by reducing the surface area which is applied on the desired cemented spot. Accordingly, several stainless steel tips of different surface areas (< 0.2826 cm²) were used depending on how strong the column was cemented. The stronger the sample, the less surface area (of the tip) was applied. In order to obtain accurate measurements, the penetrometer was calibrated with dead weights (0.5-7 kg). The readings were taken as a range of the two readings on the device before and after approximately 1 mm of the tip had penetrated into the cemented sand-column. Sometimes breakage of the cemented sample could not be avoided. In these instances, the strength was calculated according to Equations 5.1 and 5.2.

\[
\text{Unconfined compression strength (kg.cm}^{-2}) = \frac{\text{Weight}}{\text{Surface area}}
\]

\[\text{Surface area (cm}^2) = \pi \times r^2\]

Where, (kg) is the unit of the weight, and (cm²) is the unit of the surface area.

Where, (r) is the radius of the pocket penetrometer tip which was placed at the cemented surface to measure its strength.
5.3. Results

5.3.1. Experimental Setup: A series of experiments were setup to examine the effect of calcium ions and citrate salts on the attachment of bacterial cells to sand granules. Furthermore, the effect of the incubation period of the cells *in-situ* prior to the initiation of cementation reaction on the attachment was examined. The retention of bacterial cells *in-situ* was calculated by measuring the mass (by OD measurement) and urease activity (by conductivity measurement) of the inlet and outlet liquid.

5.3.2. The Effect of Premixing the Bacterial Cells in the Presence of Calcium Ions and Citrate Salt on Cell Retention and Strength

To examine the *in-situ* cell retention in the presence of CaCl$_2$, Trisodium citrate or CaCl$_2$/tri-sodium solution, the cells were premixed separately in the presence of the desired chemicals prior to the injection.

The presence of Ca$^{2+}$ ions (premixed prior to injection) resulted in 2-4 fold increase in cell retention according to urease activity and OD (Figure 5.1), whereas in the presence of citrate there was no increase in the cell retention (Figure 5.1). This lacking effect of citrate on cell attachment contradicts what has been reported in the literature; Deshpande and Shonnard (2000), for example showed that the presence of citrate increases the bacterial attachment to sand granules.

The increase in cell retention in the presence of Ca$^{2+}$ ions enabled the development of a mechanical strength of 5-15 kg.cm$^{-2}$; alternatively, no strength was produced in the absence of calcium ions.
5.3.3. The Effect of Incubating the Cells In-Situ after being Premixed with Calcium Chloride on Cell Retention and Mechanical Strength Production

To study the effect of incubating the bacterial cells in-situ on bacterial attachment to sand granules, the bacterial culture was premixed in the presence of calcium chloride prior to the injection. This mixture was incubated in-situ for 0-48 hours. It was found that by incubating the cells in-situ for 24-48 hours prior to the exposure to cementation solution; the cells attached during that time and retention was enhanced (40-64% according to OD and activity measurements) even in the absence of Ca$^{2+}$ ions (Figure 5.2). Thus, in-situ cell retention seems to be a time-dependent process. Without in-situ incubation of cells; an insignificant increase in the cell retention (7-9%) in the presence of Ca$^{2+}$ ions was observed (Figure 5.2).

Three-to-four fold increase in the mechanical strength was recorded in the cells which had been incubated in-situ in the presence of Ca$^{2+}$ ions only (31-47 kg.cm$^{-2}$, Table 5.2) as compared to the non incubated (7.5-15 kg.cm$^{-2}$, Table 5.2). Thus, it is concluded that incubating the cells in-situ after being premixed in the presence of Ca$^{2+}$ ions is needed for relatively high strength production. This strength enhancement probably was due to the formation of irreversible (firm) attachment by the presence of Ca$^{2+}$ ions and in-situ incubation of cells.
Figure 5.1: The percent retention of cells in 10 cm packed sand-columns (60 ml plastic syringe) according to OD at 600 nm (□) and urease activity (■). Cells were premixed with 18 mM CaCl$_2$ and Na-citrate separately (zero incubation). The input urease activity and OD were 18.9 mM urea hydrolysed.min$^{-1}$ and 5.9 respectively.

Figure 5.2: Effect of incubation time on cell retention in the packed sand-columns (10 cm sand-columns in 60 ml plastic syringe) according to OD at 600 nm (□) and urease activity (■). The input urease activity and OD were 19 mM urea hydrolysed.min$^{-1}$ and 6 respectively.
Table 5.2: Strength measurement of the columns which were up-flushed with cells premixed in the presence and absence of 6 mM CaCl$_2$. The cells were incubated in the columns for 0, 24, and 48 hours before up-flushing the cementation solution (1 M). The strength was measured by pocket penetrometer which was calibrated with different dead weights (Appendix E). The readings were taken as a range of the two readings before and after breaking (or immersing 1 mm of the lower end of the tip into) the cemented sand column.

<table>
<thead>
<tr>
<th>Column</th>
<th>Diameter of the tip (cm)</th>
<th>Surface area (cm$^2$)</th>
<th>Reading of device (kg.cm$^{-3}$)</th>
<th>Calibrated with dead weight (kg)</th>
<th>condition of the cemented column</th>
<th>Range of strength (kg.cm$^{-3}$)</th>
<th>Strength conversion (MPa)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absence of Ca$^{2+}$</td>
<td>0  -  -  -  -  -  -  -  -  -  -</td>
<td></td>
<td></td>
<td></td>
<td>Too soft</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Presence of Ca$^{2+}$</td>
<td>0  0.2  0.0314  0.0-0.25  0.25-0.5  +  7.5-15  0.74-1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24  0.2  0.0314  0.25-0.5  1.0-1.5  +  31-47  3.0-4.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48  0.2  0.0314  0.25-0.5  1.0-1.5  +  31-47  3.0-4.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(✧) the column was not well cemented; it was extremely soft with lots of cracks so strength cannot be measured.
(+ ) the cemented column was broken when the maximum strength was applied.
(★) converted strength into MPa by knowing that 1kg.mm$^{-2}$=9.8 MPa, then 1kg.cm$^{-2}$=0.098 MPa. Note that the measurement by the pocket penetrometer (kg.cm$^{-2}$) produced higher values than the unconfined compression strength (UCS, MPa).
Figure 5.3: The percent retention of cells in 10 cm packed sand-columns (60 ml plastic syringe) due to growing the cells in the presence of 3 mM CaCl$_2$ and Tri-sodium citrate separately according to OD at 600 nm (■) and urease activity(□). The input urease activity and OD were 18.9 mM urea hydrolysed.min$^{-1}$ and 5.9 respectively.

Table 5.3: *In-situ* cells retention due to growing the bacterial culture in the presence of Ca$^{2+}$ ions and citrates. The retained mass and urease activity within the packed sand-columns (void volume of 30 cm$^3$) was calculated as shown in chapter 4.

<table>
<thead>
<tr>
<th>Columns</th>
<th>Mass retention (mg)</th>
<th>Urease activity retention (mmol urea hydrolysed.min$^{-1}.cm^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of calcium ions</td>
<td>1.11</td>
<td>0.00160</td>
</tr>
<tr>
<td>Presence of Na-citrate</td>
<td>1.20</td>
<td>0.00100</td>
</tr>
<tr>
<td>Presence of calcium ions</td>
<td>2.04</td>
<td>0.00230</td>
</tr>
<tr>
<td>Presence of calcium ions and Na-citrate mix</td>
<td>1.53</td>
<td>0.00204</td>
</tr>
</tbody>
</table>
5.3.5. The Effect of Incubating the Cells *In-situ* after being Grown in the Presence of Ca\(^{2+}\) Ions

Previous sections have shown that cell attachment can be enhanced by a) allowing an incubation period of 24-48 hours and b) providing calcium salts during growth. To examine the combination of the two effects, cells grown in the presence of 6 mM Ca\(^{2+}\) ions were allowed to incubate after flushing into the packed sand-column. A relatively high strength value of 1400 kpa (Figure 5.4) was achieved after growing the cells in the presence of Ca\(^{2+}\) ions and incubating them for 24 hours *in-situ* before up-flushing the cementation solution. Further incubation led to a marginal increase (18%) in the mechanical strength (1700 kpa) (Figure 5.5). In the control column where the cells were not incubated, there was no strength formation. The high strength production after incubating the cells confirmed what was previously shown in section 2.3.3, i.e. that the cell attachment is time-dependent. Unfortunately, the effect of time-dependence on the quantity of the attached cells to sand granules in the case of growing the cells in the presence of Ca\(^{2+}\) ions was not studied. Further detailed investigation, however, is necessary for determining the correlation between the incubation time and quantity of cells which are attached to the sand granules.

The success in attaching the cells to sand granules directed to a new cementation technology which depends on a two-steps process. In this process, the cells were first fixed and followed by up-flushing cementation solution; thereby preventing the clogging which was observed in the one-step process (the cells were mixed with cementation solution and up-flushed directly).
Figure 5.4: The effect of growing bacterial cells in the presence of CaCl₂ and incubation period of 24 hours in-situ on the unconfined compression strength (UCS). The cemented column resulted from up-flushing the bacterial cells which were grown in the presence of 6 mM CaCl₂. Subsequent to incubating the cells in-situ, 1 M cementation solution was up-flushed. (A) Raw data (to calculate B, tested by Centre of Offshore Foundation Systems (COFS), UWA, Australia), and (B) Actual strength (kpa). On the right of the Figure the biocemented column prior (C) and subsequent (D) to performing the UCS test was shown.
Figure 5.5: The effect of growing the bacterial cells in the presence of CaCl$_2$ and incubation period of 48 hours \textit{in-situ} on the unconfined compression strength (UCS). The cemented column resulted from up-flushing the bacterial cells which were grown in the presence of 6 mM CaCl$_2$. Subsequent to cell incubation, 1M cementation solution was up-flushed. (A) Raw data (to calculate B, tested by Centre of Offshore Foundation Systems (COFS), UWA, Australia), and (B) Actual strength (kpa). On the right of the Figure the biocemented column prior (C) and subsequent (D) to performing the UCS test was shown.
5.4. Discussion

The presence of Ca\(^{2+}\) ions in the bacterial culture prior to the injection increased the cell retention and mechanical strength of a packed sand-column. For biocementing a packed sand-column, it is important to attach the bacterial cells to the sand granules to prevent washing out of cells after the up-flow of cementation solution. Poorly attached cells will be easily washed out especially if the cementation solution was up-flowed continuously. Thus, as long as the cells were retained in-situ the CaCO\(_3\) will be precipitated forming a mechanical strength. The relationship between CaCO\(_3\) precipitation in-situ and strength production is investigated in the next chapters. Moreover, the enhancement in cell retention in-situ increases the point-to-point contact. This type of contact was shown to be one of the factors that increase the strength formation as mentioned previously in chapter 4.

Further increase in cell retention and strength production (three-times) was achieved by growing the cells in the presence of Ca\(^{2+}\) ions (6 mM) and incubating them for 24-48 hours. Even in the absence of Ca\(^{2+}\) ions, there was enhancement in cell retention subsequent to the incubation of the cells in-situ forming softer cementation than the incubated cells in the presence of Ca\(^{2+}\) ions. This soft strength production in the absence of Ca\(^{2+}\) ions suggests the importance of these ions besides the incubation time for a high strength formation. The high strength formation due to incubating the cells in-situ over the non incubated ones has supported the existence of a time dependent process for attachment. Fletcher and Floodgate (1973) and Morris et al (1989) suggested that the time-dependent attachment process usually is mediated by extracellular material (EPS) that allows the cells to attach firmly to the surface. Marshall (1985) described the time dependent active adhesion model. This model proposed that the bacteria at first attach weakly followed by firm attachment through the formation of anchoring exopolymers. Costerton et al (1987) have confirmed the role of exopolymers in cell attachment through microscopic examinations.
which showed that the bacterial cells that adhere to solid surfaces are often embedded in exopolymers.

During the initial stages of *P. starania* (Wrangstadh *et al.*, 1989) have found that the presence of solid surface has enhanced the appearance of exopolymers around the cells. According to Coplin *et al* (1990), contacted surfaces might enhance the exopolymer synthesis by enhancing external signals through membrane-embedded signal-transducing proteins (METP). METPs were found to control exopolymer synthesis in several bacteria. The solid surfaces enhanced exopolysaccharide synthesis in a reversible manner (Vandervivere and Kirchman, 1993), in which exopolymer synthesis increased five-fold by attached cells compared to unattached ones.

El-Hamdaoui *et al* (2003) studied the effect of Ca$^{2+}$ ion concentrations on the establishment and development of rhizobial (bacteria that enhance the nodulation of legumes) symbiosis in *Pisum sativum* plant. They reported that high adsorption to the roots was observed when Ca$^{2+}$ ions were 2.72 mM. This cellular adsorption confirmed the findings of Smit *et al* (1989) which revealed that Ca$^{2+}$ ions enhance the bacterial attachment to the root hairs through the production of exopolysaccharide (EPS) (Bennet *et al*., 1990; Morris *et al*., 1989). Similarly, a study by Fowle *et al* (2004) of the effect of Ca$^{2+}$ ions on the attachment of *E. coli* to a quartz surface; revealed that the attachment was associated with the presence of Ca$^{2+}$ ions. They claimed that 0.01 M of Ca$^{2+}$ ions altered the electrical field of the cell wall causing the cells to attach to the mineral surface. Alternatively, Smit *et al* (1986) suggested that fibrils (appendages) are essential in the attachment of *Rhizobium leguminosarum* to pea root hair tips (caps) and to glass. One year later, Smit *et al* (1987) postulated a model for rhizobial (*R. leguminosarum* 248) attachment to pea caps. They found that fibril-negative mutants or wild-type rhizobia that grow under Ca$^{2+}$ ions deficiency were not able to form caps. Consequently, they concluded that both cellulose fibrils and the Ca-dependent adhesion(s) are
involved in the attachment process. The postulated adhesion model was a two steps model. In step one; single rhizobial cells are attached due to a Ca dependent process followed by the attachment of cellulose fibril dependent, resulting in a bacterial aggregation on the caps. The bacterial attachment to the caps is a non-specific mechanism for rhizobial cells (Morris et al., 1989). Therefore, it might be useful to relate the two steps adhesion model to the attachment of bacterial cells to the sand granules in the case of ureolytic calcite precipitation.

Some divalent salts such as SrCl$_2$ and MgCl$_2$ were successfully used to replace CaCl$_2$ in the growth medium without affecting the properties of _R. leguminosarum_ 248 to adhere to pea root hairs (Smit et al., 1987). Accordingly, there was no absolute requirement of Ca$^{2+}$ ions for cellular attachment but of a divalent cation.

The precise mechanism by which Ca$^{2+}$ ions and cellular incubation _in-situ_ enhance the attachment of microbial cells to sand granules is not clear and requires more detailed examination. Moreover, there are some questions which need to be addressed in future work, for example: Are Ca$^{2+}$ ions truly triggering the production of polysaccharide? If so what is the mechanism that directs the structure and amount of polysaccharide?
Chapter 6
Biocementation of 1 Meter Sand-Column

6.1. Effect of Sequential Loading of Bacteria and Cementation Solution on Plugging 1 m Sand-Column

6.1.1. Introduction

In previous biocementation applications all reaction components (bacteria, urea and calcium solution) were mixed and applied together. This technique has resulted in successful cementation of short columns (10 cm). However, preliminary experiments indicated that insufficient penetration of the cementation mix occurred when longer columns (1 m) were used. For practical applications, a penetration depth of several meters is desirable to enable the commercial application of biocementation (personal communication Vicky Whiffin, Geodelft, Netherlands). One of the aims of this chapter was to test whether a high penetration depth (up to 100 cm) can be accomplished by avoiding the cementation mix to plug the injection end of the column, by sequentially adding the bacteria and the cementation solution (calcium/urea mix). The idea was that bacteria could be flushed into the column first, allowing the cells to attach to the sand granules. Then the bacterial substrate (urea) and the calcium solution were supplied subsequently assuming that a sufficient amount of bacteria remains in the column rather than being flushed out with the injection of the cementation solution. This way a more uniform cementation would be expected. Thus, this chapter aims at studying the penetration depth and consequently the mechanical strength profile by sequential loading of bacteria and cementation solution (calcium/urea mix) through 1 m sand-column. In addition the distribution of the bacterial cells along the column will be examined.
6.1.2. Materials and Methods

6.1.2.1. Packing 1 m Sand-Column

The sand-column (1 m long, internal diameter 4.5 cm, PVC) was packed with 100-400 µm sand (Appendix B). It was positioned vertically. Each end of the column was fixed with a filter (to confine sand), above which gravel (approximately 2.5 cm) was placed (Figure 6.1).

Figure 6.1: Schematic diagram of the experimental set-up used for biocementing long sand columns (1 m) through sequential and combined plugging. Where "P" is a pressure pump.
6.1.2.2. Injection through Combining Bacterial Cells with the Cementation Solution (Combined Injection)

Bacterial culture (MCP11, 360 ml) with urease activity of 13.3 mM urea hydrolysed.min⁻¹ (1.2 mS.min⁻¹) and specific urease activity of 2.2 mM urea hydrolysed.min⁻¹.OD⁻¹ (0.2 mS.min⁻¹.OD⁻¹) was mixed with 640 ml of cementation solution (final concentration of 1.25 M calcium chloride and 1.7 M urea). This cementation mix was up-flushed into 1 m sand-column immediately under pressure of 5 psi for 10 min (~1 L.h⁻¹) then 7.5 psi for 3 min (~1.5 L.h⁻¹). The column was tapped smoothly to get rid of air. To be sure that the voids within the column were filled with the cementation mix, it was up-flushed until 100 ml was flushed out.

6.1.2.3. Injection through Up-Flushing the Cells First Followed by the Cementation solution (Sequential Plugging)

The 1 m sand-column was up-flushed with 600 ml bacterial culture (1.2-void volumes). The column with the bacteria inside was kept at room temperature for a period of 72 hours, after which the cementation solution (at similar concentration and flow rate mentioned above in section 6.2.2) was up-flushed.

6.1.2.4. In-Situ Cells Retention Measured by OD and Urease Activity in the Sequential Plugging

Fractions of effluent culture (50 ml) were collected from the sequential plugging column after up-flushing the cementation solution. OD at 600 nm and urease activity (by conductivity meter) were measured immediately prior to the sample collection.

6.1.2.5. U-tube Manometer Test for Measuring Calcium Carbonate Content of Cemented Packed Sand-Column

A graduated glass tube (100 ml) was connected to a piece of tubing to construct a U-tube. A vessel (50 ml) containing a smaller vessel (2.5 ml) was connected to the U-tube using a rubber stopper. A cemented sample was placed in the larger vessel, then 2 ml of 2 M HCl was
added to the smaller vessel. After closing the larger vessel by the stopper, the vessel was laid down to bring the acid in contact with the sample, and gently was shaken. The reaction of the acid with the CaCO$_3$ in the sample produces CO$_2$, which is measured by the water volume shift in the graduated glass tube under standard conditions of 25°C and 1 atm (Appendix F).

6.1.3. Results

6.1.3.1. Experimental Setup: Two 1 m packed sand-columns were cemented in parallel, using the same bacterial culture and cementation solution concentrations. The control column was up-loaded as previously described by combining the cells with the cementation solution while the test column was first up-loaded with bacteria, wait for 72 hours and then up-flushed with the cementation solution. The amount of urea hydrolysis (measured by NH$_4^+$), calcite formation (examined by acid dissolution analysis and SEM) and mechanical strength were examined.

6.1.3.2. In-Situ Cell Retention Along the 1 m Sand-Column

According to OD at 600 nm or urease activity about 39% or 57% of cells respectively were retained in-situ in the sequential flushing (Figure 6.2). In the test using combined flushing, all the bacteria were retained (no activity or turbidity in the outflow) within the 1 m column due to the immediate CaCO$_3$ formation as soon as the contact between the cells and the cementation solution has occurred as described in section 1.2.2.
Figure 6.2: The retention of cells in the sequentially flushed column measured by OD (■) at 600 nm and urease activity (□). Culture-fractions of 50 ml were collected after up-flushing the cementation solution. Note that the void volume was 500 ml, the OD and urease activity for the last 50 ml fraction were not measured as it was turbid due to the presence of cementation solution (i.e. Due to cementation reaction, CaCO$_3$ particles formed resulting in the disturbance of OD and urease activity measurements).

6.1.3.3. Urea Conversion, Calcite Formation and Mechanical Strength In-Situ

For the sequential flushing, the urea conversion (NH$_4^+$ analysis, chapter 2, section 2.2.6.2.) was almost uniform along the cemented column (Figure 6.3). Similarly, the CaCO$_3$ precipitation and mechanical strength were about uniform along this column (Figure 6.3, and Figure 6.4) implying uniform availability of calcium and urea.

In the combined flush-application, contrarily, the urea conversion, calcite precipitation and mechanical strength (Figure 6.1, column1; Figure 6.3) decreased gradually along the cemented column. The attainable high strength was limited to the area closest to the injection point (0-37cm depth, > 226 kg.cm$^{-2}$) (Figure 6.4). This strength decreased along the cemented column, until no cementation was
obtained. Accordingly, mixing the bacterial cells with cementation solution prior to the injection was inefficient to cement a column greater than 37-50 cm length (the data of the combined flushing was not mentioned in a figure or table because of the low penetration depth). It is possible that the precipitated CaCO₃ closest to injection end prevented the cells from penetrating to a distance more than 37-50 cm (Figure 6.4, column 1).

**Figure 6.3:** NH₄⁺ production (●, mM), CaCO₃ precipitation (▲, mg.g⁻¹) and mechanical strength measured by a modified penetrometer (◆, kg.cm⁻²) measurements along the sequential cemented column (1 m). The maximum hypothesized NH₄⁺ production was 3400 mM (1700 mM urea was used). The true strength value at each distance along the cemented column was greater than the one mentioned in the figure, because the strength measurement was taken as a range between the values before and after strength failure. This range of strength values were measured as shown in Table 6.1. A high degree of uniformity was observed along the cemented column.
Figure 6.4: The two attempts of cementing 1 m sand-column, combined plugging (column 1) and sequential plugging (column 2). (I) sketch (II) Images of the consolidated and unconsolidated parts along the 1 m column. (III) Magnification of the areas from A to D along the cemented column in (II) from the point of injection. The sequential column was up-flushed with 1.2-void volume cementation solution with 1700 mM urea and 1250 mM CaCl$_2$. 

No cementation

6-13 kg.cm$^2$

$>$226 kg.cm$^2$
Table 6.1: Strength measurements of the sequential flush application. The strength was measured by pocket penetrometer calibrated with different dead weights (to reduce the error encountered by the penetrometer measurement). The readings were taken as a range of the two readings before and after the failure of the cemented sand-column.

<table>
<thead>
<tr>
<th>Distance from injection point (cm)</th>
<th>Diameter of the tip (cm)</th>
<th>Surface area (cm²)</th>
<th>Reading of device (kg.cm⁻³)</th>
<th>Calibrated with dead weight (kg)</th>
<th>Range of strength (kg.cm⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.3</td>
<td>0.07065</td>
<td>2-2.5</td>
<td>4.0-4.5</td>
<td>057-064</td>
</tr>
<tr>
<td>6.5</td>
<td>0.2</td>
<td>0.0314</td>
<td>2.5-3</td>
<td>4.5-5.0</td>
<td>143-159</td>
</tr>
<tr>
<td>12.5</td>
<td>0.2</td>
<td>0.0314</td>
<td>2.5-3</td>
<td>4.5-5.0</td>
<td>143-159</td>
</tr>
<tr>
<td>19</td>
<td>0.2</td>
<td>0.0314</td>
<td>2.5-3</td>
<td>4.5-5.0</td>
<td>143-159</td>
</tr>
<tr>
<td>25</td>
<td>0.2</td>
<td>0.0314</td>
<td>2.5-3</td>
<td>4.5-5.0</td>
<td>143-159</td>
</tr>
<tr>
<td>37.5</td>
<td>0.2</td>
<td>0.0314</td>
<td>3-3.5</td>
<td>5.0-5.5</td>
<td>159-175</td>
</tr>
<tr>
<td>50</td>
<td>0.2</td>
<td>0.0314</td>
<td>3-3.5</td>
<td>5.0-5.5</td>
<td>159-175</td>
</tr>
<tr>
<td>62.5</td>
<td>0.2</td>
<td>0.0314</td>
<td>2.5-3</td>
<td>4.5-5.0</td>
<td>143-159</td>
</tr>
<tr>
<td>75</td>
<td>0.2</td>
<td>0.0314</td>
<td>2.5-3</td>
<td>4.5-5.0</td>
<td>143-159</td>
</tr>
<tr>
<td>87.5</td>
<td>0.2</td>
<td>0.0314</td>
<td>3-3.5</td>
<td>5.0-5.5</td>
<td>159-175</td>
</tr>
<tr>
<td>97</td>
<td>0.2</td>
<td>0.0314</td>
<td>3-3.5</td>
<td>5.0-5.5</td>
<td>159-175</td>
</tr>
</tbody>
</table>

For field application where deep penetration is needed, the sequential plugging is advantageous over the combined plugging. However, in the sequentially flushed column only partial urea conversion was achieved (40-48% of urea was converted, Figure 6.5). Reasons for this partial degradation of urea were unclear. The partial degradation of urea was probably due:

- The precipitation of CaCO₃ crystals around the cells could cause diffusion barriers which limit the access of substrate. This is unlikely to occur because the amount of CaCO₃ which is precipitated from one flush of cementation solution around
the bacterial cells (current study, chapter 3) could not form diffusion barrier, enabling easy access of substrate and removal of by-products. This easy diffusion was supported by the ability of reusing the cells around 30-times in the continued supply of cementation solution (details are given in chapters 7 and 8).

- Accumulation of by-products (NH$_4^+$, CO$_2$) in the microenvironment surrounding the bacteria. This is likely to happen because there was no other way to dispose of the by-products.

6.1.3.4. SEM Images of the Combined and Sequential Plugging

For comparison purpose, the distribution of CaCO$_3$ crystals at the surface of sand granules in the sequential and combined application-columns was examined. The sand granules at the injection end of the combined flush application were fully covered with CaCO$_3$ spheroids (Figure 6.6), compared to fewer separated spheroids in the sequential plugging (Figure 6.7).

![Figure 6.5: Percentage of urea hydrolyzed along 1 m packed sand-column (sequential plugging). % of urea hydrolysed was calculated from the known molar ration (1 urea: 2 NH$_4^+$) at different positions along the column a way from the point of injection. The concentration of urea in the cementation solution was 1700 mM.](image)
Figure 6.6: SEM images of the combined plugging (control column) in which the cells and cementation solution were mixed together and up-flushed immediately under pressure. The sand granules were fully covered with CaCO₃ crystals at (A) 0 cm; and (B) 25 cm depth from injection point.
Figure 6.7: SEM images of the sequential flush application at 25 cm along the 1 m column in which cells were kept for 72 hours within the column. Then the cementation solution was up-flushed. The bacteria formed CaCO$_3$ spheroids at the surface of the sand granules.
6.1.4. Conclusion

A successful bacterial cementation to a penetration depth of 1 m was achieved by sequentially flushing the bacteria and then cementation solution.
6.2. Effect of Growing Cells with Ca\textsuperscript{2+} ions on the Attachment to Sand Granules Along 1 m Sand-Column

6.2.1. Introduction

In previous biocementation experiments most of the bacterial cells (around 60%) were washed out of the sand-column after up-flushing the cementation solution (calcium/urea mix). For a successful strength production, a sufficient number of cells should be retained within the packed sand-column so that the bacterial CaCO\textsubscript{3} precipitations form bridges; binding the sand granules together. Thus, there is an inevitable need to attach the cells to the surface of the sand granules. Morris et al (1989) has pointed the effect of attaching bacterial cells to the surfaces after being grown in the presence of Ca\textsuperscript{2+} ions. Preliminary experiments on 10 cm sand-column indicated that bacterial cells attach to the sand granules. This attachment was due to growing the cells in the presence of 6 mM Ca\textsuperscript{2+} ions, and keeping the cells for greater than 24 hours within the sand. This experiment aims at studying the strength profile along 1 m depth packed sand-column subsequent to attaching the cells \textit{in-situ}. Fine sand will be used due to its particular interest to the biocementation industry. A successful biocementation in terms of strength was expected due to the attachment of cells to the sand granules.

6.2.2. Materials and Methods

6.2.2.1. Bacterial Culture Condition

The bacterial culture was grown without urea at 28\textdegree C for 24 hours. The urease activity was 11.11 mM urea hydrolysed.min\textsuperscript{-1} (1 mS.min\textsuperscript{-1}) and the specific urease activity was 2.1 mM urea hydrolysed.min\textsuperscript{-1}.OD\textsuperscript{-1} (0.2 mS.min\textsuperscript{-1}.OD\textsuperscript{-1}). Another batch of culture was grown in the presence of 6 mM CaCl\textsubscript{2}.2H\textsubscript{2}O, in which the urease activity was 9.3 mM urea hydrolysed.min\textsuperscript{-1} (0.8 mS.min\textsuperscript{-1}) and specific urease activity was 2.3 mM urea hydrolysed.min\textsuperscript{-1}.OD\textsuperscript{-1} (0.2 mS.min\textsuperscript{-1}.OD\textsuperscript{-1}).
6.2.2.2. Plugging the Packed Sand-Columns

Three parallel columns (1 m long) were packed with fine silica sand (100-250 µm), as was described in section 6.2.1. One of the sand-columns was up-flushed with 650 ml bacterial culture (1.3-void volumes and was grown in the presence of Ca\(^{2+}\)) under a constant pressure of 7.5 psi for 11:39 min (1.4 L.h\(^{-1}\)). The column with the bacterial cells inside was kept at room temperature for a period of 48 hours after which cementation solution (final concentration of 1.25 M calcium chloride and 1.7 M urea) was up-flushed.

For the other two control columns, the same procedures were applied except the treatment of the cells prior to the injection. The cells were either mixed with 6 mM Ca\(^{2+}\) ions immediately before being up-flushed or grown in the absence of Ca\(^{2+}\) ions.

6.2.3. Results

6.2.3.1. Experimental Setup: Three 1 m packed sand-columns were cemented in parallel, using the same concentration of cementation solution and the way of loading. The bacteria were up-flushed first, incubated for 48 hours followed by the application of cementation solution. The difference between the columns was the way of bacterial treatments prior to the placement. The cells were treated as follows:

- In the absence of Ca\(^{2+}\) ions;
- Mixed with Ca\(^{2+}\) ions; or
- Grown in the presence of Ca\(^{2+}\) ions.

6.2.3.2. In-Situ Cell Retention According to Optical Density (OD) and Urease Activity

To measure the retained cells within the sand-column, 50 ml fractions of effluent culture were collected continually from the three columns during up-flushing the cementation solution. The samples were stored at 4°C for several hours until processing. Cells that were pre-grown in the presence of 6 mM Ca\(^{2+}\) ions showed a 5 or 3 times increase in the
cell retention (Table 6.2). In this column, the solution in the out-let was turbid indicating the presence of cells in the solution. This means that there is a possibility of retaining the cells within a sand-column longer than 1 m. Retaining the cells along several meters is advantageous for industrial purposes.

Table 6.2: The retained OD and urease activity in the three columns (1 m). Fractions (50 ml) were collected after incubating the bacterial cells within the sand-column for 48 hours. The cells were treated differently; (A) grown in the absence of Ca$^{2+}$; (B) mixed with 6 mM Ca$^{2+}$ immediately before being up-flushed; and (C) grown in the presence of 6 mM Ca$^{2+}$. The urease activity of the bacteria used for the loading was 11.11 mM urea hydrolysed min$^{-1}$.

<table>
<thead>
<tr>
<th>Treatments of cells</th>
<th>% OD retention</th>
<th>% Activity retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grown in the absence of Ca$^{2+}$ ions</td>
<td>14</td>
<td>20</td>
</tr>
<tr>
<td>Mixed with Ca$^{2+}$ ions</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>Grown in the presence of Ca$^{2+}$ ions</td>
<td>75</td>
<td>67</td>
</tr>
</tbody>
</table>

6.2.3.3. Biocementation Process Along the 1 m-Column

The urea conversion was the highest in the column which was up-flushed with bacterial cells grown in the presence 6 mM Ca$^{2+}$ ions (Figure 6.8; Figure 6.9). It represented Four-times increase in urea conversion as compared to the absence of Ca$^{2+}$ ions and two-times increase as compared to the premixed cells with Ca$^{2+}$ ions prior to the injection. A gradient of urea conversion was observed along the cemented column with higher conversion closest to the injection point than the opposite end. This was supported by calcite formation (Figure 6.10) and mechanical strength production (Figure 6.11; Figure 6.12). The decrease in urea conversion away from the injection point could be due to:

- The supply of calcium and urea far from the injection point was low due to the high urease activity closest to the injection point. This was not likely to occur because the flow rate of the cementation solution was high (1.4 L.h$^{-1}$), therefore, the supply
of calcium and urea closest and away from the injection point was almost similar.

- Heterogeneity of cell retention in-situ due to the filtration effect by the fine sand (100-250 µm).
- Higher compaction of sand at the bottom of the column increased the possibility of calcite bridges formation at the bottom compared to the top. This compaction problem could be avoided by placing a dead weight at the top of the sand-column, which will prevent the looseness of the sand granules especially when high flow rate was applied.

![Figure 6.8: Urea conversion (NH₄⁺ analysis, according to the molar ratio of 1 urea: 2 NH₄⁺) at the end of cementation process along the three treated columns which differ from each other according to the way in which the cells were treated; grown (▲); mixed with Ca²⁺ ions (●); or in the absence of Ca²⁺ ions. Heterogeneity in the urease conversion along the fine sand-column was observed.](image-url)
Figure 6.9: Percentage Urea conversion along the 1 m sand-column for the differently treated columns. The percentage of urea conversion was calculated from Figure 6.8.

Figure 6.10: CaCO$_3$ precipitation *in-situ* along the 1 m sand-column at the end of cementation process.
Figure 6.11: Sketch of the cemented 1 m packed sand-columns by using three treatments. For these treatments, the bacterial cells were (A) column 1: grown in the absence of Ca²⁺; (B) column 2: mixed with Ca²⁺ immediately prior to the injection; and (C) column 3: grown in the presence of Ca²⁺ ions. The cementation has occurred in all columns with different strength values.
Figure 6.12: Strength profile along the cemented columns after cementation was complete. It was calculated as a range value before and after strength failure as mentioned in figure 6.3. An average value in the range of strength penetrometer measurement was taken to draw the strength versus the distance from the injection point.

6.2.3.4. SEM images

To examine the effect of Ca\(^{2+}\) ions on attaching and distributing the cells along the 1 m packed sand-column, SEM images of some locations along the cemented columns were examined. Apparently, when cells were grown in the presence of Ca\(^{2+}\) ions, the sand granules were fully covered with CaCO\(_3\) crystals (Figure 6.13) as compared to the premixed cells in the presence (Figure 6.14) or absence of Ca\(^{2+}\) ions (Figure 6.15). Thus, the attachment to sand surface due to growing cells in the presence of Ca\(^{2+}\) ions has performed. Due to the high distribution of the spheroids at the surface of the sand granules, counting the attached crystals was difficult. However, it was clear from (Figure 6.13: Figure 6.15), that the number of CaCO\(_3\) crystals at the sand surface when the cells were grown in the presence of Ca\(^{2+}\) ions was absolutely greater than the other treatments.

The SEM images in conjunction with the retention of cells in-situ confirmed the effectiveness of growing the cells in the presence of Ca\(^{2+}\) ions on the attachment of bacterial cells along the cemented column.
Figure 6.13: SEM of the packed sand-column in which the cells were grown in the presence of 6 mM Ca$^{2+}$ ions at (A) 0 cm; and (B,C) 12.5 cm. These images show white calcite layer and spherical crystals attached to the sand granules.
Figure 6.14: SEM images of packed sand-column which was up-loaded with bacterial cells pre-mixed in the presence Ca\textsuperscript{2+} ions immediately prior to the injection at (A) 0 cm; and (B) 12.5 cm. These images show small (10-25 µm) individual rhombohedral cells forming bridges binding sand granules together.
Figure 6.15: SEM images of the packed sand-column which was up-flushed with bacterial cells without being mixed in the presence of Ca\textsuperscript{2+} ions at (A) 0 cm; and (B) 12.5 cm. These images show small (6-10 µm, surrounded by circles) and low number of spheres at the surface of the sand granules.
6.2.4. Discussion

The bacterial cells were successfully attached to the sand granules throughout the 1 m sand-column, when grown in the presence of 6 mM Ca$^{2+}$ ions and incubated for about two days within the column. Due to the incubation period of the cells *in-situ*, the cells were attached to the surface of the sand granules. The attachment was evident by OD, urease activity measurements and formation of CaCO$_3$ crystals attached to the sand granules. Subsequent to this attachment the cementation solution was up-flushed as discussed in chapter 5.

The profile of the mechanical strength along the cemented column which was up-loaded with the cells grown in the presence of Ca$^{2+}$ ions was heterogeneous. This profile was confirmed by urea conversion, CaCO$_3$ content and strength production. It was found that the urea conversion, calcite and strength at the bottom closest to the injection point were higher than the other end. This heterogeneity was probably attributed to the filtration effect of the fine sand (i.e. fine sand was used due to its particular interest to the biocementation industry) as discussed previously.

In previous experiment of biocementing 1 m sand-column; homogeneous cementation was performed. The differences between the two experiments that may affect the profile of cementation were:

- The presence of Ca$^{2+}$ ions in the bacterial growth medium. This is unlikely to occur because the effect of growing bacterial cells in the presence of Ca$^{2+}$ ions on attachment is not instant. They need time to attach. Thus during up-flushing the cells through the packed sand-column, being grown in the presence of Ca$^{2+}$ ions will not affect their distribution along the column.
- The use of different sand types in both experiments. Compacted fine sand will posses small pores thus lots of cells might be retained at the bottom more than the top.
6.2.5. Conclusions

- Growing the cells in the presence of Ca$^{2+}$ ions enhanced their attachment along the 1 m sand-column and consequently the strength.
- Obtaining homogeneous cementation proved more difficult with using fine sand. Thus, using finer sand proved more difficult to obtain full penetration to 1 m.
Chapter 7
Parameters that Affect the *In-Situ* Biocementation Process

7.1. Effect of Reactants on the Biocementation Process

7.1.1. Introduction

All cementation experiments reported in the literature and in this study were run in a batch mode. This means that reaction conditions change dramatically with high calcium/urea and low NH$_4^+$ at the beginning and low calcium/urea and high NH$_4^+$ at the end. The following experiment aims to test whether:

- Continued cementation reaction can be obtained by a continuous supply of calcium/urea; and/or
- Cementation also occurs under conditions as they exist at the end of a batch (low calcium/urea and high NH$_4^+$).

The most expensive cementation reaction component is the enzyme. By having a continuous cementation process, a cost saving process will be obtained by reusing the cells several times.

7.1.2. Materials and Methods

7.1.2.1. Bacterial Growth and Placement Conditions

The detailed procedures of bacterial growth and placement through the packed sand-columns were discussed previously in chapter 5. Below is a list of bacterial conditions and placement:

- The cells (MCP11) were grown in the presence of 6 mM Ca$^{2+}$ ions.
- Urease activity of cells was 19 mM urea hydrolysed. min$^{-1}$ (1.71 mS.min$^{-1}$), specific urease activity was 5.4 mM urea hydrolysed. min$^{-1}$.OD$^{-1}$ (0.49 mS.min$^{-1}$) and OD was 3.5.
- The packed sand-columns (298 g sand, 300-400 µm) were washed with 3-void volumes water with flow rate of 1312 ml.h$^{-1}$. 

Bacterial cells were mixed with 100 mM urea and 100 mM Ca\(^{2+}\) ions immediately before being up-flushed into the packed sand-column.

Retention of cells *in-situ* was 80%.

Void volume of the packed sand-column was 60 ml.

Retention time of the cementation solution within the sand-column was 1.6 hour.

Flow rate of the cementation solution was 37 ml.h\(^{-1}\) (in soil application only slow flow rate is used).

The cells were kept *in-situ* for 72 hours and then the cementation solution were up-flushed continuously. Two mixtures of cementation solutions were up-flushed as follows:

- Continuous flow of high concentration of cementation solution (calcium/urea) with low concentration of NH\(_4^+\) ions: urea (1M), calcium chloride (1 M) and NH\(_4^+\) (100 mM) was up-flushed (for the batch treatment only 1.5-void volume was up-flushd).
- Continuous flow of low concentration of cementation solution (calcium/urea) with high concentration of NH\(_4^+\) ions: Urea (100 mM), calcium (100 mM) and NH\(_4^+\) (1.8 M) mix was up-flushed (for the batch treatment only 1.5-void volume).

**Table 7.1:** The cementation conditions of the batch and continuous fed columns. These columns were setup to examine the continuous cementation reaction.

<table>
<thead>
<tr>
<th>Sand-Column</th>
<th>Treatment</th>
<th>Concentration of calcium/urea and NH(_4^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column 1 (control 1)</td>
<td>Batch</td>
<td>High calcium/urea and low NH(_4^+)</td>
</tr>
<tr>
<td>Column 2</td>
<td>Continuous</td>
<td></td>
</tr>
<tr>
<td>Column 3 (Control 2)</td>
<td>Batch</td>
<td>Low calcium/urea and High NH(_4^+)</td>
</tr>
<tr>
<td>Column 4</td>
<td>Continuous</td>
<td></td>
</tr>
</tbody>
</table>
7.1.2.2. Urease Activity \textit{In-Situ} Calculation

The urease activity was calculated according to the ammonium ion production (1 urea: 2 NH$_4^+$), considering the liquid produced \textit{in-situ} during up-flushing cementation solution and its retention time (Equation 7.1; Equation 7.2). By integrating urease activity during the cementation process, the total amount of urea hydrolysed \textit{in-situ} could be determined.

\[
\text{In-situ urease activity (mmol}.L^{-1}.h^{-1}) = \frac{\text{NH}_4^+ \text{ concentration in the out-flow (mmol} / L\text{)}}{\text{HRT (h)}} \hspace{1cm} \text{(7.1)}
\]

Where HRT is the hydraulic retention time.

\[
\text{Liquid produced (ml)} = \text{flow rate (ml} \cdot h^{-1}) \times \text{elapsed time (h)} \hspace{1cm} \text{(7.2)}
\]

7.1.2.3. Calcium Carbonate Calculation

By considering the \textit{in-situ} ammonium produced, liquid produced, and the molar ratio of calcium ions precipitation: NH$_4^+$ production (1:2), the theoretical (calculated) CaCO$_3$ precipitation \textit{in-situ} was calculated according to the following formula:

\[
\text{CaCO}_3 \text{(mmol)} = \frac{0.5 \times \text{NH}_4^+ \text{ produced (mmol)} \times \text{liquid produced (ml)}}{1000 \text{ (ml)} \times \text{(L}^{-1}) \times \text{(L)}} \hspace{1cm} \text{(7.3)}
\]

The mass of CaCO$_3$ precipitation per gram of sand (298 g) was calculated by considering the molar mass of CaCO$_3$ and mass of the sand in the column. By cumulating the CaCO$_3$ precipitation \textit{in-situ} through up-flushing the cementation solution, CaCO$_3$ content could be determined.

7.1.3. Results

7.1.3.1. Experimental Setup: Four-columns were run in parallel to examine the effect of biocementation conditions on bacterial activity \textit{in-}
situ. The cells in the immersed column were exposed to conditions similar to those at the beginning and end of any biocementation process. One of the columns was exposed continuously to high concentration of cementation solution (calcium/urea) and low concentration of NH$_4^+$ ions and the opposite was applied to the other examined column.

7.1.3.2. Evaluating the Repeated Use of Bacteria by Continuous Flow Cementation

This experiment was aimed at examining the possibility of continuous cementation. Continued cementation was obtained by the continuous supply of high concentrations of cementation solution (Figure 7.1). The volume however, through the column was as equivalent of 30 batches.

There was a steady state in urea conversion with the maximum rate that could be achieved. Then, the ability of bacteria to degrade urea appeared to decrease gradually during the course of biocementation (Figure 7.1). At the beginning of the biocementation process, urea conversion was 100% compared to 7% at the end of the biocementation process; possible reasons for this drop in urease activity could be diffusion limitation or bacterial decay. Details of these reasons will be discussed in next chapter.

Due to the continuous cementation, higher calcium carbonates precipitation (Figure 7.2) and mechanical strength than the batch were produced (Figure 7.3). This continuously fed column was about 6-times stronger than the batch fed column (Table 7.2). This means that the bacterial catalyst can be reused many times, allow significant cost savings. Six times more strength with the same amount of bacteria kept the bacterial cost components 6-times lower. The consolidation did not significantly lower the permeability of the cemented sand which was 2.77×10^-6.
7.1.3.3. Cementation by Low Concentration of Cementation Solution and High Concentration of NH$_4^+$ Ions

To examine whether cementation occurs under cementation condition like those which are found at the end of a batch reaction; high concentration of NH$_4^+$ and low concentration of cementation solution was supplied continuously *in-situ*. Under these conditions, biocementation process continued over 70 hours with about 100% of urea conversion (Figure 7.1). Thus, the high concentration of NH$_4^+$ ions *in-situ* (almost 2 M) did not inhibit the urease activity. However, the continuous flow of low concentration of cementation solution did not provide saturating supply of urea. Thus, the bacteria close to the injection point utilised all the urea and calcium ions causing consolidation of only the bottom of the cemented column (Table 7.2). It was not possible to test the strength of this sample by UCS. When the height to diameter ratio (H/D) is too small, the shear plane passes through the end platens resulting in a higher stress levels (i.e. the error is high due to end effects) (Binaya Bhattarai, 2006, COFS, UWA, Personal communication). The area above the 2.5 cm length was not cemented similar to the control column with one flush of the cementation solution.
Figure 7.1: Urease activity (measured by ammonium analysis) during continuous biocementation process over 70 hours. The columns were treated with (a) High concentration of cementation solution and low concentration of NH$_4^+$ (1 M of urea, 1 M of calcium chloride and 100 mM ammonium chloride; ●) and (b) Low concentration of cementation solution with high concentration of NH$_4^+$ ions (100 mM of urea, 100 mM of calcium and 1.8 M ammonium chloride; ○).

Figure 7.2: Cumulative CaCO$_3$ precipitations (mg.g$^{-1}$) as a function of time. Two different concentrations of calcium/urea were tested. (A) High concentration of cementation solution (1 M calcium and urea were supplied in equimolar ratio; ●), (B) low concentration of cementation solution (100 mM calcium chloride and urea were supplied in equimolar ratio; ○).
Table 7.2: The strength measurement of the batch and continuous fed columns. Two cementation conditions were examined: (a) High concentration of calcium/urea, low concentration of NH$_4^+$ and (b) Low concentration of calcium/urea, high concentration of NH$_4^+$.

<table>
<thead>
<tr>
<th>Description</th>
<th>Type of reaction</th>
<th>Strength (Pocket Penetrometer) (Kg.cm$^{-3}$)</th>
<th>UCS (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High concentration of cementation Solution, low concentration of NH$_4^+$.</td>
<td>Batch</td>
<td>32-48</td>
<td>Too soft to be measured</td>
</tr>
<tr>
<td></td>
<td>Continuous</td>
<td>&gt; 207-229</td>
<td>4.2</td>
</tr>
<tr>
<td>Low concentration of cementation solution, high concentration of NH$_4^+$.</td>
<td>Batch</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Continuous</td>
<td>&gt; 207-229</td>
<td>Too short to be measured (2.5 cm)</td>
</tr>
</tbody>
</table>

Figure 7.3: The actual CaCO$_3$ precipitation of the columns up-flushed with a mixture of high concentration of cementation solution (1 M) and low concentration of NH$_4^+$ ions (100 mM) under (A) batch and (B) continuous mode, and a mixture of Low concentration of cementation solution (100 mM) and high concentration of NH$_4^+$ ions (1.8 M) under (C) batch and (D) continuous mode. Note: the actual CaCO$_3$ precipitation was measured as the average of top, middle and bottom of the cemented column.
7.1.4. Discussion

To test the ability of bacterial cells to be reused at a condition similar to that found at the beginning of cementation solution, high concentration of cementation solution (calcium/urea) was continuously supplied. The cells were reused around 30-times. To our knowledge, this study was the only study that examined supplying the cells in-situ continuously with cementation solution during the biocementation process.

High concentration of NH$_4^+$ ions (2 M) does not inhibit urease activity as shown by the full urea conversion (Figure 7.1), actual CaCO$_3$ precipitation (Figure 7.3), and the mechanical strength which was produced closest to the injection point (Table 7.2).

Strength production at high concentration of NH$_4^+$ ions and low concentration of cementation solution indicates that even at the end of biocementation reaction, strength can be produced.
7.2. Effect of Different Concentrations of Cementation Solution on Urease Activity, Calcium Carbonate Formation and Strength Production

7.2.1. Introduction

It was previously thought that biocementation could be obtained only when high concentration of cementation solution (calcium/urea) was used at batch conditions (Whiffin, 2004; Edward Kucharski, 2005, Calcite Technology Pty Ltd., WA, Personal communication). The range of cementation concentration in the literature was between 0.7 and 1.1 M equimolar (Whiffin, 2004) or 1.25 and 1.75 M calcium and urea respectively (Edward Kucharski, 2005, Calcite Technology Pty Ltd., WA, Personal communication). Results of the previous chapter contradict this believe by showing continuous low flow of 0.125 M cementation solution produced strength at the injection end of the packed sand-column (2.0-2.5 cm), with a complete urea hydrolysis (i.e. above 2.0-2.5 cm there was no cementation because all the calcium and urea were utilised at the bottom of the cemented column). This study is aiming at determining the best concentration of cementation solution that gives the highest strength and as a consequence the inhibitory effect of different concentrations of cementation solution on urease activity \textit{in-situ}.

7.2.2. Materials and Methods

7.2.2.1. Bacterial Growth and Placement Conditions

- Bacterial culture: Cells (MCP11) were grown in the presence of 10 \( \mu \text{M} \) \( \text{Ni}^{2+} \) ions and 6 mM calcium chloride.
- Urease activity of 24.2 mM urea hydrolysed.min\(^{-1}\) (2.4 mS.min\(^{-1}\)) and OD of 5.9.
- Specific urease activity of 4.1 mM urea hydrolysed.min\(^{-1}\).OD\(^{-1}\) (0.37 mS.min\(^{-1}\).OD\(^{-1}\)).
- Five packed sand-columns (290 g sand), with void volume of 55 ml.
- The columns were washed with water (3-void volumes).
- The cells were up-flushed into the columns with flow rate of 620 ml.h\(^{-1}\) (1.2-void volume).
- Continuous flow of cementation solution (equivalent concentration of calcium/urea): 0.125, 0.25, 0.5, 1 and 2 (309 ml.h\(^{-1}\), the proper flow rate was chosen to be greater than the retention time which was required to complete the urea hydrolysis 2.5-times. Assuming that the cementation reaction will complete within 5 hours).
- Retention time of the cementation solution within the sand-column was 0.2 hour (12 min).
- Total up-flushed cementation solution was 1391 ml.

7.2.3. Results

7.2.3.1. Experimental Setup: To obtain the best concentration of cementation solution that produces the highest strength and the inhibitory concentration, 5 packed sand-columns were run in parallel. These columns were up-loaded with cells and 66 hours later, different concentrations of cementation solutions (ranges from 0.125 to 2 M) were up-flushed continuously.

7.2.3.2. Effect of Different Concentrations of Cementation Solution on Urease Activity and Calcium Carbonate Precipitation In-Situ

The highest \textit{in-situ} activity was at 0.5 M cementation solution. Concentrations of 1 M and 2 M showed less activity (Figure 7.4; Figure 7.5) and hence less cementation (Figure 7.6; Figure 7.7) and less strength (Figure 7.8).

The reason for the lower activity when the cementation solution was higher than 0.5 M was due to inhibition and loss of activity due to the reactants rather than the products (e.g. ammonia), as the ammonia concentration was lower in the 1 M and 2 M tests.
The differences in activity was not caused by differences in urea immobilisation as in all columns more than 94% or urease activity was retained (data were not shown).

In the tests shown here, significant unused cementation solution was wasted when more than 0.5 M strength was used. However, for longer columns used, the unused cementation solution would penetrate deeper into the column. While 0.5 M was the best cementation solution strength in this experiment, a 1 M cementation solution concentration would be likely to accomplish more penetration.

![Figure 7.4: Effect of different concentrations of calcium/urea on in-situ urease activity during continuously flushed cementation columns.](image)
Figure 7.5: Ammonium content (mmol) produced over 4.5 hours of up-flushing different concentrations of calcium/urea (cementation solution). It was calculated from the ammonium analysis (1urea: 2 NH₄⁺) considering the cementation liquid which was produced within the column.

Figure 7.6: The cumulative CaCO₃ precipitation (mg.g⁻¹) as a function of time. Five different concentrations of calcium/urea (cementation solution) were tested. The cumulative carbonate increased gradually between 0.125-0.5 M and then decreased gradually when 1 and 2 M cementation solution was up-flushed.
Figure 7.7: The theoretical (■) and the actual (□) CaCO₃ precipitation in the five columns which were up-loaded continuously with different concentrations of cementation solutions ranges from 0.125 to 2 M over 4.5 hours.

Figure 7.8: The actual CaCO₃ precipitated (O, mg.g⁻¹), and the unconfined compression strength (●, MPa) of the five columns which were treated with different concentrations of cementation solution (0.125-2.0 M). The unconfined compression strength (MPa) increased with the increase in CaCO₃ precipitation.
7.2.4. Discussion

To determine the inhibitory effect of cementation solution on urease activity *in-situ* and its impact on strength and penetration depth, different concentrations of cementation solution (0.125-2 M) were used. Above 0.5 M, there was inhibition on urease activity. This inhibition on activity was reflected by the decrease in CaCO$_3$ precipitation (Figure 7.6) and as a consequence, the decrease in the attained strength (Figure 7.8).

At the non-inhibitory concentrations of cementation solution ($\leq$ 0.5 M), it is expected to obtain similar mechanical strength. However, because of calcium/urea limitation less cementation occurred.
7.3. Effect of Different Strains on Biocementation Process

7.3.1. Introduction

In nature, ureolytic activity is a general phenomenon for almost all bacteria (Bouquet et al., 1973). The bacterial cells differ in urea tolerance ex-situ and it is known that S. pasteurii is a urea tolerant strain among the ureolytic bacteria (Whiffin, 2004).

For a strong biocemented column, it is desirable to select the best strain that tolerates the biocementation conditions \textit{in-situ}. The aim of this attempt is to examine the tolerance of different ureolytic strains to the cementation condition \textit{in-situ}.

7.3.2. Materials and Methods

7.3.2.1. Bacterial Growth and Placement Conditions

- Urease activity of cells was 24.9-35.6 mM urea hydrolysed min$^{-1}$ (2.24-3.2 mS min$^{-1}$).
- Specific urease activity was 7.8-16.7 mM urea hydrolysed min$^{-1}.OD^{-1}$ (0.7-1.5 mS min$^{-1}.OD^{-1}$).
- The optical density of MCP11 (OD) was of 2.1-3.5 (Table 7.3).
- Weight of the sand was 291 g.
- Flow rate of cells through the packed sand-column was 314 ml h$^{-1}$.
- Retention of cementation solution \textit{in-situ} was 1.4-1.5 hour.
- Dilution rate of cementation solution \textit{in-situ} was 0.7 h$^{-1}$.
- Void volume was 60 ml.
- Concentration of cementation solution was 0.5 M.
- Flow rate of cementation solution was 40-42 ml h$^{-1}$ (2-times faster than using 1 M cementation solution).
- Total up-flushed cementation solution was 1718 ml (29-void volumes).
- The retained cells according to the urease activity measurements were 38-48% (less than usual, shaker problem during the cell growth).
Table 7.3: *In-situ* cell retention of different soil-isolates and *S. pasteurii* (for comparison purpose). The table shows input OD, urease and specific urease activity of the tested strains. *In-situ* retention of cells was calculated according to the input and output urease activity measurements.

<table>
<thead>
<tr>
<th>Strain</th>
<th>In-flow</th>
<th>Out-flow</th>
<th>% <em>In-situ</em> urease activity retention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD</td>
<td>Urease activity (mS.min⁻¹)</td>
<td>Specific urease Activity (mS.min⁻¹.OD⁻¹)</td>
</tr>
<tr>
<td>S. pasteurii</td>
<td>3.4</td>
<td>2.24</td>
<td>0.7</td>
</tr>
<tr>
<td>MCP1</td>
<td>2.9</td>
<td>3.2</td>
<td>1.1</td>
</tr>
<tr>
<td>MCP4</td>
<td>2.1</td>
<td>3.2</td>
<td>1.5</td>
</tr>
<tr>
<td>MCP11</td>
<td>3.5</td>
<td>2.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

7.3.3. Results

7.3.3.1. Experimental Setup: To examine *in-situ* tolerance of four ureolytic bacterial strains (MCP1, MCP4, MCP11 and *S. pasteurii*) to cementation conditions; four packed sand-columns were up-flushed with almost similar input of urease activity (Table 7.3). Then the sand-columns were up-flushed continuously with 0.5 M cementation solution continuously subsequent to incubation period (48 hours).

7.3.3.2. *In-Situ* Urease Activity of Different Ureolytic Strains

The bacterial cells tolerated the cementation conditions differently; MCP11 had the highest tolerance to the cementation conditions *in-situ* (Figure 7.9). It showed the highest *in-situ* urea conversion as compared to the other examined strains (Figure 7.9). With continued up-flushing of the cementation solution, the effect of end products (NH₄⁺, CO₂) was eliminated. Similarly, the effect of calcium concentration on decreasing urease activity was eliminated because the cells had tolerated the same concentration of calcium from the beginning of the reaction. Thus, what decreases the urease activity of different strains *in-situ* is the urease
stability under the cementation conditions to tolerate the CaCO$_3$ precipitation.

Figure 7.9: The tolerance of different strains to cementation solution which was represented as % conversion of urea supplied during continuous flow of cementation solution. Equivalent concentration of calcium/urea solution (0.5 M) was continuously uploaded. The examined strains showed different tolerance for cementation conditions.

In line with the urease activity results, CaCO$_3$ content was the highest in the column which was treated with MCP11. It was higher than the other treated columns 2 to 5 times (Figure 7.10).

There was no direct correlation between the input urease activity (the uploaded urease activity through the packed sand-column), CaCO$_3$ production \textit{in-situ} (Figure 7.11; Figure 7.12) and as a consequence strength production (Figure 7.13: Figure 7.16). Although the input urease activity of MCP1 and MCP4 strains was higher than \textit{S. pasteurii} and MCP11 strain, the former strains produced lower CaCO$_3$ content (Figure 7.12) and strength (Figure 7.16) than the later. At the same time \textit{S. pasteurii} and MCP11 had the same input urease activity but MCP11 produced higher CaCO$_3$ content (Figure 7.12) and strength (Figure 7.16) than \textit{S. pasteurii} (Figure 7.12; Figure 7.16). This ambiguous relationship between input activity and CaCO$_3$ precipitation supports the idea that the
input activity does not necessarily determine the *in-situ* activity (the actual urease activity for biocementation process which produced CaCO$_3$ and strength).

![Figure 7.10: The cumulative CaCO$_3$ precipitation per gram sand (mg.g$^{-1}$) as a function of time. (A) The equimolar calcium/urea solution (0.5 M calcium/urea) was continuously up-flushed into the immersed column with MCP1, MCP4, MCP11 and *S. pasteurii*. CaCO$_3$ content was calculated as described previously (current chapter, section 7.1.2.3)](image-url)
Figure 7.11: The calculated (■) and actual (□) CaCO₃ precipitation per gram sand in the four treated columns which were injected with *S. pasteurii*, MCP1, MCP4 and MCP11 continuously up-flushed. Considering the calcium precipitated during the batch culture.

Figure 7.12: Relationship between input urease activity and CaCO₃ formation of the different bacterial cells. These bacterial cells were up-flushed continuously with 0.5 M cementation solution (equivalent concentration of calcium/urea). The activity retention was almost the same (Table 7.3).
Figure 7.13: Unconfined compression strength (UCS) of the cemented column using MCP1 strain. (A) Row data (to calculate B, examined by Centre of offshore foundation system, UWA, Australia); (B) actual strength data (kpa); (C and D) the cemented column after performing the UCS test.
Figure 7.14: Unconfined compression strength (UCS) of the cemented column using MCP11 strain. (A) Row data (to calculate B, examined by Centre of offshore foundation System, UWA, Australia); (B) actual strength data (kpa); (C and D) the cemented column after performing the UCS test.
Figure 7.15: Unconfined compression strength (UCS) of the cemented column using *S. pasteurii*. (A) Raw data (to calculate B, examined by Centre of offshore foundation system, UWA, Australia); (B) actual strength data (kpa); (C and D) the cemented column after performing the UCS test.
Figure 7.16: The correlation between the input urease activity and strength production using *S. pasteurii*, MCP1 and MCP11 strains.
7.3.4. Discussion

The tolerance of different bacterial isolates (MCP1, MCP4, MCP11 and S. pasteurii) to cementation in-situ was examined. Ureolytic strains tolerated the cementation conditions differently in-situ. Among the examined strains, MCP11 considered to be the best strain that exhibit the highest urea conversion (Figure 7.9); leading to high strength production (30 MPa) (Figure 7.15). The low strength formations for MCP11 which were recorded previously were due to the experimental conditions. For example, low urease activity in-situ, higher concentration of cementation solution than 0.5 M, and low number of applications of cementation solution could reduce strength.

*In-situ* urease activity is the key role in increasing the strength of the cemented columns (calculated from Figure 7.9) and not the input urease activity (Figure 7.16). The increase in urease activity led to more CaCO₃ precipitation (Figure 7.11) and as a consequence more strength was produced (Figure 7.16).
7.4. Effect of Different Concentrations of Bacterial Cells on Strength Formation

7.4.1. Introduction
So far it is proven that the unconfined compression strength increased with the increase in CaCO$_3$ content in-situ, due to a significant level of urease activity. It was found that high level of pure urease activity caused a decrease in the mechanical strength of silica sand (Edward Kucharski, 2005, Calcite Technology Pty Ltd., personal communication). In this manner, it was necessary to test the effect of the increase in In-situ biomass on the mechanical strength formation. Thus, this study is aiming at:

- Determining whether low concentrations of biomass form the same strength of high concentrations if sufficient time was given to complete the cementation reaction; and
- Determining the best concentration of urease activity for production of high strength in non-continuous cementation.

7.4.2. Material and Methods

- Bacterial culture: Cells (MCP11) were grown in the presence of 10 $\mu$M Ni$^{2+}$ (filter sterilized) and 6 mM CaCl$_2$, producing urease activity of 23.3 mM urea hydrolysed.min$^{-1}$ (2.1 mS.min$^{-1}$) and specific urease activity of 4 mM urea hydrolysed.min$^{-1}$ (0.36 mS.min$^{-1}$).
- After 30 hours growth, different concentrations of biomass (5.4, 13.5, 27, 54, 108, 216, 432 g.L$^{-1}$) were concentrated by centrifugation (5000 rpm, 15 min, and 4°C). Then, the cells were resuspended with sterile saline to avoid bacterial growth in-situ.
- Seven sand-columns (11 cm long) were packed with a sand density of 1.8 g.cm$^{-3}$.
- The cells (for each concentration) with the reactants (1 M cementation solution) were mixed and up-flushed immediately under a slow flow rate (11 L.h$^{-1}$).
- Three liquid samples were taken from the cemented columns for urea conversion (ammonium analysis), soluble calcium ions
(analysed by Marine and Freshwater Research Laboratory, Environmental Science, Murdoch University), then the mechanical strength was measured by pocket penetrometer.

7.4.3. Results

7.4.3.1. Experimental Setup: Seven 11 cm sand-columns were cemented in parallel, using different concentrations of bacterial cells (from 5.4 to 432 g.L\(^{-1}\)) and 1 M cementation solution. The suspended cells were combined with cementation solution and up-loaded immediately with the high flow rate to avoid CaCO\(_3\) precipitation before injection. The mechanical strength (by pocket penetrometer), calcium precipitation (done by MFRL, Murdoch University) and urease activity (by NH\(_4^+\) analysis) were examined after giving the cells in all columns sufficient time to complete urea conversion.

7.4.3.2. Urea Conversion, Calcite Formation and Mechanical Strength

To examine the pattern of strength formation resulting from uploading different concentrations of biomass, sufficient time were allowed for the bacterial cells to complete cementation reaction. Similar urea conversion (70-86%) was recorded in all of the columns which were treated with different concentrations of biomass (Figure 7.17). The similar conversion of urea was supported by similar precipitation of CaCO\(_3\) (91-94%) within the cemented column (Figure 7.17). The urea conversion indicates less degradation which is might be attributed to the volatilization or conversion of NH\(_4^+\) into NH\(_3\). Irrespective of similar CaCO\(_3\) content in all of the columns which were treated with different concentrations of cells, it was found that the strength significantly differs. An increase in cells concentrations enhances the unconfined strength formation up to a certain level (from 5.4 to 54 g.L\(^{-1}\)) under a maximum urea conversion condition. A plateau was reached at cells concentrations between 54 and 216 g.L\(^{-1}\) to form strength of 143–287 kg.cm\(^{-2}\) (Table 7.4). Then the strength decreased dramatically forming strength of 80–127 kg.cm\(^{-2}\) (Table 7.4).
The possible reasons for low strength production at lower concentrations of cells are:

- **Size of CaCO$_3$ crystals.** It was shown previously (chapter 3) that low biomass produces small CaCO$_3$ crystals. These small crystals might result in the formation of weak bonds between sand granules.
- **Point-to-point contact.** It was found in chapter 3 that initial precipitation of CaCO$_3$ spheres was associated with single bacterial cells during crystal growth. Laterally some of the spheres might be associated with more than one cell. These spheres will form rhombohedral crystals in spherical arrangement (chapter 3) forming point-to-point contact between sand granules (chapter 4), which was proven to form unconfined compression strength of 1200 kpa. So it is expected that as the point-to-point contacts increase, the strength increases. Accordingly, the increase in biomass will enhance the mechanical strength up to a certain limit, above which another factor might interfere (e.g. type, shape or/and size of crystals) to cause further decrease in the strength.

\[\text{Figure 7.17: The effect of different concentrations of cells (}\sim 2.9-186 \text{ mM urea hydrolysed.min}^{-1}\text{) on strength formation, urea conversion and Ca}^{2+}\text{ ions precipitation during the biocementation process. The strength (Kg.cm}^{-2}\text{, }\diamond\text{ urea conversion (mM, }\bullet\text{) and Ca}^{2+}\text{ ions precipitation (mM, }\triangle\text{) —calculated from insoluble Ca}^{2+}\text{ ions analysis—after 2 weeks from the start of biocementation reaction (addition of 1 M cementation solution).}\]
Table 7.4: Strength measurements of the cemented sandy materials which were uploaded with different concentrations of ureolytic cells (MCP11, ~2.9-186 mM urea hydrolysed.min⁻¹). The strength was measured by pocket penetrometer calibrated with different dead weights as mentioned previously in chapter 6.

<table>
<thead>
<tr>
<th>Cells concentrations (g.L⁻¹)</th>
<th>Diameter of the tip (cm)</th>
<th>Surface area (cm²)</th>
<th>Reading of device (kg.cm⁻³)</th>
<th>Calibrated with dead weight (kg)</th>
<th>Range of strength (kg.cm⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4</td>
<td>0.2</td>
<td>0.0314</td>
<td>1.5-2.5</td>
<td>2.5-4</td>
<td>80-127</td>
</tr>
<tr>
<td>13.5</td>
<td>0.2</td>
<td>0.0314</td>
<td>1.5-2.5</td>
<td>2.5-4</td>
<td>80-127</td>
</tr>
<tr>
<td>27</td>
<td>0.2</td>
<td>0.0314</td>
<td>2.5-3.0</td>
<td>4.5-5.0</td>
<td>143-159</td>
</tr>
<tr>
<td>54</td>
<td>0.2</td>
<td>0.0314</td>
<td>5-5.5</td>
<td>7.0-7.5</td>
<td>223-238</td>
</tr>
<tr>
<td>108</td>
<td>0.2</td>
<td>0.0314</td>
<td>6.5-7.0</td>
<td>8.5-9.0</td>
<td>270-287</td>
</tr>
<tr>
<td>216</td>
<td>0.2</td>
<td>0.0314</td>
<td>5.5-6.5</td>
<td>7.5-8.5</td>
<td>238-270</td>
</tr>
<tr>
<td>432</td>
<td>0.2</td>
<td>0.0314</td>
<td>1.5-2.5</td>
<td>2.5-4.0</td>
<td>80-127</td>
</tr>
</tbody>
</table>

The low strength production by high concentrations of cells was probably attributed to:

- Urease inhibition due to the accumulation of a product(s), such as CO₃²⁻, NH₄⁺, or OH⁻ (pH)—this is not likely possible because urease activity was not repressed, as the cementation reaction was almost complete in-situ according to urea conversion and CaCO₃ precipitation examinations.
- Size of CaCO₃ crystals — resulting in the formation of weak bonds between sand granules. At high urea conversion powder like crystals were formed, which differ from the naturally slow precipitated calcite crystals in the limestone (Whiffin, 2004). Based on this observation, the low strength formation at very high concentration of cells (432 g.L⁻¹) was probably due to the formation of small size crystals. Further investigation is necessary to understand the precise role of the level of urease activity (different
concentrations of ureolytic bacterial cells) on the size of CaCO₃ crystals.

- Type or shape of crystals—two types of crystals (spheres and rhombohedral) are formed due to biocementation reaction. The strength of the cemented sandy material will increase with the increase in the rhombohedral crystals ratio. The high increase in the biomass probably shifts the ratio towards more spheres formation. Further investigation is necessary to understand the precise role of the level of urease activity (different concentrations of ureolytic bacterial cells) on the type or shape of CaCO₃ crystals.
7.4.4. Conclusions

- With fewer cells, the urea will be degraded with time almost to the same extent when using more cells but will not give the same strength (Figure 7.17), probably due to the size and type (or shape) of CaCO$_3$ crystals (chapter 3) and/or the point-to-point contact (chapter 4).

- In non-continuous biocementation system, the best urease activity for high strength formation was 23.3–93.2 mM urea hydrolysed.min$^{-1}$ (2.1–8.2 mS.min$^{-1}$) (Figure 7.17).

- Very Low (2.9–12.2 mM urea hydrolysed.min$^{-1}$ (0.26–1.1 mS.min$^{-1}$)) and very high (186.4 mM urea hydrolysed.min$^{-1}$ (16.8 mS.min$^{-1}$)) urease activity are not suitable for high strength formation.
Chapter 8
Feeding Ureolytic Bacteria In-Situ during Cementation Process

8.1. Feeding Bacterial Cells during Biocementation Process under Batch Conditions

8.1.1. Introduction

In previous biocementation experiments the calcium/urea solution (cementation solution), was up-flushed into the packed sand-column after 48 hours from fixing the cells to the sand granules. This approach had resulted in a successful biocementation of a 1 meter column to a moderate strength up to 79-95 kg.cm$^{-2}$. For some applications (e.g. strengthening dikes and landforms sand), an increase in the strength of biocemented samples may be needed. According to previous results higher strength was caused by more CaCO$_3$ formation, enabled by higher levels of urease activity. This increase in activity and strength in-situ could be achieved by feeding the cells during biocementation process with cementation solution supplemented with yeast extract medium, assuming that this feed will enhance the ability of microbe to degrade urea efficiently.

The attached cells could be fed through a batch mode. In this mode, the cells are subjected to unstable conditions where substrates deplete and products accumulate and which ultimately inhibits the reaction.

The aim of this experiment is to test the effect of feeding cells via the batch mode on the urease activity and hence the biocementation process.

8.1.2. Materials and Methods

8.1.2.1. Bacterial Growth and Bacterial Placement in Packed Sand-Columns

- The urease activity of the grown cells (MCP11) was 19 mM urea hydrolysed.min$^{-1}$ (1.7 mS.min$^{-1}$) and the specific urease activity was 5.4 mM urea hydrolysed.min$^{-1}$,OD$^{-1}$ (OD = 3.5).
- Cells were mixed with sodium nitrate to a final concentration of 10 mM (as an electron acceptor which can support growth to most aerobes as an alternative to oxygen respiration), and 10% of the usual cementation solution (calcium/urea) to a final concentration of 100 mM immediately before being up-flushed (to increase the retention of cells in-situ).
- The cells were up-flushed with a flow rate of 1313 ml.h⁻¹.
- Then the cells were kept for 48 hours after which 1.1-void volume of cementation solution (equivalent concentration of 1 M calcium/urea) with a flow rate of 1313 ml.h⁻¹ and a retention time of 0.05 hour (the void volume of the packed sand-column was 60 ml). The test column cementation solution contained 4 g.L⁻¹ yeast extract, while the control solution did not.

### 8.1.3. Results

8.1.3.1. Experimental Setup: Two columns were setup in parallel, and were up-flushed by cementation solution after attaching cells to sand granules. The cementation solution for one of the columns was supplemented with yeast extract medium. Retention of cells in-situ (before the start of cementation process) in both of the fed and unfed columns according to optical density (OD), and urease activity was 75-82% respectively (Table 8.1).

<table>
<thead>
<tr>
<th>Columns</th>
<th>Out-flow OD</th>
<th>Out-flow urease activity (mS.min⁻¹)</th>
<th>% OD retained</th>
<th>% urease activity retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-fed</td>
<td>2.64</td>
<td>1.4</td>
<td>75</td>
<td>82</td>
</tr>
<tr>
<td>Fed</td>
<td>2.61</td>
<td>1.4</td>
<td>75</td>
<td>82</td>
</tr>
</tbody>
</table>

Table 8.1: Percentage of cells retained in the packed sand-column after incubation period of 2 days according to OD and urease activity measurements. Cells retention was calculated from subtracting outflow from the inflow measurements. The inflow OD (600 nm) was 3.5 and the urease activity was 1.7 mS.min⁻¹ (19 mM urea hydrolysed.min⁻¹).
8.1.3.2. Actual Urease Activity Retained within the Cemented Columns (Ammonium Analysis)

In order to determine the percentage of urea conversion at the end of biocementation process, 1 ml of the liquid remained \textit{in-situ} was collected for NH$_4^+$ analysis. After four days from the cellular reaction with the cementation solution, the actual urease activity (measured by NH$_4^+$ analysis) in the fed column supplemented with growth media was increased almost twice. A complete urea hydrolysis was achieved (100%) in the fed column instead of partial urea hydrolysis (60%) in the un-fed one (Figure 8.1).

![Figure 8.1: Urea conversion (%) calculated from ammonium analysis (□) CaCO$_3$ content (■) and Unconfined compression strength (□) of the unfed and fed cells under batch conditions after the biocementation process was completed. The urea hydrolysis increased 2-folds in the fed column as compared to the unfed one leading to an increase in the precipitated CaCO$_3$ and hence the strength formation (MPa).](image)

8.1.3.3. Potential Urease Activity within the Cemented Column

To determine the potential urease activity (attached to sand granules), sand (1 g) was removed from the middle of the column and mixed with 4 ml of 1.78 M urea and the urease activity measured by conductivity increase. Potential urease activity (under urease saturation) in the column
supplemented with yeast extract medium was increased Four-folds over the un-supplemented one (Table 8.2). This means that urease activity in-situ could be enhanced by feeding the cells in-situ with yeast extract-based media. Although the reaction went to completion in the column supplemented with yeast extract medium, the cells did not lose their urease activity as they were still active after the cementation reaction had completed. The increase in potential urease activity in-situ, suggests a possibility of increasing the strength by addition of further reactants to the cementation solution.

Table 8.2: The retention of urease activity within the cemented sand-columns of the supplemented and un-supplemented with YE-medium subsequent to the completion of cementation process (i.e. measured by the increase in conductivity measurement of 1 g sand collected from the cemented column at the end of the biocementation process).

<table>
<thead>
<tr>
<th>Columns</th>
<th>Urease activity (mS.min(^{-1}))</th>
<th>Urease activity (mM urea hydrolysed.min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-fed</td>
<td>0.032</td>
<td>0.3</td>
</tr>
<tr>
<td>Fed</td>
<td>0.12</td>
<td>1.3</td>
</tr>
</tbody>
</table>

8.1.3.4. Calcium Carbonate Content

Calcium carbonate content (acid-dissolution method) in the column supplemented with yeast extract medium increased 2-folds as compared to the un-supplemented one (from 9 to 20 mg.g\(^{-1}\), Figure 8.1). Judging from the increase in calcium carbonate precipitation, an enhancement of urease activity in-situ had occurred due to supplementing the cementation solution with yeast extract medium.

8.1.3.5. Unconfined Compression Strength (UCS)

Unconfined compression strength measurements (UCS, sample was sent for testing at Dutch Geotechnical Company (GeoDelft, Delft, Holland, www.geodelft.nl) showed that strength of the fed column (supplemented with yeast extract medium) was 2-times stronger than the unfed one. Since the strength of the column supplemented with the medium was 0.2 MPa (actual measurement) as compared to the control (unfed column)
which was 0.1 MPa (calibrated from pocket penetrometer measurements as shown in Table 8.3). By using pocket penetrometer, the strength of the column supplemented with yeast extract medium (159-175 kg.cm\(^2\)) was twice that of the unfed one (79-95 kg.cm\(^2\)). Accordingly, if the former column was 0.2 MPa, then the later column will be 0.1 MPa (Table 8.4).

**Table 8.3**: Strength measurement of the unfed and fed columns. A pocket penetrometers with a tip surface of 0.0314 cm\(^2\) (tip diameter 0.2 cm) was loaded with incrementally increasing, calibrated dead weights until breakage of the sample occurred (as mentioned previously in chapter 6, Table 6.1). Unconfined compression strength (UCS) is included for comparative purposes.

<table>
<thead>
<tr>
<th>Column</th>
<th>Reading of device (kg.cm(^2))</th>
<th>Calibrated with dead weight (kg)</th>
<th>Range of strength (kg.cm(^2))</th>
<th>Breakage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfed</td>
<td>0.0-0.25</td>
<td>0.0-0.5</td>
<td>0-15</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0.0-0.5</td>
<td>1.0-1.5</td>
<td>31-47</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0.5-1.0</td>
<td>1.5-2.0</td>
<td>47-63</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>1.0-1.5</td>
<td>2.0-2.5</td>
<td>63-79</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>1.5-2.0</td>
<td>2.5-3.0</td>
<td>79-95</td>
<td>Yes</td>
</tr>
<tr>
<td>Fed</td>
<td>0.0-0.25</td>
<td>1.5-2.0</td>
<td>47-63</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0.25-0.5</td>
<td>2.0-2.5</td>
<td>63-79</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0.5-1.0</td>
<td>2.5-3.0</td>
<td>79-95</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>1.0-1.5</td>
<td>3.0-3.5</td>
<td>95-111</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>1.5-2.0</td>
<td>3.5-4.0</td>
<td>111-127</td>
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</tr>
<tr>
<td></td>
<td>2.0-2.5</td>
<td>4.0-4.5</td>
<td>127-143</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>2.5-3.0</td>
<td>4.5-5.0</td>
<td>143-159</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>3.0-3.5</td>
<td>5.0-5.5</td>
<td>159-175</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 8.4: Conversion of the strength (kg.cm\(^2\)) of the unfed and fed columns which was measured by pocket penetrometer (from Table 8.3) into UCS (MPa). The strength was converted into MPa by knowing that 1 kg.mm\(^2\)=9.8 MPa, then 1 kg.cm\(^2\)=0.098 MPa.

<table>
<thead>
<tr>
<th>Columns</th>
<th>Range of strength (kg.cm(^2))</th>
<th>Calculated (MPa)</th>
<th>UCS (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-fed</td>
<td>79-95</td>
<td>7.7-9.3</td>
<td>0.1*</td>
</tr>
<tr>
<td>Fed</td>
<td>159-175</td>
<td>15.6-17.2</td>
<td>0.2**</td>
</tr>
</tbody>
</table>

* measured by penetrometer and calibrated with the column measured by UCS.
** measured by UCS.
8.1.4. Discussion

In the unfed column, the bacterial reaction did not complete. This has been found to be due to deterioration of the enzyme activity overtime (chapter 7) such that even after 4 days of incubation the urease reaction did not complete. The addition of 4 g.L\(^{-1}\) of Yeast extract avoided this deterioration of enzyme activity, allowing the reaction to complete and more calcite being formed and more strength being generated (Figure 8.1).

The reasons for this beneficial effect of yeast extract are not known but could be due to:

- *In-situ* cell multiplication; or
- An increase in the life time of urease.

8.1.5. Conclusions

- Presence of yeast extract medium in the cementation solution increases urease activity, which in turn increases the carbonate content and the mechanical strength of the cemented column; and
- The fed cells could be re-used for further calcium carbonate precipitation because of the retained activity which was observed in the cemented column; after the cementation process was completed (i.e. not all of the urease activity attached to sand granules was used during the biocementation process).
8.2. Continuous Feeding of Bacterial Cells during Biocementation Process Subsequent to Urease Activity Decrease \textit{In-situ}

8.2.1. Introduction

In a previous biocementation attempt (Chapter 7, section 7.1.3.2), microbial urease activity decreased with time during the continuous flow of calcium/urea (cementation solution) through the packed sand-column with the attached cells. By this continuous flow of cementation solution, the cells become adapted to the bioreactor conditions in which the concentration of the cementation solution can be maintained more stable and the end products will be removed during the process of biocementation. Thus, the cells will not be exposed to substrate depletion nor inhibition by end products resulted from biocementation reaction.

Based on the idea that the presence of yeast extract (YE) in the cementation solution can enhance urease activity by growing the cells \textit{in-situ} or increasing the longevity of urease; this experiment was carried out. It was an attempt to accelerate urease activity by feeding the cells with yeast extract medium continuously subsequent to the precipitation of CaCO$_3$. This experiment aims at:

- Testing the possibility of increasing urease activity and life span after initiation of biocementation process by feeding the cells (using growth medium); and
- Examining the effect of CaCO$_3$ precipitation on the rate of urease activity change \textit{in-situ}. 
8.2.2. Materials and Methods

8.2.2.1. Bacterial Growth and Placement in the Packed Sand-Columns

- The bacterial urease activity of MCP11 was 22.22 mM urea hydrolysed.min\(^{-1}\) (2 mS.min\(^{-1}\)).
- Specific urease activity was 5.56 mM urea hydrolysed.min\(^{-1}\).OD\(^{-1}\) (0.5 mS.min\(^{-1}\).OD\(^{-1}\), OD = 4).
- Three packed sand-columns (297 g sand, 300-400 µm) were run in parallel.
- Sand-columns were up-flushed with two-void volume water under a flow rate of 638 ml.h\(^{-1}\).
- The columns were up-flushed with 1.1-times its void volume with the bacterial culture (pump flow rate was 638 ml.h\(^{-1}\)).
- The cementation solution (1 M) was up-flushed (through two stages, Figure 8.2) with a constant speed peristaltic pump. There was a drop in the flow rate of the pump over time but it stayed in general between 17-20 ml.h\(^{-1}\) (except a drop after 86 hours from the biocementation process, which was 2 ml.h\(^{-1}\)).
Figure 8.2: Method of loading the cementation solution (calcium/urea; Ca/U) into the sand-columns. The cells were exposed to Ca/U through two stages; stage 1 at which Ca/U was up-loaded continuously for 48 hours. It was followed by stage 2, at which each column was treated differently: (A) continuous flow of Ca/U; (B) continuous flow of Ca/U and YE-medium; and (C) continuous flow of substrate only (urea). Where: P stands for peristaltic pump.
8.2.3. Results

8.2.3.1. Experimental Setup: Three packed sand-columns were run in parallel to examine the possibility of controlling urease activity *in-situ* during cementation process (Figure 8.2).

8.2.3.2. *In-situ* Cell Retention According to OD and Urease Activity Prior to Biocementation Reaction

According to optical density (OD) and urease activity measurements, about 70-75% of cells were retained in the three packed sand-columns (Figure 8.2, Table 8.5). The urease activity which was retained in the columns was 16.7 mM urea hydrolysed.min⁻¹.

Table 8.5: Percentage of cells retained in the packed sand-columns subsequent to incubation period of 2 days, according to OD and urease activity measurements. Cells retention was calculated from subtracting outflow from the inflow measurements. The inflow OD (600 nm) was 4 and the urease activity was 22.22 mM urea hydrolysed.min⁻¹ (2 mS.min⁻¹).

<table>
<thead>
<tr>
<th>Columns treatment</th>
<th>Out-flow OD</th>
<th>Out-flow urease activity (mS.min⁻¹)</th>
<th>% OD retained</th>
<th>% urease activity retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium/urea</td>
<td>1.08</td>
<td>0.48</td>
<td>73</td>
<td>75</td>
</tr>
<tr>
<td>Calcium/urea and growth media</td>
<td>1.2</td>
<td>0.59</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Urea</td>
<td>1.08</td>
<td>0.48</td>
<td>73</td>
<td>75</td>
</tr>
</tbody>
</table>

8.2.3.3. Effect of the Presence of Growth Medium on Increasing Urease Activity, Longevity and Calcium Carbonate Precipitation *In-Situ*

During biocementation process, the actual urease activity used to hydrolyse urea was 335-368 mmol urea hydrolysedreactor liquid L⁻¹.h⁻¹ which decayed very quickly over time (Figure 8.6). By considering the flow rates used and the percentage conversion of urease in the columns, the actual *in-situ* urease activity could be determined (Figure 8.6). Details of the actual urease activity calculation were shown previously (chapter 7, 7.1.2.2)
In the presence of calcium and urea continuously—in which CaCO$_3$ crystals were formed—the *in-situ* urease activity decayed faster than that in the presence of urea only. The reason for this rapid drop in activity over time can not be due to the inhibition effect of end products such as NH$_4^+$ ions (generated from urea) or depletion of substrate as the cells were continuously supplied with fresh solution (cementation solution or urea only). Accordingly, it may be due to surrounding the cells by the CaCO$_3$ precipitation.

**Figure 8.6:** The actual urease activity (mM urea hydrolysed, L$^{-1}$ reactor.h$^{-1}$) during the cementation process. (A) an equimolar calcium/urea solution (1 M) was continuously loaded (●). After 48 hours from the addition of calcium/urea (area following the arrow), (B) an equimolar of 1 M calcium/urea and yeast extract based medium (○), and (C) 1M urea (▲) were loaded continuously into the packed sand-columns.

The addition of yeast extract based growth medium did not significantly increase the rate of urease activity and longevity during biocementation in the case of the presence of the medium after certain quantity of CaCO$_3$ was precipitated (Figure 8.7). This unbeneficial effect of the presence of yeast extract medium probably was due to microbial failure to benefit directly from the composites of the growth medium (mainly macromolecules), as the medium was supplied after the cells were enclosed in the CaCO$_3$ crystals (the cells will be surrounded by spherical crystals within minutes from the initiation of biocementation reaction,
chapter 3). However the column which was fed with urea only, but no calcium showed a prolonged urease activity indicating that inhibition was caused by the continued supplied of calcium. Because the calcium itself was not inhibitory at the beginning of the experiment and urease activity in the column which was fed with urea only (no calcium present in the feed) remained active in-situ longer than the columns which were also fed with calcium, it could be concluded that the process of CaCO$_3$ precipitation appeared to inhibit urease activity (Figure 8.8). There was a continuous precipitation of CaCO$_3$ within two days after which little or no further precipitation occurs. This means that the continued precipitation of CaCO$_3$ has lowered in-situ urease activity (Figure 8.8). Accordingly, the so called encrustation effect in which CaCO$_3$ crystals is precipitated at the surface of the bacterial cells is the cause for the decrease in urease activity. As derived from chapter 3 (section 3.3.3.2, Figure 3.13; section 3.3.6, Figure 3.18) the cells will be enclosed in spherical crystals with different thickness. These spheres might cause:

- Diffusion limitations; or
- The growth of rhombohedral crystals inside the spheres might damage the cells, leading to their fatality.

![Figure 8.7: Percentage conversion of urea supplied during continuous flow at unfed (●) and fed cells (○) and in the absence of calcium ions (▲). (A) equimolar calcium/urea solution (1 M) was continuously loaded (●). Arrow indicates the addition of (B) an equimolar of 1 M calcium/urea and YE-medium (○) and (C) 1 M urea (▲) were up-loaded continuously into the packed sand-columns.](image)
**Figure 8.8:** The cumulative CaCO$_3$ precipitation per gram sand (mg·g$^{-1}$) as a function of time. (A) The 1 M cementation solution (equimolar calcium/urea) was continuously loaded (●). After 48 hours from the addition of calcium/urea (area following the arrow); (B) an equimolar of 1 M calcium/urea and supplemented with YE-medium (○), and (C) 1M urea (▲) were loaded continuously into the packed sand-columns; no further CaCO$_3$ precipitation was possible as calcium ions were not available to precipitate the carbonates which are produced due to urea degradation. Inset shows the early hours where the initial urease velocity can be calculated from the slope of the linear line of CaCO$_3$ content versus time. CaCO$_3$ content was calculated as described previously (chapter 7, section 7.1.2.3)

There was insignificant difference between the continuously fed (i.e. cementation solution supplemented with yeast extract medium or urea only) and the unfed columns regarding the calculated CaCO$_3$ precipitation (Figure 8.9). At the time that the fed and unfed columns have almost the same calculated CaCO$_3$ content, the column which was fed with urea only showed a 53% decrease in actual CaCO$_3$ content (Figure 8.9). This decrease in CaCO$_3$ content was not due to crystals dissolution because of the alkalinity of solution *in-situ* (pH ~9.0 due to the buffering effect of NH$_4^+$ ions). In the columns at which the calcium and urea or urea only were up-flushed separately, there were discharge of CaCO$_3$ crystals. These
crystals appeared in the out-let as a white precipitate. The longer the up-flow is the more the loss of CaCO$_3$ will be. Therefore, the decrease in the actual CaCO$_3$ content was probably due to the discharge of CaCO$_3$ crystals.

![Graph](image)

**Figure 8.9:** The calculated (■) and actual (□) CaCO$_3$ precipitation per gram sand (mg.g$^{-1}$) in the three treated columns which were loaded continuously with calcium/urea, calcium/urea supplemented with YE-medium or urea after the cementation reaction had proceeded. Calculated CaCO$_3$ levels were compiled from NH$_4^+$ data and flow rate.

The time course of CaCO$_3$ precipitation had three distinct regions: linear increase (early stage), plateau (late stage) and curvilinear transition between the two (intermediate stage) (Figure 8.8). This linear increase was expected as the cells were grown in excess of substrate urea, and in the absence of end products (NH$_4^+$, CO$_2$). Most of the CaCO$_3$ (80-100%) was precipitated at this portion. The slowdown (curvilinear) and the prevention (plateau) of CaCO$_3$ precipitation were unexpected which might be due to the decay of the bacterial cells as discussed previously in the current section.

The permeability of the cemented sand-columns (i.e. which were uploaded continuously with calcium/urea, calcium/urea supplemented with yeast
extract medium or urea after the cementation reaction had proceeded) was $7.29 \times 10^{-8}$ (examined at Geodelft, Netherlands). Thus the content of CaCO$_3$ did not affect the permeability of the biocemented column.

### 8.2.3.4. Effect of the Presence of Growth Media Subsequent to Calcium Carbonate Precipitation on the Mechanical Strength

To determine the effect of the presence of yeast extract medium subsequent to initial CaCO$_3$ precipitation on the mechanical strength, Unconfined Compression Strength (UCS) was performed. UCS showed that supplementation with the growth medium, did not significantly increase the mechanical strength of the packed sand-column (Figure 8.10). Although the strength value of the supplemented column with the growth medium was higher (22 MPa, Figure 8.10) than the non-supplemented one (12.7 MPa), yet this putative increase in strength can't be attributed to the presence of the growth medium. It might be due to the behavior of the individual columns, as the column which was supplemented with the growth medium exhibited higher urease activity than the non-supplemented one from the start of biocementation process (Figure 8.6; Figure 8.7).

The urease experiment in the column which was provided with urea only (in the absence of calcium ions) stopped cementation after 2 days and achieved higher strength than the column which was provided with calcium and urea (the supplemented column was exposed to calcium and urea for 140 hours). This high strength production after 2 days from cementation process shows that most of the strength built up was during this period of CaCO$_3$ precipitation (Figure 8.8; Figure 8.10). Further precipitation of CaCO$_3$ in the non-supplemented column did not add much to the mechanical strength. After 2 days of cementation process the decrease in urease activity was fast (Figure 8.8). At low urease activity — as derived from chapter 3— small CaCO$_3$ crystals were found. Therefore, the insignificant increase the mechanical strength after 2 days from cementation process was probably due to the small sizes of crystals
appeared as the urease activity decelerated (i.e. the nano-particles found in SEM images in Figure 8.13; Figure 8.16).

Figure 8.10: The actual CaCO₃ precipitation per gram sand (mg.g⁻¹) versus the unconfined compression strength of the columns supplemented with urea, calcium/urea and YE-medium and calcium/urea, which were continuously up-flushed.

8.2.3.5. SEM Examination

In order to examine the effect of yeast extract medium on the composition and shape of CaCO₃ crystals, slices of the cemented sand-columns were examined by SEM and XRD (examined by Madame Castanier and Gaëlle Levrel, 2006, University of Angers, France). SEM examination showed that no obvious differences in the shapes of CaCO₃ crystals were observed in the continuously unfed and fed columns. The sand granules were fully covered by CaCO₃. From the images (Figure 8.11; Figure 8.12), it was obvious that the percentage of CaCO₃ precipitation was very high, making the sand granules hard to be recognized under scanning electron microscope. Rhombohedral crystals (Figure 8.11 B and D), small undefined nano-particles (Figure 8.13C and D; Figure 8.14; Figure 8.16) and fibre-radial structure (Figure 8.15) were formed. These nano-particles
were not found in batch biocementation trials; instead rhombohedral crystals were formed (current study, chapter 4, Figure 4.6). It seems that the nano-particles were formed at low urease activity which was recorded at the late stage of the continuous biocementation process \textit{in-situ}. This observation is in line with the presence of small oval CaCO\textsubscript{3} crystals at the end of biocementation process (current study, chapter 3, section 3.3.3.2, Figure 3.13) but not at the beginning.

Careful examination of the CaCO\textsubscript{3} crystals did not reveal bacteria or its remnants. This absence of cells or its remnants in addition to the drop in urease activity over time suggested the loss of cells during the continuous flow of cementation solution. This loss of activity could be due to bacterial loss in the outlet or within the column. Urease activity measurement showed that there was no urease activity in the outlet, therefore the recorded loss in urease activity was within the column. There were some holes (\(<1\ \mu\text{m}\)) embedded in the CaCO\textsubscript{3} crystals. Some studies considered the presence of such holes in the calcite crystals as a biosignature, indicating that the crystals were formed due to bacterial activity (Bosak, 2004). As the CaCO\textsubscript{3} crystals had precipitated at the cells surface causing diffusion difficulties, it is expected that these cells will fail to perform their metabolism. This failure might leads to cellular decay. Accordingly, the holes in the rhombohedral crystals which were found in Figure 8.12 were probably signs of bacterial cells had decayed overtime.
Figure 8.11: SEM micrographs of the continuously fed column with cementation solution supplemented with YE-medium (22 MPa). (A) The sand granules were hardly seen since they were fully covered with CaCO$_3$. (B) Magnification of rhombohedral crystals found in (A). (C) The CaCO$_3$ crystals are small, interlocated with not well recognized shape (irregular, unsystematic). (D) Magnification of the irregular crystals found in (C). (E) X-ray diffraction patterns of the CaCO$_3$ crystals, indicating normal calcite.
Figure 8.12: SEM micrographs of the continuously flow unfed column with cementation solution (12.7 MPa). (A) An overview of the dense sand grains with a good contact between crystals. (B, C and D) Magnification of CaCO$_3$ crystals found in (A). (D) Shows a gap between the sand green and the carbonate crystals which probably occurred during the thin slice preparation (C). XRD characterisation of (E) sand granule (as a reference) and (F) white crystals, indicating normal calcite precipitation.
Figure 8.13: SEM micrographs of the continuously flow unfed column with cementation solution (12.7 MPa), another field of focus. These images show the contact area between sand grains (A-B) and the presence of pseudo-spherical nano-particles (C-D) which are composed of calcium carbonate (E) according to XRD characterisation.
Figure 8.14: SEM micrographs of the continuously flow unfed column with cementation solution (12.7 MPa), another field of focus. These images show the nano-particles on the sand grain surface. The XRD characterization of the nano-particles thinner (E) and of thicker layer of the nano-particles in a siliceous environment.
Figure 8.15: SEM micrographs of the continuously flow unfed column with cementation solution (12.7 MPa), another field of focus. (A-D) Fibre-radial structure (micro spherical carbonate particles assembled in the form of micro-columns perpendicular to the growth support area). (E) XRD characterization of the fiber-radial structure confirming the calcium carbonate composition.
Figure 8.16: SEM micrographs of the continuously flow unfed column with cementation solution (12.7 MPa), another field of focus. The images show the nano-particles at sand granule surface (B) magnification of A.
8.2.4. Discussion

The aim of this experiment was to test the possibility of increasing the urease activity and longevity \textit{in-situ} by feeding the cells subsequent to CaCO$_3$ precipitation. In addition, the effect of CaCO$_3$ precipitation on the rate of urease activity change was tested. There was a gradual loss in urease activity \textit{in-situ}, in the presence or absence of CaCO$_3$ precipitation. The loss in the activity was faster in the presence of cementation solution (calcium/urea). The formation of CaCO$_3$ appeared to be detrimental to urease activity \textit{in-situ} (Figure 8.8). During urea degradation in the presence of calcium ions, the bacterial cells grow in fragile crystals where more stable rhombohedral CaCO$_3$ crystals will be precipitated when the proper supersaturation is reached (chapter 3, section 3.3.8; section 3.3.9). This precipitation of CaCO$_3$ crystals at the surface of the cells or so called "encrustation" might cause diffusion limitation or cells fatality as discussed in section 8.2.3.3. Accordingly, encrustation was suggested to be one of the factors that may lower the urease activity \textit{in-situ}. This idea of encrustation is supported by the presence of holes (Figure 8.12D) in the CaCO$_3$ crystals as discussed earlier in section 8.2.3.5.

The presence of yeast extract medium subsequent to CaCO$_3$ precipitation did not accelerate the urease activity or its longevity; this negative effect of the growth medium will provoke the following question: Does the presence of growth medium in the cementation solution from the start of biocementation reaction will accelerate the activity and life span of urease \textit{in-situ}? This question will be addressed in the next section.

One of the interesting findings was that further addition of urea (without the presence of calcium ions) after discontinuing the cementation flow may have a positive impact on strength; although the CaCO$_3$ content was less than that in the column which was continuously up-flushed with cementation solution (Figure 8.10). Excess of urea means excess of NH$_4^+$ and CO$_3^{2-}$ ions. Ammonium (NH$_4^+$) has a buffering effect, keeping the pH high all the time (around 9.0). High concentration of CO$_3^{2-}$ ions might
increase the diffusion of ions into the spherical calcite —surrounding the cells during cementation process— causing more \( \text{CaCO}_3 \) precipitation. This high strength may suggest the use of higher concentration of urea than calcium chloride in the cementation solution for future experiments. This unequal concentration of calcium/urea was suggested by Edward Kucharski (2005, Calcite Technology Pty Ltd., WA, personal communication). Kucharski has suggested the use of 1: 1.25 calcium: urea for obtaining a better strength. Thus, the following question is raised: Could the use of higher concentration of urea than calcium ions in the cementation solution will give better strength? If so, what is the proper concentration of calcium: urea that give better strength?
8.2.4. Conclusions

From *in-situ* feeding of cells after two days from the progress of biocementation reaction, one can conclude that:

- The addition of nutrient had insignificant effect on accelerating urease activity, and life time of urease.
- The CaCO₃ precipitation limits the duration of the urease activity of the bacteria.
- Greater than 90% cementation (CaCO₃ precipitation) has occurred in first 48 hours (19 flushes), after which further cementation occurred with a lower rate.
- From the fact that nonlinear relationship was existed between strength and CaCO₃ precipitation, it was concluded that not all of the precipitated CaCO₃ were contributed to mechanical strength.
8.3. Effect of Feeding Cells Continuously on Urease Activity during the Biocementation Process

8.3.1. Introduction

So far, it is known that urease activity as well as strength of a cemented column were increased 2-folds due to feeding bacterial cells during the biocementation process (batch mode) by supplementing the cementation solution with yeast extract medium, and allowing the cells to precipitate CaCO$_3$ (current study, chapter 8, section 8.1). However, by the continuous supply of yeast extract medium; the urease activity and longevity of enzyme was not increased. This supply of yeast extract medium was subsequent to the precipitation of a sufficient amount of CaCO$_3$ (current study, chapter 8, section 8.2). Consequently, the positive effect of feeding cells \textit{in-situ} under the batch culture, one would postulate that the continuous presence of yeast extract medium from the beginning of the biocementation process would somehow increase urease activity \textit{in-situ} leading to an increase in the mechanical strength.

In biocementing very long columns (several meters), the cells at the injection end will utilize most of the cementation solution causing depletion in calcium/urea solution at the opposite end. Therefore the cells far from the injection point will not be able to precipitate CaCO$_3$ crystals. Thus, there will be unequal distribution of calcium/urea along the column. To solve this inequality of calcium/urea distribution; feeding the incubated bacterial cells continuously with cementation solution supplemented with yeast extract medium after diluting the concentration of the cells (i.e. diluting urease activity) is suggested. The low activity of cells along the column might allow enough calcium/urea to be at the top of the column causing cementation. Thus, this experiment aims at examining the effect of feeding bacterial cells from the start of the biocementation process on activity and strength. Furthermore, the possibility of producing strength from diluting the cells 10-times will be examined.
8.3.2. Material and Methods

8.3.2.1. Bacterial Growth and Bacterial Placement in the Sand-Columns

- Bacterial urease activity was 23 mM urea hydrolysed.min\(^{-1}\) (2.1 mS.min\(^{-1}\)).
- Specific urease activity was 4 mM urea hydrolysed.min\(^{-1}\).OD\(^{-1}\) (0.37 mS.min\(^{-1}\).OD\(^{-1}\)).
- The cells (MCP11) were diluted 10-times in yeast extract medium without a carbon source (yeast extract and sodium acetate were not included in the medium) to be sure that they will not grow in-situ during the incubation period.
- The cells were flushed upward at a flow rate of 395 ml.h\(^{-1}\) (2.5 void volumes).
- Nourished column: Cementation solution (1 M, equivalent concentration of calcium/urea) mixed with yeast extract medium to a final concentration of 4 g.L\(^{-1}\). This mixture was applied continuously.
- Flow rate of cementation solution (12 ml.h\(^{-1}\), actual flow of solution through the sand–column).
- Retention time of cementation solution in-situ was 5 hours.
- Total volume of the cementation solution of the tested column was 1044 ml (17 flush, for a period of 87 hours, void volume of 60 ml).

8.3.3. Results

8.3.3.1. Experimental Setup: Cementation solution supplemented with yeast extract medium was up-flushed through a packed sand-column. In this column, 71% of bacterial cells were retained (Table 8.6). In parallel, a control column was up-flushed with cementation solution without the presence of yeast extract medium.
Table 8.6: Percentage of cells retained in the packed sand-column after incubation period of 2 days according to OD and urease activity measurements. Cell retention was calculated as shown previously in chapter 4 by subtracting outflow from the inflow readings. Note that the inflow OD (600 nm) and urease activity were 0.573 and 0.205 mS.min\(^{-1}\) (2.3 mM urea hydrolysed.min\(^{-1}\)) respectively.

<table>
<thead>
<tr>
<th>Columns</th>
<th>Out-flow OD</th>
<th>Out-flow urease activity (mS.min(^{-1}))</th>
<th>% OD retained</th>
<th>% Urease activity retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-fed</td>
<td>0.22</td>
<td>0.07</td>
<td>61</td>
<td>66</td>
</tr>
<tr>
<td>Fed</td>
<td>0.24</td>
<td>0.055</td>
<td>58</td>
<td>71</td>
</tr>
</tbody>
</table>

8.3.3.2. Effect of Growth Medium on the Extent and Longevity of Urease In-Situ

To examine the effect of feeding the cells from the beginning of the cementation process on urease activity, the cells were continuously supplied with yeast extract medium. *In-situ* urease activity of the continuously supplemented column with the growth medium (4 g.L\(^{-1}\)) and longevity were increased 4 and 6 times respectively over the non-supplemented (Figure 8.17). This increase in urease activity was probably due to the multiplication of cells *in-situ*. Despite the presence of cementation solution and growth medium, a gradual decrease in urease activity was observed (Figure 8.18). This decrease in urease activity was attributed either to cell death, or cell encrustation causing diffusion limitation. The presence of yeast extract medium helped the cementation process —by increasing the activity and longevity of urease as well— to a limit until another limited factor(s) interfered with the process.

Because of only partial urease hydrolysis, there was always sufficient urea presence along the column. The maximum urea hydrolysis was 25% (an equimolar 1 M calcium/urea was used) after 16 hours from cementation process had proceeded (Figure 8.17). This low percent of urease degradation assures the availability of the cementation solution for the bacterial cells at the top of the sand-column. The maximum urea hydrolysis was attributed to an actual urease activity of 50 mM urea hydrolysed.h\(^{-1}\) (Figure 8.18).
Figure 8.17: Effect of feeding cells during the process of biocementation on urease activity. The diluted cells (0.1x) were continuously subjected to (A) 1 M equimolar calcium/urea solution (●) and (B) 1 M equimolar calcium/urea solution supplemented with yeast extract-based growth medium (○).

Figure 8.18: Effect of feeding cells on the actual urease activity in-situ during the process of biocementation (measured by the ammonium produced considering the liquid produced and its retention time in-situ, (calculated as shown in chapter 7, section 7.1.2.2)). The diluted cells (0.1x) were continuously subjected to (A) 1 M equimolar calcium/urea solution (●) and (B) 1 M equimolar calcium/urea solution and YE-medium (○). The cumulated urease activity in-situ in the fed column was 300 mM urea hydrolysed.h⁻¹.
The CaCO$_3$ precipitation in the supplemented column with yeast extract medium was 13 to 15-fold higher in the fed column compared to the control (Figure 8.19). The actual and calculated CaCO$_3$ precipitations (calculated as mentioned in chapter 7, section 7.1.2.3) were almost the same (Table 8.7).

![Graph showing CaCO$_3$ content (mg.g$^{-1}$) over time (h)](image)

**Figure 8.19:** The calculated cumulative CaCO$_3$ precipitation per gram sand (mg.g$^{-1}$) as a function of time (the CaCO$_3$ which was precipitated under the batch mode —after discontinuing cementation solution flow— was not included). The cells were continuously subjected to calcium/urea solution under two conditions (A) lack (●) and (B) presence of YE-medium (○).

**Table 8.7:** The effect of supplementing cementation solution with YE-medium, on CaCO$_3$ formation and strength. The calculated and actual CaCO$_3$ content (including the precipitated CaCO$_3$ under batch mode after the flow of cementation solution was discontinued) and the unconfined compression strength of the cemented sand-columns were continuously subjected to calcium/urea solution under two conditions in the absence (un-fed) and presence (fed) of YE-medium.

<table>
<thead>
<tr>
<th>Columns</th>
<th>Calculated CaCO$_3$ (mg.g$^{-1}$)</th>
<th>Actual CaCO$_3$ (mg.g$^{-1}$)</th>
<th>Strength (MPa)</th>
<th>Permeability (m.s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un-fed</td>
<td>5</td>
<td>3.5</td>
<td>Too soft to be measured</td>
<td>Too soft to be measured</td>
</tr>
<tr>
<td>Fed</td>
<td>66</td>
<td>61</td>
<td>2.0</td>
<td>9.43×10$^6$</td>
</tr>
</tbody>
</table>
8.3.3.3. Strength Production Due to Feeding Cells *In-situ* from the Beginning of Cementation Process

The mechanical strength of the cemented sand-column with low enzyme activities and continuous supply of yeast extract medium was higher than the non-supplemented (as was shown previously in Table 8.7). In the absence of yeast extract medium and the continuous supply of cementation solution, undetectable strength was produced.

The use of low enzyme activities over longer time intervals would allow a deeper penetration of the biocementation into *in-situ* soils or very long test columns (several meters long). In soil environments only low flow rates can be used. In combination with high enzyme activities this would result in complete urea depletion a short distance from the injection point. The possibility of using lower enzyme activities and maintaining it by feeding with yeast extract medium could therefore have practical implications.
8.3.4. Discussion

To test biocementation with low concentrations of the relatively costly bacteria, the normal culture was diluted 10-times and up-flushed through the column as previously described with a continuous supply of cementation solution. The urease activity diminished to zero within about 20 hours and no detectable strength was formed. However with the presence of yeast extract in the cementation solution the urease activity was higher and lasted over 90 hours, allowing continued calcite and strength formation (Figure 8.19 and Table 8.7). The results show again that the enzyme activity decreases during the biocementation reaction but that the decrease can be slowed down by providing a suitable growth medium. This could be explained by a general decay rate of the enzyme either due to environmental conditions or to the build-up of a diffusion barrier by calcite crystal encrusting the bacterial cells. The slower enzyme deterioration in the fed column could be the result of an offset by renewed enzyme production at the same time.

The longevity of urease due to the presence of yeast extract medium in the cementation solution was confirmed by a work done to study the potential of reusing ureolytic cells through three applications of cementation solutions under a batch mode (1 M calcium/urea) (Geodelft, 2006b). Little increase in urea hydrolysis was monitored in the second and third applications. The presence of bacterial growth medium (2.5 g.L\(^{-1}\) yeast extract) caused an increase in the longevity of urea hydrolysis, which in turn cause a complete degradation for a second flush. By contrast, a complete urea hydrolysis without the addition of new bacteria or nutrient was observed when a second cementation solution was applied in another biocementation attempt (Geodelft, 2006b).

The current biocementation study did not consider the effect of the presence of organic molecules on crystal formation. However, some studies on the mechanisms related to the biomineralisation processes have shown that the use of organic molecules can control the
polymorphism, morphology and structural properties of CaCO$_3$ (Falini et al., 1994; Han and Aizenberg, 2003). Bosak and Newman (2005) studied the importance of organic substances against microbial process that control the CaCO$_3$ precipitation in solution. They have found that the growth of calcite crystals (anhydral and round edges crystals) was enhanced by the presence of organic molecules (acetate, lactate, yeast extract, cysteine-HCl and vitamin solution), exopolymeric substances and lipopolysaccharide (extracted from $D$. desulfuricans G20). In addition, they have found that kinetic control was exhibited by microbial active uptake (or release) of primary organic and inorganic metabolites. In our experiment, the yeast extract caused an increase in $in-situ$ activity, producing sufficient CaCO$_3$ precipitation to increase the mechanical strength of the cemented column.
8.3.5. Conclusions

- Feeding bacterial cells continuously *in-situ* with cementation solution supplemented with yeast extract medium during the biocementation process will maintain urease activity for long.
- Biocementation of several meters column could be achieved by using low urease activity, because of the following:
  - When cementation of 1 m sand-column was achieved it was found that the end which was away from the injection end was cemented, this means that the cementation solution could reach more than 1 m.
  - When the bacterial culture was up-flushed through the 1 m column, only part of the cells were attached while most of the cells were washed out. This means that the cells could be attached to more than 1 m.
  - When the cementation solution was continually up-flushed, it was found that the bacteria lost activity probably because of the encrustation. This means that the cells at the injection end will not utilize the substrate after losing the activity letting the cells at the far end utilize the substrate and precipitate calcium carbonate. This could allow the cementation of more than 1 m to occur.
Chapter 9
Conclusions and Recommendations for Future Work

9.1. Introduction

This study investigated a novel technology, whereby bacterial urease without additional processing was used to bind sand into hard rock while still conserving its porosity. This technology has a variety of potential applications other than consolidation of sandy material as discussed thoroughly in chapter 1.

In a previous proof of concept study, a method for sand consolidation was used which showed significant problems for in-situ application. This method depended on combining the bacterial cells with the cementation solution prior to injection showing the following problems:

- The precipitation of CaCO₃ crystals before the injection due to the cementation reaction leading to clogging of the injection end; and
- The need for high flow velocities (~1.4 L.h⁻¹) to accomplish the movement of bacteria during their reaction with the cementation solution. High flow velocities cannot be reached in most in-situ situations as the required injection pressure is impossible to maintain.

This study developed a better injection method in which the above mentioned problems were rectified. The successful injection method depends on stabilizing the cells in-situ followed by a separate flow of cementation solution.

9.2. Enrichment and Isolation

Methods to enrich and isolate Bacillus bacteria from most soil within a short cultivation period were developed. These methods were designed to select ureolytic bacteria which were suitable for biocementation condition (high pH and tolerance to high concentration of ammonium ions) and
which were superior to the existing strain of *S. pasteurii*. The high level of urease activity observed in some of the enrichment trials combined with the fact that urease activity was the key factor for successful biocementation, demonstrated that the enrichment culture can be used for biocementation process under high concentration of calcium/urea without the need of purification. Purification of cells is time consuming, laborious and costly process. Therefore, using enrichment cultures in the biocementation process could be more practical and cost effective.

The ureolytic isolates have produced sufficient enzyme concentration in their growth medium to produce strength without requiring costly cell concentration. Some of these isolates throughout this study were examined for their suitability for biocementation. The microbial calcite precipitation isolates (MCP6 and MCP11) successfully formed rocks from sandy materials while (MCP4) cemented only the areas close to the injection point. Therefore, MCP4 is recommended to be used for surface coating compared to other isolates which can be used for strength purposes.

Urease activity of the successfully isolated strains throughout this study was consistent. Accordingly, there was no problem encountered with the consistency of urease activity production as compared to the use of *S. pasteurii* in sand consolidation (Whiffin, 2004).

### 9.3. Calcium Carbonate Crystal Formation

As a general phenomenon, ureolytic bacteria in the presence of high concentration of calcium and urea form spherical CaCO\(_3\) deposits (fragile) likely to be vaterite (Figure 3.3; Figure 3.5: 3.7), followed by rhombohedral calcite crystals (Figure 3.20, video clip saved as spheres to rhombohedral.avi). At high degree of supersaturation and alkalinity, the metastable phase (vaterite) precipitates faster than the stable phase (calcite). As soon as the supersaturation drops, the vaterite will be transformed into calcite spontaneously. The precipitation of calcite lowers
the ionic activity product (IA) below the solubility product (ksp) of vaterite; causing the dissolution of vaterite (details are given in chapter 3).

There was no sign of cells at the surface of rhombohedral calcite crystals, which indicates that these types of crystals were chemically precipitated at high level of supersaturation. So the spherical localized area may form the suitable conditions of supersaturation and alkalinity that enable CaCO$_3$ to precipitate. This alkaline localized area close to the cell surface is suggested to be due to active movement of calcium ions through Ca$^{2+}$/2H Pump (Hammes and Verstraete, 2002).

Many of CaCO$_3$ spheres were associated with single cells during precipitation and others were associated with single cells only at the beginning of cementation reaction. After the precipitation of spheres, their size increased at a rated dependent on the cell concentrations. The initial formation of spheres due to single cell, and the increase in size by the increase in cell concentrations probably support the idea that the localized spherical crystals can form due to the activity of a single cell whilst its increase in size is probably due to the activity of urease in the immediate surroundings of the crystal.

9.4. High Strength Production at Limited Costs

The present study provides a method of producing high-strength cement products. This is the first study to achieve a high-strength bio cementation of sand up to 30 MPa unconfined compression strength (UCS). It is the result of the continued cementation reaction obtained by a continuous supply of calcium and urea to the cells, which were attached to the surface of sand granules. The continued cementation produced strength improvements in sand granules with 240-270 fold increase in UCS. Continued cementation allowed the cells to be reused. Reusing the cells is a cost-effective process since the bacterial cells are considered to be the most expensive component in the biocementation process.
The highest strength throughout this study was attained in the absence of yeast extract medium in the cementation solution. It was found that the continued cementation in the presence of yeast extract medium increased the strength several folds (Table 8.7). This is believed to be due to counter-acting the deterioration of enzyme activity (chapter 8). Accordingly it is expected that the high strength value (30 MPa) could be further increased by feeding the cells *in-situ* continuously in the presence of yeast extract medium.

The continuous flow of cementation solution along the cemented packed sand-columns for several days without clogging difficulties (i.e. the flow rate was not lowered by the effect of the CaCO$_3$ precipitation), indicated that the permeability of the cemented sand granules was not greatly affected. The permeability of the strong cemented sand was within the standard values (i.e. the suitable decrease in permeability is by a factor of 5-VWS) (Geotechnieck, 2004). It ranges between $7.29 \times 10^{-8}$ and $9.43 \times 10^{-6}$ m.s$^{-1}$, and its changes was not related to the CaCO$_3$ content. The retention of permeability allows the water to move easily through the consolidated sand, preventing the deterioration of the material due to moisture logging. Moisture logging is considered to be one of the problems encountered for ordinary cementation process (Tiano *et al*., 1999).

**9.5. Depth of Cementation**

Penetration depth is the distance along a packed sand-column that can be penetrated by bacterial cells and cementation solution to cement the sand granules. Penetration depth is of significance for field soil stabilization attempts where depth of several meters may need to be reached. Previous studies of cementing packed-sand columns (porous material) have shown low penetration depths ranging from 0.5 to 170 mm (Castanier *et al*., 2000a; Rodriquez-Navarro *et al*., 2003; Whiffin, 2004), due to clogging the cemented product close to the injection end resulted from the method of injection. This method depends on combining the cells with cementation solution allowing the cementation reaction to occur in a
limited depth. In the current study, a penetration depth of 1 m was achieved and by applying the technology in pilot scale trials, columns 5 m length and blocks of 1 m$^3$ have now been successfully produced with the technology developed here (Leon A. van Paassen, 2008, Geodelft, personal communication). In the current technology penetration depth requires that the urease is immobilized and urea solution can flow past at a rate that is faster than its conversion by the enzyme, such that it can reach deeper levels before being converted by the bacteria. In this particular case lower numbers of bacteria may be of benefit. The success in increasing the penetration depth was due to the way that the cells and cementation solution were applied. The cells —as discussed in chapter 6— were up-flushed first followed by cementation solution (sequential flushing). Moreover, the penetration depth could be further increased by the continuous flow of cementation solution, which provided the calcium and urea to the end opposite to the injection point. High penetration depth is made possible by the gradual decrease in in-situ urease activity, which enables the cells close to the injection end to lose their activity gradually allowing calcium/urea to move further into the column. The loss of the urease activity in-situ was attributed to "encrustation" in which CaCO$_3$ precipitation probably caused a diffusion difficulty of cementation reactants (calcium and urea) or a loss of the bacterial cells. This encrustation will therefore prevent the cementation reaction from proceeding enabling deeper penetration.

In the field application, low flow rate and biocementation of several meters of porous materials are needed. The slow flow rate of cementation solution allows the attached cells to degrade urea completely and precipitate CaCO$_3$ along short distances of the porous medium. This unbalanced calcium/urea distribution along the column resulted in unconsolidated sand at the end of the column compared to the beginning. For a successful penetration depth in the field, inhibitory concentration of cementation solution (1-1.25 M) continuously up-flushed is recommended. The inhibitory concentration of cementation solution will lower the urease activity at the point of injection allowing the calcium and urea to move
further producing CaCO$_3$ precipitation and strength along the packed porous material.

**9.6. Calcium Carbonate (Calcite) and Strength Correlation**

The possible relationship between the strength development and the concentration of the actual CaCO$_3$ precipitation was investigated. Non-linear correlation between UCS and CaCO$_3$ precipitation was found which indicates that the amount of CaCO$_3$ precipitation not always determines the mechanical strength of the cemented column (Appendix G).

During *in-situ* biocementation process, the spheres increased in size (1-25 µm) leading to rhombohedral crystal precipitation. As the spheres increase in size, the shear wave velocity increased until it reached the maximum (800 m.s$^{-1}$) when maturation stage was reached (i.e. 100% rhombohedral crystals formation). Because it was proven that the rhombohedral crystals were formed from or within the spheres (chapter 3), the increase in spherical size leads to the increase in the size of rhombohedral crystals arrangement (cluster of rhombohedral crystals in a spherical arrangement). Therefore, the size and the type of CaCO$_3$ crystals participate in determining the strength formation.

Concentration of cells within the sandy materials can control the strength. It was found that different concentrations of cells produced different strength after sufficient time was given for all concentration to reach the maximum urea conversion rate. A range between 22 and 93 mM urea hydrolysed.min$^{-1}$ (2 and 8.4 mS.min$^{-1}$) formed strength, below and above which there was low strength formation. However, clear conclusions about reasons for low strength formation at low urease activities can not be made as typically low levels of urease activity faded away during the cementation trial leading to incomplete conversion of the cementation solution and less total calcite formation. Further work is needed to investigate whether consistent low levels of urease activity can also form high strength. This could be done by supplying a low level of yeast extract
medium into the column to enable bacteria to replenish its urease activity. This low strength formation at very high urease activity could be due to the expected smaller size of CaCO\textsubscript{3} crystals. Also here further investigation is needed. Moreover, the increase in cells concentration will enhance the potential cementation sites for CaCO\textsubscript{3} precipitation as discussed in chapter 4. Images showed that rhombohedral crystals are arranged in spherical arrangements, forming point-to-point contact between the sand granules (Figure 4.6, Table 4.3). The strength of the cemented sand was obvious when the crystals were in the rhombohedral shape. Therefore, it is suggested that point-to-point contact between sand granules that are bridged by crystals play a role in the mechanical strength formation.

9.7. Strength Development Measured by Shear-Wave Velocity

The strength development during biocementation reaction \textit{in-situ} was monitored online by shear wave velocity. Initially a dramatic increase in strength development was observed until a steady state was achieved. The strength was developed within the first 4-5 hours of the biocementation reaction. The stiffness of the consolidated column increased 11-16 times. Although the shear wave velocity was a useful method for monitoring the development of strength produced by bacterial cells, it did not estimate the actual strength of the biocementation column. By relating the strength development to the crystal shape, it was evident that the strength production was due to the rhombohedral crystals since this type of crystals were formed mostly when the strength reached the maximum value (Table 4.3).

The shear wave experiment was conducted without an attempt of immobilising the bacteria to the sand granules. It is recommended that this trial to be repeated using the immobilising technique discovered in this study. This technique depends on growing the cells in the presence of 6 \( \mu \text{M} \) Ca\textsuperscript{2+} ions, and incubating them \textit{in-situ} for 24-48 hours, thereby allowing the cells to attach to sand granules. Moreover, the cementation
solution should be applied continuously while monitoring the high-strength development online by shear wave velocity.

9.8. Key Factors that Affect Biocementation Process

The parameters that affect CaCO$_3$ precipitation in solution or on agar dishes are well studied (Dick, 2006; Sangwal, 1993). These studies have concluded that CaCO$_3$ precipitation is controlled by pH increase, urease activity (ability of urea conversion), supersaturation, production of extracellular polymeric excretions (EPS), biofilm formation, surface potential ($\zeta$-potential) and precipitation of dense crystal layers.

To our knowledge, the current study is the only study that investigated some of the parameters that affect the in-situ CaCO$_3$ precipitation leading to high strength of a consolidated packed sand-column. The key factor in producing a strong biocemented product was the in-situ urease activity. Higher urease activity in-situ produced higher strength. The in-situ activity is controlled by:

- The concentrations of cementation solution in which a concentration higher than 0.5 M is considered to be an inhibitory concentration in-situ.
- The tolerance of the ureolytic strains to cementation process in-situ. It was found that different bacterial strains tolerated the cementation conditions differently, precipitating different CaCO$_3$ content leading to different levels of strength formation. The best isolate in terms of cementation condition tolerance was MCP11 which also showed the highest urea conversion rate in-situ.
- Encrustation as mentioned previously in this chapter.

Thus, one of the advantages of the consolidation of porous material by the use of ureolytic bacteria is that the strength of the biocemented product is controllable by controlling the in-situ urease activity.
9.9. Recommendations

Biocementation technology is a promising technology due to its suitability to field application. To a certain extent it leads to the conservation of porosity, high penetration depth, and a variable degree of strength production of a packed sand-column. However it also results in an environmental problem due to its high production of ammonium. Ammonium which is an end product of urea degradation might leak into the ground water causing environmental problems. To solve this problem, biocementation using other C-sources such as organic acids instead of urea is recommended as this would avoid the production of ammonium.

Isolating ureolytic bacteria for biocementation process is a laborious and expensive process. Because enrichment culture exhibited high urease activity and the \textit{in-situ} urease activity is the key factor in cementation process, it appears worthwhile to test the use of enrichment cultures for consolidation purposes instead of isolating a pure strain.

In the case of cementation of several meters in the real field where only very low flow rates of solutions can be used, high conversion rate of urea at the injection end caused a depletion of reactants at the opposite end. Therefore, \( \text{CaCO}_3 \) precipitation will decrease gradually, until no cementation will occur further into the reaction path. To produce more homogenous cementation and avoid the low penetration depth, it is perceivable to use a urease inhibitor to reduce urea conversion rate in the injection end so that other bacteria further along the packed sand-column access to reactants precipitating \( \text{CaCO}_3 \). The natural tendency of decay of urease activity during the cementation reaction could be used for advantage in this context. High penetration depth could be achieved by the use of more than 0.5 M cementation solution, as this concentration will inhibit the \textit{in-situ} urease activity at the injection end more than further down in the column.
Ureolytic bacteria might find applications in the medical field. The decomposable, porous nature of CaCO₃ spheres could make them suitable to encapsulate micro and macro molecules. In addition, the spheres are easily decomposed to release the drugs into the patient's body. It is recommended to study further the spheres formation by ureolytic bacteria and its role in encapsulating medical molecule/drugs.
References


Bang, S.S., and Ramakrishman, V. (2002). Microbial Application in Strengthening of Sandy Sub-Bases and in Remediation of Concrete Cracks, Proposal Submitted to National Research Council, USA.


and *Proteus vulgaris* for Cultured Human Renal Proximal Tubular Epithelial Cells. *Infectious Immunology* 59:2036-2042.


## Appendices

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<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
</tr>
</thead>
<tbody>
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<td>Appendix A</td>
<td>Ammonium Determination by a Modified Nessler Analysis</td>
</tr>
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<td>Appendix G</td>
<td>Correlation between Calcium Carbonate Precipitation and Unconfined Compression Strength formation</td>
</tr>
</tbody>
</table>
Nessler method was modified by whiffin (2004). This modified method was used to determine ammonium concentration. Analytical grade NH₄Cl was used for the standards.

![Graph showing standard curve of ammonium concentration (mM) versus absorbance at 425 nm for the modified Nessler analysis by Whiffin (2004).]

**Figure A.1:** Standard curve of ammonium concentration (mM) versus absorbance at 425 nm of the modified Nessler analysis by Whiffin (2004).

A linear relationship between NH₄⁺ concentration and absorbance (425 nm) in the range of 0-0.06 mM, which is expressed by:

\[ y = 24.011x \quad (R^2 = 0.9962) \]
Appendix (B)

Sand Size Distribution
(Chapter 2, Section 2.3.3; Chapter 6, Section 6.1.2.1)

Silica white sand (90-400 µm) was used for consolidation by ureolytic bacteria. The sand was manufactured by Cook Industrial, Minerals Pty. Ltd.

Figure B.1: Grain sand distribution (90-400 µm) manufactured by Cook Industrial, Minerals Pty. Ltd.
The extracted DNA was used in PCR amplification to target the bacterial 16S rRNA gene using eubacteria-specific primers designed by Muyzer et al (1993) as described in chapter 2, section 2.2.4. Nucleotide sequences of the PCR products were determined (Figure C.1). Putative identification of each sequence was carried out using Basic Local Alignment Search Tool (BLAST) (Table C.1).

```plaintext
ggctcaggacgaacgcggcgtgcctaanacatcgcaagtcgagcgaacagaagaggagcttgctcctct
gacgttagcgccgcatgggtgagtaacacagccccagacgctgcgtagtccgtagctcatcgtagctcagaagatggggcccagccgcatagtccgtagctcatcgtagctcatcg
gctggcagctgcatagctggctgcatagctggctgcatagctggctgcatagctggctgcatagctggctgcatagctggctgcatagctggctgcatagctggctgcatagctgg
```

**Figure C.1:** Direct sequencing of the 16 rRNA gene of the soil isolate MCP11.

**Table C.1:** The most similar already published sequences to the soil isolate MCP11 according to NCBI.

<table>
<thead>
<tr>
<th>Bacterial type</th>
<th>Partial sequence length</th>
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<th>Gaps</th>
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<tr>
<td><em>Bacillus sp. CPB 2</em></td>
<td>1320</td>
<td>1286/1288 (99%)</td>
<td>0/1288 (0%)</td>
</tr>
<tr>
<td><em>Bacillus aquamarinus</em></td>
<td>1508</td>
<td>1448/1507 (96%)</td>
<td>4/1507 (0%)</td>
</tr>
<tr>
<td><em>Bacillus pasteurii</em></td>
<td>1431</td>
<td>1301/1345 (96%)</td>
<td>4/1345 (0%)</td>
</tr>
</tbody>
</table>
Appendix (D)

ESEM Micrographs of Silica Cemented Sand by Bacterial CaCO₃ Precipitation
(Chapter 4, Section 4.3.3)
Figure D.1: ESEM micrographs (A-G) of silica sand cemented by bacterial CaCO$_3$ after 2 weeks from the completion of the cementation reaction (A-F). The CaCO$_3$ was precipitated as rhombohedral crystals in spherical arrangement forming point-to-point contacts between the sand granules.
To have accurate measurements for the strength by pocket penetrometer, the penetrometer was calibrated with different dead weights. This calibration was done with the use of 2 mm tip.

\[ Y = 1.5585x + 0.0207 \quad (R^2 = 0.9968) \]

**Figure E.1:** Unconfined compression strength (kg cm\(^{-1}\)) measured by modified pocket penetrometer versus the calibrated pocket penetrometer with dead weights.
Determination of Calcium Carbonate Content by U-tube Manometer
(Chapter 6, Section 6.1.2.5)

Calcium carbonate (CaCO₃) was measured by acid dissolution test (U-tube manometer). The reaction of the acid with the precipitated CaCO₃ precipitates produces CO₂, which is measured by water volume shift in a graduated glass U-tube.

\[ y = 0.1805x \]  \hspace{1cm} (R² = 0.9978)

Figure F.1: Standard curve for gas production from acid dissolution of CaCO₃ at 25°C, 1 atm.
Appendix (G)

Correlation between Calcium Carbonate Precipitation and Unconfined Compression Strength formation
(Chapter 9, Section 9.6)

CaCO$_3$ content of biocemented columns was calculated by acid dissolution method while strength formation was examined by unconfined compression strength (UCS).

Figure G.1: The correlation between the mechanical strength and CaCO$_3$ precipitation in-situ of different cemented sand-columns. The densities of the columns were considered for comparison (CaCO$_3$ (mg.g$^{-1}$) was divided by the density (g.cm$^{-3}$)).