FACTORS INFLUENCING THE MICROBIAL CALCIUM CARBONATE PRECIPITATION

PERFORMANCE IN SANDS

By

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Abstract

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Microbial-induced calcium carbonate precipitation is a relatively new ground improvement technique employed by geotechnical engineers. This technique uses non-pathogenic bacteria, that are found naturally in the soil environment, to cement sand particles together at their contacts. This thesis describes the experimental study undertaken to research the influence of various factors on cementation of MICP-treated soil catalyzed by bacillus pasteurii and urease. Some of these factors include the number of nutrient cycles, concentration of the calcium chloride solutions, the number of injections, and sand particle size. Finally the objective of my research is to have a better understanding of the effects of various parameters on the biological cementation.

Ottawa sand has been used for all the experiments that are presented herein. *Sporosarcina pasteurii* (ATCC-6453), a urease-producing bacteria, was used for the bio-treatment of the Ottawa sands. The microorganisms were introduced to the soil by suspending them in the urea solution, and over time they were supplied with necessary nutrients via nutrient cycles through a peristaltic pump and syringe. A scanning electron microscopic examination was performed to study the microstructure of the soil samples, over a range of curing periods, for any cementitious bonds that may have formed.

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Chapter 1

Introduction

This thesis describes the results of an experimental study conducted for the purpose of examining the influence of various factors that affect the microbiologically-induced calcium precipitation. The bio treatment was done using the bacteria called *sporosarcina pasteurii* (ATCC 6453). The sand used for this experiment was Ottawa sand, as it has been used by various other researchers. The bacterial solutions were introduced into the sample using either a peristaltic pump or syringe, and over time they were supplied with nutrient cycles.

MICP is a bio-geochemical process that precipitates calcium carbonate within the soil matrix. It binds sand particles together at particle-particle contacts and thus results in increasing the strength and stiffness of the soil. Subsurface microbes can promote MICP by increasing the alkalinity accomplished by reaction networks like urea hydrolysis and sulfate reduction (Dejong et al 2010, Van passen et al 2010). In this research, urea hydrolysis was not used to induce calcium precipitation. The precipitation was done by the hydrolyzing urea, which produced ammonium and bicarbonate ions, thereby increasing the pH (Fujita et al 2008).

The bacteria can be found naturally; however, these microbial communities need to be augmented to support MICP in the field. If there are few ureolytic bacteria in the subsurface, then it has to be augmented with nutrient cycles.

The experiments that were conducted in this research were augmented with ureolytic bacteria so that natural bacteria could multiply under the same conditions. Bbacteria has been used for a very long time for microbial calcium carbonate cementation in oil recovery (Ferris and Stehmeier, 1992); ground water treatment (Mitchell and Ferris, 2005); and bioremediation (Ferris, 2003); restoring cracks in

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concrete (Gollapudi et al 1995; Ramachandran et al., 2001); increasing shear strength of the soil (Dejong et al., 2006); bio-clogging and bio-cementation (Ivanov and Chu, 2008); increasing the bearing capacity of soil (Bianco and Madonia, 2007); and Bio-bricks (Dosier, 2010, unpublished). The research described herein focuses on cementation by calcite formation, which is aided by the calcium carbonate producing bacteria, and on how the various parameters affect the cementation. The effects of microbial calcite cementation on cohesion less soils were examined by employing triaxial tests.

This thesis is arranged in the following order. Chapter 2 presents the literature review, summarizing the effects of cementation on soil behavior. Chapter 3 describes the materials and methods that were used in the laboratory testing, specifically the bacterial culturing and feeding techniques. Chapter 4 represents the experimental results; i.e., SEM images. Finally, Chapter 5 presents all the conclusions that were obtained from these tests.

Chapter 2

Literature Review

Soil improvement techniques require evolution in order to ensure effective and efficient improvement.   Simultaneously, they need to possess sustainable and environment-friendly characteristics. Commonly, cementing agents are injected into soil pores to bind the particles together. Those cementing materials include micro-fine cement, epoxy, acrylamide, polyurethane, silicates, etc. (Karol, 2003). However, the use of these artificial injection formulas often modifies the soil pH and contaminates groundwater due to their toxic and hazardous characteristics. In recent years, a relatively green and sustainable soil improvement technique, termed as *Microbially Induced Calcite Precipitation (MICP)* has been introduced. This technique utilizes a biochemical process in the soil to improve its engineering properties (i.e., strength and impermeability). The applications of this technique have shown promising achievement in diverse fields; i.e., concrete strength improvement and durability, brick durability, soil (or sand) strength, and sand impermeability.

The main objective of this paper is to provide an overview of the factors affecting the MICP in soil. A laboratory study was conducted to investigate the influence of various factors on MICP-treated soil catalyzed by bacteria and urease. These factors include bacteria concentration, reactant concentration (urea and calcium), reaction time, number of life cycles, and type of sand and apparatus. The calcite formed in the treated soil was further examined using a Scanning Electron Microscope (SEM).

2.1 Soil cementation

Cementation can be broadly classified into natural cementation that contains minerals such as iron, alumina, carbonate, organic matter, and artificial cementation in

which cementing agents are added such as lime, calcite, and Portland cement, gypsum (Carraro and Salgado).

2.1.1 Naturally cemented soils

Naturally cemented soil can be achieved in many ways. Some cases involve deposition of cementing agents over the deposits of sands, mostly at shallow depths, and in some other cases, sand grains can be transferred by streams and then deposited. Cementation can be done by weathering byproducts and can also occur through chemical deposition for in-place cementation. These naturally cemented soils can be found in various places and are generally attributed to precipitation of calcite cementation (Saxena, 1978). They can usually be found in the earth's crust. The program of research described herein also focuses on cementation caused by calcite formed with the help of calcium carbonate-producing bacteria.

2.1.2 Artificially cemented soils

Portland cement specimens have ductile yield, while calcite-cemented samples exhibit brittle yield. Ismail et al (2002) carried out triaxial tests on specimens that were treated with various cementing agents and found that even the type of cement has an effect on the shear behavior of the soil, but the density at a given cementation does not affect the volumetric response. According to Rotta, the primary yield stress is a function of void ratio and cement content under compression. The isotropic compression is inversely proportional to void ratio and is dependent upon the cement content.

Many investigations described herein depict difficulties encountered when testing soils. Many of those difficulties were due to the influence of various effects that were caused during treatment. As an example, the loss attributed to specimen stiffness was due to the result of the breakage of cement bonds at inter-particle contacts (Santamarina, 2000).

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Artificial ground improvement techniques have many potential benefits. Many researchers have employed many different techniques, and one of the researchers (Ismail, 2002) used a chemical cementation process called CIPS (calcite in-situ precipitation system) developed by CSIRO, the division of exploration and mining in Australia. It's a water-based, non-toxic low viscous cementing technique that is achieved by flushing chemicals into the pores. This results in calcite precipitation, which increases the mechanical strength of soils. This strength is based on many factors such as individual grain strength, soil density, decreasing particle size, and shape of the grains (round or angular).
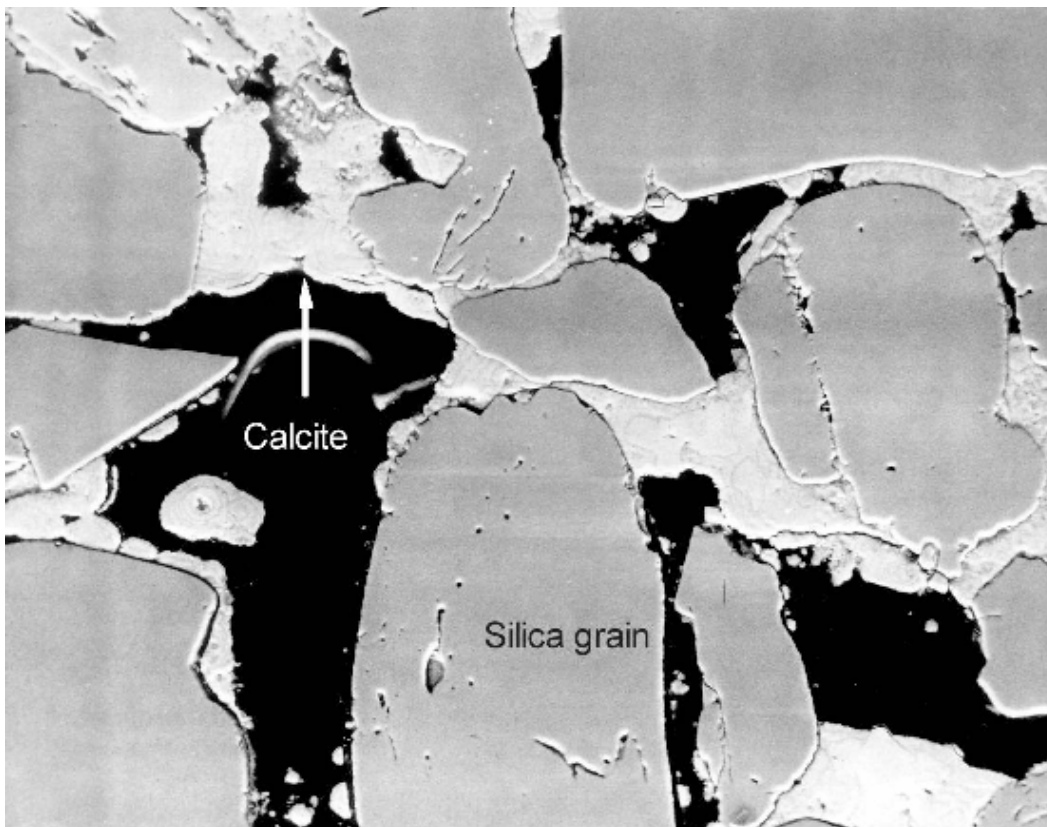
Fig 2-1: Cross-section of silica sand treated with the Calcite In-Situ Precipitation System (Whiffin, 2004)

2.1.3 Bio-induced cementation

The three main groups of organisms that can induce MICP through their metabolic processes are (i) photosynthetic organisms, (ii) Sulphate-reducing bacteria, and (iii) organism's involving the nitrogen cycle. Photosynthetic organisms in aquatic environments cause the most common form of MICP. When this reaction occurs in the presence of calcium ions, calcium carbonate is produced.

$$CO_2 + H_2O \rightarrow (CH_2O) + O_2$$
$$2HCO_3^- \leftrightarrow CO_2 + CO_3^{2-} + H_2O$$
$$CO_3^{2-} + H_2O \leftrightarrow HCO_3^- + OH^-$$
$$Ca^{2+} + HCO_3^- + OH^- \rightarrow CaCO_3 + 2H_2O$$
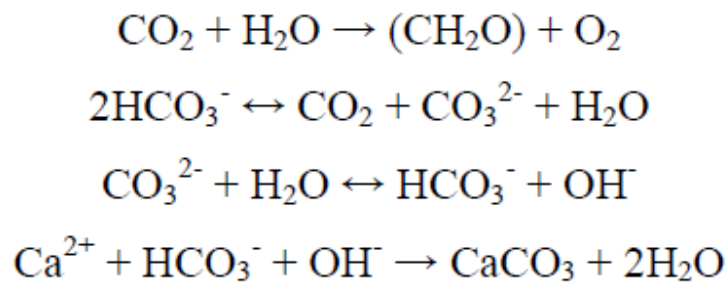
Heterotrophic organisms can also precipitate calcite by the production of carbonate ions and modification of the environment to favor precipitation. Organisms involving the nitrogen cycle, via ammonification of amino acids, nitrate reduction, and the hydrolysis of urea can also induce MICP. The simplest of all of the mechanisms described for MICP is the hydrolysis of urea by the enzyme urease, which results in the production of carbonate ions in the presence of ammonium.

2.2 Role of biological processes

Biological activity can facilitate the control and management of the timing rate and chemical network reaction, and the byproducts can improve the soil properties. The activity looks attractive based on the presence of the bacteria in the subsurface and the time period over which they have been active. The microbes used for the biomediation are very small; therefore, a large number of microbes are used for bio mediation. More than 10^9 cells per gram of soil can exist in the top layer of soil (Whitman, 1998). As the

depth decreases, the population size also gets decreased. At around 30m of depth, the concentration of about 10^6 cells per gram can be found (Whitman, 1998).

In biomediated soil improvement, the chemical reaction network is regulated mainly to control the timing of reaction, which is enabled by the introduction of chemicals into the subsurface. The population of bacterial cells in-situ is either stimulated through the nutrient injections or augmented by additional microbe injections. In both ways, the objective is to increase the microbial population by increasing the microbial concentration to the required level. The final and desired rate of calcite precipitation is finally governed by the rate of metabolic processes and by the available chemicals.

2.2.1 Soil and Bacteria

Among all the microorganisms, bacteria are the most abundant in soil and range from 10^8 to 10^{10} per gram of dry soil at the ground surface, with the population concentration generally decreasing with depth. The percentages of different types of bacteria commonly found in soil are provided in the below table. The microorganisms are also recognized for their influence on the mineral formation for a wide variety of minerals such as oxides, phosphates, sulfates, and silicates (Fortin, 1997). The chemical transformation of metals and ions in soil is mediated by soil microorganisms. Precipitation of silica dioxide, which glues soil particles together (Ivanov and Chu, 2008), and the precipitation of calcium carbonate by the microbial hydrolysis of urea (Dejong et al., 2006) are the most commonly occurring precipitation processes in nature.

Based on the studies by Mitchell and Santamarina, the soil bacteria cannot pass through pores smaller than 0.4 µm, while fungi and protozoa require pores greater than 0.6 µm. The bacteria can vary significantly in shape, being either round, spiral, or rod shaped (Fritzes, 2005).

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Table 2-1: Relative percentages of different types of bacteria found in the soil (Alexander, 1977)

Genus	Percentage (%)
Arthrobacter	5 to 60
Bacillus	7 to 67
Pseudomonas	3 to 15
Agrobacterium	>20
Alcaligenes	2 to 12
Flavobacterium	2 to 10
Others	<5
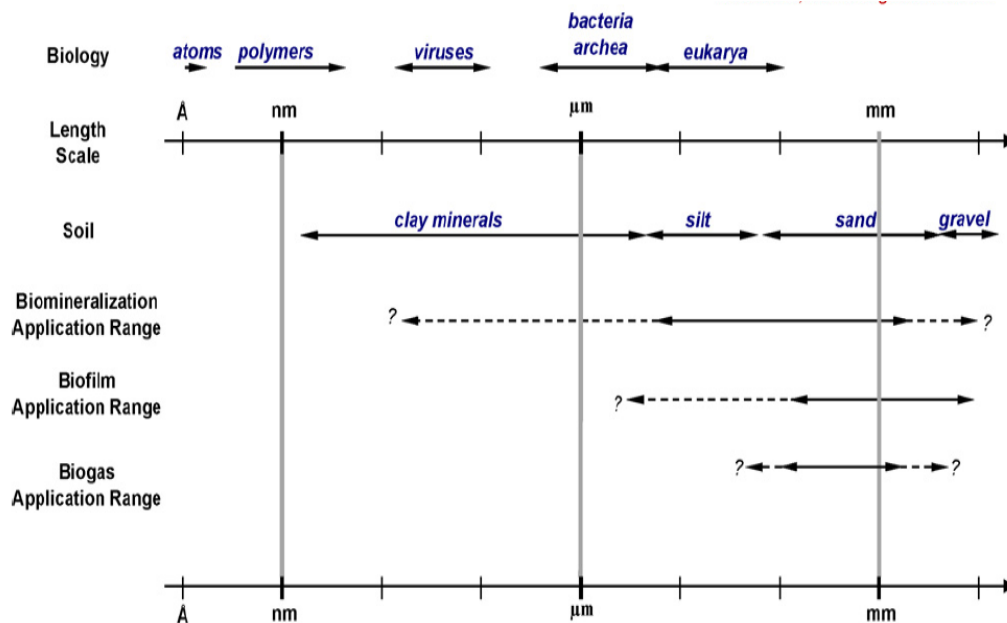
Fig 2-2: Size comparison between soil particles and microorganisms (Dejong, 2010)

2.2.2 Biomediated Calcite Cementation

Calcite precipitation can result from the interaction of *bacillus pasteurii*, urease, and ammonia through a series of complex biochemical reactions (Bang et al, 2000). Calcite is the most abundant soil carbonate source and is mostly formed in the root zones. *Bacillus pasteurii* is the most abundant alkalophilic soil microorganism and plays an important role in cementation by producing urease, which hydrolyses urea to ammonia and carbon dioxide (Sarda, 2009). Ammonia increases the pH of the surrounding soil, inducing calcium precipitation.
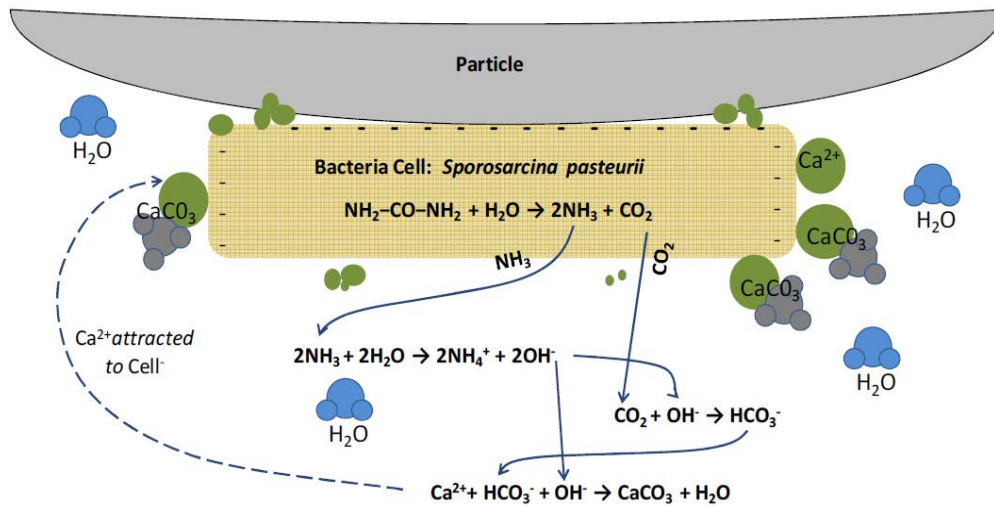
Fig 2-3: Overview of bio-mediated calcite precipitation using ureolysis (Montoya, 2008)

2.2.2.1 Bio-geo-chem calcite precipitation

Sporosarcina pasteurii is an alkalophilic soil bacterium which contains very high urease enzymes and undergoes metabolism by consuming urea within the microbe and decomposing it into ammonia and carbon dioxide (Ferris, 1996). Two chemical reactions spontaneously take place: ammonia into ammonium (NH_4^+) and carbon dioxide (CO_2), and carbonic acid into carbonate and bicarbonate ions. The generation of hydroxyl ions

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helps to increase the net pH through the production of ammonium, thus creating an alkaline environment for the precipitation of calcite.

2.2.2.2 Alternative methods of inducing calcite precipitation

Alternative biological processes that can increase the pH for calcite precipitation are desirable due to the efficiency of natural soil microbial communities. One of the primary alternatives is denitrification, followed by iron and sulfate reduction. Ureolysis will be predominant as long as urea exists and manipulates the reaction changes of the environmental conditions of a system by increasing the pH inhibiting other competitive techniques (Pikuta, 2007). The change in free energy at standard conditions for hydrolysis is low when compared to other processes. The main challenges are the production of ammonia and controlling the cycle of nitrogen. The production of urea from the area of urea depletion will react with oxygen that is present outside the zone of bio-cementation, resulting in nitrification. If there is a oxidizable carbon source, denitrification would be expected to dominate. This is an anaerobic process, thus possibly be advantageous for inducing at greater depths. The control of carbon dioxide production is a challenge since every mole of acetate produces two CO_2 moles. Denitrification will be dominant as long as the nitrate is available. Then iron plays a major role because solid iron oxyhroxides is the dominant form of iron in soils. Once iron is depleted, sulfate-reducing mechanisms may prevail, but it is the least energetically favorable of these processes. The electron uptake capacity, based on sulfate, can be greater than Iron if the soils are near seawater (Schink, 1999).

The primary challenges for these alternatives are the slower rate of creating and sustaining the supersaturated conditions; however, some of them are outweighed as the researchers are successful in producing favorable byproducts such as the gas generation

of denitrification that can reduce the saturation level, thus reducing the liquefaction potential.

2.3 Microbial-induced calcite precipitation (MICP)

2.3.1 What is microbial-induced calcite precipitation (MICP)?

MICP is a bio-geochemical process that precipitates calcium carbonate within the soil matrix and binds sand particles together at particle-particle contacts, resulting in an increase of the strength and stiffness of the soil. The bio-treatment is done using the bacteria called *sporosarcina pasteurii* (ATCC-6453), which can be found naturally. These micro-organisms are cultivated under aerobic conditions. The suspension containing the bacteria is injected into the soil and supplied with a solution of urea and calcium chloride.

2.3.2 Mechanism of bio-cementation

In marine environments, calcite forms when water saturates calcium carbonate. Calcite can also precipitate due to a chemical reaction of soil grains near a water-based boundary at the surface. There are many factors, such as the physical and chemical composition, texture, environment, and stabilization that can affect the mechanism of the cementation process. Calcite is the most common carbonate form and is typically found in root zones or places where CO_2 concentrations are high. A series of bio-chemical reactions, such as *Bacillus pasteurii,* the most abundant alkalophilic soil microorganism, plays an important role by producing urease that hydrolyzes urea in cementation.

According to Sarda (2009), this bacterium has high urease production and ammonia content that increases the pH of the surrounding soil through calcium carbonate precipitation (Bang, 2001; Sarda, 2009). Hence, it has been used for many calcium carbonate cementation studies.

In the following paragraphs, the experimental study will be described more clearly. It should be noted that *Bacillus pasteurii* has now been renamed as *sporosarcina*

pasteurii (Mitchell and Ferris, 2006). The solution that is provided to the bacteria for nutrition, known as nutrient solution, contains $NaHCO_3$, NH_4Cl, $CaCl_2$, urea, and nutrient broth (Difco and Bacto). *Sporosarcina pasteurii* uses urea as its energy source by producing ammonia (NH_3) and carbon dioxide (CO_2), thus increasing the pH of the surrounding surface under favorable conditions. This can be explained by the following equation:

$$NH_2–CO\text{-}NH_2 + H_2O \rightarrow 2NH_3 + CO_2$$

$$\text{(Urea)} \qquad\qquad \text{(Ammonia)}$$

Urea hydrolysis causes two reactions to naturally occur in the presence of water, converting ammonia and carbon dioxide into ammonium (NH_4^+), and carbonic acid (HCO_3). Ammonium production increases the pH, causing an increase in hydroxide ions (OH^-). As these bacteria prefer a higher pH environment (Ozdogan, 2010), the increase in pH is an ideal environment for the bacteria to react and feed on the urea.

$$2NH_3 + H_2O \rightarrow 2NH_4^+ + 2OH^-$$

$$CO^2 + OH^- \rightarrow HCO_3^-$$

The increase in pH causes the calcium that comes from the dissolved $CaCl_2$ ions to react with carbonic acid and hydroxide ions to form calcium carbonate ($CaCO_3$). During this process, the bacteria attracts grain to the surrounding soil, as it carries a negative charge outside its cell wall. Grains carry high nutrient concentration (Dejong et al., 2010). Simultaneously, calcite bonds take place at particle-particle contacts, which has an overall cementation between sand particles.

$$Ca^{2+} + HCO^{3-} + OH^- \rightarrow CaCO_3 + H_2O$$

2.3.3 Bacillus pasteurii

Urease-producing bacteria can be divided into two distinct groups, based on their urease response to the ammonium: those whose urease activity is not repressed, and those whose urease activity is repressed. During bio cementation, high concentrations of urea are hydrolyzed. Only those microorganisms whose urease activity is not repressed by ammonium are useful. The organism must also meet the requirements for safe environmental applications, as well as meeting the needs for biocementation. In order to safely release an organism into the environment, it must be non-pathogenic, and should not contain any transferable elements that may increase the pathogenicity of environmental strains. From the perspective of both biocementation and environmental constraints, *Sporosarcina pasteurii* has the potential of being a good urea source for biocementation,

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

Fig 2-4: Microorganisms with urease activity (Whiffin, 2004)

2.3.4 Urea Hydrolysis

Urea hydrolysis is the most easily controlled of the carbonate-generating reactions. It has the potential to produce high concentrations of carbonate within a short time. Hydrolysis of urea is an irreversible reaction in which urea reacts with water to form ammonium and carbonate.

$$CO(NH_2)_2 + 2\,H_2O \xrightarrow{\;urease\;} 2\,NH_4^+ + CO_3^{2-}$$
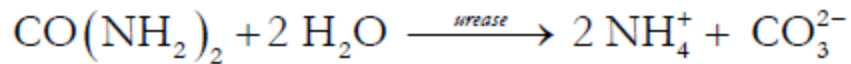
At neutral pH, bicarbonate (HCO_3^{3-}), rather than carbonate (CO_3^{2-}), is the dominant carbonate species. It causes a rise in pH to maintain charge balance. As a result of the increasing pH, ammonium (NH4+) starts to dissociate to ammonia (NH3) until equilibrium is reached. Chemical hydrolysis of urea is a very slow process. The enzyme urease catalyzes the reaction significantly up to 10^{14} times faster. Urease is a commonly found enzyme in many organisms, including many bacteria, some yeast, and several higher plants (Whiffin 2004).

The rate at which urea in aqueous solution is hydrolyzed per gram of dried biomass differs from species to species. The urease is available in different forms, varying from grinded bean meal to a purified powder. Jack Bean is the most common source of commercially available urease (Whiffin 2004). The most commonly used bacterial source of urease is *Sporosarcina pasteurii.* This micro-organism, formerly known as *Bacillus pasteurii*, is also well known for its high ureolytic activity and is used in this study. The hydrolysis of urea generates carbonate ions at a 1:1 molar ratio. If a higher urea concentration is present, the $CaCO_3$ can be significantly increased, which is one of the key parameters for calcite precipitation. The hydrolysis of urea is ideal for high rates of MICP because it not only provides a higher pH, but it also generates carbonate production.

2.3.5 Growth of calcite crystals

Once stable nuclei are formed, they start growing. Different CaCO3 mineral types can be formed, each having a different crystal lattice and solubility product and resulting from a different growth mechanism (mononuclear, polynuclear, spiral growth) as a function of supersaturation, temperature, and the solution chemistry. At high

supersaturation, precipitation of metastable phases (amorphous $CaCO_3$, $CaCO_3.H_2O$ and vaterite) is kinetically favored over the more stable mineral calcite, which is also directly formed at lower supersaturation levels (Kralj,1990). At low temperatures, ikaite ($CaCO_3.6H_2O$) can be a stable mineral phase (Gal,1996).

The crystal growth rate for each of these mineral phases or growth mechanisms is directly related to the supersaturation in the solution. For large crystals, the supersaturation at the surface of the crystal can be lower than in the liquid. When crystals become large, the crystal growth can become limited by diffusion. When the crystal remains in contact with the solution, they dissolve when the ionic activity product in the solution drops below the solubility product. In this way, a crystal undergoes several transitions. Metastable mineral phases, like amorphous calcium carbonate and vaterite, are formed at high supersaturation and eventually dissolve and reprecipitate as the more stable calcite.

<u>2.4 Factors affecting the performance of MICP</u>

2.4.1 Nutrients

Nutrients are the energy source for bacteria; hence, the supplement of common nutrients to the soil specimen during the soil treatment process is essential. Previous reports suggested adding 3 grams for one liter of nutrient broth (nutrient Bacto) to the treatment solution to sustain the growth and viability of urease-producing bacteria. These nutrient supplements can ensure that the bacteria sustains long enough and supports the calcite precipitation so that it achieves the desired level of improvement.

2.4.2 Bacterial compatibility

The geometric compatibility of urease-producing bacteria is critical whenever the transportation of bacteria within the soil is required for soil treatment. Depending on the microbe size and soil composition, pore size can affect the passage. Bacteria are the

most abundant microorganisms in soil. Their sizes generally range from 0.5 to 3.0 μm. Soil microbes travel across the pore throats between soil particles, either by propelled movement or by diffusion. A significant amount of silt and clay in soil would have an inhibitory effect on the bacteria's movement. This inhibitory effect obstructs the bacteria distribution in soil. It is thus essential to take into consideration the type of soil, its pore throat size, and the size of the bacteria when selecting the appropriate type of bacteria for MICP treatment.

2.4.3 Reactant concentration

A solution that contains equimolar of both reactants would provide better conversion to calcite. In terms of weight, the stoichiometric ratio of 2.5 for urea and calcium chloride is critical in order to achieve complete production of calcite. The concentration of reagents and salinity influence the MICP process. A higher concentration of urea and calcium chloride extends the amount of composited calcite. A lower concentration of urea and calcium chloride contributes to a satisfied level of urea decomposition into ammonia. Urease is still available for the MICP process at high salinity, but the ratio of calcite precipitated and the calcite composition decreased with an increase in the reactants' concentrations. A higher composition of calcite can be achieved by adding a high concentration of reagents, provided that the urease enzyme is introduced into the soil, but not produced in situ by the ureolytic bacteria. Repeated injections of reagents into the soil would increase the composition of calcite.

2.4.4 Bacterial fixation and distribution in Soil

The urease-positive bacteria should be distributed evenly and fixed in place when they are injected into soil. Improper injection methods might cause the bacteria to be located only in a certain part of soil or to be flushed out from the soil. According to the study by Harkes, the injection of undiluted bacteria suspension, followed by one pore

volume of fixation fluid (high saline), could successfully retain almost all bacteria suspension in the sand bed. Low ionic and adsorption strength of bacteria in low salinity solutions allow them to travel great distances.

2.4.5 pH

The calcite precipitation commences when urea is decomposed by the urease enzyme. The urease enzyme is produced by microbial metabolic activities and, as a result, urea hydrolysis is preferable around the cell. Like all other enzymes, the urease enzyme is only active at a certain range of pH. Stocks-Fischer et al. stated that the optimum pH for the urease enzyme is in the range of 7.5 to 8.0, and this finding is further supported by the works of Evans and Arunachalam. Stocks-Fischer found that the urease activity increased rapidly from pH 6.0 to 8.0. Many of the researchers found that the urease activity is high at pH 8 and decreases gradually.

2.4.6 Reaction time

MICP through bacteria is a complex biochemistry and transient process. Reaction time is one of the key factors for the MICP. MICP-treated sand samples were prepared to study the influence of reaction time on MICP and soil properties. The samples had reaction times varying from 3 days to 7 days, but were not strong enough to withstand remove from the molds immediately after the end of the nutrient cycles. Based on the results of various studies, both the MICP-treated efficiency and specific urease activity increased when the bacterial optical density was increased. When the MICP reaction time was extended, there was an increase in the precipitation rate, and the rate during the initial days is higher than the latter days. This may be explained by the fact that the bacteria had already been wrapped by calcite crystals and the enzyme activity was reduced. Moreover, the precipitated $CaCO_3$ reduced the pore volumes of the

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samples and even blocked the pores, which reduced the effective contact between the cementation media and urease, thereby bringing down the MICP bonding efficiency.

2.4.7 Bacteria cell concentration

A higher bacterial cell concentration supplied to the soil sample increases the amount of calcite precipitated from the MICP process. The rate of urea hydrolysis has a direct relationship to the bacterial cell concentration, provided sufficient reactant concentrations are available. High bacterial concentrations produce more urease per unit volume to induce urea hydrolysis. Stocks-Fischer suggested that the bacteria cells served as nucleation sites for calcite to precipitate in biochemical reaction. From SEM images, they discovered that the nucleation of calcite takes place at the bacteria cell walls, which is one of the key factors for calcite precipitation. Calcite precipitation is associated directly with the concentration of *Sporosarcina Pasteurii*.

2.5 Method used in introducing bacteria to the soil

Different researchers have used various methods to introduce bacteria into soil specimens. One commonly-used method is to use a peristaltic pump for pumping the bacterial solution into the soil. After careful examination of the physical and chemical properties of the microbial calcite precipitation process by different microbiologists, Dejong (2006) described a method to achieve natural calcite cementation within loose sand, using the bacteria (*sporosarcina pasteurii*) and the liquid growth medium containing urea and calcium chloride. Test specimens were prepared for un-cemented and cemented treatment. The details regarding the flow rate, the time interval between the nutrient cycles, and the setting time will be explained in further slides which are given below.

After introducing the bacterial solution, the soil specimen remained undisturbed for a minimum of 4 hours, after which the nutrient cycles were applied to feed the

bacteria. The entire process was divided into three steps; namely, the setting period, cementation period, and curing period. The setting period is the period during which the sample is allowed to react with the microbes, the cementation period is the period during which the nutrient injections are applied for the cementation of the sample, and the curing period is the period during which the sample is allowed to cure after the experiment.

Dejong et al., (2006) and Whiffin et al., (2007) performed microbial calcite cementation in a similar fashion by using a peristaltic pump for introducing bacteria via fluid circulation. Different apparatus, such as a PVC tube, split mold, and acrylic tube were used to test the calcite cementation, using microbial solutions.

According to Gollapudi (1994) microbial calcite cementation can be used to reduce the porosity for highly permeable soils. A fluid known as urea-H_2CO_3-$CaCl_2$ contained suspended bacterial cells and was used to introduce the bacteria to the sand. This creates slurry, which can later be used to make columns. An additional fluid nutrient broth was applied by gravity infiltration during the curing time. A control sample was made each time in order to compare the results.

Sarda (2009) showed successful bio-calcification in brick, and thus demonstrated the favorable effects of improving the durability of bricks by reducing the water absorption. The bacterial solution was made using sporosarcina pasteurii, and the oven-dried bricks were immersed in the solution in order to inoculate with bacterial cells. After an incubation period of 24 hours, the nutrient solution, made up of an urea and calcium solution, was added, and the bricks were cured for more than 4 weeks. After curing, the bricks were dried at room temperature, weighed, and tested for their water absorption capacity. They were then compared with the control samples to check the absorption rate. The absorption rate for treated samples was less than that for the controlled

samples, and this was believed to be because of the microbiologically-aided calcite cementation process.

Fisher (1999) also achieved calcite cementation using the same bacteria and the liquid medium. He prepared the test samples by suspending the bacterial cells in a urea-$CaCl_2$ medium and mixing it with sterile sand. A control specimen was also prepared separately. These columns were fed continuously, by gravity, with a urea-$CaCl_2$ medium that contained 25.2 mM of $CaCl_2$. After 10 day days of curing, the samples were dried and subjected to further analysis with the help of X-ray diffraction and microscope examination. The X-ray diffraction analysis indicated that approximately 30% of the total weight of the sand column was treated by bacteria, while no calcite was detected in the sample without the bacteria cells. Fisher et al. (199) also observed that the calcite cementation growth was significantly faster than that of chemical cementation.

Table 2-2: Summary of literature review on different approaches of introducing bacterial solution (Ozdogan, 2010)

Authors	Soil Type & Microorganism	Medium	Setup	Nutrient Cycles
Gollapudi et al. (1994)	Sand *Bacillus pasteurii*	Urea-H_2CO_3-$CaCl_2$	Soil mixed with medium Packed into a column	By gravity (4 days)
Fischer et al. (1999)	Sand *Bacillus pasteurii*	Urea- $CaCl_2$	Soil mixed with medium Packed into syringe column	By gravity (10 days)
Dejong et al. (2006)	Ottowa sand *Bacillus pasteurii*	Urea- $CaCl_2$	Sand column	Peristaltic pump (4 ml/min) (3-4 days)
Whiffin et al. (2007)	Itterbeck sand, *Bacillus pasteurii*	Urea- $CaCl_2$	Sand column (5 m PVC tube)	Pump (1L/hour)
Jonkers et al. (2009)	Cement stone, *Bacillus pseudofirmus*	N/A	Cement stone mixed with bacteria cell	No nutrient cycle 9,22,42, and 153 days (curing period)
Sarda et al. (2009)	Bricks, *Bacillus pasteurii*	Urea- $CaCl_2$	Dried bricks immersed in bacterial medium	4 weeks

<u>2.6 Overview of biomediated soil improvement</u>

A biomediated improvement system broadly refers to a chemical reaction network, managed and controlled within the soil through biological activity, whose byproducts alter the engineering properties of the soil.

2.6.1 Chemical reaction networks

A number of chemical reactions have been identified to date, even in the absence of microbial biological activity; however, in these cases, the soil properties were not improved. There is a potential of altering the engineering properties of soils by utilizing the byproducts produced from these network of chemical reactions. These byproducts include gas generation, inorganic precipitation, and organic precipitation.

2.6.2 Upscaling

The two main reasons for upscaling a ground improvement technique are treating a large area of soil uniformly and enhancing the permeability of treated soil. However, the deposition of soils and their chemical-biological relationships are very complex, and the desired results are difficult to achieve. Science and engineering concepts have to be optimized before gaining a maximum uniformity and understanding these mechanisms is possible.These include the process of execution during treatment and health monitoring of treated samples. The other challenges include the transportation of nutrient solutions, recirculation of bacterial media, reducing byproducts (undesired) and limiting the treatment duration. This upscaling procedure requires a thorough understanding of fundamental science and engineering from particle contact to field application.

2.6.3 Process monitoring

As the main objective is about improving the soil properties, there is a need to determine the methods by which the byproducts of a given bio-mediated process can alter the engineering properties of soils. Three primary methods are employed to measure the geophysical properties; i.e., using shear waves, compressional waves, and resistivity (the inverse of conductivity). As the methods involve low strain magnitudes, the soil will be undisturbed for measurements during the treatment process.

Both biological and chemical components are vitally important. The biological component is all about microbial concentration, activity state and potential and nutrient concentration. The chemical component is all about pH, concentrations of chemicals, and conductivity. The methods that are used to assess these components do not require discrete samples but are generally well established in respective fields (Madigan, 2003).

The real time information is not attainable, as the labor is intensive, and destructive invasive sampling may be required. There is a need for developing a way to understand the chemical, biological, and geotechnical parameters of soils for a better process monitoring of a given bio-mediated process. Geophysical methods are the best as of now since they provide an indication of how the soil properties are being altered.

2.6.4 Microbe soil size compatibility

One of the factors that determines uniformity of the treatment depends on the size compatibility between the microbes and the soil in which they are injected. Since there are many types of soil sizes, ranging from coarse to fine grained soils, microbes are capable of traveling through different soil types. The relatively small sizes, typically between 0.5 to 3 μm, are advantageous (Madigan and Martinko, 2003). The size of the pore throats within the soil matrix plays a crucial role, as they cause restriction to microbes moving from one pore space to another (Mitchell and Santamarina, 2005).

The effective treatment depends on the fraction of the microbes acting at the particle-particle contacts; however, the mixing of the microbes with the soil can vary with the wide variety of soil samples amenable to treatment into pure clays. In addition to the size compatibility, the triggering chemical reactions and their corresponding byproducts should also be considered in order to study how an aggregation of byproducts may facilitate microbes and be able to migrate through treated soil. The estimation of aggregate size, the reduction in the pore throat size due to the accumulation of precipitation around it, and degradation of microbial communities are all vital to the process. Therefore it seems that bio-mineralization can be applied to a broad range of soils, but further studies are required. Biofilms have successfully formed in sand and coarse gravels in engineered systems (Perkins, 2000). Based on this information and studies, limited applications have been observed and a broader range is possible.

2.7 Previous research

For almost a decade, the microbial carbonate precipitation has been used in industries as a mineral-plugging material, immobilizing contaminants in surface ground water treatment. Some of the popular applications are remediating cracks in concrete and granite (Gollapudi, 1995; Ramachandran, 2001), bio-clogging and bio-cementation (Ivanov and Chu, 2008), increasing the shear strength of soils (Dejong, 2006; Canakci and Cabalar, 2003), and remediation of filling cracks in concrete (Jonkers, 2008; Bang, 2001; Ramakrishnan, 2008).

2.7.1 Stiffness

The increase in stiffness by bio-mediated calcium precipitation can be observed with the help of bender elements. Experiments have shown an increase in the shear wave velocity up to 1200 m/s, which corresponds to "Rock" according to the National Earthquake Hazards Reduction Program (NEHRP, 2003). Dejong (2006) demonstrated that microbial-cemented specimens exhibited increased strength when compared to uncemented specimens, and that the shear behavior of the bacterially-cemented specimens was similar to that of gypsum-cemented soil. Some shear strength improvements were achieved using bacteria indirectly, such as through the use of biopolymers, which are polymers produced by living organisms. The sand was subjected to different levels of biopolymer treatment produced by the bacteria named *Xanthomonas compestris*. Samples treated with different biopolymer contents were cured 7, 28, and 50 days. They found that the shear strength of the sand increased as the biopolymer supply was increased. The soil particles were held together by a bio-polymeric material known as xanthan gum because of its adhesion (Canakci and Cabalar, 2003). These results also support the possibility of using bio-polymer-forming bacteria for ground improvement applications in geotechnical engineering.

2.7.2 Porosity

There will be reduction of pore throats as the calcium precipitation takes place in the pores within the soil particles, thus preventing the water flow (Whiffin, 2007). Results from others' triaxial tests show that the treated sand strength, stiffness, and porosity were all significantly affected by the calcium carbonate precipitation, and the porosities of the bio-treated samples were 90% smaller than the untreated specimens.
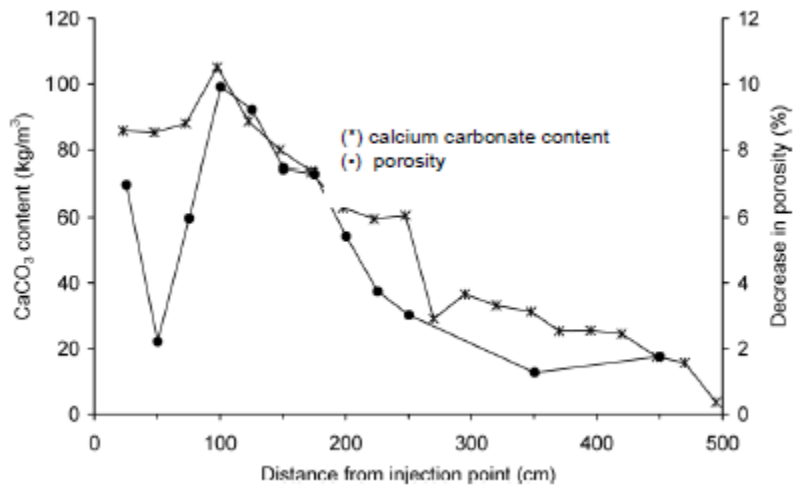
Fig 2-3: Calcium carbonate content and porosity vs. the distance from the injection point along the column length (Whiffin, 2007)

2.7.3 Microbial calcium carbonate as a protection agent

Recent investigations have also shown that the microbial calcite precipitation can be used as a self-repairing agent for use in sustainable concrete (Jonkers, 2009). Bacteria-treated concrete samples were examined to check the viability of bacteria and cement, the pore size distribution within the samples, and the effect of an added agent on the stiffness and self-healing properties. Ordinary Portland cement has been used to mix with the bacteria suspension and then cured at different times. Compressive strength results showed that, as a result of the bacterial process, the pore diameter size

decreased with the specimen age. With the aid of the microbial calcite precipitation, the corrosion effect can also be reduced, which helps to protect cement-based buildings from being corroded (chunxiang, 2009).

Chapter 3

Experimental Methods and Materials

This chapter explains the details of the testing program and discusses how to achieve cementation of sand, using the bio-mediated cementation process. In the following sections, a detailed description of the soil used in this study is presented.

3.1 Bacteria and growth conditions

A urea-hydrolyzing bacteria named *sporosarcina pasteuriiI*, formerly known as *Bacillus pasteurii* (ATCC -6453), was used for this experiment which was grown at 30°C in an ammonium yeast extract medium. The details of the components and the mixing proportions are detailed in the following sections.

This solution is referred to as the test media or growth media throughout the thesis report. The bacteria was inoculated in the test media from the stock culture and incubated at 30°C inside the incubator for 48-72 hours.

3.1.1 Bacteria (Microorganism)

The bacteria, *sporosarcina pasteurii* (ATCC-6453), used in this study were an isolated culture, and the source was the American-type culture collection (ATCC). It was cultured in an ammonium yeast medium under aerobic conditions in a laboratory in the Life Science Building (Room number LS 216) that belongs to University of Texas, Arlington.

Constituents	Amount
Ammonium Sulfate $(NH_4)_2SO_4$	10 g
Yeast Extract	20 g
Agar	20 g
Tris Buffer Solution (pH 9.0)*	1 L

* Comprised of distilled water, tris, and hydrochloric acid.

Fig 3-1: Components of the NH4-YE Agar Plate Medium (Ozdogan, 2010)

3.1.2 Making test media (Tris buffer solution)

The purpose of making the tris buffer was to adjust the pH of the distilled water used in the agar plate medium. As mentioned in the above table, a 0.13 molar tris buffer was made according to the following steps.

Step 1: 15.75 grams of tris were put into 500ml of distilled water, and using 1M hydrochloric acid (HCL), it was titrated until it reached a pH of 9.

Step 2: The solution was separated into equal parts into three beakers, and $(NH_4)_2SO_4$ (10 g), yeast extract (20 g), and Agar (10 g) were added to each of the beakers.

Step 3: The three resulting solutions were sterilized in an autoclave at 120°C for 15 minutes.

Fig 3-2: Preparation of Tris buffer solution a) Components autoclaved separately b) Solution of mixed components

3.1.3 Making Bacterial solution

The preparation of the bacterial solution is explained in the following steps:

Step 1: Immediately after the autoclaving, the contents of the three beakers were mixed together, making one solution, resulting in an ammonium-yeast agar medium.

Step 2: The solution was poured into culture plates.

Step 3: The solution was poured under laminar flow, using the hood, to eliminate the risk of contamination prior to the introduction of the bacterial cultures.

Step 4: The plates were solidified approximately 15 minutes after pouring the solution.

Step 5: One loopful was taken from the stock bacteria and streaked onto each plate, and each plate was incubated for 48-72 hours at 30°C.

Step 6: Some of the single colonies transferred from the plate to the culture tube contained the ammonium yeast medium or the test media once the colony growth of the bacteria had occurred.

Step 7: The culture tube was then incubated for 24-36 hours at 30°C at 200 rpm (Lo bianco and Madonia, 2007).

Step 8: Once the incubation was done, each tube was centrifuged at 8000 rpm for 10 minutes in order to separate the supernatant. The supernatant was removed by pouring it into a separate flask, and the remaining bacterial pellet was used for the bacterial treatment process applied to the soil sample.
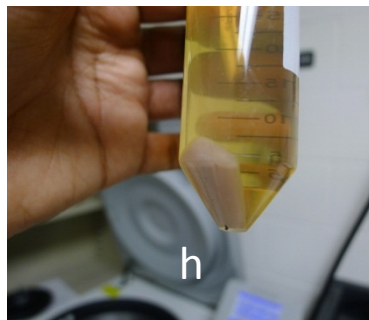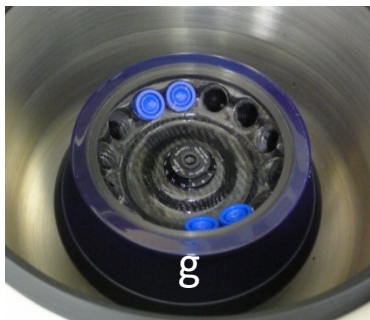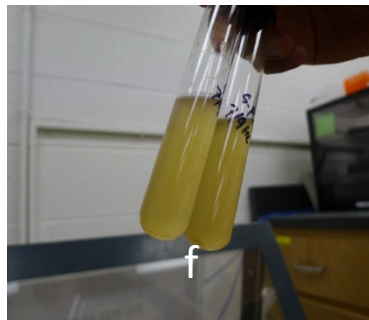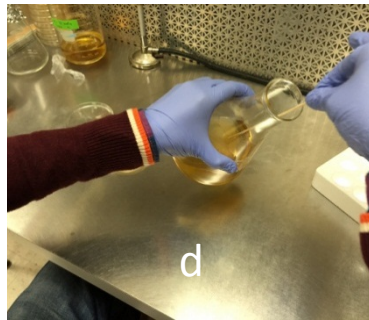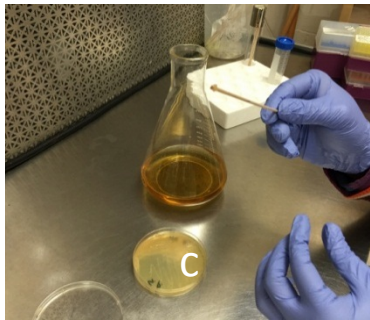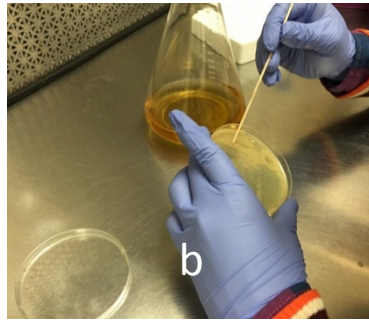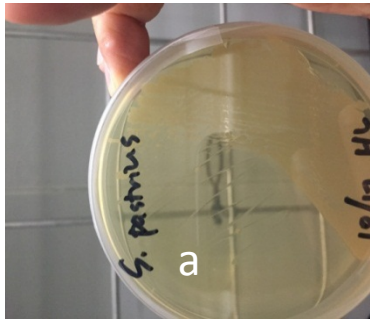
Fig 3-3: Preparation of bacterial solution: a) Ammonium-yeast agar plates, b) Bacteria streaking technique, c) Bacterial colonies, d) Colonies being mixed with ammonium yeast solution, e) Incubating solution for 24-36 hours, f) After incubation, g) Centrifuging the solution, h) Bacterial pellet

3.1.4 Gram Staining Procedure

The main purpose of conducting the gram-staining technique was to identify any unknown bacteria. It provided a means to divide bacteria into two groups; namely, gram positive and gram negative, depending upon the differing abilities of the bacteria in these groups. Gram positive turns purple as soon as it is exposed to alcohol, while gram negative bacteria decolorizes the purple color. In order to make the decolorized cells visible, safranin was applied, which converted the gram negative cells to pink. Since *sporosarcina pasteurii* is known to be gram-positive bacteria, this procedure was used to check whether it had been contaminated. The following steps guided the gram-stain procedure

1) A thin smear of the bacteria was put on a microscope slide and air dried. (The bacteria that were used in this step were obtained directly from the bacteria-culturing plate.)

2) The smear was covered with crystal violet, it sat for 30 seconds, then was rinsed with distilled water.

3) The smear was covered with Gram's iodine, and it sat for 30 to 45 seconds before the solution was poured off.

4) The slide was held at an angle, and one drop of 95% ethyl alcohol was added to the smear to decolorize it.

5) The smear was covered with safranin for 45 seconds, rinsed gently, and left to dry at room temperature.

6) The slide was examined with a microscope, using oil immersion at 1000X magnification.
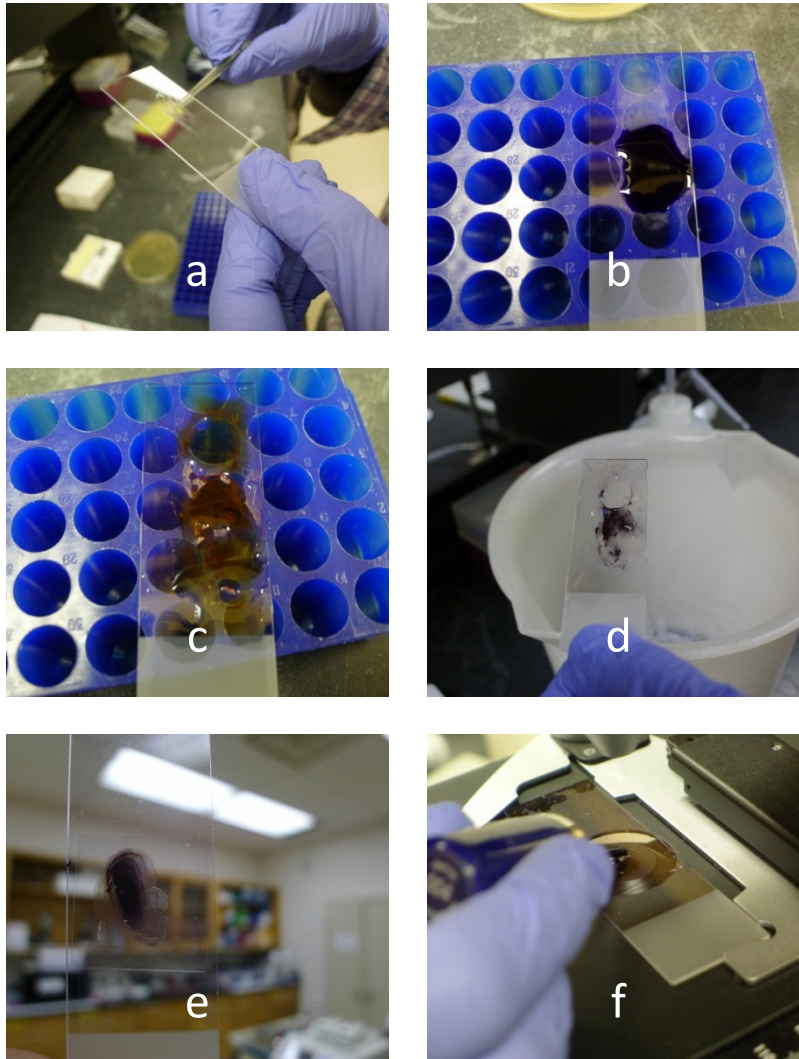
Fig 3-4: Gram-staining procedures; (a) Smear preparation, (b) Covering the
smear with crystal violet, (c) Covering the smear with Gram's iodine
(d) Decolorizing the smear with 95% ethyl alcohol, (e) Covering the
smear with safranin and (f) The stained smear for microscopic examination

3.1.5 Role of a phosphate buffer solution (PBS)

PBS has many uses because it is isotonic and non-toxic to most cells. These
uses also include substance dilution and cell container rinsing. PBS is also used to
disengage attached and clumped cells. The preparation of a phosphate buffered saline
(PBS Buffer) is described as follows:

Step 1: Dissolve the following in 800ml distilled H_2O

8g of NacL

0.2g of KCL

1.44g of Na_2HPO_4

0.24g of KH_2PO_4

Step 2: Adjust pH to 7.4 with HCL.

Step 3: Adjust volume to 1L with additional distilled H_2O.

Step 4: Sterilize by autoclaving.

After getting the bacterial pellet from the centrifugation, the PBS solution is added to it and mixed thoroughly, followed by centrifugation at 8000 rpm for 10 minutes so that the bacterial cells are completely mixed with the PBS, and the pellet can be obtained easily.
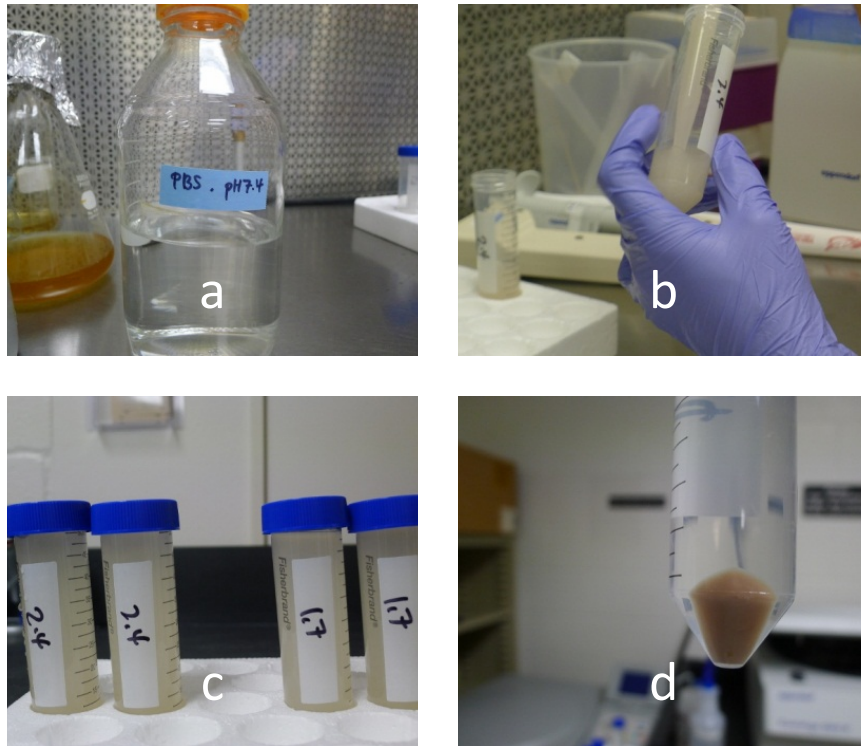
Fig 3-5: PBS treatment procedure: a) PBS solution, b) Mixing the bacterial pellet with the PBS solution, c) Mixed solution of pellet and PBS, d) Bacterial pellet after centrifugation

3.1.6 Making nutrient solution

The nutrient solution was prepared in the following way:

STEP 1: The following ingredients were mixed to create the urea medium solution: nutrient Bacto (3 g), urea (20 g), NH_4Cl (10 g), $NaHCO_3$ (2.12 g), and 500 mL distilled water.

STEP 2: Each of the solid ingredients were mixed thoroughly in 500 mL of distilled water until they dissolved.

STEP 3: More distilled water was then added to reach the final required volume (1 L). After autoclaving, the pH of the urea medium was measured and found to be 8.0.

STEP 4: After autoclaving, the resulting 1 liter solution was divided into 100 mL batches.

STEP 5: The pH of one of the 100 mL urea medium solution batches was then adjusted by stirring the solution to aerate it, until the pH increased from an initial value of 7.0 to approximately 8.5, as measured using a pH meter.

STEP 6: The remaining 100 mL solution batches were stored for later use. The 100 mL of aerated solution was used for the experiment.

Fig 3-6: Nutrient solution
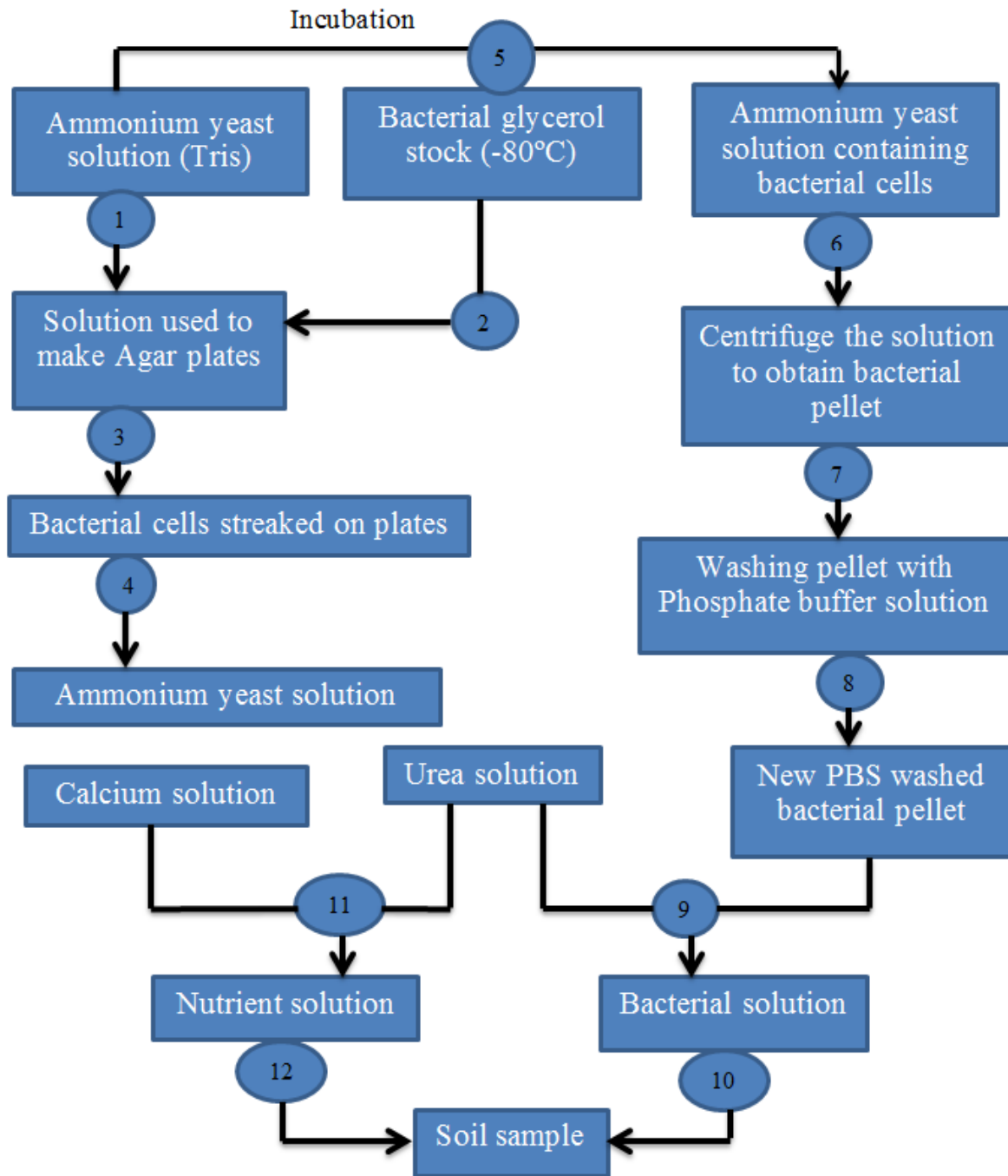
Fig 3-7: Flow chart of bio-cementation treatment

3.1.7 Bacteria counting

Bacteria can be counted and the approximate concentration of bacteria to be added to each specimen can be determined using the spectrophotometer method. The Spectronic Genesis 5 model spectrometer was used for this method. The step-by-step procedure explaining the technique is given below

Step 1: A 10 mL sample of the bacteria culturing solution from the bacteria-culturing flask was placed into a sample cell, and the sample cell was placed into the spectrophotometer. The cover was then closed.

Step 2: The absorbance or optical density of the bacteria in suspension was read at a wavelength of 580 – 600 nm, and was used to determine the approximate concentration of bacteria in the solution.
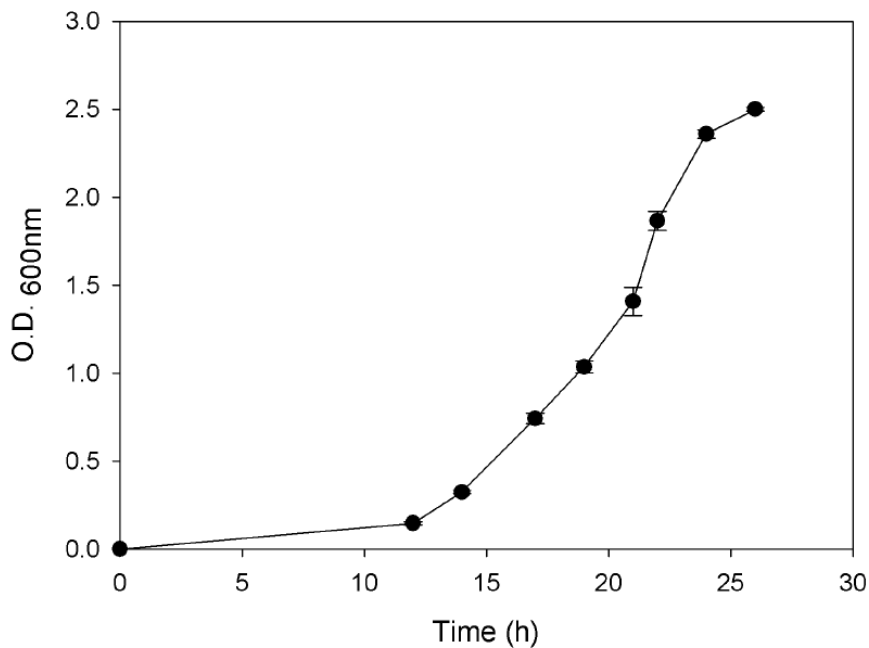
Fig 3-8 : Growth curve for sporosarcina pasteurii

Fig 3-9: Bacteria counting technique: a) Spectra Genesis 5 spectrometer, b) Absorbance at 600 nm wavelength

3.2 Soil specimen properties

Both Ottawa 20-30 and Ottawa well graded sand were used throughout the experiment. The grain-size distribution of these sands was determined in accordance with ASTM D 422, and the results are shown the below figure. Based on the results, the 20-30 sand was a poorly graded medium-to-coarse sand passing 100 percent of particles through the U.S sieve number 16 (1.18 mm), 2 percent of particles passing the U.S sieve number 30 (0.6 mm). The well graded sand was poorly graded medium-to-fine sand, 98 percent passing the U.S sieve number 30 (0.6 mm), and 2 percent of particles passing through the U.S sieve number 50 (0.15 mm). It's white in color and round in shape, The average uniform coefficients (Cu) for 20-30 and for well graded sand are 1.61 and 1.8, respectively.

Fig 3-10: Grain size distribution for Ottawa 20-30 and Ottawa well graded sand

Table 3-1: General Characteristics of Ottawa Sand

Parameter	Ottawa 20-30 sand	Ottawa well graded sand	Ottawa 50-70 sand
Specific gravity (G_s)	2.65	2.65	2.65
D_{50} (mm)	0.78	0.35	0.22
C_u	1.23	2.22	1.6
C_c	0.99	1.25	1.0
Mineralogy	Quartz	Quartz	Quartz
Soil description (USCS)	Poorly graded sand (SP)	Well graded sand (SW)	Poorly graded fine sand (SP)

Note: The Gs of 2.65 for Ottawa sand was assumed. The Ottawa 50-70 sand values were in the same range as those observed by different researchers for varying grain sizes. Since the grain sizes do not appear to affect significantly these values, this assumption does not significantly affect the conclusions of this study and is, therefore, believed to be a reasonable approach.

3.3 Specimen preparation (air-pluviation method)

Air pluviation was chosen for the specimen preparation method in this study because it represents natural deposition, in contrast to other commonly used sample preparation methods. With this method a sample can be prepared to the desired relative

40

density though it has disadvantages such as particle segregation and uniform distribution (Polito, 1999).

<div align="center">3.4 Bacterial Treatment Procedure</div>

The bacterial treatment was done in two different ways. In the first, the bacterial solution was mixed with the urea media and calcium chloride. In the second, the bacterial solution was mixed with the urea media, and calcium chloride was added separately right before pumping it into the soil specimen.

3.4.1 Mixing the bacterial pellet with urea media and adding calcium chloride separately right before pumping the solution into the soil specimen

After preparing the specimen, the bacterial treatment was applied to the sand before strength testing. Once the specimen was ready, the bacterial solution was introduced through a solution containing urea and calcium chloride at room temperature. The introduction of the bacterial solution into the soil specimen was done by taking the bacterial pellet from the centrifugation and adding it to the nutrient solution of required quantity. The suspended bacterial pellet solution was then added to the calcium chloride solution, right before adding it to the soil sample. The combined urea, calcium chloride, and bacterial pellet was then quickly pumped into the specimen. After a certain retention period, the nutrient treatments, consisting of urea and calcium chloride, were pumped. The nutrient treatments were applied periodically over the course of 5-6 days. The following table shows the description of the mixing proportions of different components in making the solutions required for the bio-cementation process.

Table 3-2: Components of bio-cementation treatment solution

Name of solution	Components
Urea medium	Nutrient Bacto
	Urea
	Ammonium chloride
	Sodium bicarbonate
	Deionized water
Bacterial	*Sporosarcina pasteurii*
	Urea medium
Nutrient	Urea medium
	Calcium chloride
	solution
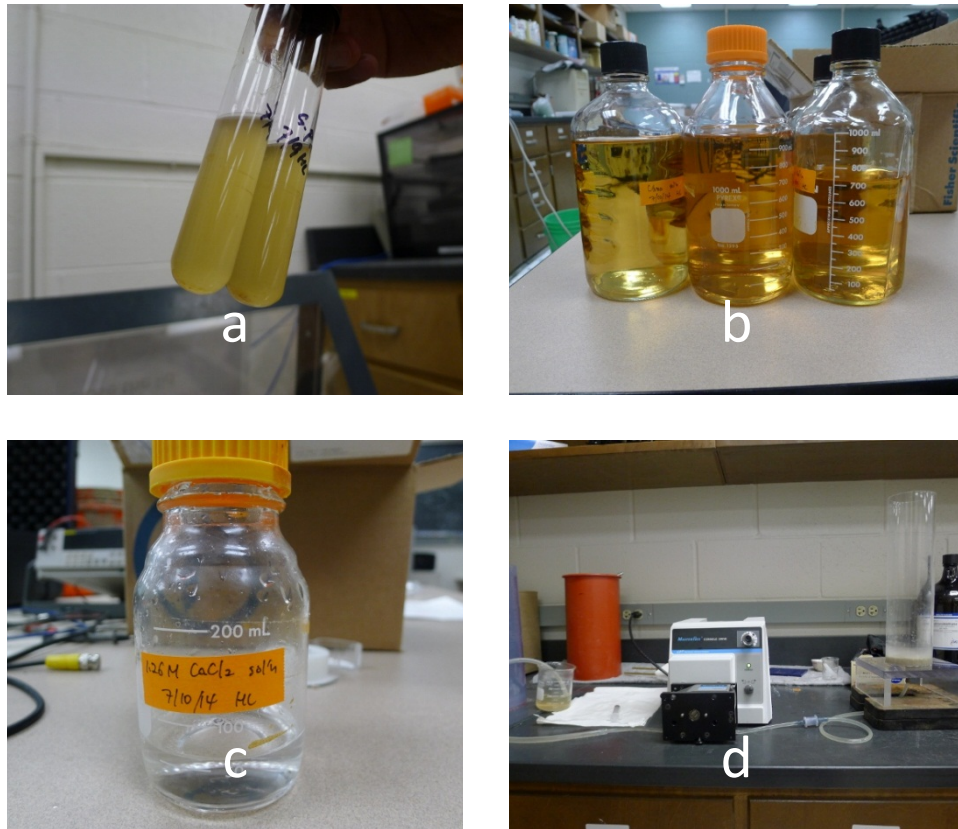
Fig 3-11: Preparation of nutrient media: a) Bacterial solution, b) Nutrient media without calcium chloride, c) Calcium chloride solution, d) Solutions being pumped by peristaltic pump

3.4.2 Mixing the bacterial pellet with urea media and calcium chloride together

The bacterial solution was prepared just as it was described in section 3.6.1, but the calcium chloride was added to the urea media while stirring, during its preparation, instead of adding it separately right before it was pumped into the soil specimen.
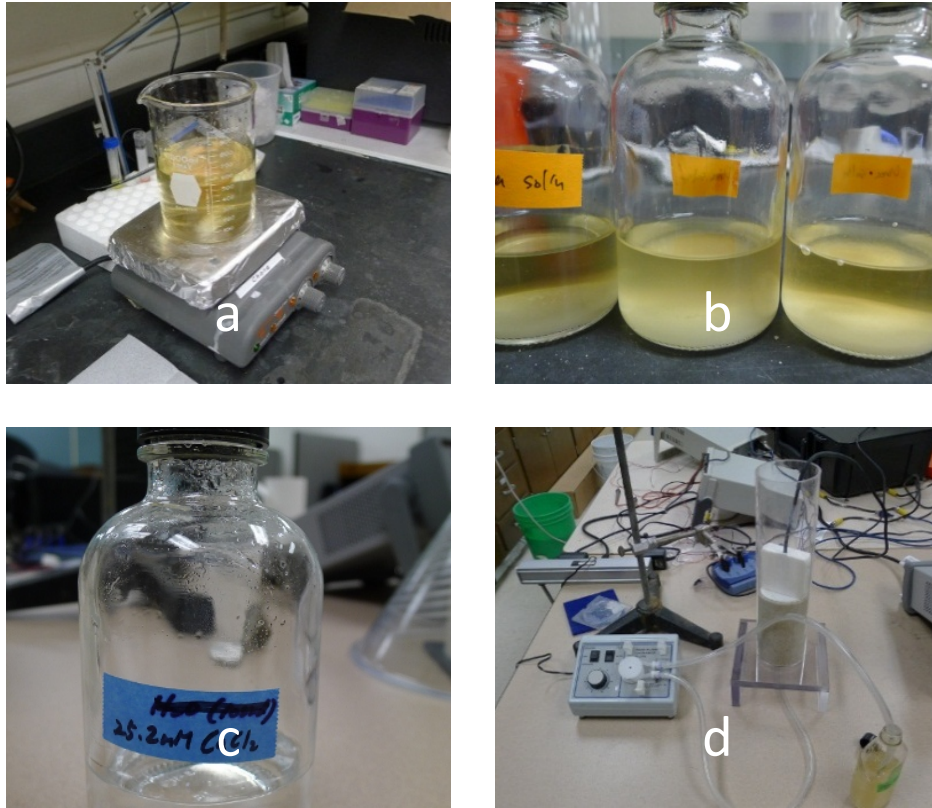
Fig 3-12: a) Nutrient solution with all the components being mixed using magnetic stirrer, b) Nutrient media mixed with calcium chloride (25.2 mM), c) Calcium chloride solution, d) Solutions being pumped by peristaltic pump

3.5 Details of Experiments Conducted to Check the Influence of Different Parameters on Bio-Cementation

3.5.1 Effect of Application of drainage facility on bio-cementation

Two different experiments were conducted to check the effect of cementation on providing drainage. One used a mold, and the other used an acrylic tube which had the drainage facility on the bottom. 50 grams of sand were put in the acrylic tube and the mold, and the solutions were pumped from the top.

Table 3-3: Experimental details of the influence of drainage on bio-cementation performance

Microbial information	Mold (sample 1)	Acrylic tube (sample 2)
Bacteria Name	ATCC-6453	ATCC-6453
Soil Type	Ottawa 20-30	Ottawa 20-30
Weight of soil sample (grams)	100	50
Direction of pumping	Top	Top
Method used for pumping solutions	Syringe	Syringe
Bacterial concentration (OD)	0.8	0.8
$CaCl_2$ concentration (mM)	25	25
Flow rate(mL/min)	4	4
Nutrient cycles	9	9
Volume of bacterial treatment (mL)	50	50
Volume of nutrient treatment (mL)	50	50
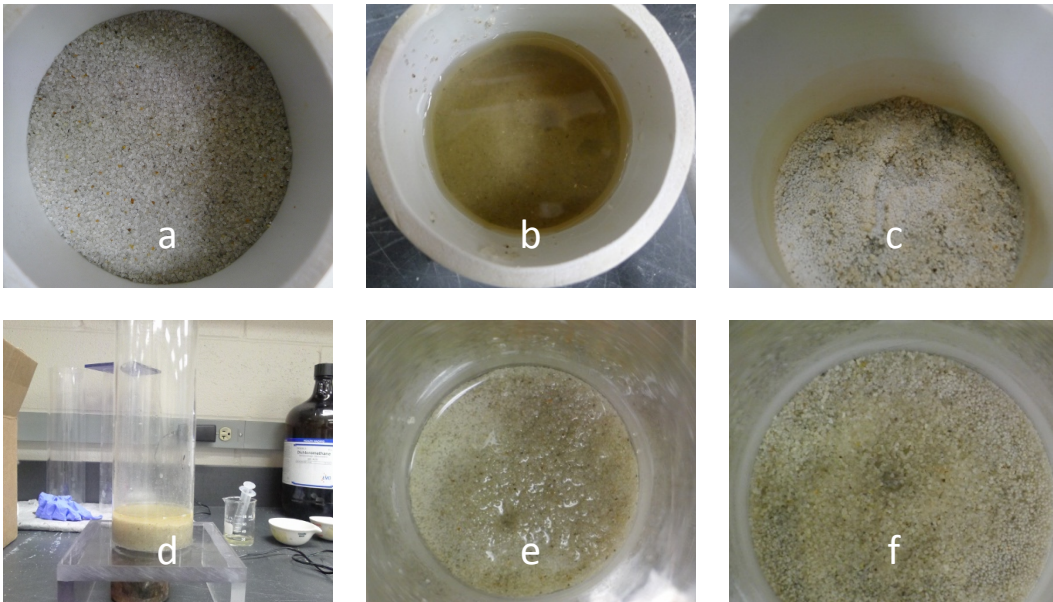
Fig 3-13: Effect of bottom drainage on cementation: a) Before treatment (mold), b) During treatment (mold), c) After treatment, d) Acrylic tube, e) During treatment, f) After treatment

3.5.2 Effect of particle size on bio-cementation

The particle size varies from coarse-grained sands and gravels to fine-grained silts and clay extensively in-situ. The effectiveness of the MICP is dependent upon the soil's permeability, which should be sufficiently permeable for the chemicals to be injected and an adequate number of the soil's contacts available within the soil matrix. The more the particles contact within the soil matrix, the larger the effect of precipitation. The strength and the stiffness of the calcium precipitation is greater at the particle contacts. The well graded dense sands have more particle contacts, hence better precipitation and more constant relative density than loose, poorly graded sands. Based on a study of a variety of soil particle sizes and gradations, the effectiveness of MICP was greater for soils which contained silica as a dominate mineral. According to Rebata-landa (2007), the coarser and well graded sands have better precipitation than very fine,

poorly graded sands, and in very coarse soils, it takes a long time to form a bond between the soil particle contacts.

In order to check the effect of different particle sizes on cementation, three sands were chosen; namely, Ottawa 20-30 (US silica, quarry in Ottawa, IL), Ottawa 50-70 (US silica, quarry in Ottawa, IL), and well graded sand (US silica, quarry in Ottawa, IL).

Table 3-4: Experimental details of the influence of particle size on bio-cementation performance

Microbial information	Ottawa 20-30 (sample 3)	Well graded sand (sample 4)
Bacteria Name	ATCC-6453	ATCC-6453
Weight of soil sample (grams)	50	50
Apparatus used	Mold	Mold
Direction of pumping	Top	Top
Method used for pumping solutions	Peristaltic pump	Peristaltic pump
Bacterial concentration (OD)	0.8	0.8
$CaCl_2$ concentration (mM)	25	25
Flow rate(mL/min)	4	4
Nutrient cycles	9	9
Volume of bacterial treatment (mL)	50	50
Volume of Nutrient treatment (mL)	50	50

Fig 3-14 Description of particle size experiment: Ottawa sand a) sample of Ottawa sand, b) Sample saturated with bacterial solution, c) During cementation, d) End of test, e) Sample of well-graded sand, f) Application of nutrient solution, g) In between nutrient cycles, h) End of test

3.5.3 Effect of urea source on bio-cementation

In order to check the effect of optical density (O.D) and phosphate buffer solution (PBS) on cementation, two different optical densities were tested. The bacteria pellet was washed in the PBS solution before pumping it directly into the sample. Ottawa 20-30 sand was used for this experiment. The experiment was conducted in two different apparatus, one using the acrylic tube (internal diameter 3 inches, external diameter 3.25 inches, length 1 foot), and the other in a syringe.

Table 3-5: Experimental details of the influence of optical density on bio-cementation performance

Microbial information Bacterial optical density (O.D)	1.7 (Test 5)	1.1 (Test 6)
Bacteria name	ATCC -6453	ATCC- 6453
Soil type	Ottaw a 20-30	Ottawa 20-30
Weight of soil sample (grams)	130	150
Apparatus used	Acrylic tube	Syringe
Direction of pumping	Bottom	Top
Method used for pumping solutions	Syringe	Syringe
CaCl$_2$ concentration (mM)	25	25
pH	7.5	7.5
Flow rate(mL/min)	2	4
Nutrient cycles	9	6
Volume of bacterial treatment (mL)	100	100
Volume of Nutrient treatment (mL)	100	100
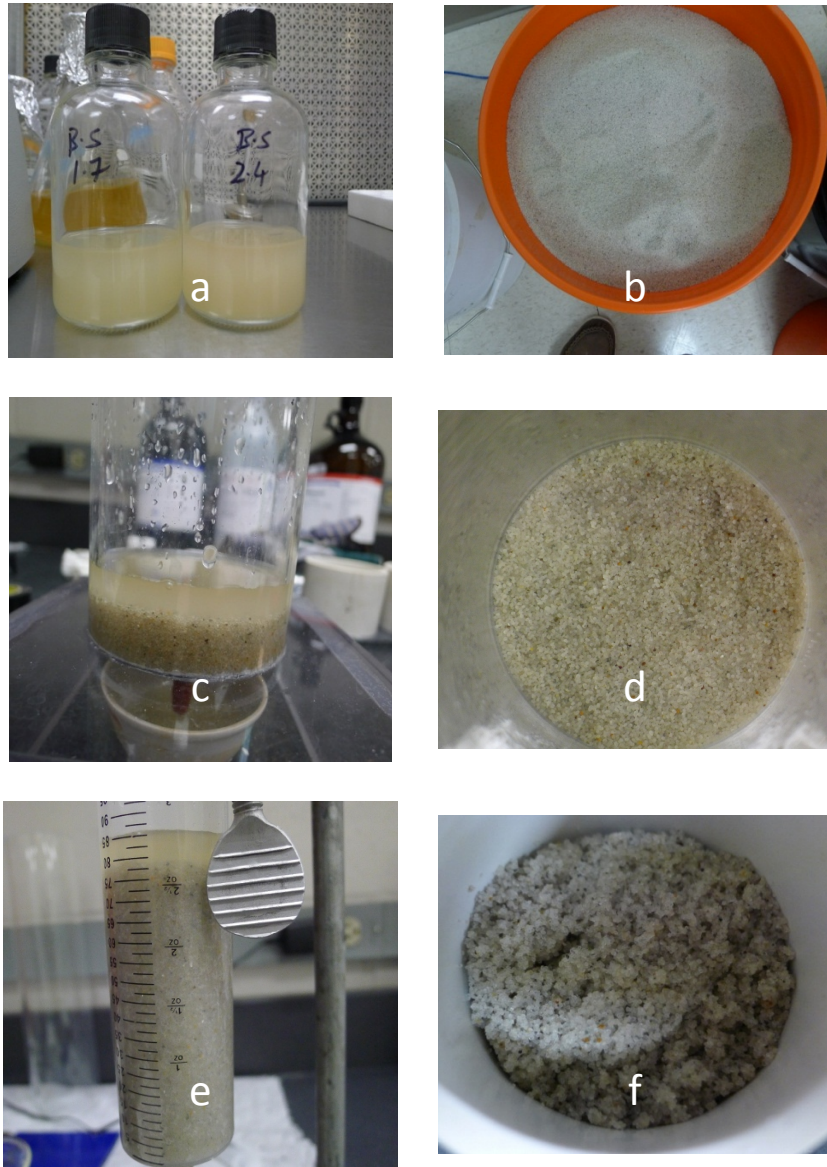
Fig 3-15: Effect of PBS and OD on cementation: a) PBS washed solution, b) Ottawa 20-30, c) Sample being treated in acrylic tube, d) Failed sample after treatment (acrylic tube), e) Sample being treated in syringe, f) Failed sample after treatment (syringe)

3.5.4 Effect of number of life cycles or nutrient injections on bio-cementation

In order to check the number of life cycles on cementation, the experiment was conducted in a way similar to that described in the above section. The experimental procedure was conducted in the following way:

3.5.3.1 Experimental procedure

The experimental solutions, such as the bacterial, nutrient and calcium solutions, were prepared in a way similar to that explained in sections 3.1.3 and 3.1.6. The solutions were pumped from the bottom, using the peristaltic pump. The setup included connectors, an acrylic tube, a syringe, and a peristaltic pump. The acrylic tube was used to hold the Ottawa sample, and the syringe was used for the well graded sample. After the first injection of the bacterial solution, the sample was kept undisturbed for 4 hours, then the nutrient cycles were started at intervals of 3 hours.

3.5.3.2 Curing

The experiment lasted for 35 cycles, which took approximately one week, then the samples were kept inside the moisture room for curing. Further details of the experiment, such as the flow rate, type of sand, pH, life cycles, volumes, and concentrations of solutions are given in the following table.

Table 3-6: Experimental details of the effect of number of life cycles on the bio-cementation performance

Microbial information	Ottawa 20-30 (Test 7)	Well-graded sand (Test 8)
CaCl$_2$(mM)	25	25
Optical Density(OD)	0.9	0.9
PBS washed	yes	yes
pH	8	8
Volume of each cycle (B.S)	100 ml	50 ml
Volume of each cycle (N.S)	100 ml	50 ml
Bacterial solution(ml/min)	10	10
Nutrient solution (ml/min)	4	4
Life cycles	35	35
Sample weight (grams)	125	62
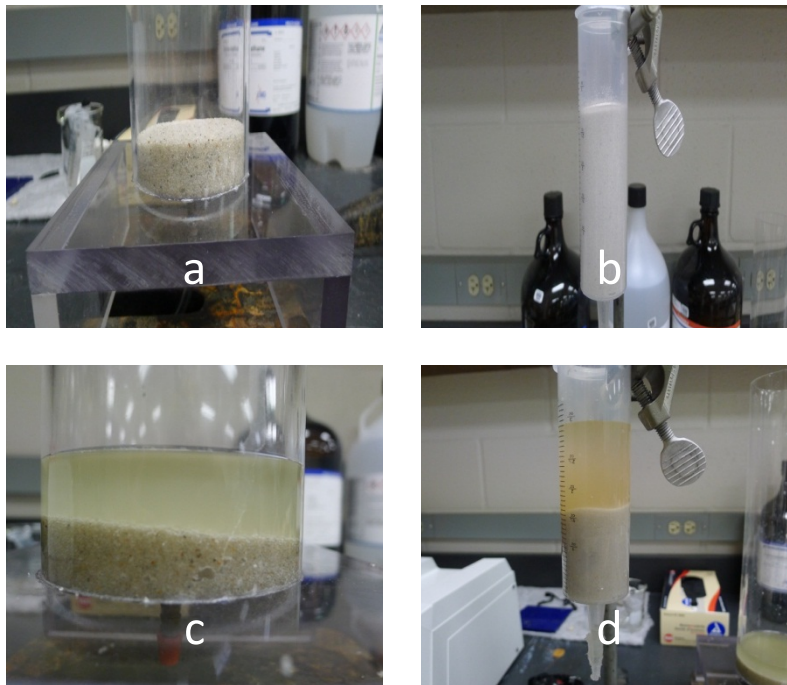
Fig 3-16: a) Ottawa 20-30, b) well-graded sand, c) Ottawa sand completely immersed in bacterial solution, d) Well-graded sand completely saturated in bacterial solution
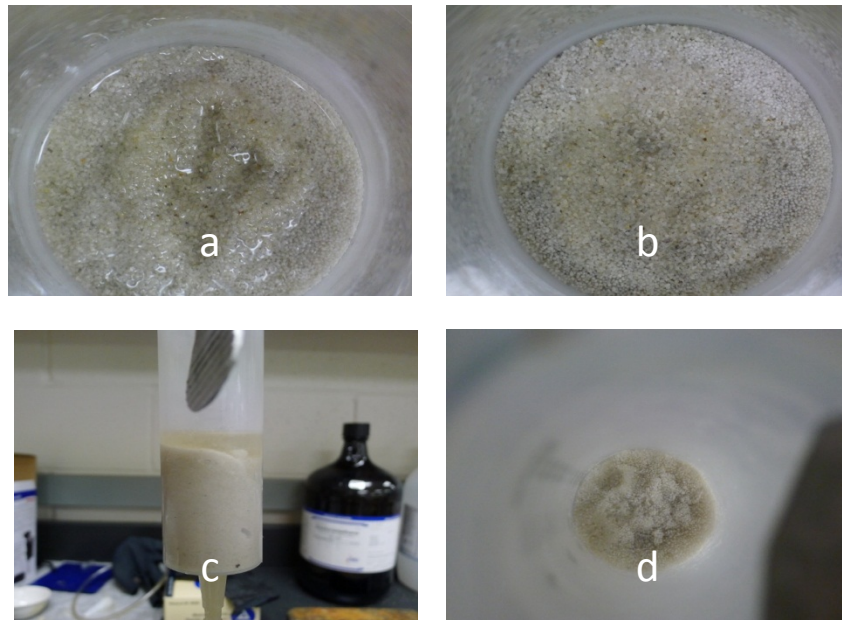
Fig 3-17: a) During treatment (Ottawa 20-30), b) After treatment (Ottawa 20-30), c) During treatment (Ottawa 20-30), d) After treatment (well graded sand)

Fig 3-18: Samples being cured in the moisture room a) Ottawa sand b) Well-graded sand
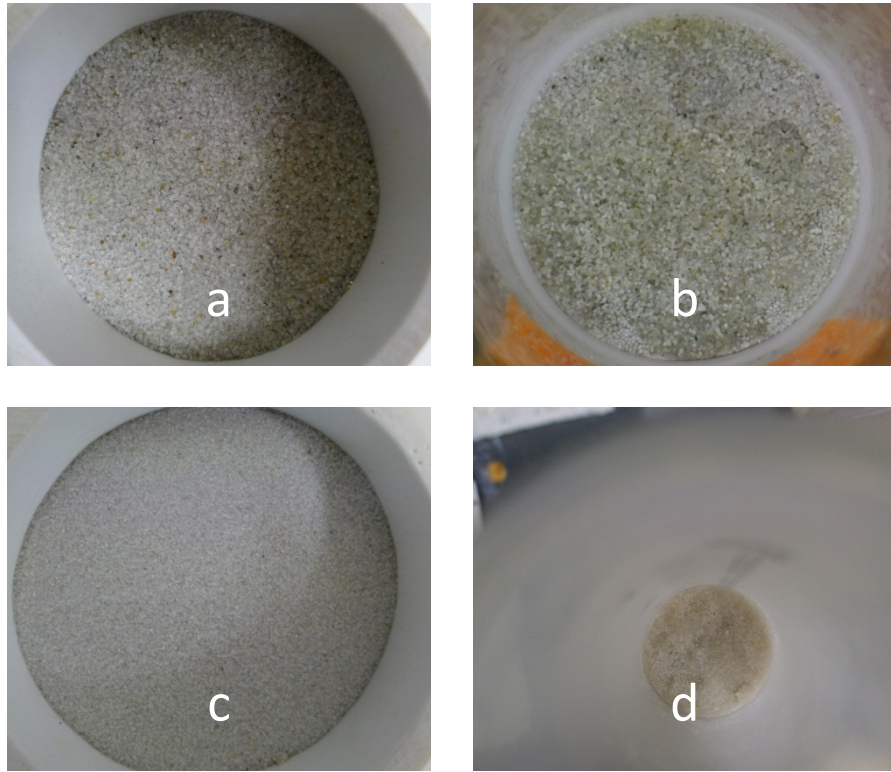
Fig 3-19: (Ottawa sand): a) Before treatment, b) After treatment (Well-graded sand), c) Before treatment, d) After treatment

3.5.3.3 Effect of calcium concentration on bio-cementation

In order to check the effect of different calcium concentrations on cementation, four different types of calcium concentrations were selected (25mM, 50mM, 0.25M, 0.5M). The details of the experimental procedure are as follows.

3.5.4.1 *Experimental procedure*

The experiment was conducted using the same bacterial and nutrient solutions as described in the previous sections, but different calcium concentrations were used for each sample. The number of nutrient cycles was limited to 7 rather than 35, as in the previous experiments. as we were just attempting to compare the calcium effect on

cementation. The amount of NH4CL, NaHCO3, urea, and nutrient broth were calculated

for a volume of 2500 ml since there were more samples this time, and a greater volume

of solutions needed to be pumped.

Table 3-7: Experimental details of the effect of different cementation concentrations on bio-cementation performance

Parameter	sample 9	sample 10	sample 11	sample 12
Soil type	Fine sand	Fine sand	Fine sand	Fine sand
Sample weight(grams)	50	50	50	50
Optical density	1	1	1	1
PBS wash	Yes	Yes	Yes	Yes
Urea (grams)	20	20	20	20
NH$_4$CL (grams)	25	25	25	25
NaHCO$_3$ (grams)	5.3	5.3	5.3	5.3
Nutrient broth(grams)	3	3	3	3

Table 3-8: Different concentrations of calcium solution used for nutrient cycles

sample	Calcium concentration (mM)	Nutrient cycles	Treatment duration (hrs.)
9	25	7	2
10	50	7	2
11	250	7	2
12	500	7	2

Table 3-9: volume of calcium chloride dihydrate added to 50ml of nutrient solution

Volume of calcium chloride (grams) dihydrate solution (per 100ml of deionized water)	Volume of calcium solution added for each 50 ml nutrient solution (ml)	Calcium concentration (mM)
18.5	1	0.025
18.5	2	0.05
73.5	2.5	0.25
73.5	5	0.5

Fig 3-20: Experimental setup Images of calcium concentration influence on bio-cementation
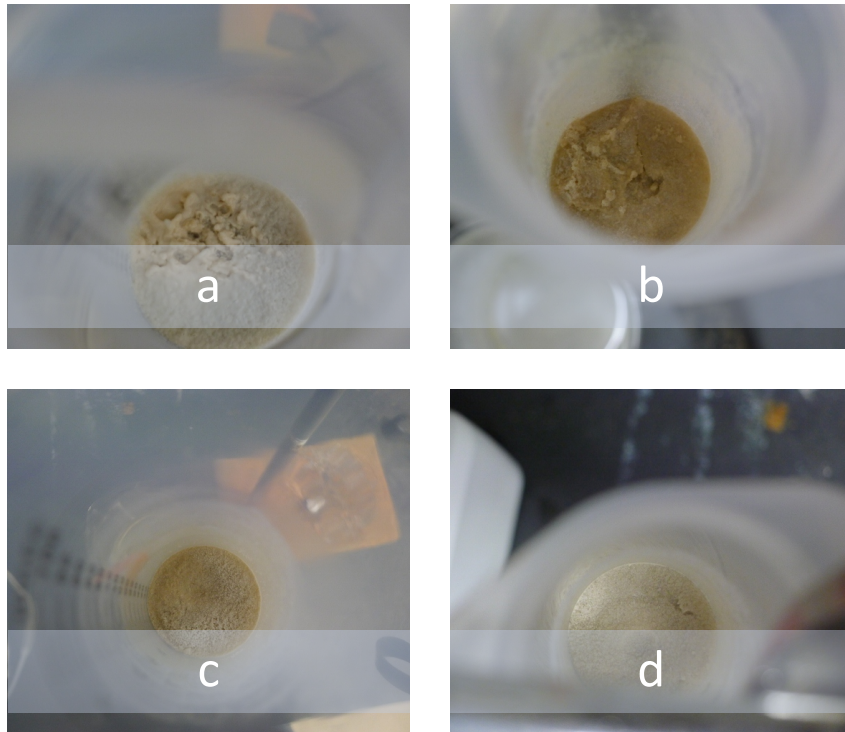
Fig 3-21: Treated samples of different calcium concentrations a) 0.5M b) 0.25M c) 0.05M d) 0.025M

3.5.6 Effect of single vs. multiple bacterial injections on bio-cementation

To obtain a uniform precipitation, the bacterial cells should permeate the entire sample so that they can react with the calcium ions. Based on the above results and tests, however, there was always better precipitation on the top layers than on the bottom layers, which obstructed the further flow of bacterial cells. Clogging of more bacterial cells took place on the top, which resulted in non-uniform precipitation. One new thing that was introduced in this experiment was the magnetic stirrer, which was used while adding the calcium chloride solution to the urea solution while making the nutrient solution. The calcium ions were mixed more uniformly throughout the nutrient solution because of using the magnetic stirrer. In order to verify this effect, an experiment was conducted

using both single and multiple injections of bacterial injections. The details of the experimental procedure are given in the following sections.

3.5.6.1 Single injection

Plastic syringes with 100-mL volume were used as test soil columns. They had an internal diameter of 3.0 cm and a height of 15 cm. The syringes were filled with 50 grams of Ottawa 20-30 sand. The sand was washed with tap water to remove any air voids and lightly compacted to make the sample denser so that less time was required for the bacteria to make the bonding by calcium precipitation between the soil particles. Plastic tubing was connected at the bottom for drainage of nutrients, and sand was packed in the columns in the presence of the bacterial liquid medium (1 PV with OD of 0.8).

The bacterial solution was injected at a rate of 5 ml/min and left undisturbed for 6 hours to allow time for the bacteria to attach itself to the sand grains. The retention time was varied by injecting urea-CaCl$_2$ liquid media of a given molar concentration and leaving it for different time durations to react. The injection was made by adding new liquid media at the top soil boundary under gravity. During the retention stage, the sand was always kept slightly overtopped with a liquid to ensure that it remained saturated at all times. Loss of evaporation and leakages were regularly checked. After the predetermined retention time, the old liquid medium in the specimen was replaced with a new one. This process of injection-retention was performed several times to provide a certain mass input of liquid media into the specimens.

3.5.6.2 Multiple Injections

Plastic syringes with 100-mL volume, internal diameter of 3.0 cm and a height of 15.0 cm were used as test soil columns. First, coarse sand (Silica 12-20 sand) was placed in the bottom of the syringe up to 1.0 cm high, and then the Ottawa 20-30 sand of

80 grams was placed in the syringe. The sand was rinsed with water several times, to eliminate air bubbles between sand particles. The velocity of the peristaltic pump connecting the syringe (at the top of the cylinder) was adjusted to inject bacteria liquid at 5 mL/min flow rate. After injecting the bacteria liquid, the bottom injection was sealed quickly and left undisturbed for 2 hours to allow more microbial adsorption to the sand particles. After 2 hours, the seal mouth was opened so that the microbial solution could completely drain out from the sand column. Then, 10 mL/min flow rate of nutrient solution was injected into the sample, and the bottom injection port was sealed immediately. After 2 hours of waiting for nutrient solution to be completely drained out of the sand column, another batch of nutrient solution was applied, then blocked for a retention period of 12 hours so that the microbes could react with nutrient solution. The above mentioned step was repeated until the solution was no longer through the sand column.

Table 3-10: Experimental details for the effect of single vs. multiple bacterial injections

Microbial information	Ottawa 20-30 (Test 7)	well graded sand (Test 8)
$CaCl_2$(mM)	25	25
Optical Density(OD)	0.9	0.9
PBS washed	yes	yes
pH	8	8
Number of bacterial injections	1	4
Number of nutrient injections	13	10
Bacterial solution(ml/min)	5	5
Nutrient solution (ml/min)	10	10
Apparatus used	Syringe	Syringe
Sample weight (grams)	50	80
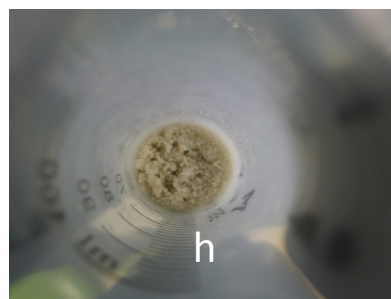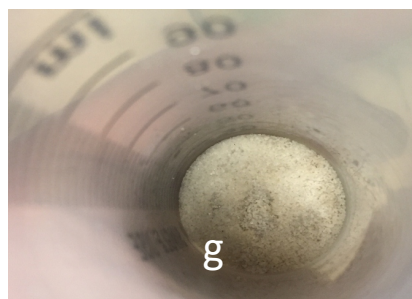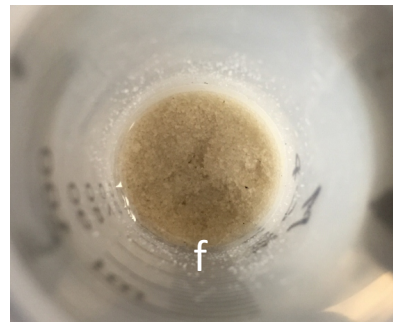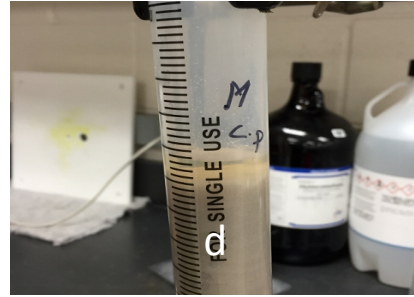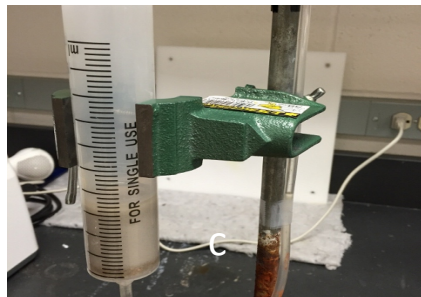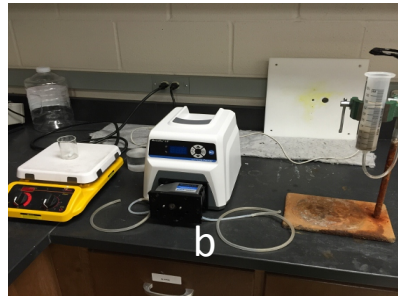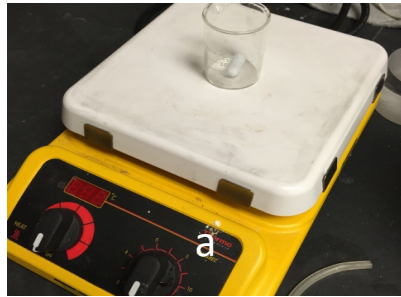
Fig 3-22: a) Magnetic stirrer, b) Experimental setup, c) Single injected sample, d) Multiple injected sample, e) During treatment of single Injections, f) During treatment of multiple injections, End of test, g) Single injected, h) Multiple injected

Fig 3-23: Images of sample after curing 16 days: a) Sawing bottom part of syringe, b) Extracting the sample, c) Single bacterial injection, d) Multiple bacterial injections

3.6 Repeatability test

For the first time, a hardened sample was the result of the test of single vs. multiple bacterial injections. To confirm the results, the test was repeated and simultaneously, a new sand sample (Ottawa 50-70) was used to check the particle size effect. The experimental details are given in the following table.

Table 3-11: Experiment test details for repeatability test

Microbial information	Ottawa 20-30 (Test 7)	well graded sand (Test 8)
$CaCl_2$(mM)	25	25
Optical Density(OD)	0.9	0.9
PBS washed	yes	yes
pH	8	8
Number of bacterial injections	8	8
Number of nutrient injections	15	15
Bacterial solution(ml/min)	5	5
Nutrient solution (ml/min)	10	10
Apparatus used	Syringe	Syringe
Sample weight (grams)	80	80

Fig 3-24: a) Ottawa 50-70, b) Well graded sand, Test setup, c) Ottawa 50-70, d) Well graded sand In-between cycles, e) Ottawa 50-70, f) Well graded sand
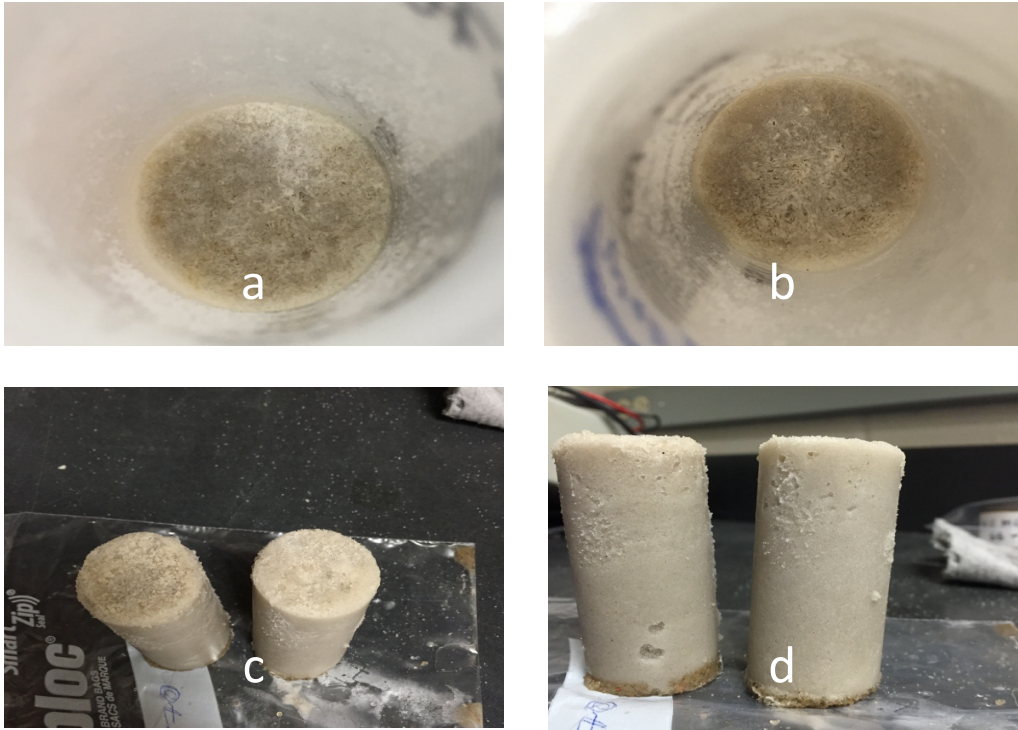
Fig 3-25: Sample images cured for 7 days: a) Ottawa 50-70 sand, b) Well graded sand,
c) Top view of both samples, d) Side view of both samples

Chapter 4

Results and Discussion

4.1 Discussion of results of all tests

4.1.1 Effect of providing bottom drainage on bio-cementation

Based on the results, the bottom drainage resulted in providing better precipitation than the mold. This was because when the solu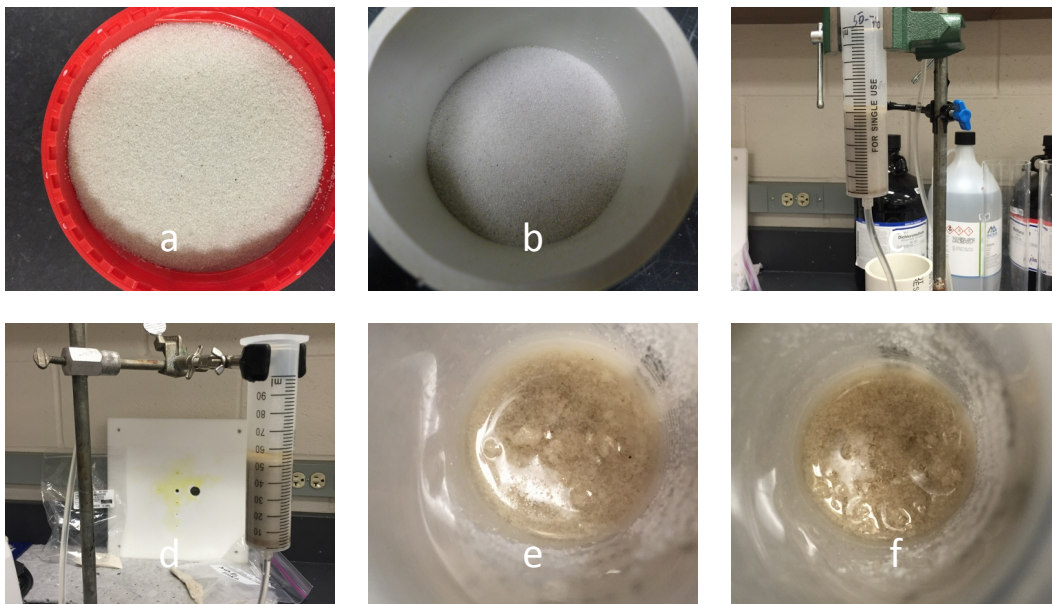tions were flowing from the sample, all the nutrients and the calcium ions permeated the sample rather than becoming clogged on top due to the fixed layer at the bottom.

4.1.2 Effect of particle size on bio- cementation

Compatibility between the soil grain characteristics and bacteria size is an important factor for MICP treatment. The soil pores should be of sufficient size to allow the bacterial transportation (Mitchell and Santamarina, 2005), with 50–400 µm reported as the most favorable soil particle size range for bacterial activity in the pores (Rebata-Landa, 2007). Results indicate that the soil permeability has great influence on cementation. The permeability of Ottawa 20-30 sand is higher than that of well graded sand, which makes it conducive for penetration of the cementation media into the specimens, promoting the MICP process. Rebata-Landa (2007) conducted research on the effect of soil type on the MICP and indicated that the soil type had two kinds of limitations on MICP. In very fine soil particles, the MICP is hindered by permeability. While in coarse soils, a thin layer of mineral precipitation cannot cement particles together. In this paper, the D_{50} value of Ottawa sand and well graded sand were 0.7 mm and 0.35 mm, respectively, which was double, and thus the grain size shown great effect on MICP. In addition to the soil permeability, the differences in mineralogy may also

affect the chemical reaction network. Hence, in the present study, the well graded sand provided better precipitation than the Ottawa 20-30 sand.

4.1.3 Effect of urea source on bio-cementation

The result in the cementation pattern was clear from the figures, as there was no precipitation at all. The PBS treatment helped the pellet wash off any other bacteria present and adjust to the outside atmosphere by balancing the pH of the solution. Since there was no urea medium in the bacterial solution, most of the bacterial cells might have been either dead or inactive. Since there was no urea source in the bacterial solution, there was no production of ammonium or carbonate ions, resulting in no precipitation of calcium.

4.1.4 Effect of number of life cycles or nutrient cycles on bio-cementation

From the figures, there is a clear indication of calcium precipitation so it was also clear that the higher number of nutrient cycles, the better the cementation will be. However, it also depends up on the concentrations of the solutions that are being used. Higher hydrolysis rates lead to higher pH and more small crystals, while higher concentrations of urea and calcium chloride lead to lower pH values. The pH, being a result of the speciation, quickly rises until critical supersaturation is reached and precipitation is initiated. Then, the pH drops to about neutral, where it stays until all substrates are depleted. Microbial-induced hydrolysis of urea and precipitation of calcium carbonate require high urea and calcium chloride concentrations and high hydrolysis rates to minimize the treatment time and number of flushes required for sufficient soil stabilization.

4.1.5 Effect of calcium concentration on bio-cementation

The presence of calcium ions also affects the urease activity, as clearly depicted by the figures. There was little effect at lower calcium chloride concentrations, up to 50

mM, but it increased as the calcium concentrations rose. Where urea hydrolysis was almost completely inhibited by the calcium source being either calcium chloride or calcium nitrate, the activity rate decreased. Although high concentrations of cementation media can improve the MICP treatment effect and mechanical properties to some extent, only a portion of calcium ions was precipitated by MICP. The results indicate that an increase in concentration of cementation media is limited in its effectiveness to enhance MICP. This observation shows that urea and calcium ions in a cementation solution have not been fully utilized when the concentration is higher, probably because the MICP process was limited by enzyme quantities and reached the maximum urea hydrolysis. Another cause is that the high concentration of calcium chlorine in the cementation solution reduced the urease activity of the bacterial enzymes.

4.1.6 Effect of single vs. multiple bacterial injections on bio-cementation

The results of this effect were very important, as they helped us reach some important conclusions. They confirmed that a single bacterial solution injection was not enough to get a uniform and well-cemented sample. The reason behind this conclusion is the fact that the bacteria precipitates calcium carbonate in the presence of calcium ions, and when more nutrient cycles were applied, more bacterial cells reacted with the calcium ions and were flushed out with the nutrient cycles. Hence, it is recommended to apply multiple injections of bacterial solution frequently, in the middle of nutrient cycles, with specific time intervals, so that more microbes can be retained within the soil sample that can help in getting a uniformly cemented sample.

Table 4-1: Ideal range of parameters based on the results of all experiments

Test number	Effective parameter	Ideal range for good bio-cementation
Test Group (1,2)	Provision of drainage	Based on the results, the bottom drainage resulted in giving better precipitation than the mold.
Test Group (3,4)	Particle size	Well graded medium-to-fine grained sand.
Test Group (5,6)	Bacterial optical density (O.D$_{600}$)	0.8-12
Test Group (7,8)	Number of nutrient cycles	12-15
Test group (9,10,11,12)	Calcium concentration (mM)	25-50
Test group (13,14)	Single vs. multiple bacterial injections	Multiple bacterial injections (at least 4 for every 2 nutrient cycles)

Table 4-2: Geotechnical parameters for all experiments

Test sample number	Type of apparatus used	Type of sand	Sample weight (grams)	Diameter (cm)	Height (cm)	Density (gm/cm^3)	Void ratio
s 1	Mold	Ottawa 20-30	100	7.6	1.2	1.73	0.34
s 2	Acrylic tube	Ottawa 20-30	100	7.6	1.2	1.73	0.34
s 3	Mold	Ottawa 20-30	100	7.6	1.2	1.73	0.34
s 4	Mold	Well graded	100	7.6	1.2	1.73	0.34
s 5	Acrylic tube	Ottawa 20-30	125	7.6	1.6	1.83	0.36
s 6	Syringe	Ottawa 20-30	150	3.5	9	1.73	0.34
s 7	Acrylic tube	Ottawa 20-30	125	7.6	1.6	1.83	0.36
s 8	Syringe	Well graded	62	3.5	4	1.61	0.39
s 9	Syringe	Well graded	50	3.5	3	1.73	0.34
s 10	Syringe	Well graded	50	3.5	3	1.73	0.34
s 11	Syringe	Well graded	50	3.5	3	1.73	0.34
s 12	Syringe	Well graded	50	3.5	3	1.73	0.34
s 13	Syringe	Well graded	50	3.5	3	1.73	0.34
s 14	Syringe	Well graded	80	3.5	5	1.66	0.37
s 15	Syringe	Well graded	80	3.5	5	1.66	0.37
s 16	Syringe	Ottawa 50-70	80	3.5	4.8	1.66	0.34

Table 4-3: Microbial information of all experiments

Effective parameter	Test sample number	Optical Density of bacteria (O.D)	Calcium concentration (mM)	Number of bacterial injections (Volume in mL)	Number of nutrient injection (Volume in mL)
Drainage facility	1(No drainage)	0.8	25	1 (50)	9 (50)
	2	0.8	25	1 (50)	9 (50)
Particle size	3 (Ottawa 20-30)	0.8	25	1 (50)	9 (50)
	4 (Well graded)	0.8	25	1 (50)	9 (50)
Urea source	5	1.7	25	1 (100)	9 (100)
	6 (No urea)	1.1	25	1 (100)	6 (100)
Number of nutrient injections	7	0.9	25	1 (100)	35 (100)
	8	0.9	25	1 (100)	35 (100)
Calcium media concentration	9	1	25	1 (50)	7 (100)
	10	1	50	1 (50)	7 (100)
	11	1	250	1 (50)	7 (100)
	12	1	500	1 (50)	7 (100)
Number of bacterial injections	13	0.8	25	1 (Slightly overtop)	13 (Slightly overtop)
	14	0.8	25	4 (Slightly overtop)	10 (Slightly overtop)
Repeatability test	15	0.8	25	8 (Slightly overtop)	15 (Slightly overtop)
	16	0.8	25	8 (Slightly overtop)	15 (Slightly overtop)

## 4.2 Microstructure examination of samples using SEM images

The microscopic examination of soil is useful for understanding the relationship between the soil structure and its mechanical behavior. It also helps to provide strong proof of the cementation formed due to the production of calcium carbonate. Previous investigators also performed microscopic examinations, using scanning electron microscopy (SEM) (Bang, 2000Mitchell and Ferris, 2006 and Dejong, 2006). The following are the some of the associated photos taken using the SEM machine (Hitachi S3000N FE SEM) from material science engineering department at the University of Texas, Arlington in a similar fashion to check the effect of calcium concentration on cementation. The after cured samples were silver coated in order to get better quality image of nucleation sites and bacteria at low magnification.
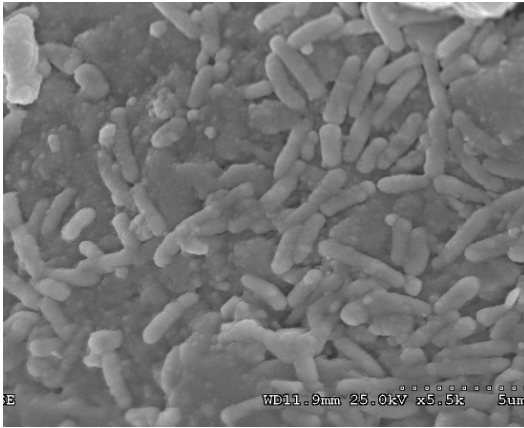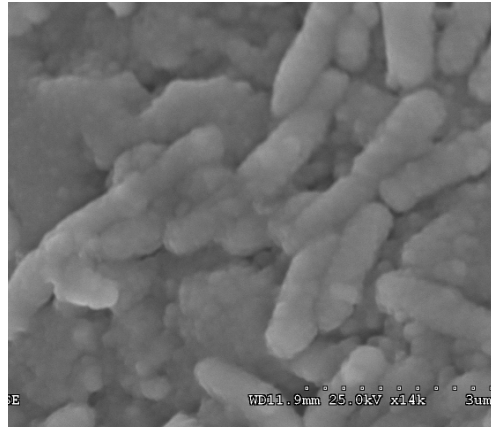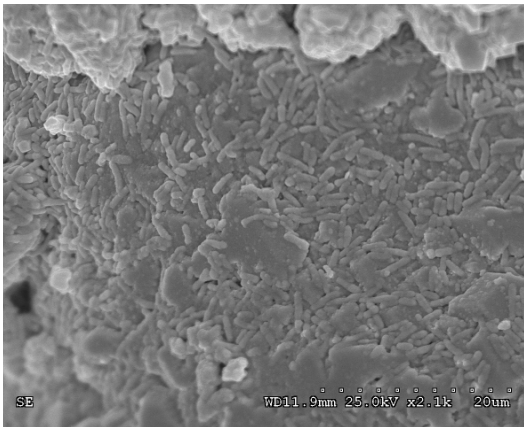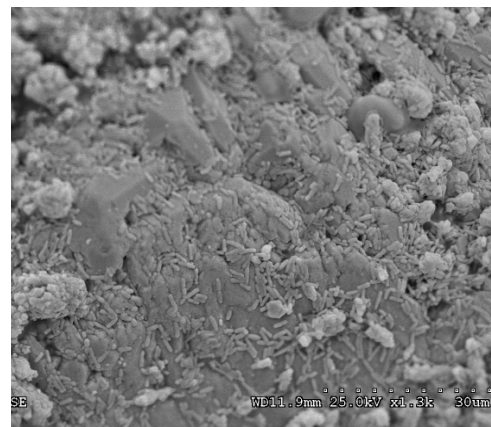
Fig 4-1: Images of bacteria taken at different magnification lengths (0.025M) a) 5μm b) 3μm c) 20μm d) 30μm
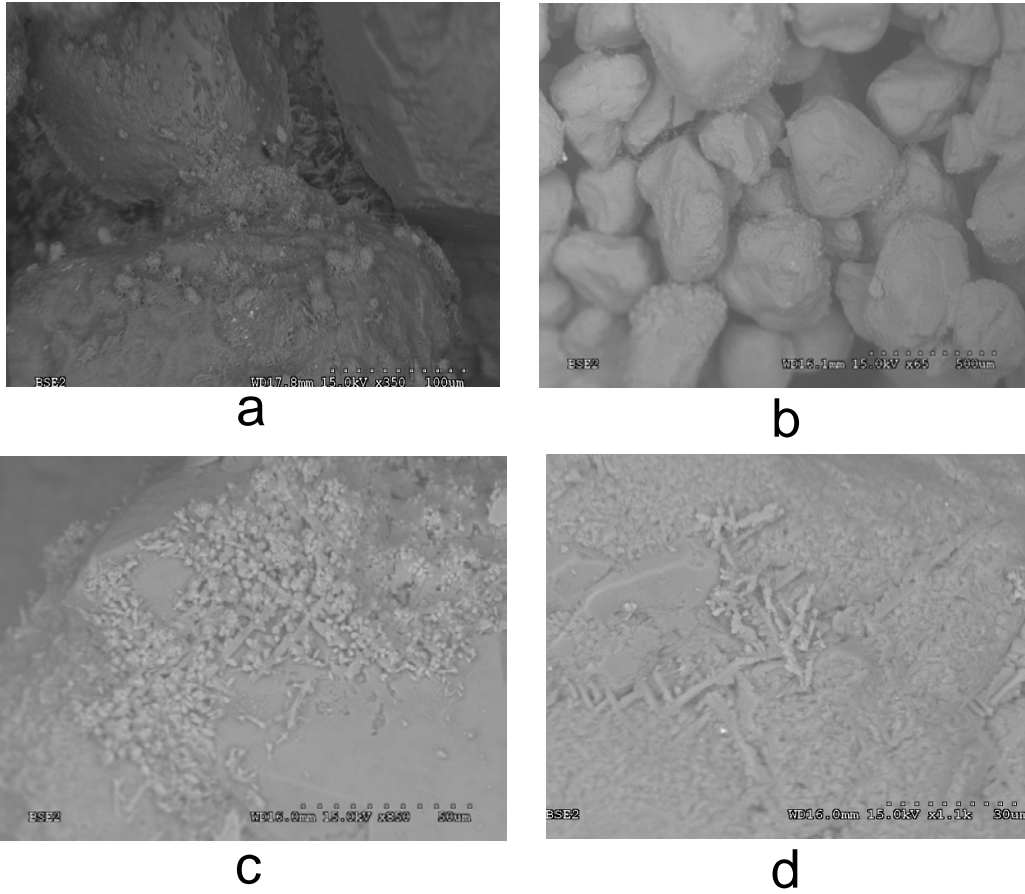
4.2.1 Before curing period

a

b

c

d

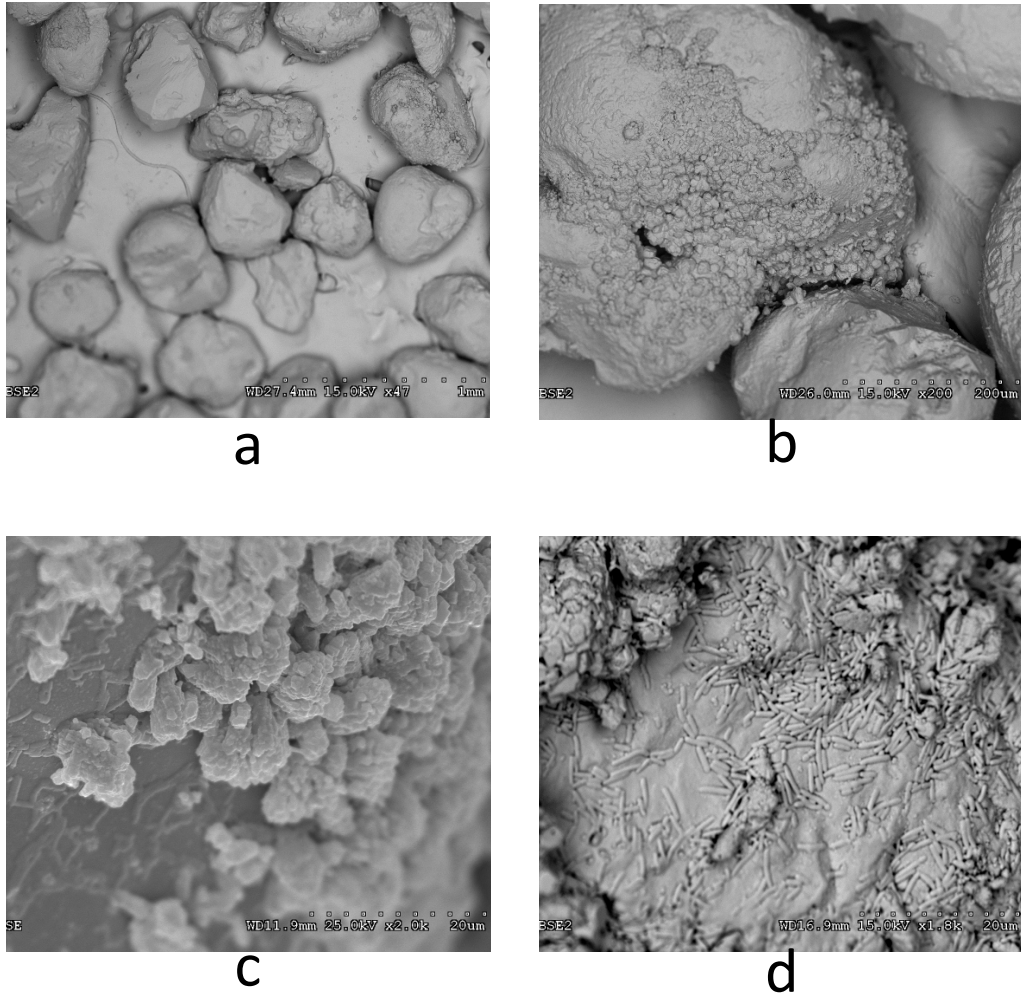Fig 4-2: Images of bio treated samples without curing a) 100µm b) 500µm c) 50µm d) 30µm

Fig 4-3: Images of bio treated sample after cured (4 days) (0.025M) a) calcite bonding between sand particles b) calcite precipitation over the sand particle c) Nucleation sites d) Bacterial traces

Chapter 5

Conclusions

MICP is a complex bio-chemical process that utilizes the urea hydrolysis that takes place between the sand particles for improvement of soil engineering properties. There is an increasing need for a ground development method, and one of the methods is to improve the strength of soil particles by utilizing the cementation technique. Even though there are various chemical methods available that are currently in practice, many of them have adverse environmental effects. This research objective is to have a better understanding of the effects of various parameters on the biological cementation and on achieving microbial cementation using soil microorganism *sporosarcina pasteurii* and a liquid growth medium containing urea and calcium chloride.

The result of these tests showed that all of the factors, such as the number of injections, bacterial O.D, concentrations of solutions, number of nutrient cycles, and particle sizes have an obvious effect on the MICP process and vary the relationship between the bacteria and urease. Even though an increase in the concentration of solutions and bacterial optical density increases the urease activity and precipitation rate, there remains the problem of getting uniform precipitation due to the accumulation of bacterial cells and clogging of chemicals on the top layer. The effect of particle size on cementation also gave an important conclusion that the particles should be neither very fine nor very coarse for a good cementation.

The SEM images clearly showed the bacterial cells within the soil matrix and the significant cementation that took place at the contact points between the sand particles. There was a clear void image for the untreated soil sample. These SEM images also proved that longer curing periods can result in better cementation, as the number of voids that are being filled up are higher when compared to the samples that are not cured.

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Based on the results of all the above described tests it, is recommended that the soil should be fine to medium coarse and that the concentrations of solutions should be lower, with a larger number of nutrient cycles, to achieve a successful and homogenous precipitation. The findings of this study indicate that the MICP is one of the best alternative ground improvement techniques due to its high efficiency and low cost within the geotechnical applications.

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