AN EXPERIMENTAL INVESTIGATION FOR IMPROVEMENT OF SANDS USING MICROBIALLY INDUCED CALCIUM CARBONATE PRECIPITATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
THE MIDDLE EAST TECHNICAL UNIVERSITY

BY

YILMAZ EMRE SARIÇİÇEK

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CIVIL ENGINEERING

SEPTEMBER 2016

Approval of the thesis:

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ABSTRACT

AN EXPERIMENTAL INVESTIGATION FOR IMPROVEMENT OF SANDS USING MICROBIALLY INDUCED CALCIUM CARBONATE PRECIPITATION

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September 2016, 113 pages

The development of innovative solutions to improve the engineering characteristics of sands becomes an emerging field of Geotechnical Engineering, as the environmental aspects, the economic feasibility and application practicability are concerned. In this scope, many improvement methods have been proposed to satisfy sustainability constraints, to reduce the overall the cost of construction and to be applied safely in the field. Among those, the use of biological means as derived from the nature gets popular which generally works through the process called Microbially Induced Calcium Carbonate Precipitation (MICP). Research studies indicate successful application of MICP based improvement using various types of bacteria and on several soils. Given the proven performance of MICP, this study aims to examine the MICP process thoroughly by comparing the calcium carbonate precipitation ability of widely studied bacteria, i.e., *Sporosarcina pasteurii* (ATCC 11859) and relatively under-recognized bacteria, i.e., *Bacillus licheniformis* (ATCC 14580) to outline the formation characteristics. For this purpose, two different sands

are tested for observing precipitation behavior using syringes and then with a series of unconfined compression tests. Furthermore, various factors such as relative density of sands, concentration of substances for nutrition of bacteria, treatment numbers and durations are examined. The improvement in the strength characteristics of these soils are also interpreted with the use of sophisticated techniques such as X-ray diffraction, scanning electron microscopy and energy dispersive X-ray spectroscopy. Successful results of this study highlight many questions regarding MICP application in sands. With that, this study opens new horizons for future works.

Keywords: Microbially Induced Calcium Carbonate Precipitation, *S. pasteurii*, *B. licheniformis*, Unconfined Compression Test

ÖZ

KUMLARIN İYİLEŞTİRİLMESİ AMAÇLI MİKROBİYOLOJİK TABANLI KALSİYUM KARBONAT ÇÖKELMESİNİN DENEYSEL ARAŞTIRMASI

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Eylül 2016, 113 sayfa

Geoteknik Mühendisliğinde, kumların iyileştirilmesi için, çevresel koşullar, ekonomik uygunluk ve uygulamadaki kolaylıklar düşünülerek önerilen yenilikçi yöntemler sürekli olarak gelişme göstermektedir. Bu bağlamda sürdürülebilirlik kısıtları göz önünde tutularak, maliyeti düşürmek ve sahada güvenle uygulamak amacıyla birçok yöntem önerilmiştir. Bunların arasında, biyoloji bilimine dayanan, Mikrobiyolojik Tabanlı Kalsiyum Karbonat Çökelmesi (MTKÇ) sonucu zemin iyileştirme, özgün bir yöntem olarak karşımıza çıkmaktadır. Yapılan araştırmalar MTKÇ tabanlı, çeşitli bakteriler ve zeminler kullanılarak yapılan birçok başarılı uygulamayı göstermektedir. Bu çalışma, başarısıyla bilinen MTKÇ yönteminin oluşum özelliklerini, daha önceden çok defa araştırılmış olan bir bakteri olan *Sporosarcina pasteurii* (ATCC 11859) ile nispeten oldukça az araştırılmış *Bacillus licheniformis* (ATCC 14580)'in kalsiyum karbonat çökeltme yetenekleri karşılaştırılarak, ortaya koymayı hedeflemektedir. Bu amaçla iki farklı kum, çökelme davranışını gözlemlemek adına şırıngalar kullanılarak ve sonrasında serbest basınç

deneyleri ile test edilmiştir. Çalışmada ayrıca kumların göreli sıkılıkları, bakteri besinlerinin madde yoğunlukları, besleme sayısı ve süresi gibi çeşitli değişkenler araştırılmıştır. Mukavemette görülen iyileşmeler aynı zamanda X-ışını saçılımı, taramalı elektron mikroskobu ve enerji dağılımı X-ışını spektroskopisi gibi ileri teknikler de kullanılarak araştırılmıştır. Elde edilen başarılı sonuçlar, MKTÇ'ye bağlı olarak ortaya çıkan birçok soruyu cevaplamıştır. Bununla beraber, bu çalışma, ileride yapılacak çalışmalar için yeni ufuklar açacaktır.

Anahtar Kelimeler: Mikrobiyolojik Tabanlı Kalsiyum Karbonat Çökelmesi, *S. pasteurii*, *B. licheniformis*, Serbest Basınç Deneyi

Dedicated to my family…

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor Asst. Prof. Dr. Onur Pekcan for his support, guidance, comments, patience and friendship throughout this study and graduate years. During the research, he has always encouraged me to "think differently".

I would like to express my gratitude to Prof. Dr. Ayşe Gül Gözen and Dr. Rafig Gurbanov for introducing me to the mysterious world of bacteria. I am indebted to Rafig for his efforts and patience in experiments and for his friendship. Also, members of Lab 255 are appreciated as I had the chance to work with. Bacteria provided by Assoc. Prof. Dr. Bülent İçgen are appreciated.

I would also like to thank Asst. Prof. Dr. Nabi Kartal Toker for his invaluable suggestions throughout my graduate years.

I am thankful to Yağızer Yalçın for his ideas and help in the study. I would like to thank Kamber Bilgen and Ulaş Nacar for their contributions to my experiments.

I have to express my gratitude to my office-mates Gizem Can and Batuhan Tezcan for all the memories we had in Office 102.

I am grateful to Ayfer and İbrahim Atabey for believing in me.

I wish to thank all my colleagues in AI2LAB for their support and understanding. Also, METU BAP and MERLAB are appreciated.

The financial support provided by The Scientific and Technological Research Council of Turkey (TÜBİTAK) through scholarship (2228-A) is appreciated.

I would like to specially thank Sinem Songür for her unconditional and never ending support, patience and understanding.

Finally, I owe my deepest gratitude to my mother Ferruha, my father Necmi, my sisters Ayşe and Elif and my brother Kürşad for their continuous support and belief.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
EDS	Energy-dispersive X-ray spectroscopy
MICP	Microbially Induced Calcium Carbonate Precipitation
OD_{600}	Optical Density at 600 nm wavelength
PPRC	Polypropylene Random Copolymer
RIR	Reference Intensity Ratio
SEM	Scanning Electron Microscopy
UCS	Unconfined Compressive Strength
XRD	X-ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 Overview

Improving the physical characteristics of ground by modifying its engineering properties has been a challenge for geotechnical engineers. The use of conventional methods such as direct application of cementitious materials, modified byproducts of factory waste, for instance, fly ash, marble dust, etc. has been the conventionally preferred methods when strengthening the soils. However, with the increase in the awareness of public to use environmentally friendly materials to keep the ecosystem healthy and therefore to contribute to the sustainability of nature, the use of biological agents started to spread as an alternative soil improvement method. Especially at the laboratory level, there has been continuous scientific progress, which also needs to be reflected in practice. Among the biological improvement agents as additive materials, Microbially Induced Calcium Carbonate Precipitation (MICP) and its applications in the field of Civil Engineering, is one of the most interesting research topics recently. The studies related to MICP, originate many challenging questions that can be solved using an interdisciplinary approach at the intersection of Biology and Engineering. It is not surprising that many researchers have directed their interests to this subject. Because not only it is promising for eliminating the possible drawbacks of improvement techniques, especially in terms of environmental issues, sustainability etc., but also the nature itself may provide the key substance, bacteria, in great quantities. The amount of organisms is as high as 10^9 to 10^{12} per 1 kilogram of soil around the ground level (Mitchell & Santamarina, 2005). The bacteria to be used for calcium carbonate precipitation can be found and isolated from lake bottoms, caves, soil etc.

A pioneering research related to microbially induced calcium carbonate precipitation indicate the complexity of the process (Stocks-Fischer et al., 1999). This study has become one of the milestones in the field together with a previous work (Ferris et al., 1996) in terms of the amounts and conditions of substances and a specific bacterium- *Sporosarcina pasteurii* (*S. pasteurii*) required for an efficient calcium carbonate precipitation by urea hydrolysis.

The progress of the studies in time has brought about diversity of bacteria. Different species of *Bacillus*, namely *S. pasteurii* (Chou et al., 2011; DeJong et al., 2006; Feng & Montoya, 2016; Ferris et al., 1996; Montoya & DeJong, 2015; Stocks-Fischer et al., 1999; Whiffin et al., 2007), *Bacillus cereus* (Han et al., 2013; Maheswaran et al., 2014), *Bacillus licheniformis* (*B. licheniformis*) (Vahabi, Ramezanianpour, Sharafi, et al., 2015; Vahabi, Ramezanianpour, & Noghabi, 2015), *Bacillus sphaericus* (De Muynck et al., 2010), *Bacillus subtilis* (Gat et al., 2014; Parks, 2009) and *Bacillus megaterium* (Soon et al., 2014) have attracted the interest of many researchers either for calcium carbonate precipitation or control/comparison of the precipitation abilities of bacteria. *Proteus vulgaris* (Whiffin, 2004) and *Idiomarina insulisalsae* (Oliveira et al., 2014) have also been subject to research studies. In successful attempts of ureolytic bacteria for calcium carbonate precipitation, the existence of urease enzyme, acting as a bio-catalyzer for urea hydrolysis, is the first step of the processes that eventually lead to calcium carbonate precipitation.

Research studies reveal various possible applications of microbially induced calcium carbonate precipitation for a wide range of engineering applications such as applicability of MICP in petroleum fields (Ferris et al., 1996), or in structural elements, for instance, concrete, mortar cracks (Achal, 2010; Kim et al., 2011), ornamental stone (De Muynck et al., 2010) and bricks (Sarda et al., 2009). A significant number of studies deal with major geotechnical problems. Research studies on permeability, settlement, bearing capacity and liquefaction have been among the subjects investigated recently. Researchers have utilized various laboratory techniques with an intention that for successful field applications verification by laboratory tests is a prerequisite. Popularity of the non-destructive testing methods and in situ measurements are promising for feasibility of the MICP

in the field. Computer aided modelling with numerical methods (Barkouki et al., 2011) and discrete elements (Feng et al., 2014) of the processes are also promising.

To generalize MICP for field conditions, the background of the process has to be understood clearly and conditions for the variables that affect the behavior have to be investigated carefully. For this purpose, factors such as the soil itself, bacteria, injection, medium and pH deserve careful attention. Sands and especially a standard silica sand, named Ottawa Sand (Karatas, 2008), constitute the important part of literature. Other defined sands from various countries have also been studied. Few studies regarding organic soil (Sidik et al., 2014), gravel (van Paassen et al., 2012) and tropical residual soil-silt (Soon et al., 2014) make a small fraction of the soil types within the literature related to MICP. All or some of the followings have been the primary issues in various studies: concentrations of the substances in the medium, flow rates and environmental factors (Al Qabany et al., 2012; Martinez et al., 2013; Mortensen et al., 2011).

1.2 Research Question & Objective

With the concerns of sustainable, economical and environmentally friendly ground improvement, this thesis focuses its attention on the usage and comparison of two species of *Bacillus*, namely *S. pasteurii* and *B. licheniformis* for MICP in the laboratory environment:

- to provide an idea about the MICP process and compare the calcium carbonate precipitation ability of *S. pasteurii* and *B. licheniformis* and observe its formation characteristics using sands in syringes,
- to demonstrate the increase in the strength of lab specimens and quantify the amount of improvement if possible by unconfined compression tests, and
- to further investigate the effects of parameters such as different bacteria, different sands, different concentrations of nutrients, relative density, number and duration of treatments.

1.3 Scope and Method

Understanding the concepts of MICP and making critical judgements about the improvement of soils treated with this microbiological method is not an easy task as too many factors are involved, which requires investigating various concepts from different fields such as Biology, Chemistry and Civil Engineering. Apart from many different possible mechanisms related to these fields, this study focuses on more concrete concepts. With that, the scope of this study is limited with the investigation on the effects of two different bacteria, on two different sands, prepared at different relative densities and tested in syringes and unconfined compression tests in the laboratory. In addition, for the interpretation of the results, the use of advanced characterization techniques such as X-ray diffraction, scanning electron microscopy and energy dispersive X-ray spectroscopy are kept in the scope.

1.4 Thesis Outline

Considering the statement of the problem, the objectives of the research and its scope are defined in this chapter, the rest of thesis is organized as follows:

- Chapter 2 provides the literature work related to MICP including its theoretical foundations, treatment methods, and the studies related to laboratory and field tests used to improve the soil samples through this technique.
- Chapter 3 introduces the small scale experiments in syringes for determining the calcium carbonate precipitation. Together with different bacteria and sand types, the effects of substance concentration are investigated in the chapter.
- Chapter 4 presents the laboratory experiments on sand specimens improved with the bacteria to determine the strength characteristics and quantify the amount of improvement from engineering point of view. The effect of various factors including bacteria, sand type, number and duration of treatments are also discussed in the chapter.

- Chapter 5 provides the summary, concludes the study and highlights some future directions related to laboratory experiments, the employed method and the means used to improve the soils microbiologically.

CHAPTER 2

MICROBIALLY INDUCED CALCIUM CARBONATE PRECIPITATION

The increasing world population together with urbanization and necessity for highways, railways, airports, dams resulted in growing construction industry. The problems due to insufficient ground conditions in the construction fields have made engineers invent and implement improvement techniques in years. Such a big construction industry, eventually, lead to a huge market of ground improvement techniques, the application of which may result in serious amount of budgets. For example, more than US$6 billion are being spent per year for over 40 000 ground improvement projects (DeJong et al., 2010).

Kirsch and Sondermann (2001) classified the ground improvement techniques currently in use in Table 2.1. Based on their types, these techniques serve for different purposes. Out of all these, cement-based improvement techniques require meticulous attention because of their possible negative effects on environment such as generation of large amounts of carbon dioxide in production phase (Park et al., 2014) or permanent soil pollution (Fauriel & Laloui, 2011). In addition, some of the substances used in grout injections were reported to be toxic and responsible for changing the pH level in subsurface (Fritzges et al., 2006).

The negative effects and concerns on environmental sustainability paved the way for new, efficient and more environmentally friendly techniques such as utilization of biological processes for ground improvement. Microorganisms can be manipulated for bio-aggregation of soil, bio-crusting, bio-coating, bio-clogging, bio-cementation, partial bio-desaturation of saturated soil, bio-encapsulation and bio-remediation (Stabnikov et al., 2015).

Throughout this chapter, the focus is on the literature studies regarding Microbially Induced Calcium Carbonate Precipitation, the backbone of thesis, and the tests on samples which were improved by this technique.

Table 2.1 Rough classification of ground improvement techniques (Kirsch & Sondermann, 2001)

Type	Technique
Compaction by static methods	Pre-loading
	Pre-loading with consolidation aid
	Compaction grouting
	Influencing ground water table
Compaction by dynamic methods	Vibro-compaction
	Compaction using vibratory hammers
	Dynamic compaction (drop weight)
	Compaction by blasting
	Air pulse compaction
Reinforcement with displacing effect	Vibro stone columns
	Sand compaction piles
	Lime/cement columns installed by displacing methods
Reinforcement without displacing effect	Mixed-in-place methods
	Jet grouting
	Permeation grouting
	Ground freezing

2.1 Microbially Induced Calcium Carbonate Precipitation

In the literature, there has been a growing number of studies regarding Microbially Induced Calcium Carbonate Precipitation especially for the last ten years. Depending on the scope of the studies, names such as Microbially Induced Calcite Precipitation (MICP), Enzyme Induced Carbonate Precipitation (EICP), Bio-Mediated Calcite Precipitation, Bio-Cementation and Plant-Induced Calcite Precipitation were adopted by various researchers. The usage of bacteria or urease enzyme, the type of calcium carbonate (calcite, vaterite or aragonite) result in different naming of the

processes which are indeed very similar. Calcite, vaterite and aragonite are polymorphs of calcium carbonate with different stabilities (Hamdan et al., 2011). In the following paragraphs and rest of the thesis, all of these studies are included under the umbrella of Microbially Induced Calcium Carbonate Precipitation (MICP).

The abundance of bacteria in the soil, their survival ability in harsh conditions such as high salinity, pH values from less than 2 to higher than 10, high pressure and temperature ranges from freezing to boiling make bacteria suitable for usage under various conditions (Mitchell & Santamarina, 2005).

In-situ microbial stimulation or injection of microbes to the ground together with necessary chemicals form the basics of bio-mediated soil improvement (DeJong et al., 2010). There are multiple possible first-step processes that eventually result in calcium carbonate precipitation. DeJong et al. (2010) stated these alternatives as urea hydrolysis (ureolysis), denitrification, iron reduction and sulfate reduction. Also they added that whenever there was urea, urea hydrolysis would become the dominating mechanism for the precipitation. Most commonly encountered process in the literature is urea hydrolysis. A relatively small number of studies investigated the denitrification (Hamdan et al., 2011; van Paassen et al., 2010).

Parks (2009) suggested some of the advantages of urea hydrolysis-based approach as low price of urea and existence of ureolytic bacteria abundance in soil. DeJong et al. (2010), by emphasizing an alkalophilic ureolytic bacterium, *S. pasteurii*, outlined the urea hydrolysis and calcium carbonate precipitation, respectively (Figure 2.1). According to the researchers the reactions take place as follows:

- The bacterium *S. pasteurii* consumes urea ($CO(NH_2)_2$) by using urease enzyme inside the cell and as a result ammonia (NH_3) and carbon dioxide (CO_2) form (Equation 2.1),

$$CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2 \quad (2.1)$$

- Following diffusion of ammonia and carbon dioxide to the surrounding medium, in the presence of water, conversion of ammonia to ammonium (NH_4^+) and carbon dioxide to bicarbonate ions make up the second stage of chain (Equations 2.2 and 2.3),

$$2NH_3 + 2H_2O \rightarrow 2NH_4^+ + 2OH^- \quad (2.2)$$

$$CO_2 + OH^- \rightarrow HCO_3^- \quad (2.3)$$

- OH^- ions from the conversion of ammonia to ammonium lead to alkaline environment and bicarbonate formation,
- Eventually, the calcite is formed by using the calcium ions (Ca^{2+}) in the medium and bicarbonate (Equation 2.4).

$$Ca^{2+} + HCO_3^- + OH^- \rightarrow CaCO_3 + H_2O \quad (2.4)$$

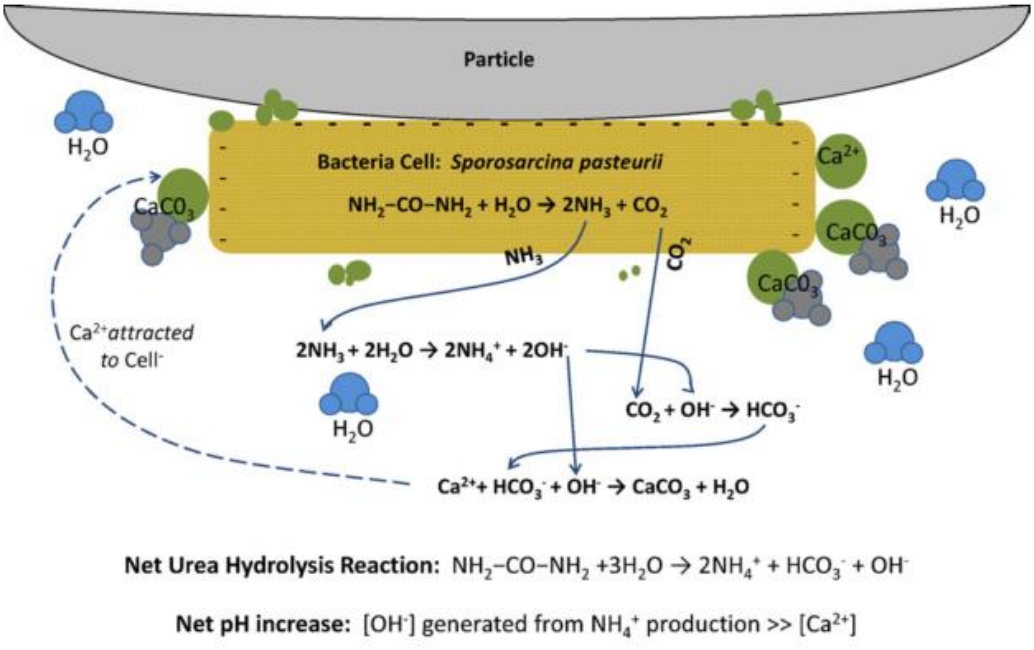


Figure 2.1 Bio-mediated calcite precipitation using ureolysis

(DeJong et al., 2010)

The release of OH^- ions results in increase of pH and this at the end creates supersaturated conditions that is required for calcium carbonate precipitation (DeJong et al., 2010).

Additionally, almost all bacterial surfaces have electronegativity (Schultze-Lam et al., 1996). This is crucial for attracting calcium ions to the cell wall.

Also, van Paassen (2009) stated the functions of microorganisms as carbonate production, alkalinization and acting as nucleation sites.

Hammes and Verstraete (2002) addressed the important factors behind calcium carbonate precipitation as the calcium and dissolved inorganic carbon concentration, pH and the nucleation site availability.

Stocks-Fischer et al. (1999) pointed out the complex nature of MICP compared to simpler chemically induced calcium carbonate precipitation. For comparison, chemically induced calcium carbonate precipitation, by alternating pH value, was carried out by using water and urea-$CaCl_2$ medium without any microorganism. At pH 9, higher amount of Ca^{2+} precipitated microbiologically than chemically.

2.1.1 Bacteria Types and Applications

Most of the studies in the literature dealt with the bacterium *S. pasteurii* (Chou et al., 2011; DeJong et al., 2006; Feng & Montoya, 2016; Ferris et al., 1996; Montoya & DeJong, 2015; Stocks-Fischer et al., 1999; Whiffin et al., 2007) and many of them investigated the success of this bacterium in the soils. However, neither the bacteria are limited to *S. pasteurii* nor the applications are only on soils. There are many bacteria, isolated from soil, water and sewage that can be used for urea hydrolysis. Besides, studies dealing with urease enzyme itself, which catalyzes the urea hydrolysis, and use of ureolytic and non-ureolytic bacteria for comparison of calcium carbonate precipitation ability exist in the literature.

Whiffin (2004) compared the suitability of many bacteria in terms of bio-cementation. Together with environmental concerns and limitations, two bacteria, namely *S. pasteurii* and *Proteus vulgaris (P. vulgaris),* were investigated in detail.

Accordingly, *S. pasteurii* met the criteria such as high urease activity, economical favorability and compatibility in non-sterile conditions, better than *P. vulgaris*. Also the study pointed out that the urease enzyme of *S. pasteurii* was active and stable at 4°C up to 25 days.

DeJong et al. (2006) stated one of the advantages of *Bacillus pasteurii* (*S. pasteurii* was formerly named as *Bacillus pasteurii*) as their tendency of not to aggregate. This provides higher cell surface to volume ratio which is important for efficiency of cementation.

B. licheniformis is widespread bacterium in the environment and used for agricultural and commercial uses (Rey et al., 2004). Gaiero (2014) cited two works in the thesis, while one did not find urease genes in *B. licheniformis*, the later one described an isolate of bacterium as ureolytic. Other studies comparing the genome of different *B. licheniformis* strains exist in the literature (Dhakal et al., 2014). Vahabi et al. (2015) worked on *B. licheniformis* AK01 strain and compared its performance with some other bacteria. Satisfying results were obtained as the strain was more effective than *S. pasteurii* DSM-33 for calcium carbonate precipitation in the study.

Bacillus sphaericus (De Muynck et al., 2010), *Bacillus megaterium* (Soon et al., 2014), *Idomarina insulisalsae* (Oliveira et al., 2014) are other microorganisms mentioned in the literature.

The use of ureolytic and non-ureolytic bacteria together also attracted the attention of researchers. Gat et al. (2014) searched for the effects of *S. pasteurii* (ureolytic) and *Bacillus subtilis* (*B. subtilis*, non-ureolytic) on the MICP process. In their findings, they concluded that the existence of *B. subtilis* in addition to *S. pasteurii* resulted in acceleration in the process by acting as extra nucleation sites.

Utilization of bacteria under different conditions is also within the scope of research studies. Chou et al. (2011) were interested in the effect of *S. pasteurii* state, namely growing, resting or dead cells, on the improvement of soils. Growing cell conditions performed much better considering the minor improvements that could be attributed to resting or dead cells. Yasuhara et al. (2012) used urease enzyme without bacteria claiming more straightforward application. Kavazanjian and Hamdan (2015) also

worked with urease enzyme excluding bacteria indicating that the possible limitations related to microbiological processes were eliminated.

The nature provides many strains of bacteria that can be utilized for microbially induced calcium carbonate precipitation. Studies of Kim et al. (2011) and Liet al. (2011) indicate the possible usage of newly identified bacteria in the nature.

As for possible applications of MICP, many studies were published in various fields. For instance, Ferris et al. (1996) investigated the usage of this method in petroleum fields for plugging zones against water permeability. Studies on strength of treated concrete samples, mortars etc. were also performed by researchers (Achal et al., 2011; Kim et al., 2011). Ornamental stone protection (De Muynck et al., 2010) and improvement of properties of brick strength (Sarda et al., 2009) are other examples of applications.

MICP opens the door to ground improvement for soils and foundations. Major geotechnical problems such as high permeability, settlement, bearing capacity insufficiency, liquefaction etc. have been investigated in years with increasing number in addition to concerns like dust control (Meyer et al., 2011), wind erosion (Anderson et al., 2014) and soil internal erosion (Jiang et al., 2014). At that point, the importance of soil compatibility comes into the picture, which is discussed in the next section.

2.1.2 Soil Compatibility

Bacteria show variations in their shapes: Spherical, rod-like or spiral. The diameters of the cells are usually within 0.5 to 3 μm. One of the main factors that affect the movement of bacteria inside the soil pore is the narrow pore throats. Also, electrical interactions, surface roughness and fluid chemistry affect the movement and attachment of the bacteria (Mitchell & Santamarina, 2005). Depending on the smaller soil particles, the pore throats form the lower limit of the applications (DeJong et al., 2010).

Most of the studies in the literature mentioned successful treatments in sands, especially on Ottawa Sand, a standard silica sand (Karatas, 2008).

Rebata-Landa (2007) studied the effect of soil geometry in bio-cementation. Kaolinite, silt, fine sand, glass beads in various sizes, coarse sand and fine gravel were tested. Based on the results, kaolinite sample did not show cementation and the coarse grains were de-bonded. The author suggested that the results in the very fine soil arose from inactivity of bacteria and insufficient nutrient percolation. The failure in coarse soil was due to inadequacy of cementation for large grains (Figure 2.2). The finding related to fine soil is similar to the lower limit suggested by DeJong et al. (2010).

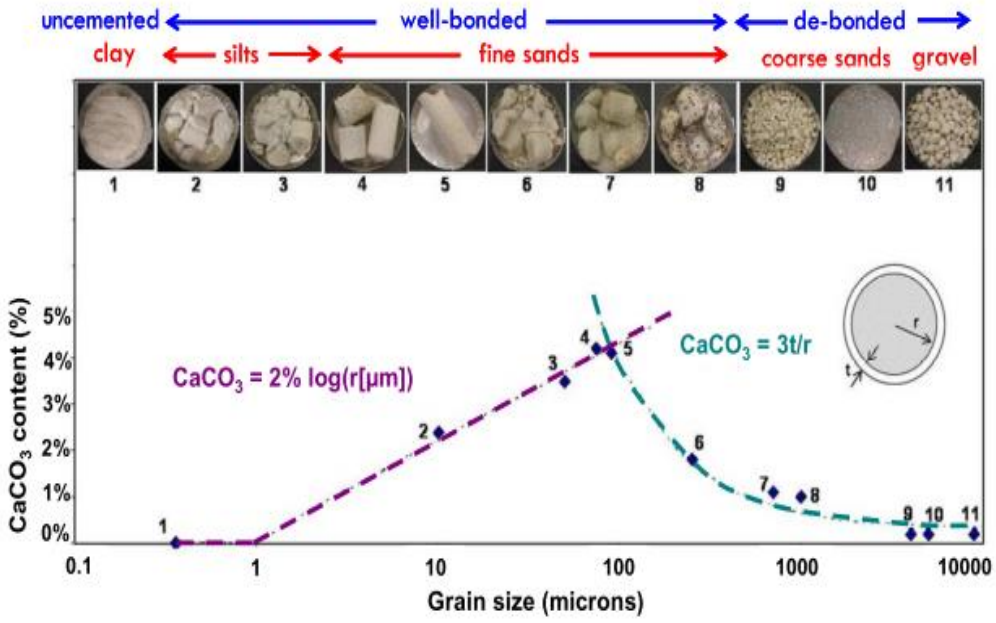


Figure 2.2 $CaCO_3$ content and models as a function of the grain size

(Rebata-Landa, 2007)

Soon et al. (2014) studied the suitable conditions for improvement of a tropical residual soil which was classified as silt (ML). In the end, they obtained significant values in terms of strength.

van Paassen et al. (2012) executed tests on gravel and pointed out that shear strength of gravel could be increased though homogeneity problem and low levels of cementation were obtained.

An organic soil classified as peat was subject to experiments in the research of Sidik et al. (2014). They pointed out the possibility of $CaCO_3$ precipitation in organic soils. Also 10-fold reduction of coefficient of permeability after bio cementation was reported.

Kavazanjian and Hamdan (2015) proposed possible usage of urease enzyme without bacteria when geometrical limitations were encountered thanks to smaller size of enzyme and its solubility in water.

The effect of different mineralogy of the soils also attracted researchers. Mortensen et al. (2011) worked on sands which had high amounts of silica, calcite, feldspar and iron oxide. Eventually, they stated that different mineralogies did not show suppressing effect on processes. Besides, because of the possible direct effect of mineralogy on thermodynamics of reactions in the system, some soils rich in certain minerals could favor precipitation more. As an example, soils that are already calcium carbonate rich could provide ideal areas for new precipitation.

2.1.3 Treatment Media and Factors Affecting Efficiency

As can be inferred from Equations 2.1 to 2.4, the treatment media consist of urea ($CO(NH_2)_2$) and calcium source mainly. In the studies related to urea hydrolysis, urea and calcium source are provided to the bacteria. Additionally, nutrient broth (growth media for microorganisms), ammonium chloride (NH_4Cl) and sodium bicarbonate ($NaHCO_3$) are other substances that were frequently used in the experiments. Sodium bicarbonate and ammonium chloride, with the required concentrations, in the medium were meaningful in terms of pH stabilization of a medium (Al Qabany & Soga, 2013).

The amounts of substances used in a medium, treatment durations, injection times of bacteria and nutrients showed variation in the studies. As one of the pioneering

researchers, Stocks-Fischer et al. (1999) used 3 grams of nutrient broth, 10 grams of ammonium chloride (187 mM) and 2.12 grams of sodium bicarbonate (25.2 mM) in addition to 20 grams of urea (333 mM) per 1 liter of glass distilled water. The amount of $CaCl_2$ to be added to the medium, was selected as 25.2 mM in soil column tests to have conditions similar to those in the nature.

Many researchers adopted the quantities suggested by Stocks-Fischer et al. (1999). Al Qabany and Soga (2013) investigated the calcium carbonate precipitation on sand samples treated by equimolar urea-calcium chloride solutions at different concentrations (0.1, 0.25, 0.5 and 1 M). Lower chemical concentrations led to stronger samples.

Rebata-Landa (2007) carried out experiments on sand mixed with bacteria inside the syringes. Refreshing the urea-$CaCl_2$ media (Table 2.2) in every 4 days, treatments, up to 64 days, were added to the syringes in 5 combinations. In each application a pore volume of nutrients was introduced. While the first three combinations yielded minor amounts of $CaCO_3$ concentrations, samples 4 and 5 had more calcium carbonates with sample 5 having the highest amount of $CaCO_3$ precipitated (Figure 2.3).

Table 2.2 Urea and $CaCl_2$ amounts per liter of solution in the different nutrient combinations (Rebata-Landa, 2007)

Nutrient combination	Urea (g)	$CaCl_2$ (g)
#1	20	1.4
#2	20	2.8
#3	20	5.6
#4	10	25
#5	15	37.5

While Rebata-Landa (2007) injected nutrients in 4 days intervals, some other researchers adopted smaller intervals between the injections. Considering the importance of speeding up the processes in the field, faster applications gained value.



Figure 2.3 Effects of time and nutrient type on cementation

(Rebata-Landa, 2007)

Feng and Montoya (2016) performed triaxial shear strength tests on soils treated with chemical injections, the recipe of which was given in Table 2.3. Accordingly, after waiting for a certain time for attachment of bacteria to the soil, for the case of light cementation, 10 injections of cementation medium were applied. For moderate cementation 20 injections and for the heavy cementation 40 injections were done with constant intervals between each treatment. For injections of bacteria and nutrients to the soil, a peristaltic pump with adjustable constant flow rate was used.

Table 2.3 Injection Media Recipe (Feng & Montoya, 2016)

Chemical Name	Biological Injection Concentrations (mM)	Cementation Injection Concentrations (mM)
Urea	333	333
Ammonium Chloride	374	374
Calcium Chloride	0	50

Ozdogan (2010) adopted the components of bacterial treatment as in Stocks-Fischer et al. (1999). The first cementation injection following the bacterial injection was performed 4 hours later and the subsequent cementation injections were performed in every 2 hours for 3-4 days.

The findings of the experiments by Martinez et al. (2013) indicated that injection of bacteria ($7*10^5$ cells/ml) at a rate of 10 ml/min for 1.5 pore volumes and 6 hours waiting time before the next injection was the optimum application for uniformity. Also, urea to calcium ratio was suggested to be greater than 1. Finally, stopped flow injection (waiting times between subsequent treatments) was found to be more effective than continuous injection. In the case of continuous injection, plugging of the columns could occur.

Previously, Stocks-Fischer et al. (1999) mentioned the complexity of MICP. In addition to concentrations of substances in recipes, treatment durations, waiting times, the activity of urease enzyme, amount of bacteria, temperature, biotic conditions, calcium sources and pH can have regulatory effects in the efficiency of microbiological process.

Whiffin (2004) stated that the rate of urea hydrolysis was affected mainly by the concentrations of urea and calcium. The urease enzyme had an optimum working temperature of 70°C. Between 15 and 25°C, the activity was stable. The enzyme could work up to concentrations of 3 M for urea and 2 M for calcium. Also, the author mentioned the activating effect of increasing urea concentration and inhibiting effect of increasing calcium source concentration up to certain levels, on urease activity. Mortensen et al. (2011) showed that ammonium concentration in their study

did not have effect on urease activity. Additionally, they concluded that higher concentrations of ammonium chloride could be utilized to delay the precipitation, which leads to more uniform distribution.

Martinez et al. (2013) stated that in a lower pH medium, the urea hydrolysis was slower compared to higher pH. In their experiments Stocks-Fischer et al. (1999) found out that the microbiologically induced calcium carbonate precipitation started at pH 8.3±1.0 and completed at pH 9.0.

The urea hydrolysis owes its importance to be a controlling factor also in terms of morphology. According to van Paassen (2009), while at high hydrolysis rates, spherical vaterite crystals were common, the occurrence of rhomboidal calcite were more possible at low rates. Beside many regulatory effects, the author showed that the faster injection rates of bacteria resulted in less attachment to the soil.

Apart from calcium chloride, other calcium sources were also subject to research. Cheng et al. (2014) obtained successful results in terms of sand strength by using the seawater as a calcium source and suggested that this could open the way for applications, especially in marine environments. Park et al. (2014) used calcium chloride, calcium hydroxide and calcium nitrate as calcium sources. They found out that calcium chloride was more effective than the others since its solubility was almost 100 times greater than the others. So, more ions were provided in the water by calcium chloride.

In summary, a researcher should think carefully on the factors affecting the calcium carbonate precipitation. Optimization of different factors would be beneficial for the most efficient ways of increasing durability.

2.2 Testing Microbially Treated Soils

For the occurrence of calcium carbonate precipitation to be meaningful, the results of tests on soils should have satisfying results. There are many studies in the literature that suggest ways to measure the success of MICP. In the following parts, the

information regarding monitoring calcium carbonate occurrence and strength tests on treated sands are presented.

2.2.1 Monitoring Calcium Carbonate Occurrence

Scanning electron microscopy (SEM) is one of the most widely referred tools by the researchers in this field. This powerful tool enables visualization of calcium carbonate particles. The arrangement of particles, crystal shapes of calcium carbonate and even bacteria can be monitored at different magnifications. SEM images presented by Lin et al. (2015) on a treated soil reveal good examples of occurrences like contact cementing, surface cementing and different morphologies (Figure 2.4). Additionally, energy dispersive X-ray spectroscopy (EDS) gives results in element basis. An example of EDS mapping of silicon and calcium elements were provided by the same researchers (Figure 2.5). Throughout the experiments silica sands soaked in acid were used.

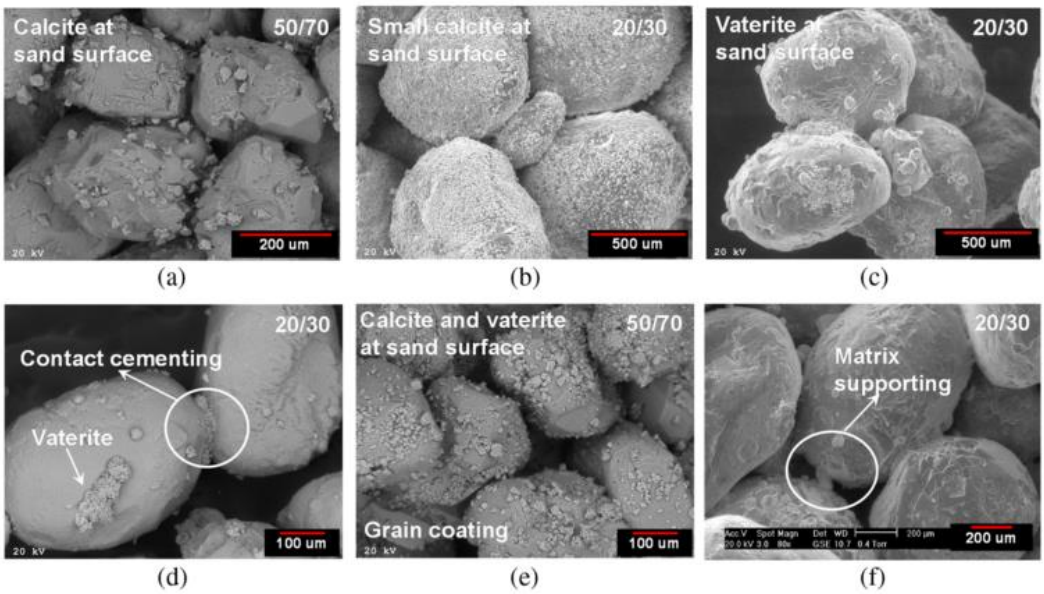


Figure 2.4 SEM Images with $CaCO_3$ morphologies and distributions

(Lin et al., 2015)

X-ray diffraction (XRD) is another tool that can be used for detection of crystalline material. For XRD scanning, the samples are prepared in powdered form in small amounts. As for the SEM images, amount of the samples that could be tested is also very limited. Monitoring the whole sample may require many small parts of samples to be tested or the use of other techniques.

Non-destructive testing by shear wave velocity measurement was also used in the determination of level of cementation in the samples. The bender elements were implemented in the sand sample in the study of Montoya and DeJong (2015). Based on the shear wave velocity measurements, they inferred the level of cementation. The values of shear wave velocities corresponding to cementation levels were used as a reference (Figure 2.6).

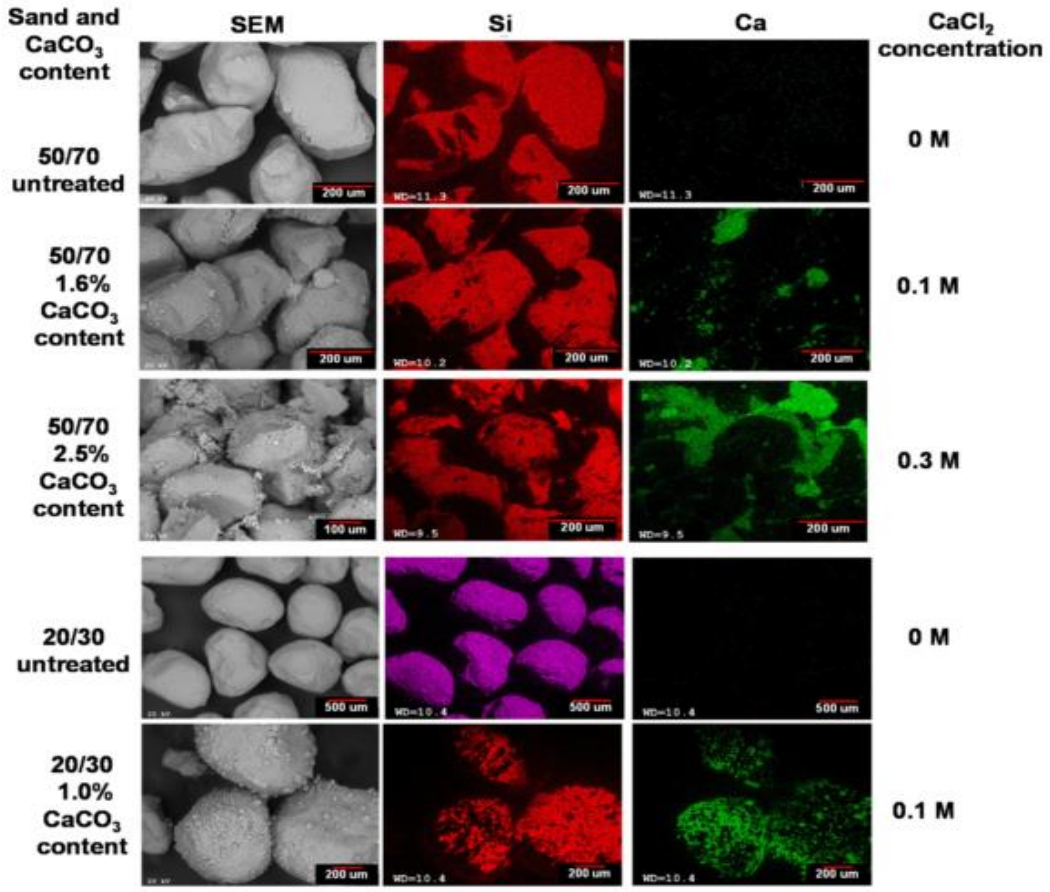


Figure 2.5 SEM and EDS mapping images of untreated and treated sands (Lin et al., 2015)

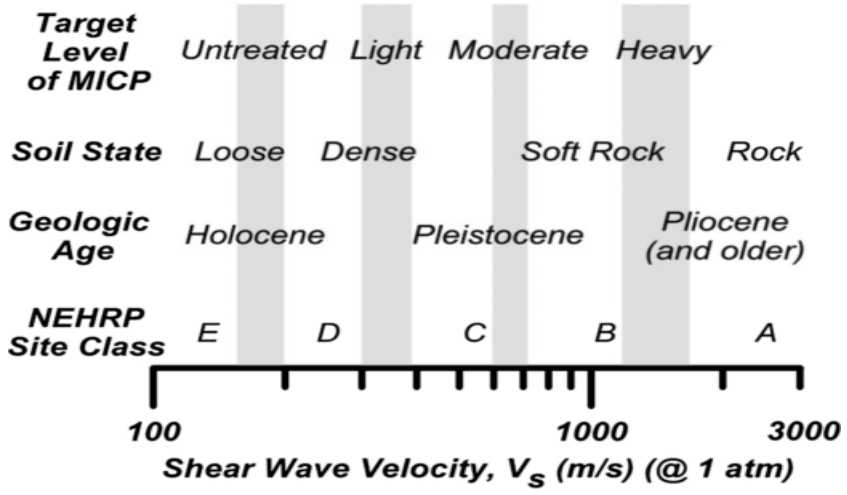


Figure 2.6 Target MICP cementation level & shear wave velocities

(Montoya & DeJong, 2015)

Similarly, in the large scale study of van Paassen et al. (2010b) geophones to transmit data were implemented in the sand container.

Another common method is the washing of samples with hydrochloric acid (HCl). Different researchers used different molarities of acid, yet the main purpose in all such studies is to detect amount of $CaCO_3$ quantity that were present in the samples. Together with some other cleaning operations, the mass difference between the soils before and after HCl washing indicated the mass of calcium carbonates in the soil. (Feng & Montoya, 2014).

2.2.2 Strength Tests on Microbially Treated Soils

From engineering point of view, the strength of treated sand samples is important in measuring the level of success. In addition to achieving calcium carbonate precipitation, the behavior of the treated soil when exposed to loading needs to be considered.

The grain contacts of particles provide nutrients for bacteria in greater quantities compared to the other surfaces of particles and this leads to the preference of bacteria

to settle at these points more (DeJong et al., 2010). An engineer certainly prefers to have stronger particle to particle contacts. However, expecting all precipitations to occur at the contact points is far from realistic. Because bacteria tend to settle more at contacts, thinking of an equal distribution of calcium carbonate all around the particle surfaces is also another issue that does not reflect the real conditions. In Figure 2.7, the possible distribution of calcium carbonate is shown (DeJong et al., 2010).

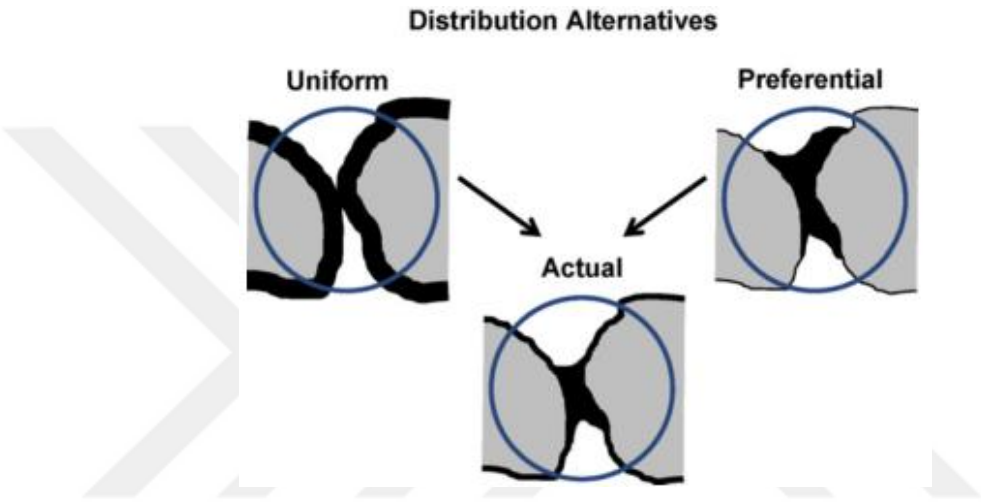


Figure 2.7 Distribution alternatives of calcium carbonate (DeJong et al., 2010)

Precipitation of the calcium carbonate once at the required points would provide the necessary conditions in the long term. Because, the dissolution of calcium carbonate is generated in the scenarios that an acidification occurs in the system or acidic groundwater penetrates constantly (van Paassen et al., 2010). In the micro scale, Lin et al. (2014) investigated the tensile and shear strength of calcite that precipitated between two glass beads by microbially induced precipitation. They measured the shear strength and tensile strength of the bond as 616.5 kPa and 41.1 kPa, respectively.

For numerical quantification of improvement and to reflect the engineering characteristics of treated specimens, triaxial compression tests (Feng & Montoya, 2016; Lin et al., 2015; Montoya & DeJong, 2015; Ozdogan, 2010), direct shear tests

(Chou et al., 2011; van Paassen et al., 2012), california bearing ratio (CBR) tests (Chou et al., 2011) and cyclic triaxial shear tests (Burbank et al., 2013) on treated soils exist in the literature. Also, a research by Feng et al. (2014) dealt with implementation of Discrete Element Method (DEM) for working on micromechanics of a treated soil under different loading/unloading stress paths. The details related to unconfined compression tests in the literature are examined next in detail.

van Paassen et al. (2010b) applied treatment methods in a relatively greater area. A 100 m^3 container was filled with Itterback sand and treatments (5 m^3 bacteria, 96 m^3 urea – CaCl2 medium) were provided via 3 injection wells for 16 days. For drainage, 3 wells were provided on the opposite side. For flow field, the levels of injection and drainage wells were arranged. Following the removal of the loose sand after treatments, approximately 40 m^3 of cemented soil was obtained. It was indicated that the flow field had shaped the cemented body (Figure 2.8). Tests in the body showed improvement in stiffness of sand.

Laboratory tests are the prerequisites of successful applications in the field. However, these tests do not ensure the desired results in the field all the time. Therefore, the test results have to be confirmed using field applications.



Figure 2.8 Cementation in a large scale experiment (van Paassen et al., 2010b)

2.2.2.1 Review on Unconfined Compression Tests

The second part of experimental applications in this thesis deal with Unconfined Compressive Strength (UCS) values of the treated samples. Hence, a greater portion of the strength tests presented here is devoted to the applications and results of unconfined compression tests.

In the tests of van Paassen et al. (2010b) samples taken from certain points were also subject to unconfined compression tests. Values within the range of 0.7 and 12.4 MPa were obtained. The amount of calcium carbonates ranged from 12.6% to 27.3%.

Oliveira et al. (2014) conducted tests on poorly graded sand with some minor amounts of gravel and silt. They mixed treatment medium, bacteria with sand in PVC molds with 70 mm diameter/140 mm height and left for curing for certain times. Then, the samples were trimmed and tested with a strain rate of 1%/min. Based on the experiment results (Figure 2.9), they could compare the effect of two different bacteria and concluded that relatively smaller values in UCS values could be attributed to pH value (8.8) of the soil and temperature (20±2°C).

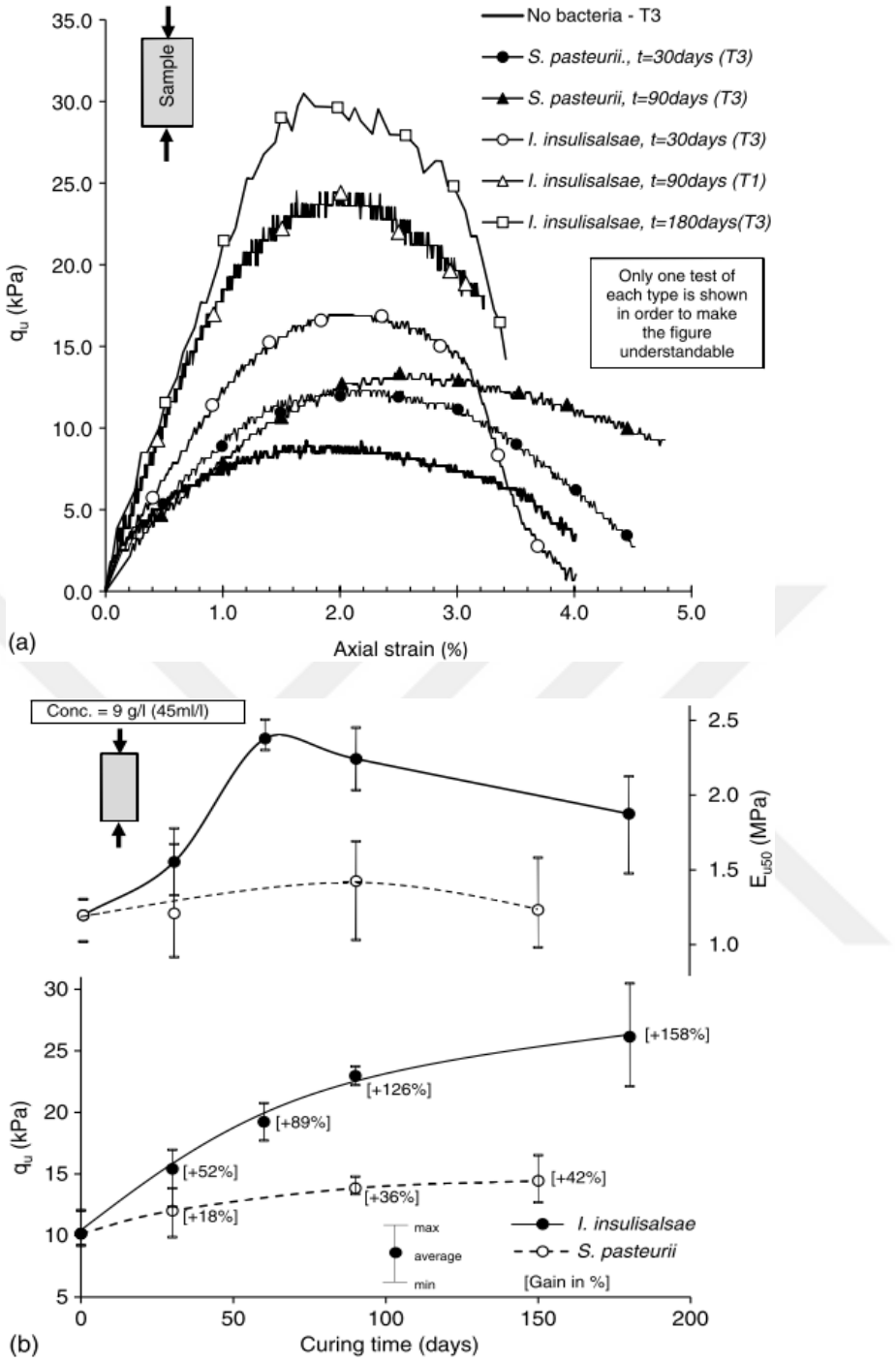


Figure 2.9 UCS test results (a) Stress-strain curves, (b) q_u and E_{50} values with curing time (Oliveira et al., 2014)

In the experiments of Cheng et al. (2014), where seawater was used as calcium source, the results of unconfined compression tests on oven dried samples (50°C)

had a maximum value of 300 kPa. Seawater had 10 mM calcium ions and during preparation equal amounts of urea was added. In order to reach to a value of 300 kPa, approximately 200 flushes of treatments were required. The comparison of these tests were done with cementation solutions which had 1 M urea and 1 M $CaCl_2$ (Figure 2.10).

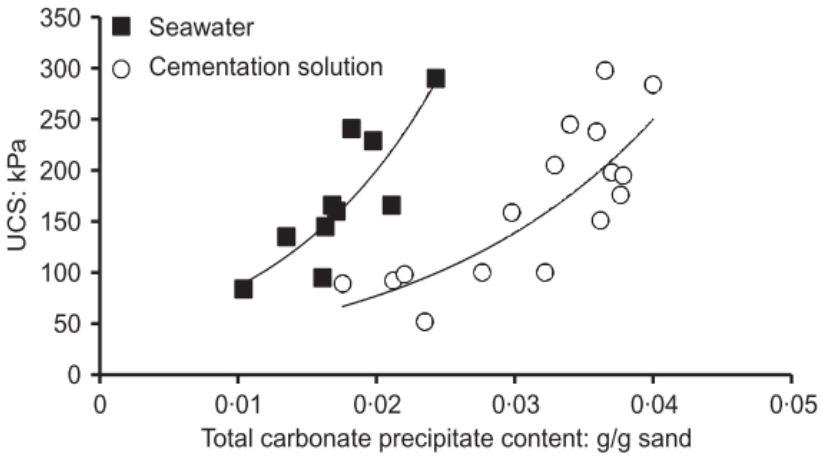


Figure 2.10 UCS strength of sand samples treated with seawater and high-concentration cementation solution (Cheng et al., 2014)

The experiments carried out by Al Qabany and Soga (2013) have given clues about the importance of molarities of the substances. 0.1 M, 0.25 M, 0.5 M and 1 M urea – calcium chloride solutions were used for sample dimensions with diameters of 35.4 mm, 100 mm, and heights of 100 mm and 250 mm respectively. The samples were prepared inside cells with 20 kPa pressure for confinement. 60 oven dried samples were tested with a strain rate of 1.14 mm/min. In the end, solutions with lower concentrations provided the most homogenous and strongest samples. Many samples treated with 1 M concentrations revealed null strength because of homogeneity problems. Additionally, they concluded that with the exception of a few samples, the strength increase was more in dense samples than in loose samples. Test results and their comparison with the study by van Paassen (2009) were provided by the authors (Figure 2.11 and 2.12) although different setups were adopted in these two studies.

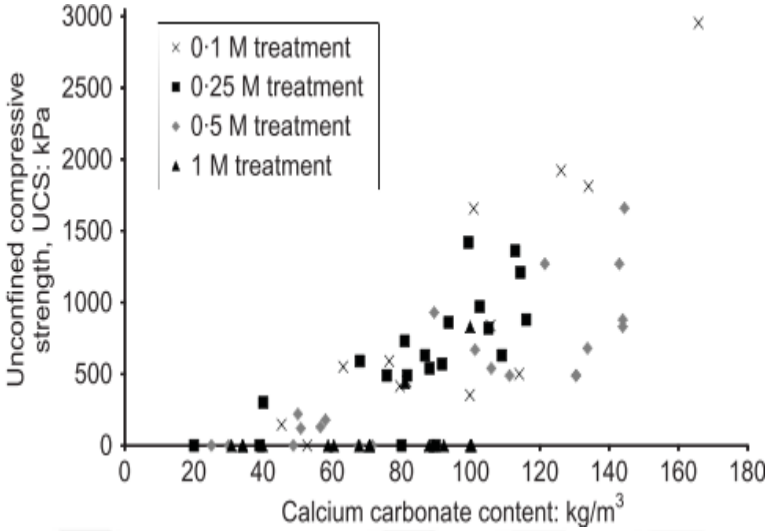


Figure 2.11 UCS test results for various treatments

(Al Qabany & Soga, 2013)

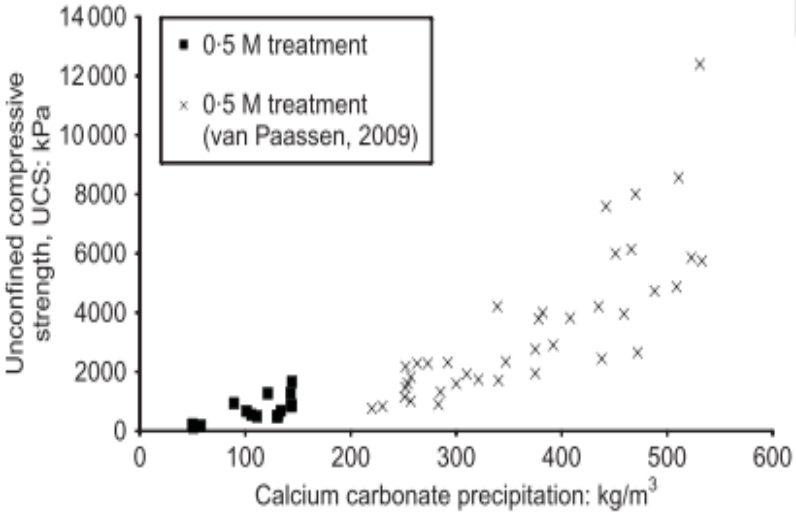


Figure 2.12 Comparison of test results with van Paassen (2009)

(Al Qabany & Soga, 2013)

Rowshanbakht et al. (2016) prepared silica sand columns with dimensions of 36 mm diameter and 200 mm height at different densities. Equimolar calcium chloride and urea injections were applied for 3 times in every 24 hours. Samples taken from top and bottom of the columns were tested and for greater proportion of the tests, the samples from the top, which was far from the injection point, had greater strength values. The authors thought that this had resulted from more homogeneity at the top in terms of calcite. Additionally, the denser the soils, the greater the UCS values were obtained (Figure 2.13). Treated samples had UCS values from 100 to 500 kPa.

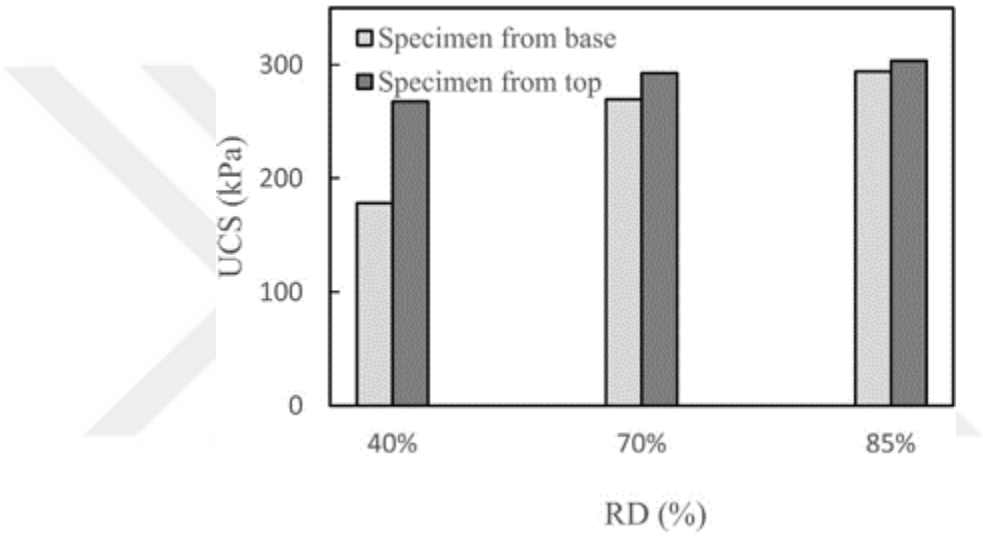


Figure 2.13 Relative density effect on UCS values (Rowshanbakht et al., 2016)

OD_{600} is basically a value that measures the absorbance of light of 600 nm wavelength which is indicator of amount of cells (van Paassen, 2009). Many studies in the literature adopted OD_{600} values within the range of 0.8 and 1.2 (Lin et al., 2014; Montoya et al., 2013; Montoya & DeJong, 2015; Al Qabany et al., 2012). Some smaller or higher OD_{600} values based on the purpose of usage were mentioned in other studies (Rong et al., 2012; Warren et al., 2001). Bacterial dilution could be applied whenever necessary for adjusting density. Rong et al. (2012) acquired UCS values up to 6.1 MPa. In their study, bacteria culture had an OD_{600} value of 2.1 and

2 M calcium solution was used. After 7 days of treatment, the samples were oven dried at 60°C. The failure mode of some sand samples are given in Figure 2.14.

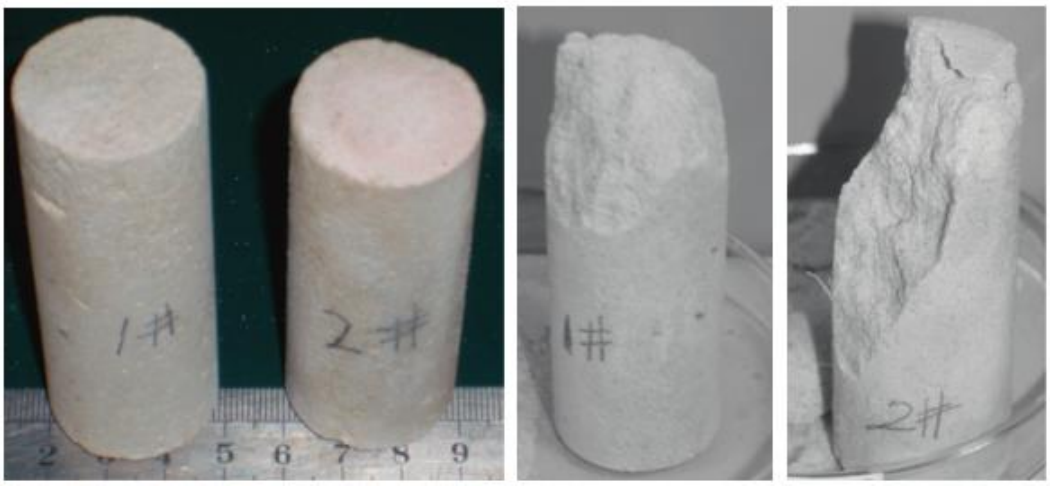


Figure 2.14 Failure mode of sand (Rong et al., 2012)

Montoya et al. (2013) had experiments performed on unsaturated sands. 1 M urea and 0.25 M calcium chloride were flushed for 40 times in 3 to 6 hour intervals in free-draining conditions. Out of 4 sand columns, two of them were feasible to be tested. Those two columns had UCS values of around 5 kPa.

Yasuhara et al. (2012) made use of urease enzyme without bacteria in the experiments. Mixing enzyme with Toyoura sand was followed by equimolar urea – calcium chloride injection while the sample was exposed to 50 kPa confining pressure. Subsequent treatments (4 or 8 times) were applied in every 2 hours. In the end, UCS values of 435 to 1620 kPa were obtained on dry samples.

In a study by Neupane et al. (2015), sand specimen was prepared inside a steel drum with 57 cm by 60 cm. Equimolar urea and calcium chloride together with urease enzyme (no bacteria) were injected from the center of the specimen with an injection tube. In total, 60 liters of medium was injected at two stages. Then, blocks with 3 cm diameter and 6 cm height were extracted for testing. Accordingly, UCS values up to 380 kPa were obtained.

The study by Kavazanjian and Hamdan (2015) is valuable to show the feasibility of testing samples which were prepared as mixed and injected columns. The injected columns showed greater cementation in the vicinity of injection tube. However, the samples could not be tested. Mixed column sands had better results in terms of feasibility.

Park et al. (2014) had used different calcium sources in their experiments by using plant extract mixed with soil. The test results indicated UCS values of 30 to 317 kPa. Air dried specimens with 5 cm diameter and 10 cm height were sheared by 1%/min axial strain.

The study of Li et al. (2015) indicated that UCS values of treated samples could be further increased by adding fibers to the samples.

Shahrokhi-Shahraki et al. (2014) had their experiments in single stage. After injection of bacteria, waiting for retention time and injection of urea-calcium chloride was done. Curing the samples for 21 days, the samples, which were enclosed by membrane, were sheared at the axial strain rate of 0.36%/min. They obtained UCS values between 50 to 240 kPa. Also, the authors provided some of the unconfined compressive strength test results in the literature (Table 2.4)

Table 2.4 Some MICP studies in the literature (Shahrokhi-Shahraki et al., 2014)

Reference	Soil type	Cementation concentration: M	Number of injection cycles	UCS: kPa	Permeability reduction: %
Whiffin et al. (2007)	Itterbeck sand, D_{50} =0.165 mm	1.1	1	0-500	22-75
Yasuhara et al. (2011)	Sand	0.5 , 1.0	4-8 for UCS 1-4 for permeability	373-1500	60-70
van Paassen (2011)	Gravel	NR	1-4	15-40	NR
Palmėn (2012)	Quartz sand, D_{50}=0.85 mm	NR	1 5	200 2600	NR
Soon et al. (2013)	Sand, D_{50}=0.52 mm	0.25	8	50	90

NR, not reported; UCS,unconfined compressive strength.

CHAPTER 3

CALCIUM CARBONATE PRECIPITATION IN SYRINGES

In this chapter, the first step of experiments for determining the calcium carbonate precipitation is introduced. From civil engineering perspective, Microbially Induced Calcium Carbonate Precipitation (MICP) is important whenever the precipitation shows reasonable changes in the behavior of soil in terms of strength. For determining this, the most suitable way to measure strength of the soil is to perform common soil mechanics experiments either in the laboratory or in the field or sometimes both. However, to continue with large scale experiments, initially a researcher needs to understand what lies behind this process. Since numerous factors such as soil type, mineralogy, temperature, pH, chemicals, competition between bacteria etc. affect the success of MICP, to provide an idea about this process and observe its formation characteristics to outline the future studies, small scale laboratory experiments are necessary.

Considering the above objective, through the use of two bacteria and two different sands with different amounts of chemical substances, the precipitation behavior was monitored in different ways in the laboratory. Experiments were carried out in syringes within various periods. The progress of the experiments was dependent mostly on the outcomes of previous ones.

3.1 Sand Properties

In Chapter 2, an emphasis on the success of microbiological treatments in sands was given. Therefore in this chapter, for observing precipitation in syringes, sands were used.

The first type of sand was obtained from METU Civil Engineering Department Hydromechanics Laboratory. The source of this sand was in the vicinity of Ankara, Turkey and it was provided to the laboratory by a local producer. Another sand type used in experiments was Quartz Sand which had been already purchased from POMZAEXPORT Mine Industries & Trade Company for a previous study (Ahmadi-Adli, 2014). For the remaining of this thesis, these sands will be referred to as Sand 1 (sand from Ankara) and Sand 2 (Quartz Sand).

Some geotechnical properties, i.e., grain size distribution, minimum and maximum void ratios in dry conditions and specific gravity of solids of Sand 1 were obtained using the relevant ASTM Standards (ASTM D6913, ASTM D4254, ASTM D7382 and ASTM D854). The laboratory experiments for dry minimum and maximum void ratios of Sand 2 gave same results reported in Ahmadi-Adli (2014). The other geotechnical properties of Sand 2 were obtained from that study. Information regarding Sand 1 and Sand 2 is given in Table 3.1 and Figures 3.1, 3.2, and 3.3.



Figure 3.1 Sands used in the experiments (Left: Sand 1, Right: Sand 2)

Table 3.1 Properties of sands used in the experiments

	Sand 1	**Sand 2**
D_{60} (mm)	0.50	0.202
D_{30} (mm)	0.30	0.14
D_{10} (mm)	0.18	0.09
C_u	2.78	2.24
C_c	1	1.08
Soil Class.	SP	SP
G.S	2.67	2.66
e_{max}	0.80	1.00
e_{min}	0.50	0.62
$\rho_{d,max}$ (g/cm^3)	1.785	1.648
$\rho_{d,min}$ (g/cm^3)	1.481	1.332

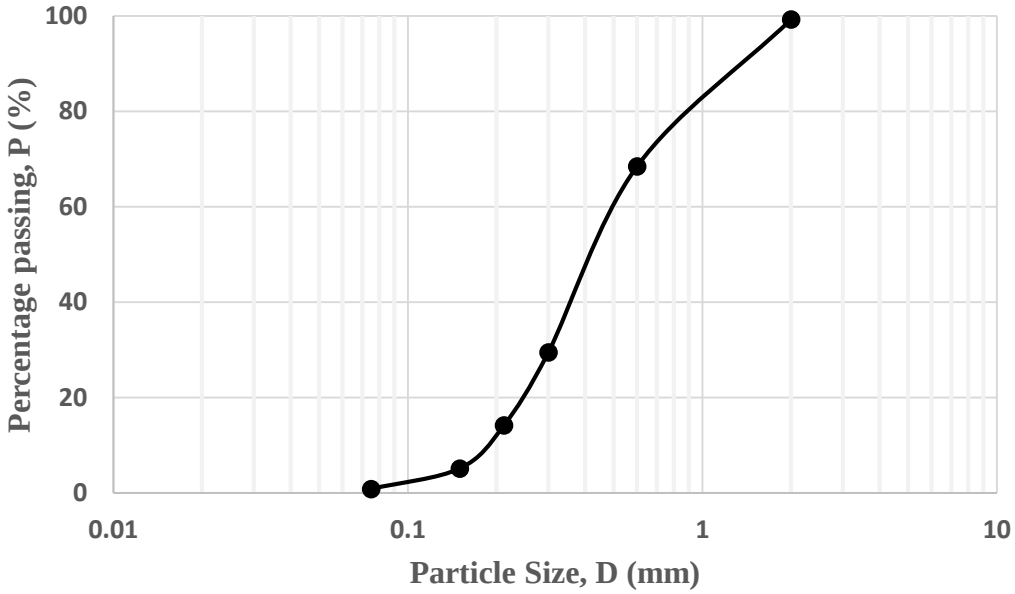


Figure 3.2 Grain size distribution of Sand 1

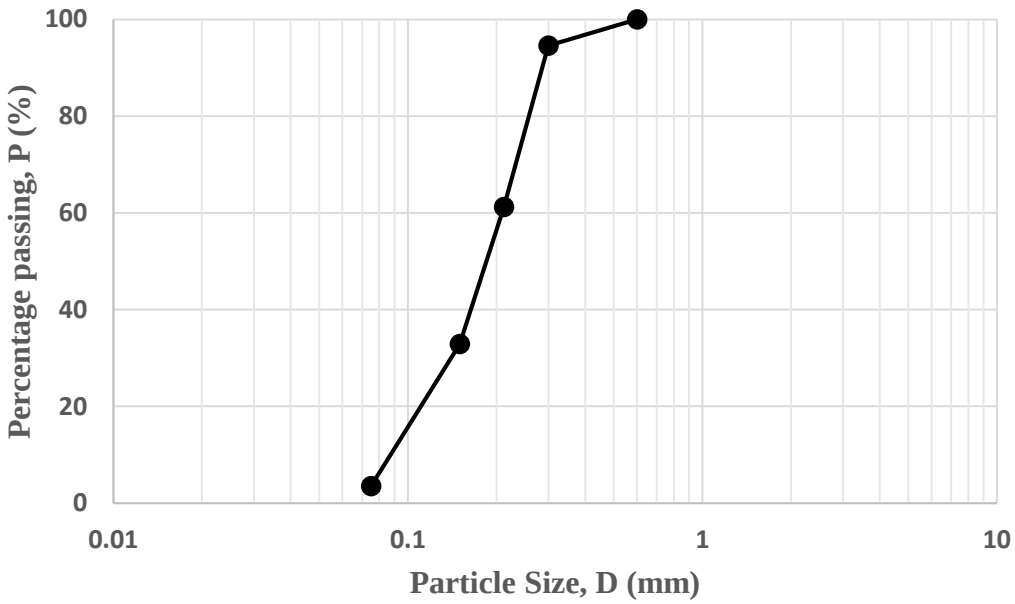


Figure 3.3 Grain size distribution of Sand 2 (Ahmadi-Adli, 2014)

Throughout the experiments, the sands were not exposed to pre-sterilization techniques such as autoclave sterilization. Before their usage, sands were kept in oven at least overnight at 105 °C.

3.2 Bacteria and Growth Conditions

The bacteria used in the experiments are *Sporosarcina pasteurii* [American Type Culture Collection (ATCC 11859)], abbreviated as *"S. pasteurii"* or "s.p" and *Bacillus licheniformis* (ATCC 14580), abbreviated as *B. licheniformis* or "b.l" in the thesis, with Biosafety Levels of 1, which indicate suitability for working in laboratory conditions. These bacteria were purchased from LGC Standards GmbH. The bacteria were imported as freeze-dried, shipped at room temperature and stored at 2 to 8 °C. The microscopic views of *S. pasteurii* ATCC 11859 and a strain of *B. licheniformis* are given representatively in Figures 3.4 and 3.5, respectively.



Figure 3.4 *S. pasteurii* ATCC 11859 micrograph (from http://2011.igem.org)

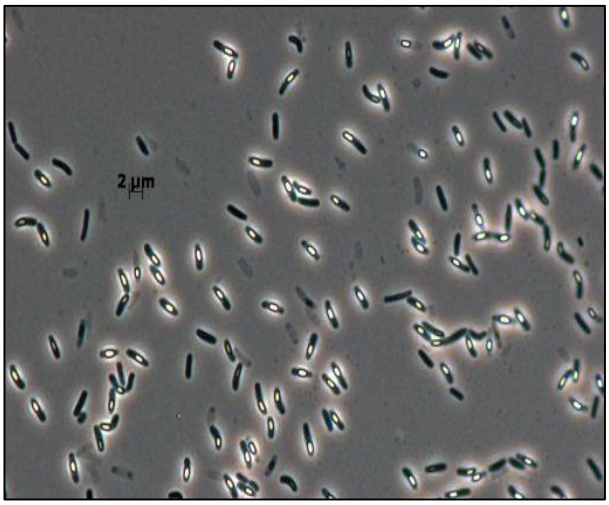


Figure 3.5 *B. licheniformis* micrograph (from http://bacdive.dsmz.de)

Following the receipt of bacteria, the propagation procedures suggested by ATCC Product Sheets were carried out.

The culture media were prepared by using the recipes provided by ATCC. In Table 3.2, the components used for culture media are provided. Ammonium-yeast extract medium (20 g of yeast extract, 10 g of ammonium sulfate ($(NH_4)_2SO_4$) and 15.75 g of Tris buffer per liter, *Sigma*) was used as a culture medium for *S. pasteurii*. 20 g of yeast extract was dissolved in 100 ml of distilled water. 10 g of ammonium sulfate in 100 ml of distilled water and tris buffer in 800 ml distilled water were prepared. Characteristic components of this medium were sterilized (121°C, 15 min) separately, were allowed to cool and combined in a 1000 ml sterile glass bottle. The pH of this medium was measured as around 9.0, which is suitable for *S. pasteurii* growth. Nutrient broth (5 g of peptone from meat and 3 g of meat extract per liter, *Merck*) was used as a culture medium for *B. licheniformis*.

Table 3.2 Ammonium-yeast extract medium components for *S. pasteurii* and nutrient broth medium components for *B. licheniformis*

Bacteria	Components	Amount (per liter)
S. pasteurii	Yeast Extract	20 g
	$(NH_4)_2SO_4$	10 g
	Tris Buffer	15.75 g
B. licheniformis	Peptone	5 g
	Meat Extract	3 g

The remaining procedure was outlined as follows: In these culture media, *B. licheniformis* was grown at 37°C in an orbital shaker (*Zhicheng*, China) at 200 rpm for 24 hours at a final optical density (OD_{600}) of approximately 1 using UV-visible spectrophotometer (*UV-5100*, *SOIF*, China). For the case of *S. pasteurii*, growth at 30°C in an orbital shaker at 200 rpm for at least 48 hours and similarly at a final

OD_{600} of 1 was performed. Then, the bacterial cells were collected by centrifugation (*Spectrafug 6C, LABNET*, USA) at 4000 g for 20 min in 15 ml volumes. All bacterial procedures were performed in sterile conditions under a laminar flow hood (*Esco*, USA) in METU Biological Sciences Department Microbial Ecology Laboratory.

Following the centrifugation, the supernatants in tubes were removed and the remaining harvested bacteria were stored at -20°C until use.

Figures 3.6 to 3.11 show some stages of preparation of bacteria for experiments and the equipment used for the experiments:



Figure 3.6 Bacteria inoculation to the medium



Figure 3.7 An orbital shaker (from http://www.bio-equip.cn/)



Figure 3.8 OD_{600} measurement with 2 ml sample after growth in orbital shaker



Figure 3.9 Pouring bacteria culture to the 15 ml tubes for centrifuging



Figure 3.10 Centrifugation equipment and tubes placed inside

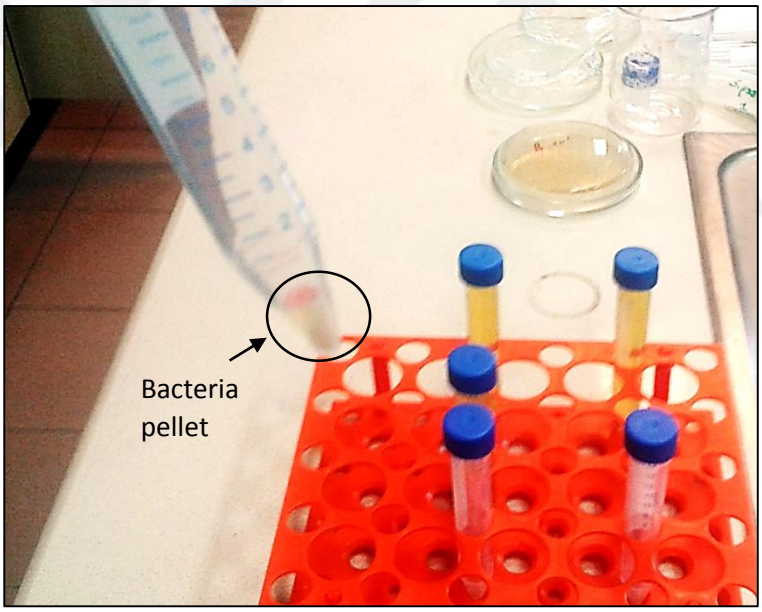


Figure 3.11 Removal of supernatant and bacteria pellet for storage

3.3 Preparation of Urea Medium

In the second step of MICP after bacteria preparation, the nutrients for bacteria are provided. Although the medium consists of not only urea but also some other

components, for simplicity and agreement with the many studies in the literature, the whole medium is named as urea medium. For the concentrations of the substances in the medium, the study of Stocks-Fischer et al. (1999) was used as reference. In their study the authors expressed the reason for selecting those molarities as representing the natural conditions. Also, since there are numerous studies that adopted different molarities of substances especially in terms of calcium source, another medium with a slight difference was prepared, the details of which are explained in the next section.

The urea medium was prepared using 3 grams of nutrient broth, 10 grams of ammonium chloride (NH_4Cl) and 2.12 grams of sodium bicarbonate ($NaHCO_3$), which were dissolved in deionized water. After distributing the solution to the flasks, the media were autoclaved (121°C, 15 min) for sterilization. The urea solution was prepared separately since the heat (autoclaving) decomposes the structure of the urea. 20 grams of urea ($CO(NH_2)_2$) was dissolved in 25 ml deionized water and filter-sterilization (0.22 μm filter, *Pall*, USA) was carried out. Then, the urea solution was added to the autoclaved media after the temperature of media decreased to 30-40°C. The amounts of components in the urea medium are provided as the requirement for a total of 1 liter of medium. Depending on the used amount, different quantities can be utilized provided that the molarities given in Table 3.3 are satisfied.

In addition to urea medium, calcium chloride ($CaCl_2$) solution was prepared by dissolution of 14 grams of $CaCl_2$ per 100 ml distilled water. Next, the solution was autoclaved (121°C, 15 min). Figures 3.12 to 3.14 show some parts of the procedure mentioned above:

Table 3.3 Urea medium components and their concentrations

Urea Medium Components	Amount (g/L)	Concentration
Urea ($CO(NH_2)_2$)	20	333 mM
Ammonium Chloride (NH_4Cl)	10	187 mM
Sodium Bicarbonate ($NaHCO_3$)	2.12	25.2 mM
Nutrient Broth	3 g	



Figure 3.12 Dissolution of components (except urea) with magnetic stirrer

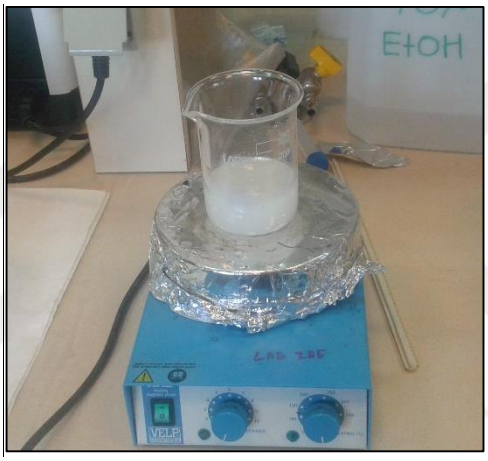


Figure 3.13 Dissolution of urea with magnetic stirrer



Figure 3.14 Urea filter-sterilization

3.4 Treatments and Sand Columns

This section is divided into three parts: Set 1, Set 2, and Set 3.

3.4.1 Experiment Set 1

The main purposes of Set 1 are:

- To observe behavior of different bacteria in calcium carbonate precipitation
- To identify the possible effect of inclusion of $CaCl_2$ in initial treatment
- To understand the effects of increasing $CaCl_2$ molarity.

To provide better attachment of bacteria or to prevent early precipitations and initial clogging, researchers adopted different strategies for inclusion of $CaCl_2$ to the system. While Harkes et al. (2010) injected fixation fluid after initial treatment, Mortensen and DeJong (2011) did not inject $CaCl_2$ in initial treatment with bacteria. Some other researchers added $CaCl_2$ in all their injections. The above studies highlighted the need to observe the effect of $CaCl_2$ inclusion of in initial treatment. Also, there are studies with different amounts of $CaCl_2$ in treatments. While many followed the procedure similar to Stocks-Fischer et al. (1999), some much higher amounts of $CaCl_2$ were used in some studies (Al Qabany & Soga, 2013). Those studies have brought open questions, which can be addressed through observing the effect of increasing $CaCl_2$ amount in experiments.

The experiments explained in this part were formed in a progressive manner. In other words, a set was constructed based on outcomes of the preceding experiments. The starting point in all sets was adding urea medium and $CaCl_2$, whenever necessary, to bacteria pellets in initial treatments and making subsequent treatments without bacteria.

In pilot experiments, *S. pasteurii* and *B. licheniformis* together with urea medium and $CaCl_2$ were added to soil samples prepared in beakers. The main purpose in these experiments was to observe the behavior of injected bacteria. For new injections, the liquid in the beaker was drained from top by using micropipette and fresh medium was added. However, this could result in the drainage of bacteria that did not settle in the soil either. Also, since the previously added liquid was trapped inside the soil

pores and not accessible for drainage, the addition of fresh medium could not be useful for bacteria (Figure 3.15).



Figure 3.15 Pilot Experiments in beakers

Therefore, the first outcome related to experiments was to provide the drainage of liquid whenever necessary.

Syringes were commonly used in the experiments for observing MICP (Inagaki et al., 2011; Meyer et al., 2011; Rong et al., 2012; Stocks-Fischer et al., 1999; Whiffin, 2004).

In this study, the precipitations were investigated in sands placed in 50 ml syringes. In order to avoid possible clogging, filter paper was placed at the bottom of syringes. The details of sand column preparation of Set 1 are as follows (Figure 3.16):

- Around 50 grams of Sand 1 with a target relative density of 40% was used.
- 3 sand columns for *B. licheniformis* and 3 sand columns for *S. pasteurii* were prepared.

After preparation of sand columns, treatment procedure was carried out in general as follows:

- The already obtained bacterial pellets were added to the urea media (1 pellet and 14 ml urea medium - ≈1.1 Pore Volume (PV) - for each sample).

- Then, necessary amounts of $CaCl_2$ (280 and 560 μl) were added from stock solution to these urea media to obtain 25.2 and 50.4 mM of $CaCl_2$ final concentrations, respectively.
- The solutions for initial treatment (bacteria + urea medium + $CaCl_2$) were added slowly and carefully to sand specimens from the top for Samples 2, 3, 5 and 6 by using micropipettes. For Samples 1 and 4, $CaCl_2$ were not included in initial treatments.
- The second treatments (14 ml) were performed 6 hours (retention time) after the initial treatments to provide sufficient time for the attachment of bacteria to the sand grains. These treatments consisted of urea medium and $CaCl_2$ (urea medium + $CaCl_2$ is referred shortly as cementation medium). During treatment the drainage of old medium from the bottom of syringe was provided. The same procedure (again without bacteria) was repeated 21 times for 6 days. For these subsequent cementation treatments, 3-4 hour intervals were acquired daily.
- In order to remove the effluents, flushing of the specimens with water were performed two times at the 5th and 13th days with amounts of 10 ml and 20 ml respectively.
- Most of the time, the bottom of the syringes was enclosed with paraffin between the treatments to keep the columns slightly immersed in medium.

The treatment strategy and details of experimental samples regarding bacteria and $CaCl_2$ concentration in treatments are provided in Tables 3.4 and 3.5.

Table 3.4 Treatment Strategy for Set 1

Number of Treatments						
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 13
4	4	4	4	Flushing with water & 2	4	Flushing with water

Table 3.5 Samples in Set 1

		Initial Treatment		Subsequent Cementation Treatments	
Samples	**Sand**	**Bacteria**	**$CaCl_2$**	**Bacteria**	**$CaCl_2$**
Sample 1	Sand 1	*S. pasteurii*	-	-	25.2 mM
Sample 2	Sand 1	*S. pasteurii*	25.2 mM		25.2 mM
Sample 3	Sand 1	*S. pasteurii*	50.4 mM		50.4 mM
Sample 4	Sand 1	*B. licheniformis*	-		25.2 mM
Sample 5	Sand 1	*B. licheniformis*	25.2 mM		25.2 mM
Sample 6	Sand 1	*B. licheniformis*	50.4 mM		50.4 mM

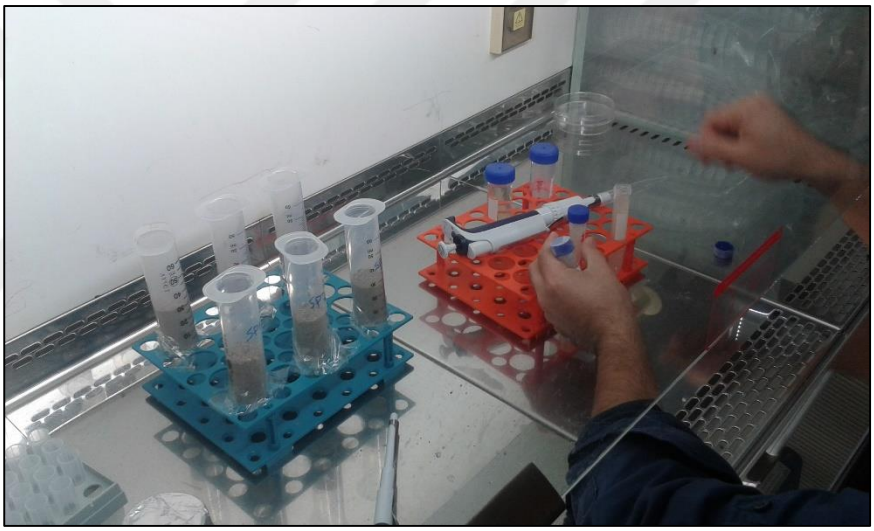


Figure 3.16 Sand columns and media for initial treatments in Set 1

3.4.2 Experiment Set 2

In Set 2 experiments, the main purpose was (in addition to purposes of Set 1) to investigate the precipitation behavior in a different type of sand in terms of mineralogy. The details of sand column preparation in this set are as follows:

- Around 39 grams of Sand 2 with target relative density of 40% was used.

- In Set 2 experiments, same treatment strategy with Set 1 was selected (Table 3.6). The pore volumes were kept the same as Set 1 to inject the same amount of medium to the samples (Figure 3.17).

Table 3.6 Samples in Set 2

		Initial Treatment		Subsequent Cementation Treatments	
Samples	**Sand**	**Bacteria**	**$CaCl_2$**	**Bacteria**	**$CaCl_2$**
Sample 1	Sand 2	*S. pasteurii*	-	-	25.2 mM
Sample 2	Sand 2	*S. pasteurii*	25.2 mM		25.2 mM
Sample 3	Sand 2	*S. pasteurii*	50.4 mM		50.4 mM
Sample 4	Sand 2	*B. licheniformis*	-		25.2 mM
Sample 5	Sand 2	*B. licheniformis*	25.2 mM		25.2 mM
Sample 6	Sand 2	*B. licheniformis*	50.4 mM		50.4 mM

3.4.3 Experiment Set 3

In Set 3 experiments, the main purposes were:

- To observe the effects of initial saturation of samples with water (2 pore volumes of water was used for this purpose)
- To understand the effects of mixing bacteria together (7 ml from each)

Mitchell and Santamarina (2005) had indicated optimum saturation level of 60 to 80% for aerobic microbial activities. Also, Martinez et al. (2013) mentioned the importance of water in the soil for flow field control. For this reason, experiments were carried out with addition of water prior to treatments. Additionally, usage of two different bacteria attracted the attention of researchers for a search of accelerated precipitation (Gat et al., 2014). The purpose of mixing bacteria in this study was to observe any possible physical changes in shorter time. The details of sand column preparation are as follows:

- Around 50 grams of Sand 1 and 39 grams of Sand 2 with target relative density of 40% were used.

- 50.4 mM $CaCl_2$ concentration was provided in all treatments (Table 3.7). Number of treatments and volume of each treatment (14 ml) were the same as the previous sets.

Table 3.7 Samples in Set 3

		Initial Treatment		Subsequent Cementation Treatments	
Samples	**Sand**	**Bacteria**	**$CaCl_2$**	**Bacteria**	**$CaCl_2$**
Sample 1	Sand 1	*B. licheniformis*	50.4 mM	-	50.4 mM
Sample 2	Sand 1	*S. pasteurii*	50.4 mM		50.4 mM
Sample 3	Sand 1	*Mixture*	50.4 mM		50.4 mM
Sample 4	Sand 2	*B. licheniformis*	50.4 mM		50.4 mM
Sample 5	Sand 2	*S. pasteurii*	50.4 mM		50.4 mM
Sample 6	Sand 2	*Mixture*	50.4 mM		50.4 mM



Figure 3.17 Set 2 (Right) and Set 3 (Left) sand columns

3.5 Results and Discussions

Following the end of experiments, the verification of $CaCO_3$ precipitation was necessary. SEM, EDS and XRD analyses were selected as representative methods

for determination of the existence of $CaCO_3$ precipitation. All these operations were performed in Central Laboratory, METU. For SEM and EDS analyses, samples obtained from the vicinity of the middle of specimens were oven dried overnight as initial trials with relatively wet samples did not provide satisfactory results (Figure 3.18). Figure 3.19 shows the specimens coated with gold-palladium (Au-Pd) tested via FEI Quanta 400 F with EDAX equipment. SEM images with various magnifications were obtained. In addition, samples embedded in epoxy resin were tested and compositional mapping of samples were obtained for Set 1.



Figure 3.18 Sand samples prepared for SEM analysis

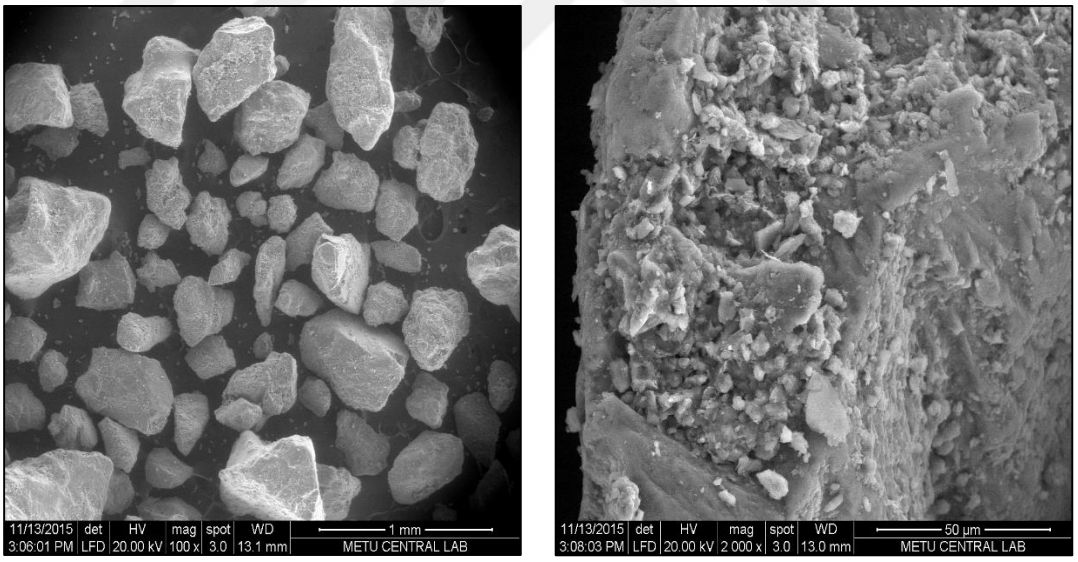


Figure 3.19 Au-Pd coating of samples

In XRD analyses in addition to overnight drying, the samples were tested in powdered form. Both quantitative and qualitative determination of mineral composition was aimed. XRD analyses were fulfilled with Rigaku Ultima-IV, Cu anode (40 kV and 30 mA), within a scan range of 2-90° with 0.02° sampling step. Quantitative results were obtained with the help of Reference Intensity Ratio (RIR) method in Set 1. The mineral phases observed in XRD scans were used during the implementation of RIR method.

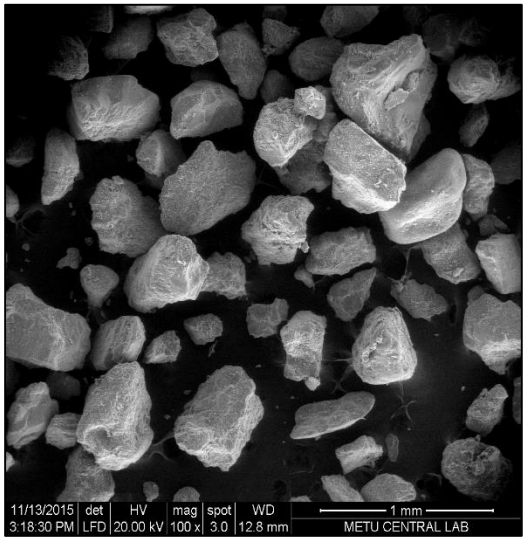
3.5.1 Results and Discussion of Set 1

The SEM images and some EDS mappings of untreated Sand 1 and samples of Set 1 are provided in Figures 3.20 to 3.27, with different magnifications (magnification factors are provided at figure captions). When present, the microbe beds and $CaCO_3$ formations are indicated in these figures.

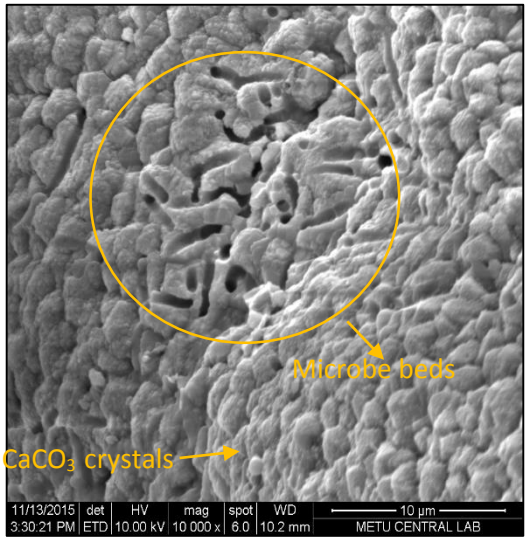


(a) **(b)**

Figure 3.20 SEM images of untreated Sand 1 (a) 100x (b) 2000x

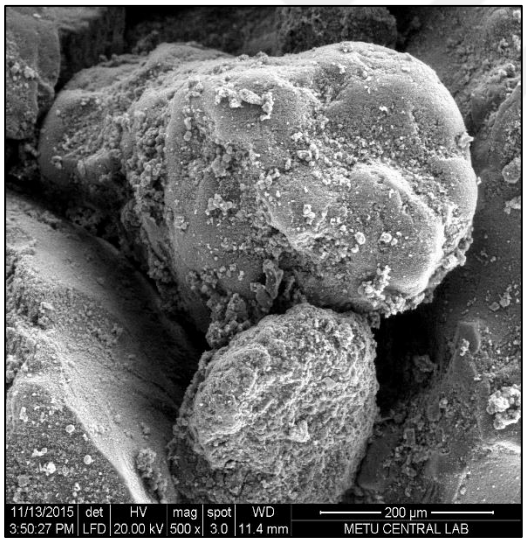


(a)

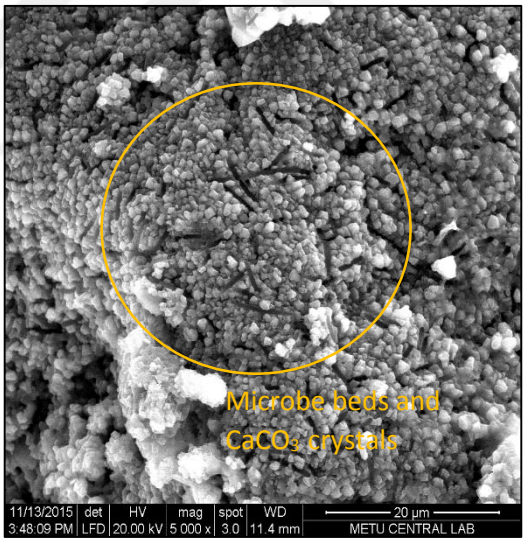


(b)

Figure 3.21 SEM images of Set 1 Sample 1 (a) 100x (b) 10000x

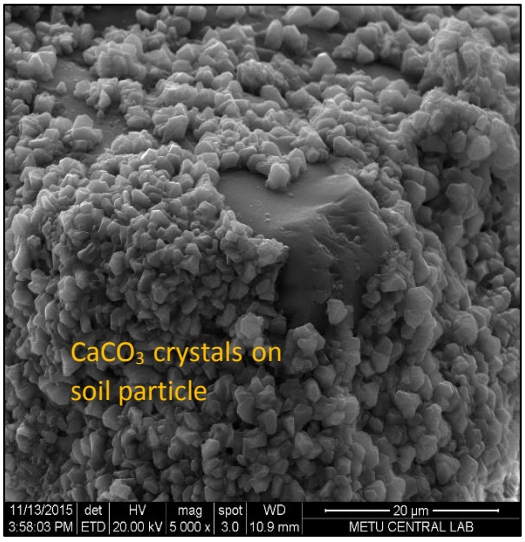


(a)

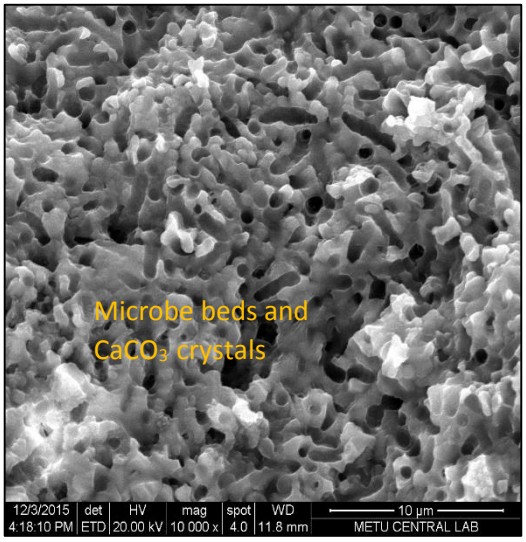


(b)

Figure 3.22 SEM images of Set 1 Sample 2 (a) 500x (b) 5000x

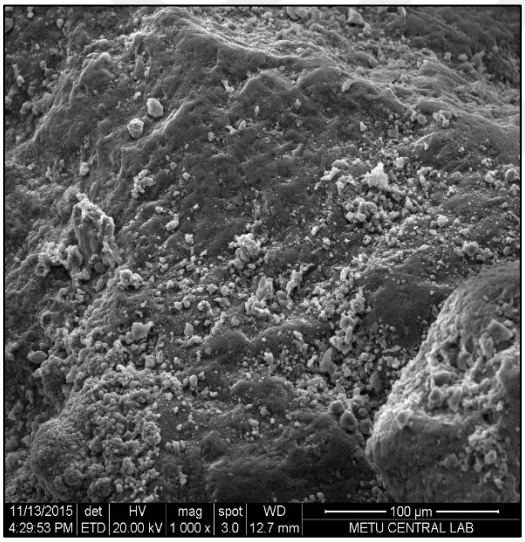


(a)

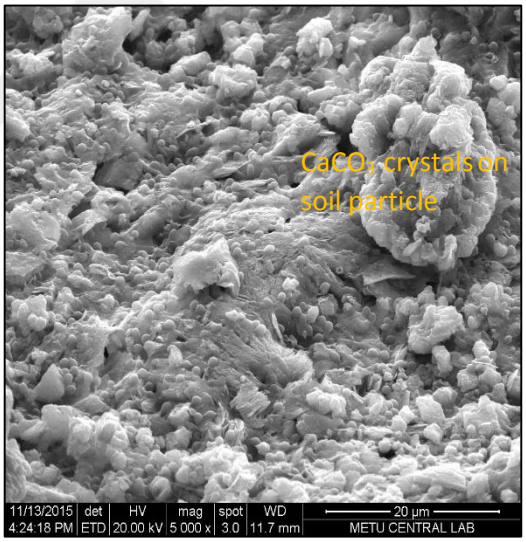


(b)

Figure 3.23 SEM images of Set 1 Sample 3 (a) 5000x (b) 10000x

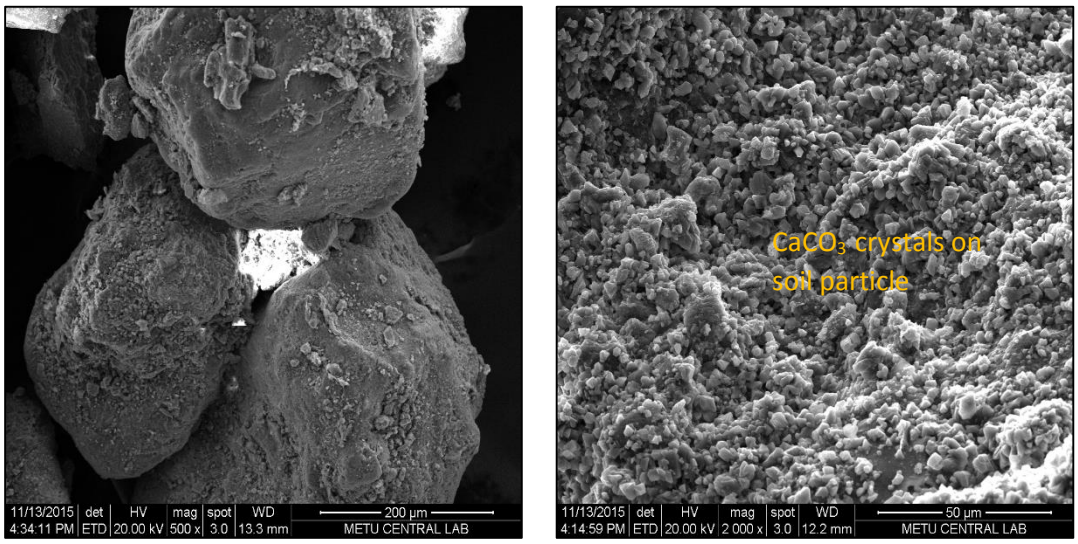


(a)



(b)

Figure 3.24 SEM images of Set 1 Sample 4 (a) 1000x (b) 5000x



(a) **(b)**

Figure 3.25 SEM images of Set 1 (a) Sample 5, 500x (b) Sample 6, 2000x

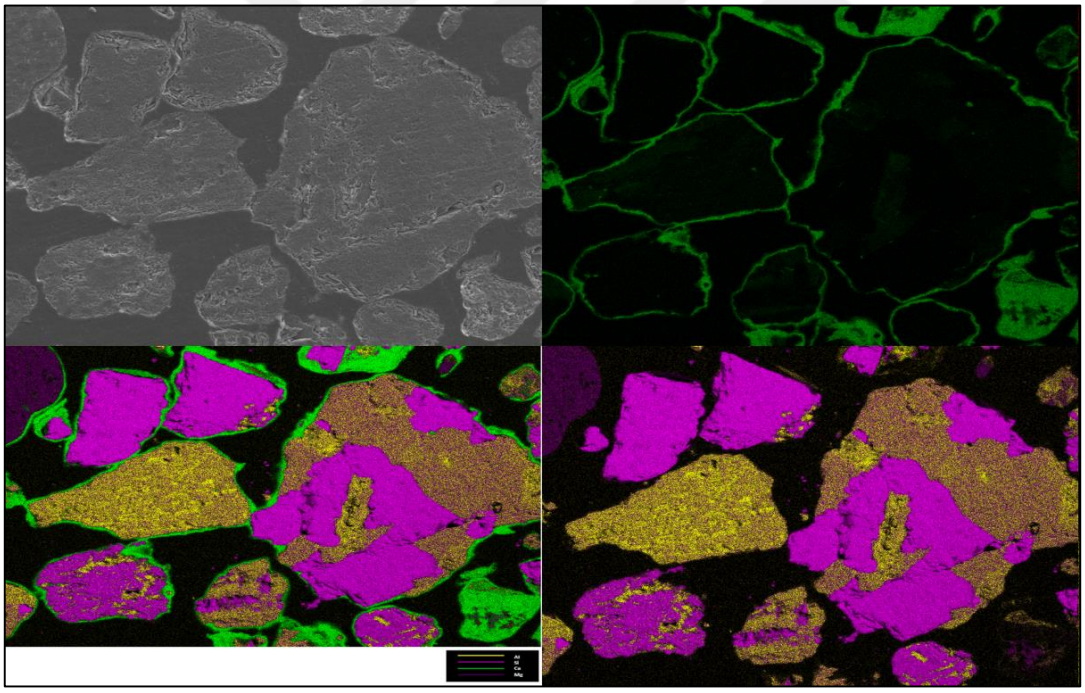
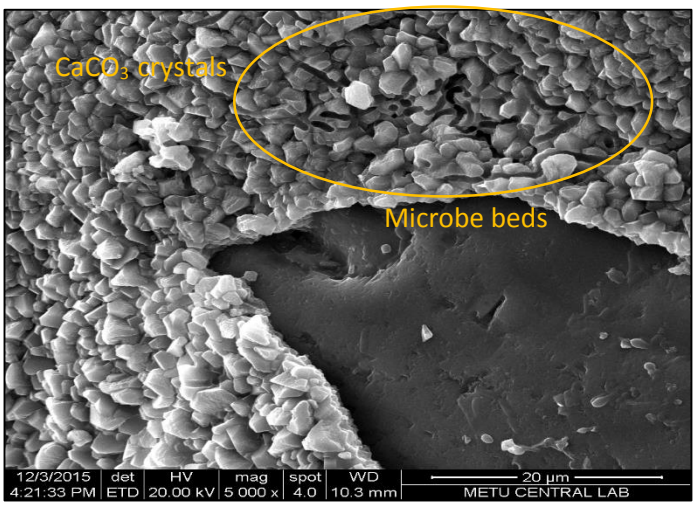
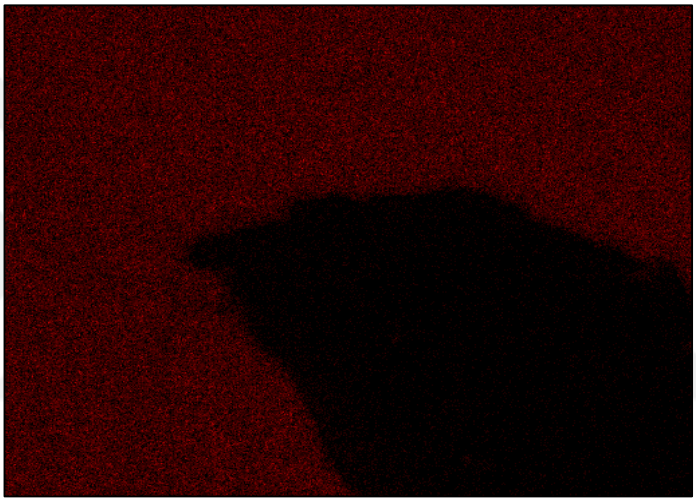


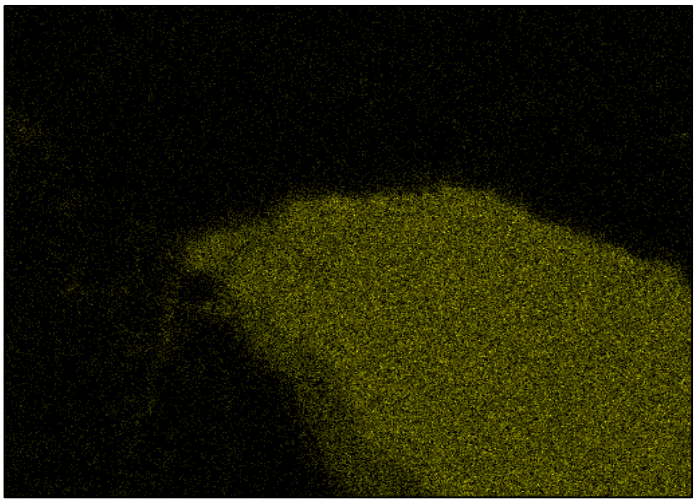
Figure 3.26 Epoxy resin embedded sample SEM and EDS Mapping, Set 1 Sample 3 (from top left in clockwise direction: 1-SEM Image, 2-Ca Mapping, 3- Al, Si, Mg Mapping, 4- Al, Si, Ca, Mg Mapping)



(a)



(b)



(c)

Figure 3.27 (a) SEM and (b) Ca, (c) Si EDS mapping images of Set 1 Sample 3

The table for quantification of minerals in samples is provided in Table 3.8. In this table, the numbers indicate the percent weight. Error ratios are not included in the table. Qualitative analyses were carried out separately and the mineral phases observed in those analyses were used in RIR method implementation.

Table 3.8 XRD – RIR Results

	SiO_2	Anorthite	Calcite	Albite
Untreated Sand	43	55	2.5	-
Sample 1	42	-	8.5	50
Sample 2	41	-	10.7	48
Sample 3	57	15	15.8	12.2
Sample 4	60	33	6.5	-
Sample 5	26	-	4.4*	70
Sample 6**	32.7	-	4.6	63
***** Magnesium Calcium Carbonate ** Repeated				

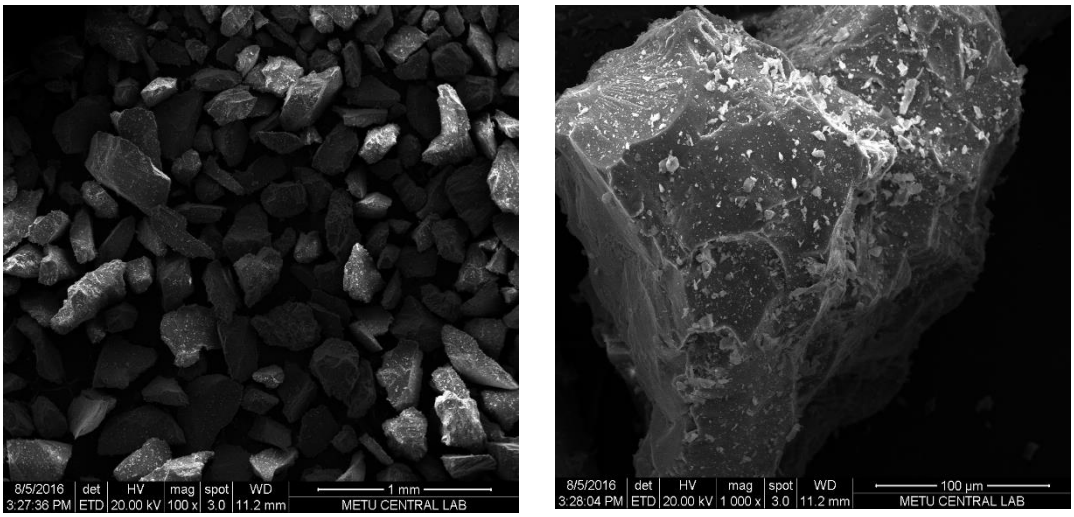
Based on the results, overall discussion for Set 1 is as follows:

- Throughout the treatments, the effect of inclusion of $CaCl_2$ in initial treatments did not show an apparent difference either physically or in SEM and XRD results.
- EDS monitoring on samples from different points revealed that in all samples calcite existed. However, XRD results indicated that untreated Sand 1 also had calcite in its mineralogical composition. The mineral detection indicated that Sand 1 consisted of SiO_2, anorthite, albite and calcite. Anorthite and albite are end members of feldspars. In sample 5, the calcium carbonate was detected as magnesium calcium carbonate.
- The natural occurrence of calcite in Sand 1 may lead to question the source of calcite particles as geological or microbially induced. However, in Figures 3.21 to 3.23, microbe beds together with calcite particles prove the existence of microbially induced calcite precipitated. Additionally, Figure 3.27 is significant as it clearly shows microbe beds and the border between silicon and calcium which belong to sand and calcium carbonate, respectively.

- Figure 3.26 presents the monitoring of sample 3 embedded in epoxy resin. Calcite on surfaces of some particles may have been removed during preparation. However, this figure clearly indicates calcite particles at the sides. Since bacteria prefer to settle at particle contacts (DeJong et al., 2010), the possibility of MICP in addition to already existing calcite is supported through this figure.
- The abovementioned figures represent samples treated with *S. pasteurii*. In addition, although RIR method does not provide certainty for percentages of minerals, still there is an increasing trend of calcite quantity as molarity of calcium increased in samples treated with this bacterium. In the samples treated with *B. licheniformis* no such trend was observed.

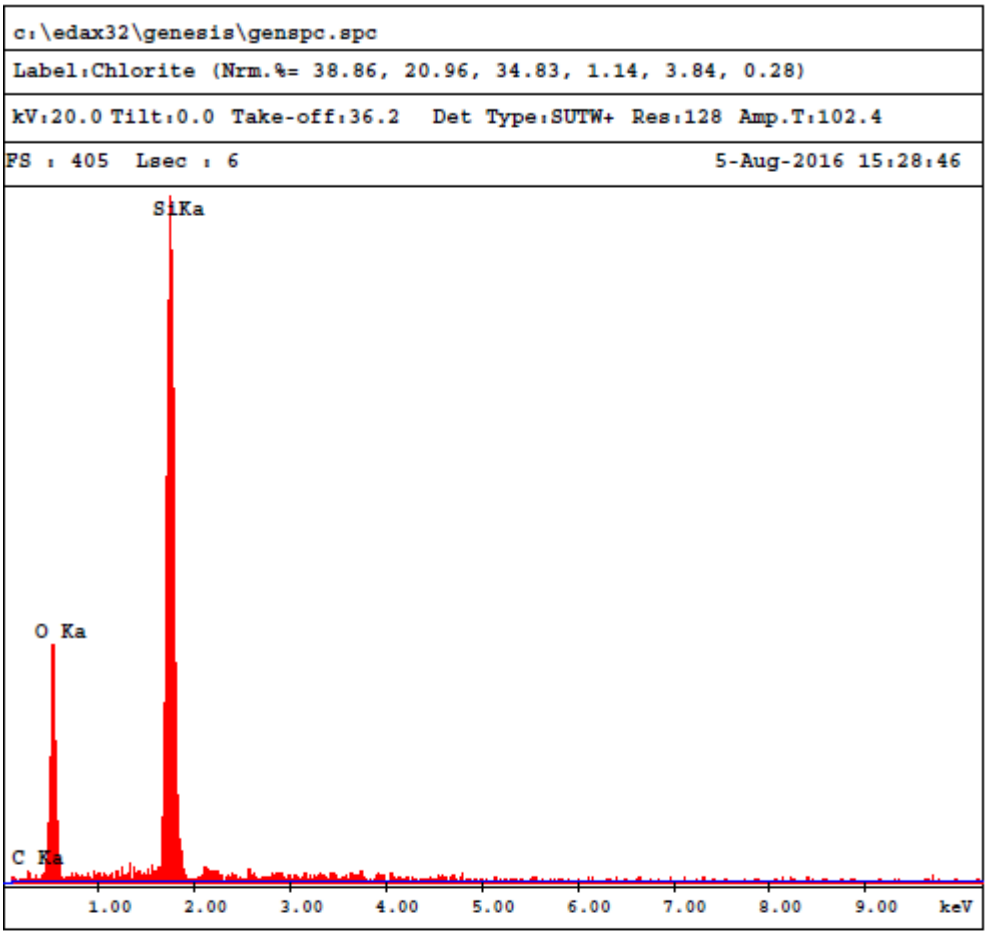
3.5.2 Results and Discussion of Set 2

The natural existence of calcite in Sand 1 indicated the accuracy of selecting sand consisting of quartz mineral (Sand 2) in Set 2. By doing so, the calcium carbonate particles that were precipitated due to microbiological processes could be visible. Sample SEM images and the results obtained from EDS of untreated Sand 2 and some samples are provided in Figures 3.28 to 3.36. When present, the microbe beds and $CaCO_3$ formations are indicated in these figures.



(a) **(b)**

Figure 3.28 SEM images of untreated Sand 2 (a) 100x (b) 1000x

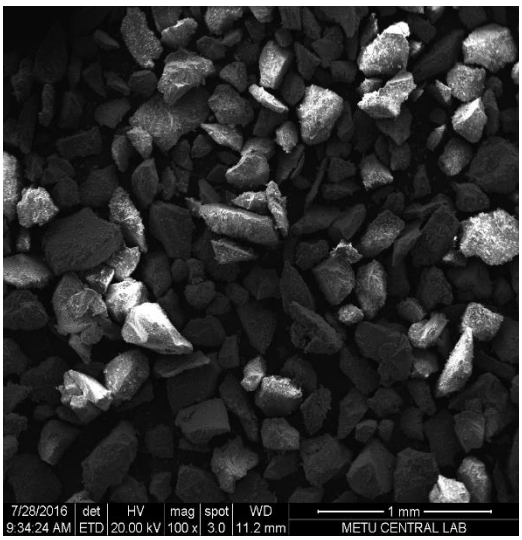


EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

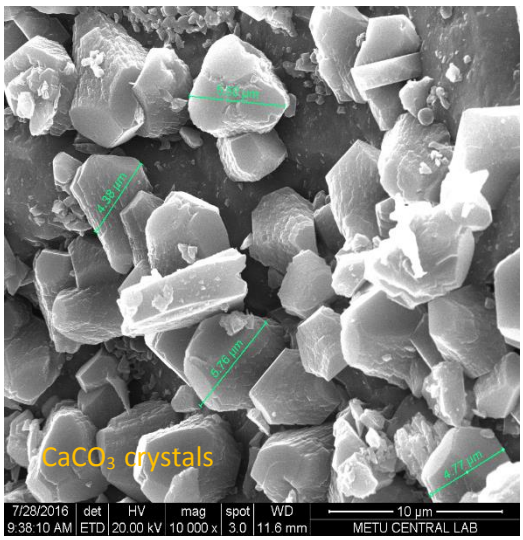
Element	Wt %	At %	K-Ratio	Z	A	F
C K	5.78	9.61	0.0069	1.0377	0.1151	1.0004
O K	43.34	54.16	0.1327	1.0203	0.2998	1.0005
SiK	50.88	36.22	0.4278	0.9779	0.8597	1.0000
Total	100.00	100.00				

Element	Net Inte.	Bkgd Inte.	Inte. Error	P/B
C K	2.05	0.95	38.46	2.17
O K	103.17	0.95	3.94	109.17
SiK	440.54	1.42	1.90	310.78

Figure 3.29 The result of EDS analysis of untreated Sand 2 sample

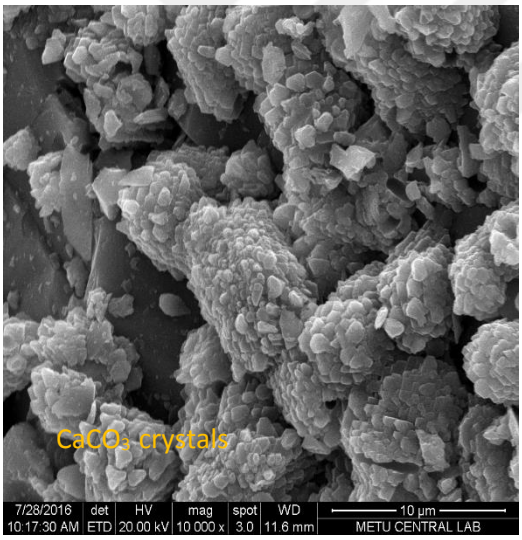


(a)

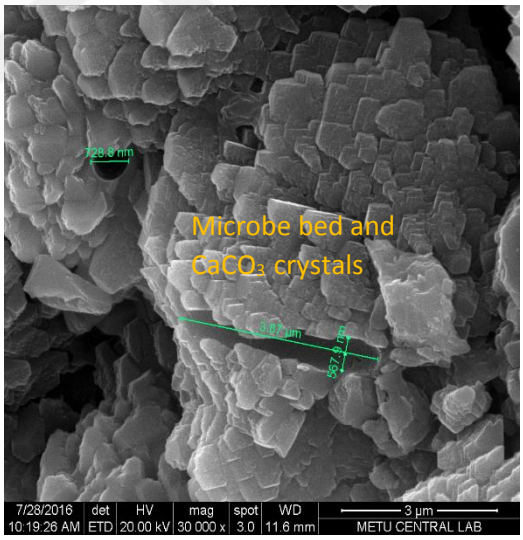


(b)

Figure 3.30 SEM images of Set 2 Sample 1 (a) 100x (b) 10000x



(a)

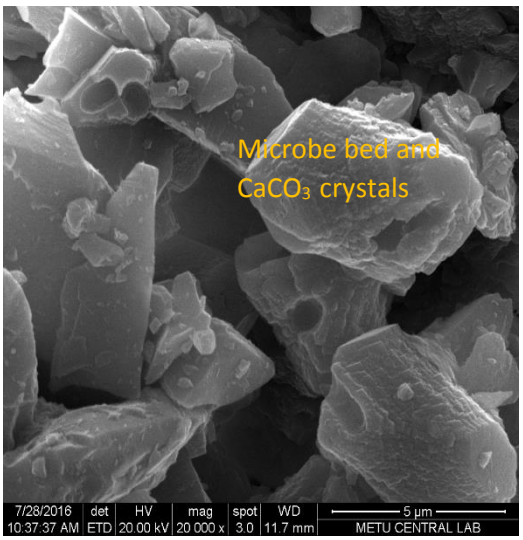


(b)

Figure 3.31 SEM images of Set 2 Sample 2 (a) 10000x (b) 30000x

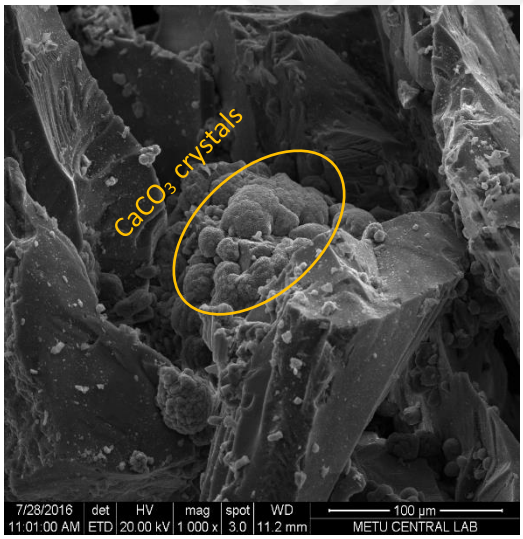


(a)

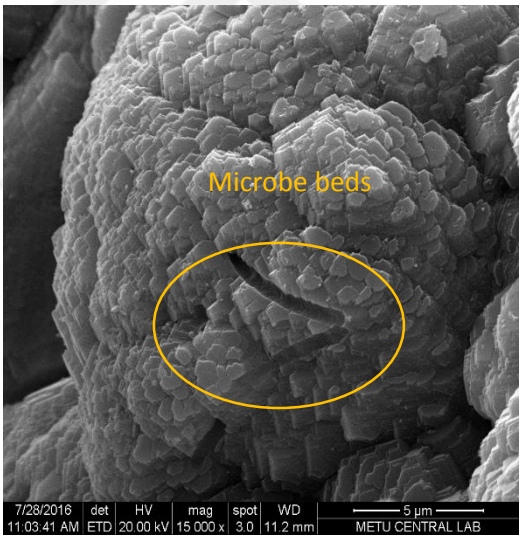


(b)

Figure 3.32 SEM images of Set 2 Sample 3 (a) 10000x (b) 20000x

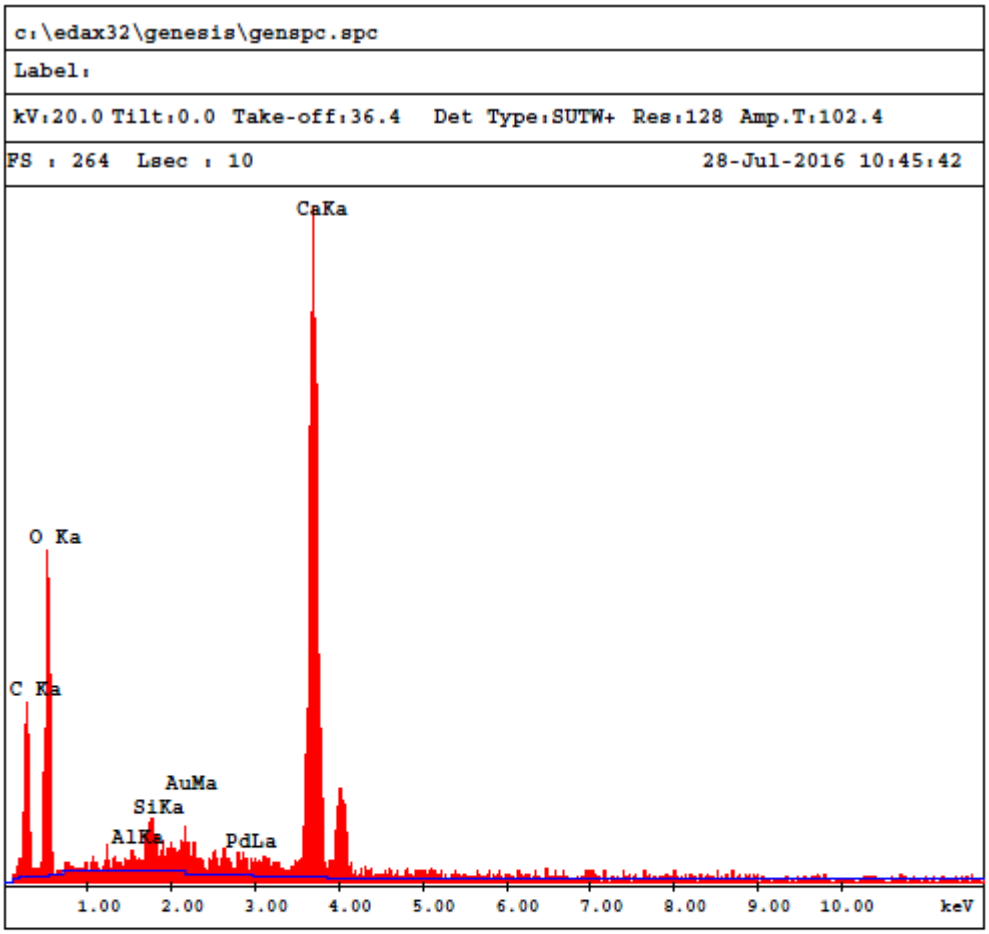


(a)



(b)

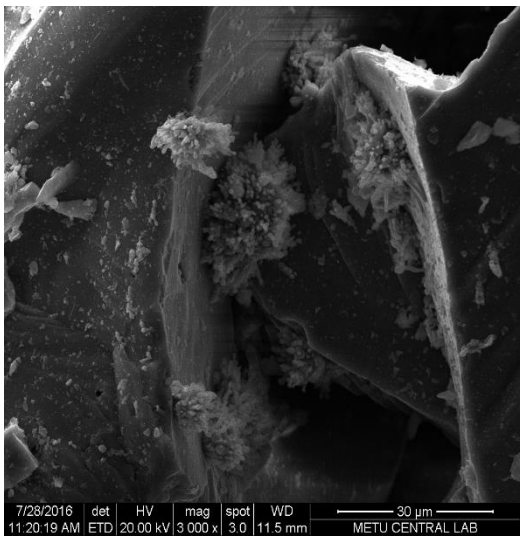
Figure 3.33 SEM images of Set 2 Sample 4 (a) 1000x (b) 15000x

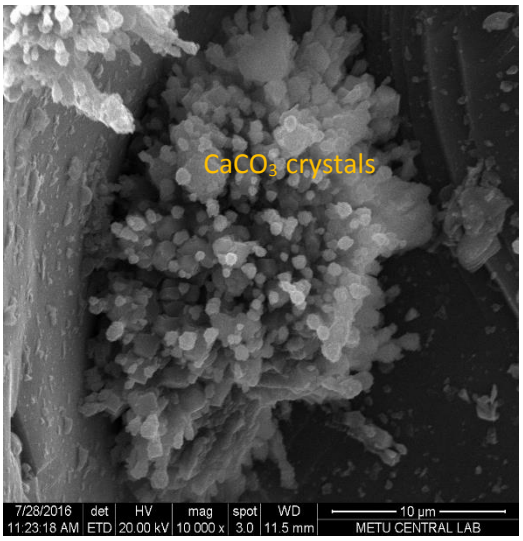


EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	22.82	36.25	0.0953	1.0430	0.4000	1.0007
O K	39.47	47.07	0.0603	1.0255	0.1491	1.0001
AlK	0.35	0.25	0.0021	0.9549	0.6254	1.0030
SiK	1.27	0.86	0.0094	0.9827	0.7484	1.0050
AuM	3.13	0.30	0.0266	0.6918	1.2275	1.0016
PdL	1.41	0.25	0.0124	0.7762	1.0934	1.0392
CaK	31.56	15.02	0.2963	0.9548	0.9832	1.0000
Total	100.00	100.00				

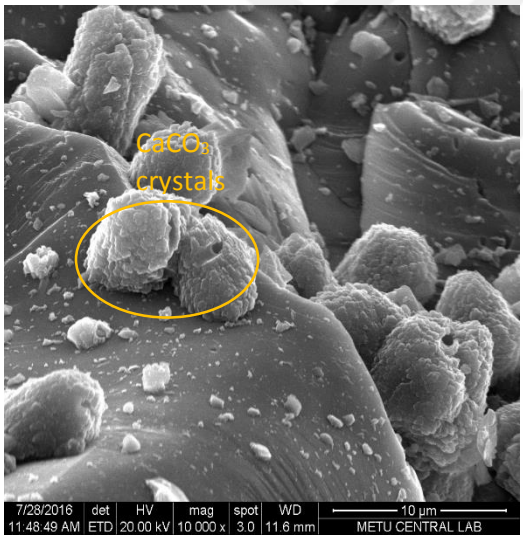
Figure 3.34 The results of EDS analysis of sample from Set 2 Sample 4

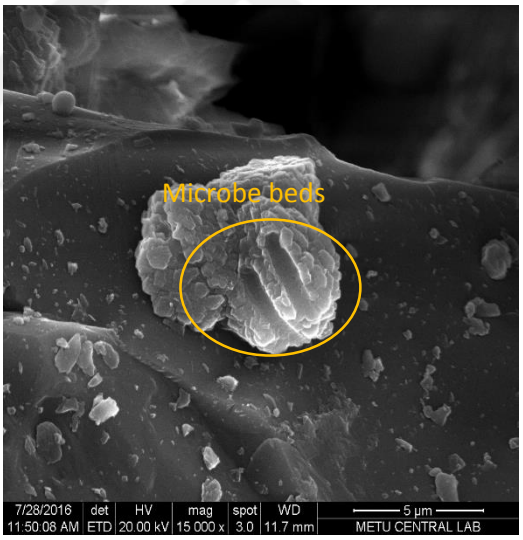




(a) **(b)**

Figure 3.35 SEM images of Set 2 Sample 5 (a) 3000x (b) 10000x





(a) **(b)**

Figure 3.36 SEM images of Set 2 Sample 6 (a) 10000x (b) 15000x

Overall discussion for Set 2 is as follows:

- The precipitation behavior could be monitored in Sand 2.
- Calcium carbonate particles were observed in all samples including samples treated with *B. licheniformis*. EDS analyses taken from certain points confirmed these occurrences. Figure 3.33 shows an example for sample treated with *B. licheniformis*.
- During SEM monitoring, the overall appearance of calcite in samples 1, 2 and 3 (treated with *S. pasteurii*) was more frequent compared to samples 4, 5 and 6 (treated with *B. licheniformis*). In samples with *B. licheniformis*, the calcite particles were visible in limited areas. Also, the peaks observed in XRD analyses that indicate the existence of calcite were more common in the former group of samples mentioned above (Figures 3.37 and 3.38)

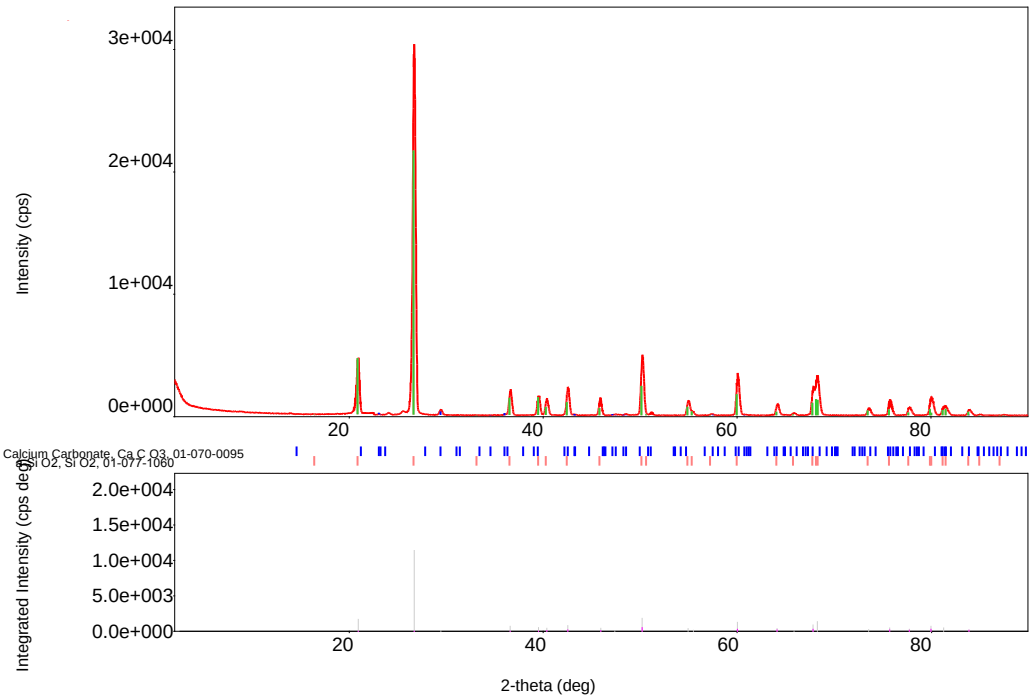


Figure 3.37 XRD result of Set 2 Sample 3

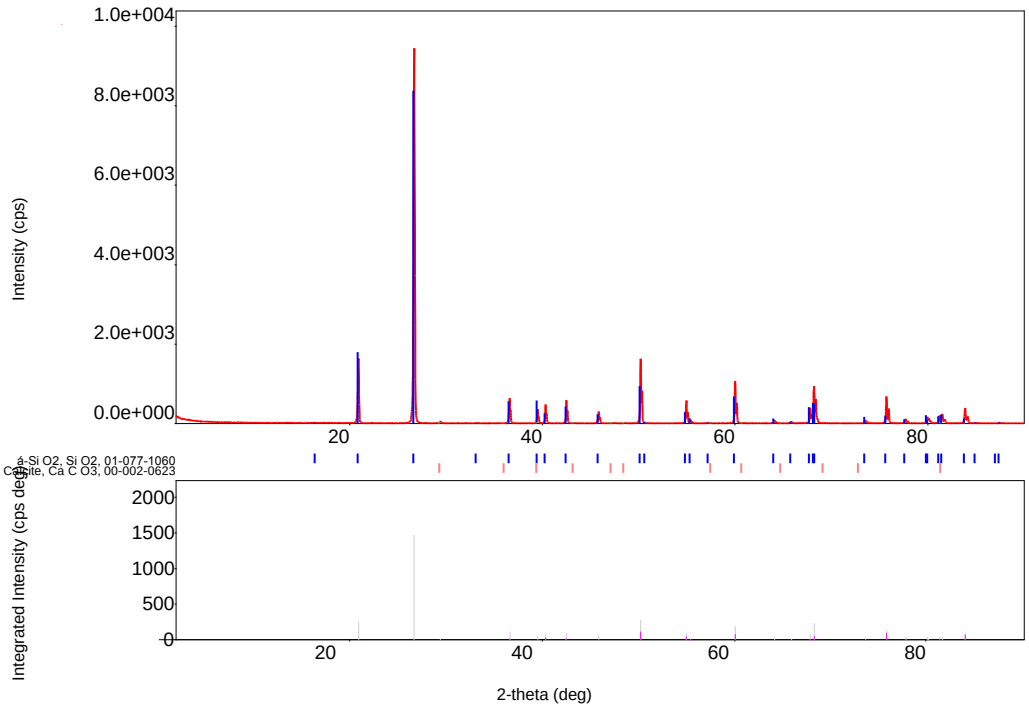
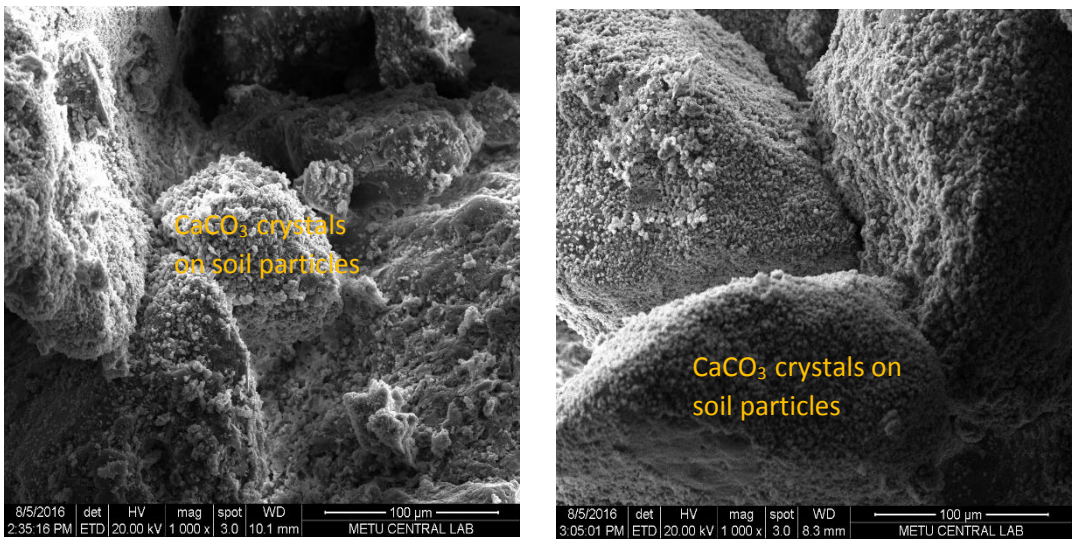


Figure 3.38 XRD result of Set 2 Sample 5

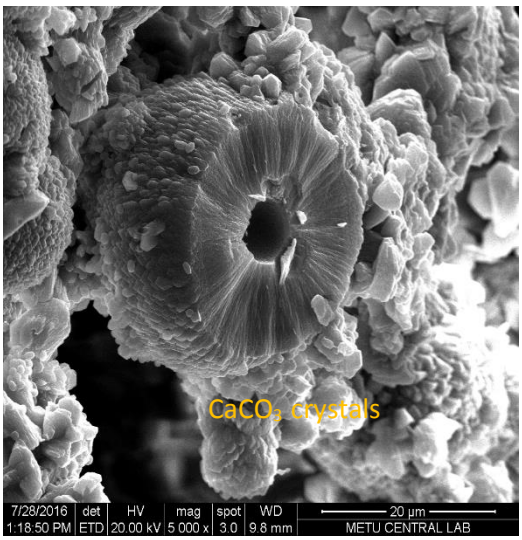
3.5.3 Results and Discussion of Set 3

In this part, the SEM images of samples in Set 3 are provided in Figures 3.39 to 3.42. When present, the microbe beds and $CaCO_3$ formations are indicated in these figures.

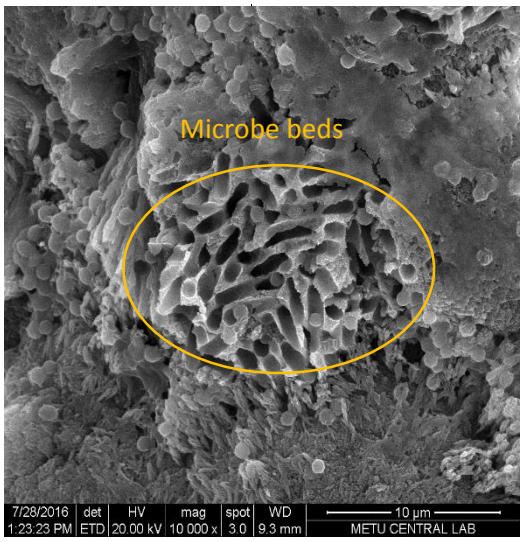


(a) **(b)**

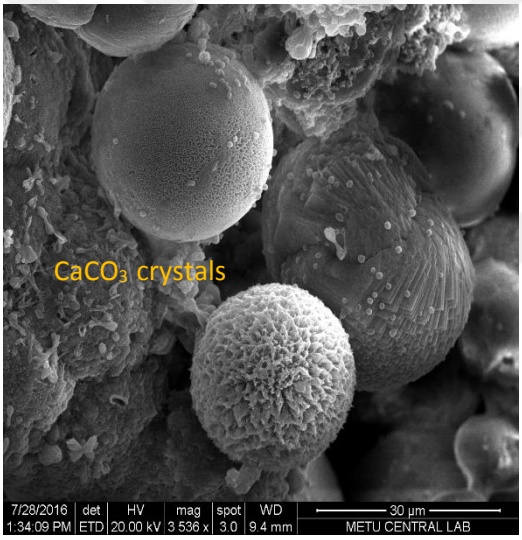
Figure 3.39 SEM images of (a) Set 3 Sample 1 (b) Set 3 Sample 2



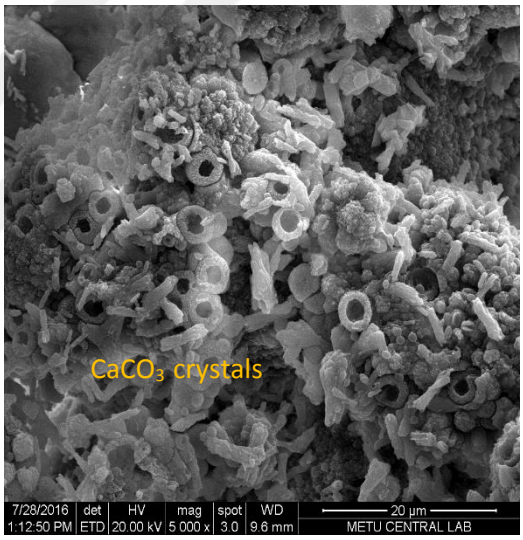
(a)



(b)

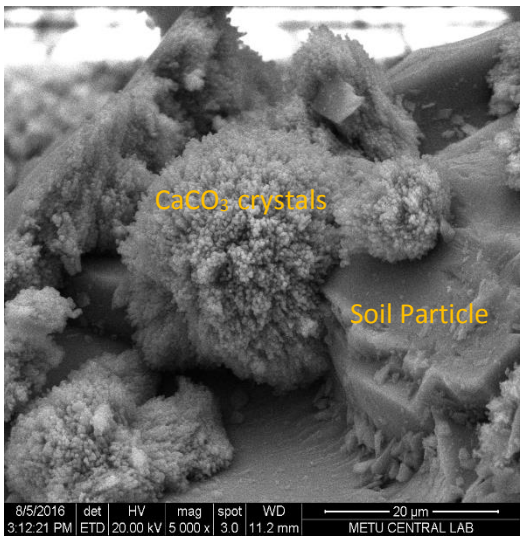


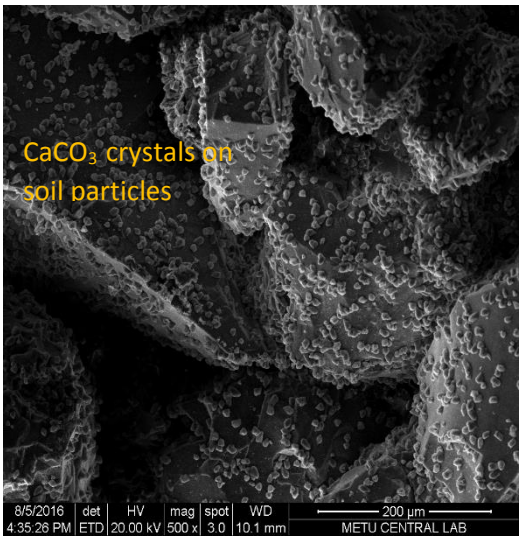
(c)



(d)

Figure 3.40 SEM images of Set 3 Sample 3





(a) **(b)**

Figure 3.41 SEM images of (a) Set 3 Sample 4 (b) Set 3 Sample 5

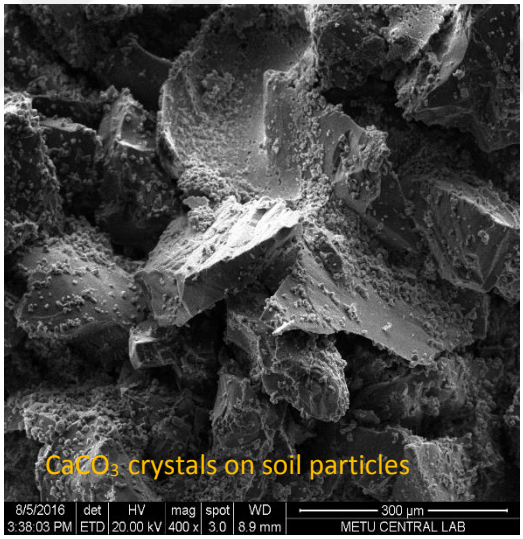


Figure 3.42 SEM image of Set 3 Sample 6

Overall discussion for Set 3 is as follows:

- The inclusion of water might have enhanced the activity of bacteria as aimed
- In Sample 3, mixture of bacteria in Sand 1, calcium carbonates with different morphologies were observed (Figure 3.40). Physically, the top portion of syringe had acquired greater strength compared to other syringe samples. The

extrusion of the top portion from the syringe required some effort and sample from top was examined in SEM analyses. The sizes of calcium carbonate particles can be seen by comparing the scales in the different figures. The reason for this kind of occurrences could be accumulation of too much nutrient or bacteria at a certain location.

- The calcium carbonate particles in soil samples treated with *S. pasteurii* seemed more than those treated with *B. licheniformis* quantitatively during SEM monitoring similar to the previous sets.
- The calcium carbonate particles observed in Sand 2 treated with *B. licheniformis* seemed to be similar in shape in Set 2 (Figure 3.35) and Set 3 (Figure 3.41-a).

CHAPTER 4

UNCONFINED COMPRESSION TESTS ON TREATED SANDS

In this chapter, the strength characteristics of sands improved with the bacteria are investigated using the laboratory experiments. The main purpose is to demonstrate the increase in the strength of lab specimens and quantify the amount of improvement if possible.

From Geotechnical Engineering perspective, the strength and deformation characteristics of soils improved with MICP method have been traditionally measured using various types of soils tests, as summarized in Chapter 2. Those tests vary from the simplest ones such as the permeability tests, unconfined compression tests (Cheng et al., 2014; Kavazanjian & Hamdan, 2015; Li et al., 2015; Montoya et al., 2013; Neupane et al., 2015; Oliveira et al., 2014; van Paassen et al., 2010b; Park et al., 2014; Al Qabany & Soga, 2013; Rong et al., 2012; Rowshanbakht et al., 2016; Shahrokhi-Shahraki et al., 2014; Yasuhara et al., 2012), California bearing ratio test (Chou et al., 2011), etc. to more complicated ones such as static (Feng & Montoya, 2016; Lin et al., 2015; Montoya & DeJong, 2015; Ozdogan, 2010) and cyclic triaxial tests (Burbank et al., 2013). In Chapter 3, the improvement effects of two different bacteria were studied on two different sands using visual and mineralogical experiments. However, those tests do not outline the engineering characteristics of improved sands fully, but, they focused more on the optimization of the improvement process. For this improvement to be quantified numerically or in terms of engineering parameters, this chapter presents the unconfined compression tests made with sands improved using bacteria.

In order to characterize improvement in terms of strength, the laboratory tests were carried out in two sets: Set 1 and Set 2. The main differences between these sets were based on treatment duration and the number of treatments, i.e., repetitions for cementation injections. Set 1 is mainly intended to understand the contribution of the cementation to improvement on the strength properties of two different sands, namely Sand 1 and Sand 2, the properties of which were provided in Chapter 3, using two different bacteria. These sands were prepared at targeted two different relative densities, 40% and 70%, respectively. Based on the results obtained from Set 1, Set 2 was prepared, but this time the same samples were treated with less number of treatments. In addition, some minor modifications were implemented in Set 2 to improve the test procedures. The details of laboratory tests and the specimens used in Set 1 and Set 2 are given in Table 4.1 and Table 4.2.

Table 4.1 Treatment details of Set 1 and Set 2

	TREATMENT DURATION	**NUMBER OF TREATMENTS**
SET 1	7 days	20
SET 2	3 days	10
	TESTED SAMPLES*	**NOT-TESTED SAMPLES***
SET 1	1, 2, 3, 4, 5	6, 7, 8
SET 2	1, 2, 4, 5, 6, 7, 8	3

*See Table 4.2

Table 4.2 Sample details for Set 1 and Set 2

Sample No	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sand	Sand 1	Sand 1	Sand 1	Sand 1	Sand 2	Sand 2	Sand 2	Sand 2
Bacteria	*S. pasteurii*	*B. licheniformis*	*S. pasteurii*	*B. licheniformis*	*S. pasteurii*	*B. licheniformis*	*S. pasteurii*	*B. licheniformis*
Relative Desity (%)	40	40	70	70	40	40	70	70
Soil Mass (g)	314.8	314.8	332.6	332.6	286.3	286.3	304.9	304.9
Urea Medium in each treatment (ml)	90	90	80	80	100	100	90	90
$CaCl_2$ in each treatment(ml)	3.6	3.6	3.2	3.2	4	4	3.6	3.6
Initially Flushed Water (ml)	160	160	150	150	180	180	165	165

4.1 Experimental Setup and Specimen Preparation

Under normal circumstances, the unconfined compressions tests cannot be carried out on sands, as they have no cohesion to hold them tight and there is no confining pressure during the test. However, with the number of cementation injections, sands are expected to gain strength which will make application of unconfined test possible.

In the literature, there are examples of laboratory test samples that were not suitable for testing either due to chemical concentrations which lead to different precipitations than usual (Al Qabany & Soga, 2013) or disturbance during the extraction of sample from the molds (Montoya et al., 2013) or low levels of cementation (van Paassen et al., 2012). In addition, some researchers preferred to use grease on inner sides of the molds to make sample extraction easier (Soon et al., 2014), whereas some others used full contact flexible molds made of geotextile (Li et al., 2015).

In our experiments, Polypropylene Random Copolymer (PPRC) pipes with 75 mm outer diameter and 50 mm inner diameter were used. The preparation of molds was done for Set 1 as follows (Figure 4.1):

- PPRC pipes were cut into molds with approximate dimensions of 107 mm height and 50 mm diameter.
- Because of the possible danger of difficulty in extracting samples from the molds, each mold was halved along its longitudinal axis in METU Mechanical Engineering Department. Initial trials had resulted in deformations in the circular cross sections of the molds. To keep the circular sections as they are, the thinnest cutting equipment were preferred.
- Again for minimizing the dangers in extracting samples and for suitable treatment strategy, bottom caps were employed for molds. These caps were already available in the METU Civil Engineering Department Soil Mechanics Laboratory. For fitting caps to molds, the outer diameters of the molds near the bottom were decreased in the lathe.

- Holes in the centers of molds were opened and a suitable apparatus for injection or drainage of media was mounted to molds.
- For keeping two half of molds rigid during treatments, metal clamps were used.



(a)



(b)

Figure 4.1 (a) Molds prepared for samples and (b) Bottom cap

- Inner surfaces of the molds were lined with filter paper. The reason for this was to be able to extract samples from the mold easily since there was a possibility of treated sand sticking to the sides of the mold. The trials before the experiments about the quality of designed system showed that using filter papers is reasonable.
- Porous stones were placed at the top and bottom of the molds to provide uniform flow as much as possible (Al Qabany & Soga, 2013). Also, filter papers were placed to the top of porous stone at the bottom and to the bottom of porous stone at the top. Porous stones at the bottom had thicknesses around 6 mm. They were fitted to the lowermost parts of molds. Hence, the mold heights were determined to be 107 mm. Previously, the bacteria permeability of a porous stone was tested by checking the $O.D_{600}$ value of the liquids that were injected and passed. There was not much difference between the two measurements.
- Pilot experiments revealed that the sides of the molds and connection points of caps and molds were prone to excessive amounts of drainage of liquids. To keep the quality of injections and media inside the molds, teflon bands were wrapped around the bottom of molds between the mold and cap. Additionally, a paste that prevents leakage was used against liquids on outer sides of the molds at drainage points.

The experience gained in the Set 1 opened the way for making mold usage more viable. As was given in Table 4.1, 3 experiments out of 8 samples were not suitable for testing as there were problems related to extraction of soil specimens. The testability success depended on some changes in molds. Based on observations in Set 1, in addition to aforementioned steps, a few more steps were introduced in Set 2:

- One of the most important differences between Set 1 and Set 2 was the usage of aluminum foil additionally in the latter one. Although they had good performance in general, at some point, removing the filter papers from the molds were difficult. To recover the sample easily, aluminum foil between filter paper and mold was used.

- Another reason for sample loss was attributed to connection points of the two sides of the molds. The connection points were carved for around a millimeter and smoothened. To block sample enlargement to these carved points, paste was spread along the joint of the molds (Figure 4.2).
- Keeping molds in the oven for a long time deformed the molds of the first set slightly. For this set, the molds were given shape in lathe one more time.
- In the first set, adherence of the porous stone to the sides of mold was one of the critical problems that was encountered. To prevent this, the sides of the porous stones were grinded so that they could easily pass the cross section of the mold. During preparation of sand samples, in order to save the position of porous stone, small bands were used between sides of the mold and porous stone.
- After experiments in Set 1, the porous stones were washed with alcohol and distilled water. Additionally, they were boiled before the next usage.



(a) **(b)**

Figure 4.2 (a) Carving the connection edge and (b) paste on the inner edges of mold

The sand samples were used in dry condition and again no pre-sterilization process was applied. The required soil masses were determined based on the targeted relative density values of sands. During the preparation of sand columns, in order to obtain homogenous samples, undercompaction method was used (Germaine & Germaine, 2009; Ladd, 1978). The method considers the fact that the bottom layers were compacted more with the compaction of upper layers. Hence, adjustment of layer heights was made for a homogenous application. The samples were prepared in 8 layers with heights of 13 mm for the bottom and around 12.4 mm for the other layers. For the calculation of these heights, the bottom layer undercompaction value was chosen to be 4% as stated by Germaine and Germaine (2009) as applicable for many soils. These undercompaction values decrease linearly to zero for the uppermost layer.

4.2 Treatment Procedure

After the preparation of samples for testing, all the samples were positioned on a previously manufactured table with holes, which fit to bottom of molds (Figure 4.3)



Figure 4.3 Molds positioned for treatments

Instead of an adjustable pipette, in this part of experiments peristaltic pump (LONGER Model BT100-1F Dispersing Peristaltic Pump with YZ1515X heading) was used for injections.



Figure 4.4 Beaker, peristaltic pump and molds

For the case of injections in samples treated with *S. pasteurii,* silicon tubing #25 with an inner diameter of 4.8 mm was used. Similarly, for the case of injections in samples treated with *B. licheniformis* silicon tubing #16 with an inner diameter of 3.1 mm were used. The reason for using different tubing was to block possible intervention of two bacteria in media. The calibration of peristaltic pump for required application rates was done before starting treatments.

The injection directions were reversed compared to the experiments in Chapter 2. Both the design of the molds and the possibility of bacteria moving out rapidly in downward injection due to gravity led to this decision. In these samples, because of the upward flushing, there could be a danger of sand moving upwards. The porous stones and filter paper on top could be also beneficial for blocking this situation.

Before starting treatments, two pore volumes of deionized water was flushed through each specimen at a rate of 10 ml/min. The amounts of water flushed were provided

in Table 4.2. The flushing was done for saturation of the samples (Lin et al., 2015; Al Qabany & Soga, 2013). This helps relatively controlled flow field (Martinez et al., 2013).

The treatments used in the samples for unconfined compression tests were basically the same with treatments in Set 3 of experiments in syringes. The molarities of the substances and final optical density of bacteria were kept the same with that set , i.e., - 333 mM urea, 50.4 mM $CaCl_2$, 3 g nutrient broth, 25.2 mM $NaHCO_3$, 187 mM NH_4Cl, OD_{600} = 1 and initial injections had $CaCl_2$ as well. Inevitably, using greater amounts of sands required usage of more amount of substances and more bacteria pellets. As in previous experiments, approximately 1.1 pore volume in initial treatment and the same amount of cementation media were injected into molds for subsequent treatments (Table 4.1). All the nutrients were prepared in METU Biological Sciences Department Microbial Ecology Laboratory and transported to METU Civil Engineering Department Soil Mechanics Laboratory. Since the amounts required for the experiment setup for a day was greater than a few liters, the media were prepared almost on a daily basis. The media were kept in refrigerator if the preparation was made a day before the experiments. The urea medium (Figure 4.5) and calcium chloride were mixed in beakers before injections via a peristaltic pump.



Figure 4.5 Adjustment of urea medium amount for treatment

In the first set of experiments, slightly less number of injections than experiments in syringes were performed. 20 treatments were applied in 7 days for Set 1 (Table 4.3). The number of treatments is similar to the one proposed by Feng and Montoya (2016) for moderately cemented soils although some differences in treatments exist between this study and theirs. Starting with the initial treatment (consisting of bacteria, urea medium and calcium chloride), after a retention time of 6 hours, two cementation media were injected into the soil with 3 hours interval in between. In the following days, 4, 3, 4, 1, 2 and 3 injections were applied respectively. In the days with multiple injections, each consecutive injection had a 3-hour interval as well. In the 5th day 3 hours before the injection, flushing of the samples with deionized water with around 0.7 pore volumes was done for cleaning the substance that did not interact with other substance and the effluent. For the injection of initial treatment media, a rate of 5 ml/min was adopted. For all other injections, 10 ml/min rate was used. The rate of injections was adopted based on many studies in the literature. There are examples of smaller and higher injection rates. For example, in one study, higher injection rates of bacteria resulted in more bacteria flushing out from the system compared to lower injection rate conditions (van Paassen, 2009).

Table 4.3 Treatment strategy of Set 1

	Number of Treatments						
Before	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
- Bacteria Harvesting -Mold and Sample Preparation -Media Preparation	3	4	3	4	Flushing with water & 1	2	3

In the bacterial growth stage, although centrifuged twice, some of the bacteria pellets did not accumulate at the bottom of the tubes. Therefore, in Set 1 the sample numbers 3 and 7 had bacterial injections given as a liquid. The optical density measurements

of these bacteria samples were done as in other samples. Following this injection, the cementation media were also injected immediately.

During the application of samples 1 and 5, white deposits occurred whenever the calcium chloride was added to the beaker in initial injections (bacteria, urea medium and calcium chloride). Following the termination of the tests, the white colored occurrences were visible beneath the porous stones. These occurrences could be early precipitated calcium carbonates. It is important to note that number 1 and number 5 samples were treated with *S. pasteurii* (Figure 4.6).

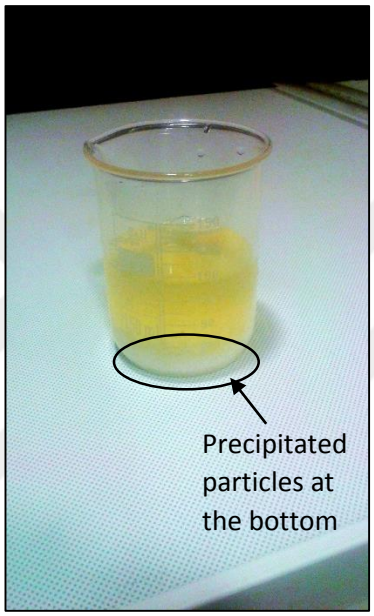


Figure 4.6 Precipitated particles at the bottom of beaker

Following the injections, the tubing connection parts of molds were sealed with paraffin and then removed before the new injection. While removing the seals at the bottom before the new injections, there were considerable drainage of liquids especially from samples 3 and 7 compared to other specimens. This could be attributed to possible clogging, precipitation etc.

In Set 2, the number of treatments and durations differed from Set 1. The sample numbers, names, bacteria types, relative density values and injection rates were the same with the corresponding ones in Set 1.

In Set 2, all the bacteria pellets had accumulated well at the bottom of the tubes.

In samples 1 and 3, the same white precipitates were visible in the beaker whenever bacteria, urea medium and calcium chloride were mixed in the first injection. Therefore, as a precaution, inclusion of calcium chloride to the beaker was a little bit delayed for the remaining samples. For the samples treated with *B. licheniformis*, no such observations were made.

Treatment strategy for Set 2 is provided in Table 4.4. Feng and Montoya (2016) had around 10 injection cycles to obtain a lightly cemented specimen in their study.

Table 4.4 Treatment strategy of Set 2

	Number of Treatments		
Before	**Day 1**	**Day 2**	**Day 3**
-Bacteria Harvesting -Mold and Sample Preparation -Media Preparation	3	4	3

4.3 Unconfined Compression Tests, Results and Discussions

Following the last treatments to the samples and drainage of liquids from the molds, molds were left for air drying initially. In Set 1, after 5 days of air drying time, the samples were still wet. Therefore, the samples were kept in oven at 60°C for 3-4 more days. The molds were positioned in the oven with clamps loosened. In Set 2, the air drying process and oven drying process were made for shorter time and after a few days from the last treatment, the samples were ready for testing. Samples 6 and 8 were kept inside the oven one more day since before testing there was moisture on filter papers. All samples were tested in dry conditions. After the tests, the moisture contents of the samples in both sets were determined to be negligible.

For tests, Wykeham Ferrance Tritech 10 was used with a proving ring number 15197 and a proving ring constant of 0.075 kg/div (Figure 4.7). Although there is not any official standard for unconfined compression tests on microbially treated soils,

ASTM D2166 – Standard Test Method for Unconfined Compressive Strength of Cohesive Soils and literature were used as guide whenever necessary in determination of specimen dimensions (approximately, 50 mm diameter and 100 mm height) and strain rate (0.5 mm/min).



Figure 4.7 Unconfined Compression Test equipment

In each set, the samples were tested with filter paper to prevent leakage of sands from the sides due to heterogeneous precipitation or during extraction of samples from the molds. Two edges of the filter paper were separated to eliminate the confining effect of filter paper during shearing.

In Set 1, samples 6, 7 and 8 were not suitable for testing. Extraction of sample 7 required considerable effort which may indicate heavy precipitation of $CaCO_3$ at some certain points.

In Set 2, only sample 3 was not tested. The innovative modifications in this set decreased the number of losses. Samples treated with *S. pasteurii* released strong smell during the experiments. On the other hand, samples treated with *B. licheniformis* did not smell as much as *S. pasteurii*. During the experiments, when necessary, smoothening of the upper surfaces were made by pouring untreated sand from the top. Figures 4.8 to 4.11 show experiments and Table 4.5 summarizes the results. Additionally, bar charts in Figures 4.12, 4.13 and 4.14 show test results.

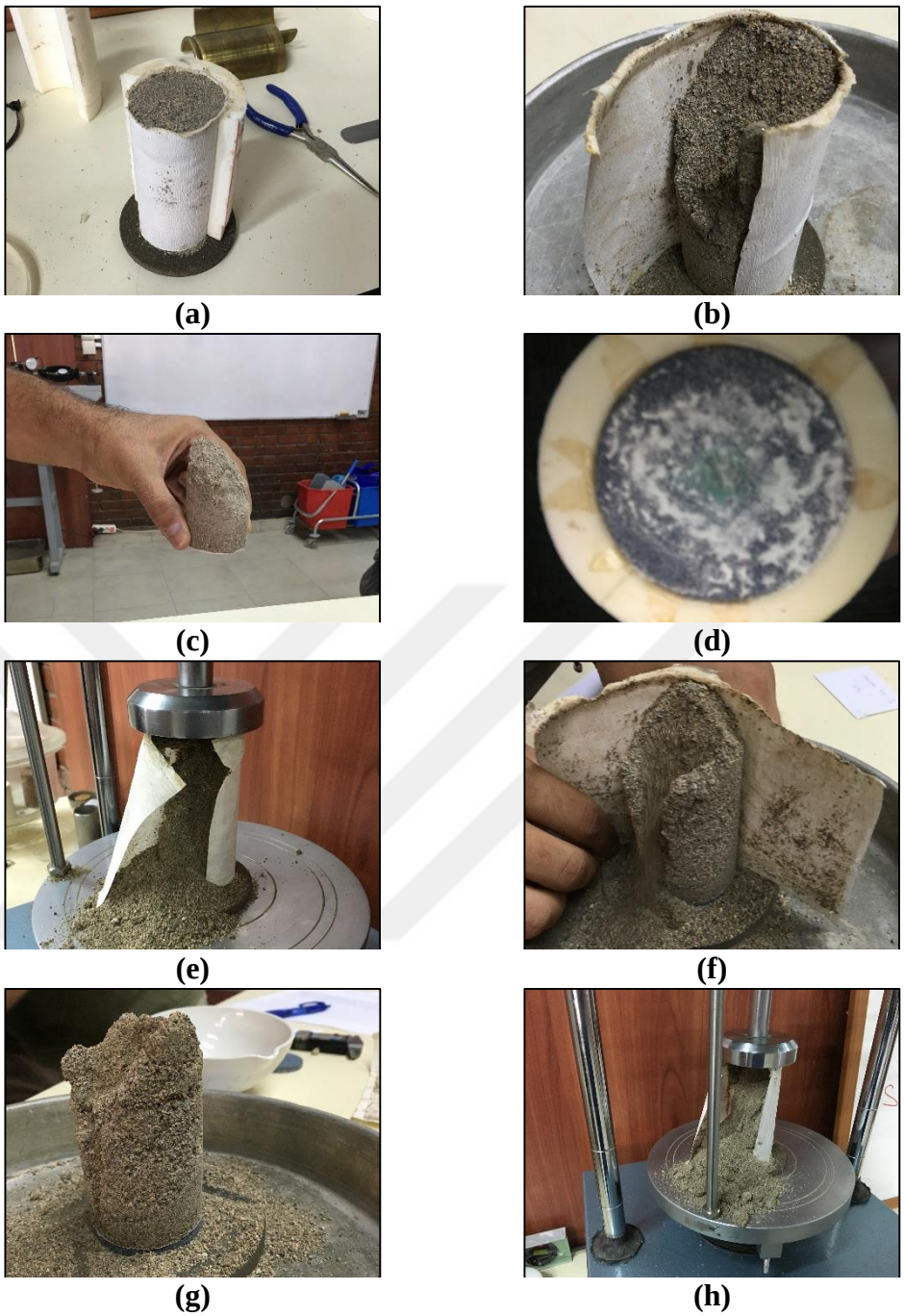


Figure 4.8 Set 1 (a) Sample 1 during extraction, (b) and (c) Sample 1 after testing, (d) bottom of porous stone, Sample 1, (e) Sample 2 during testing, (f) and (g) Sample 3 after testing, (h) Sample 4 after testing

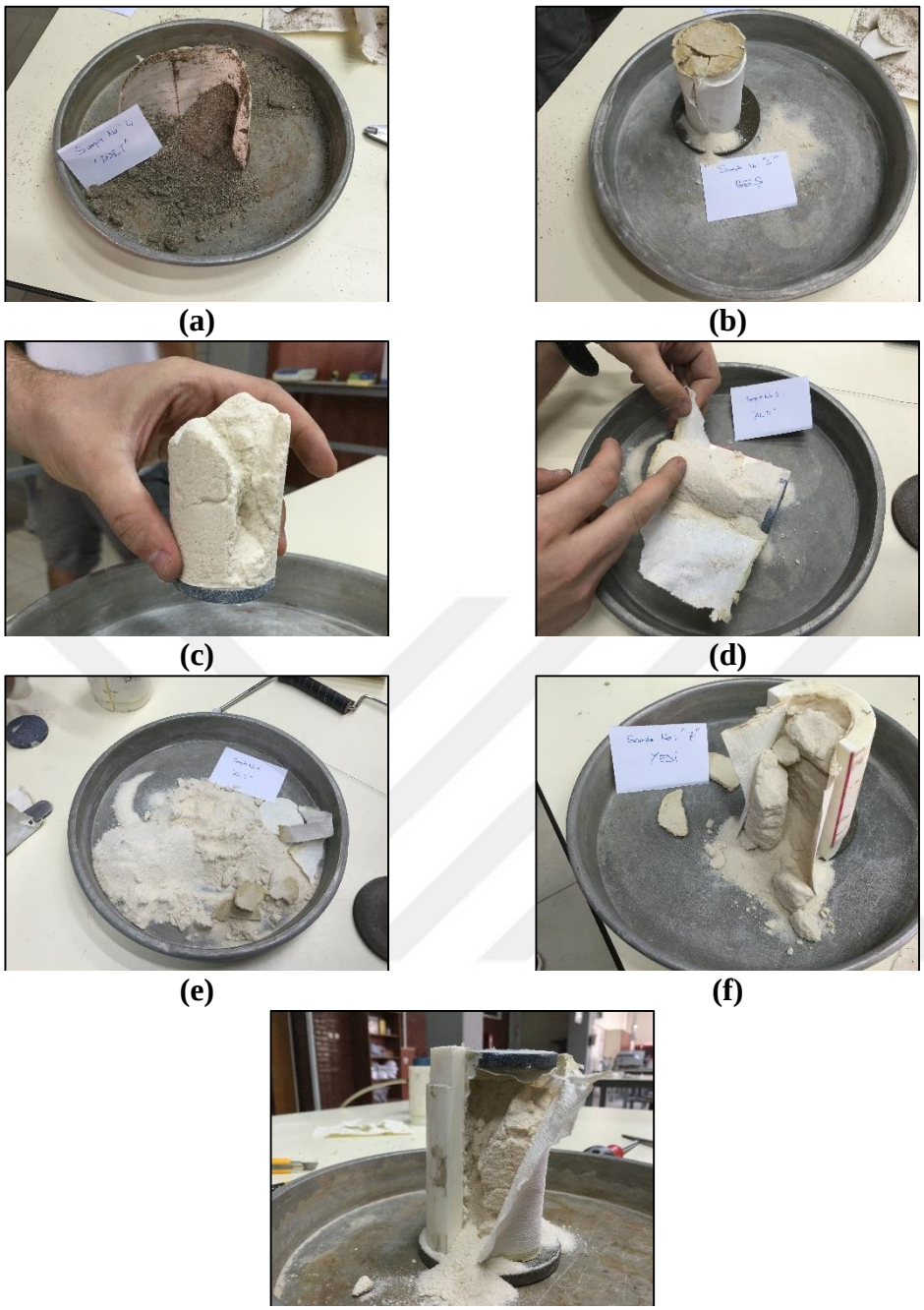


Figure 4.9 Set 1 (a) Sample 4 after testing, (b) and (c) sample 5 after testing, (d) and (e) Sample 6 after testing, (f) Sample 7, (g) Sample 8

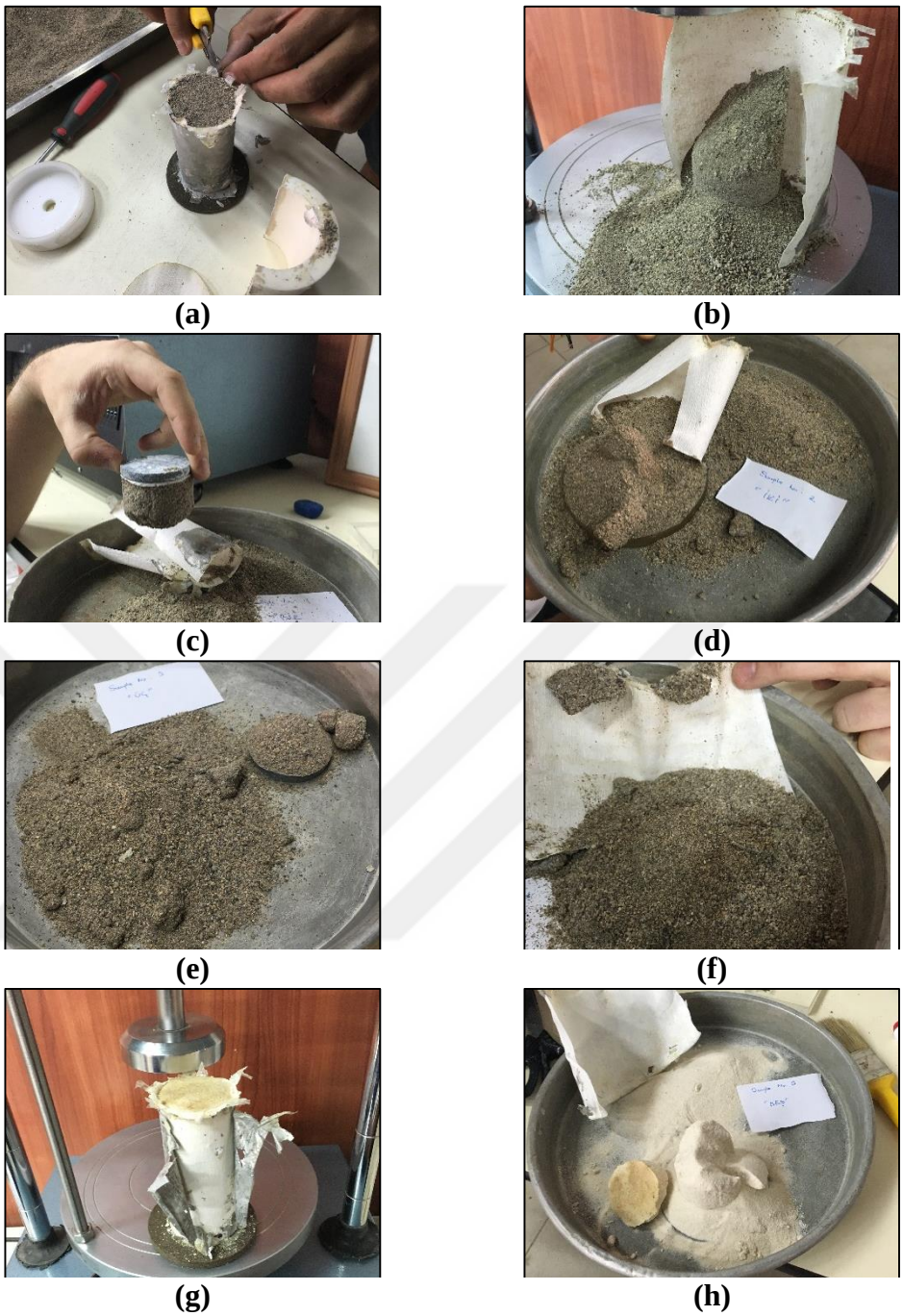
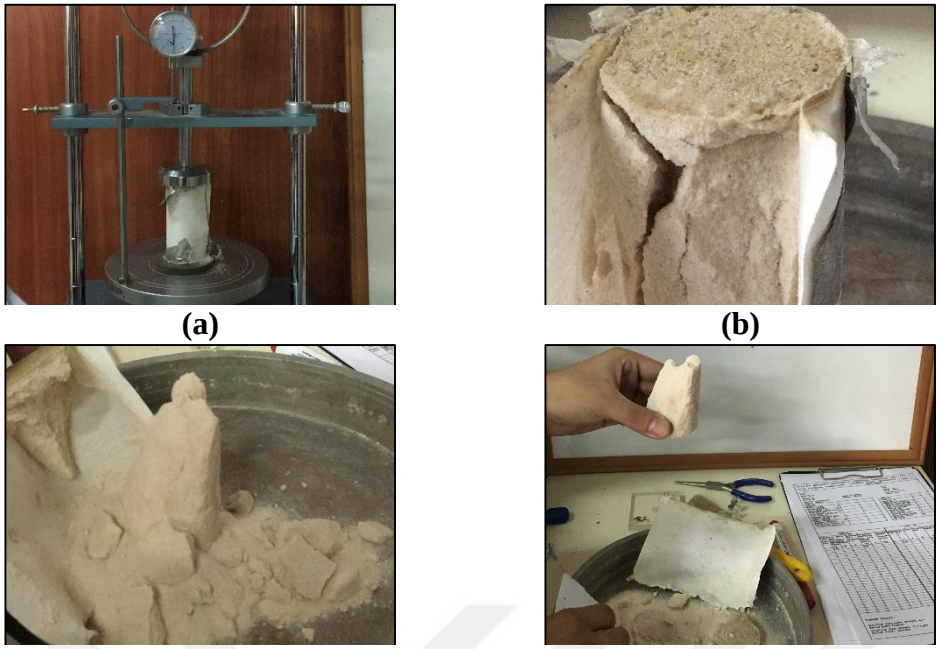


Figure 4.10 Set 2 (a), (b) and (c) preparation of Sample 1 and after testing, (d) Sample 2 after testing, (e) Sample 3 after testing, (f) Sample 4 after testing, (g) Sample 5 prepared for testing, (h) Sample 5 after testing



(a) **(b)**

(c) **(d)**

Figure 4.11 (a), (b), (c) and (d) Set 2 Sample 7

Table 4.5 Summary of Unconfined Compression Test Results

		Sample Dimensions		Strength Properties	
SET 1		**D(mm)**	**H (mm)**	**UCS, q_u (kPa)**	**Strain (%)**
Sample 1	Sand 1, s.p, D_r = 40%	49	101	53	1,8
Sample 2	Sand 1, b.l, D_r = 40%	49	101	18	1,0
Sample 3	Sand 1, s.p, D_r = 70%	49	101	50	2,3
Sample 4	Sand 1, b.l, D_r = 70%	49	101	25	1,1
Sample 5	Sand 2, s.p, D_r = 40%	49	101	57	3,6
SET 2		**D(mm)**	**H (mm)**	**UCS, q_u (kPa)**	**Strain (%)**
Sample 1	Sand 1, s.p, D_r = 40%	51	101	15	0,9
Sample 2	Sand 1, b.l, D_r = 40%	49	101	6	0,5
Sample 4	Sand 1, b.l, D_r = 70%	49	100	19	0,5
Sample 5	Sand 2, s.p, D_r = 40%	50	101	16	1,4
Sample 6	Sand 2, b.l, D_r = 40%	49	101	23	0,4
Sample 7	Sand 2, s.p, D_r = 70%	50	101	55	1,2
Sample 8	Sand 2, b.l, D_r = 70%	49	101	13	0,2

Note 1: Diameters and heights were measured by vernier caliper and rounded to the nearest integer.

Note 2: Strain values at the peak strength are provided.

Note 3:"Sample 1, Sand 1, s.p, D_r=40%" indicates the first sample which was prepared by using Sand 1 with a relative density of 40% and treated with *S. pasteurii* , b.l is used for samples with *B. licheniformis* .

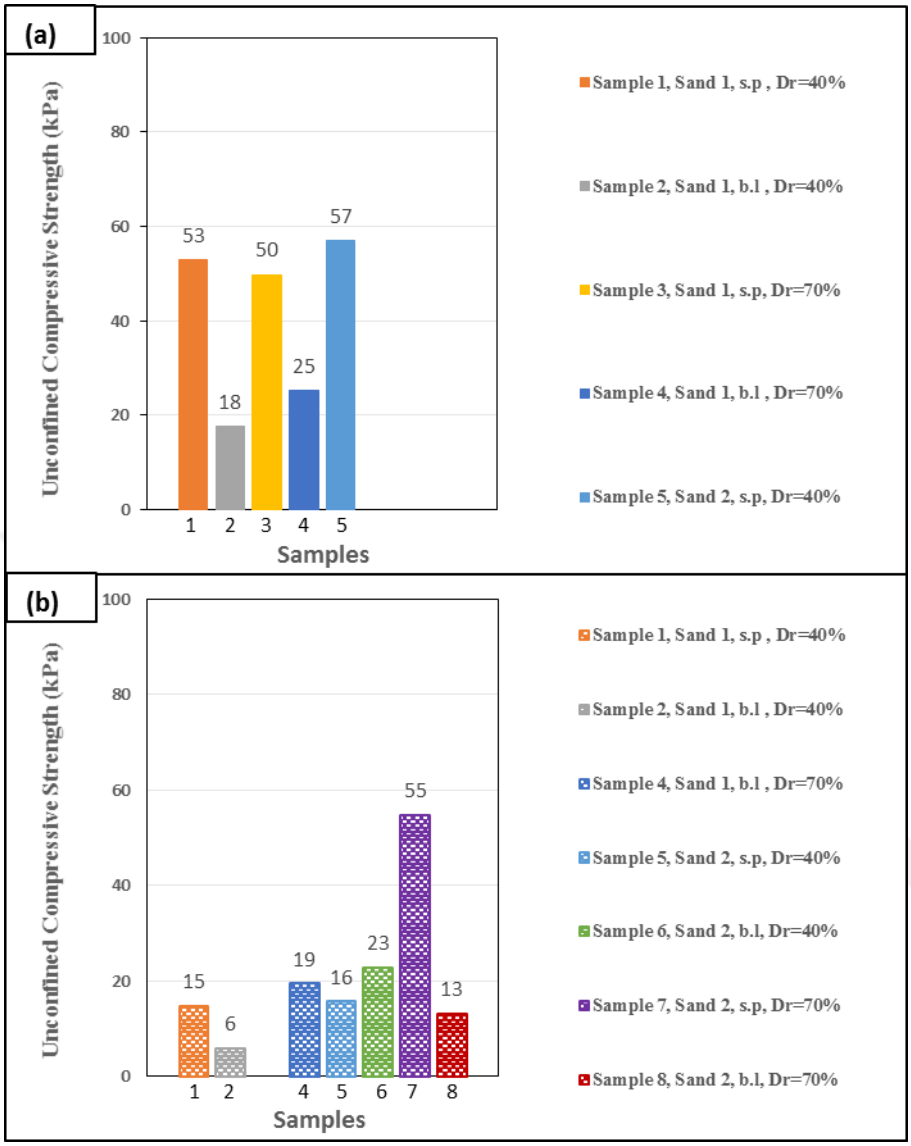


Figure 4.12 (a) Set 1 and (b) Set 2 Results ("Sample 1, Sand 1, s.p, D_r=40%" indicates the first sample which was prepared by using Sand 1 with a relative density of 40% and treated with *S. pasteurii*)

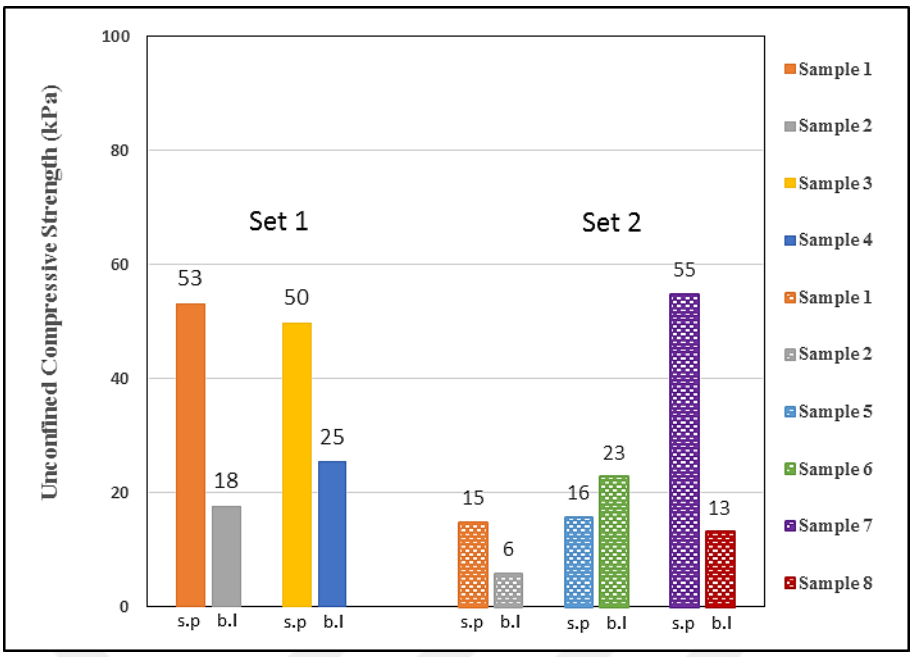


Figure 4.13 Comparison of UCS values based on bacteria (solid fills denote Set 1 and pattern fills denote Set 2, samples with *S. pasteurii* are abbreviated as s.p and samples with *B. licheniformis* as b.l)

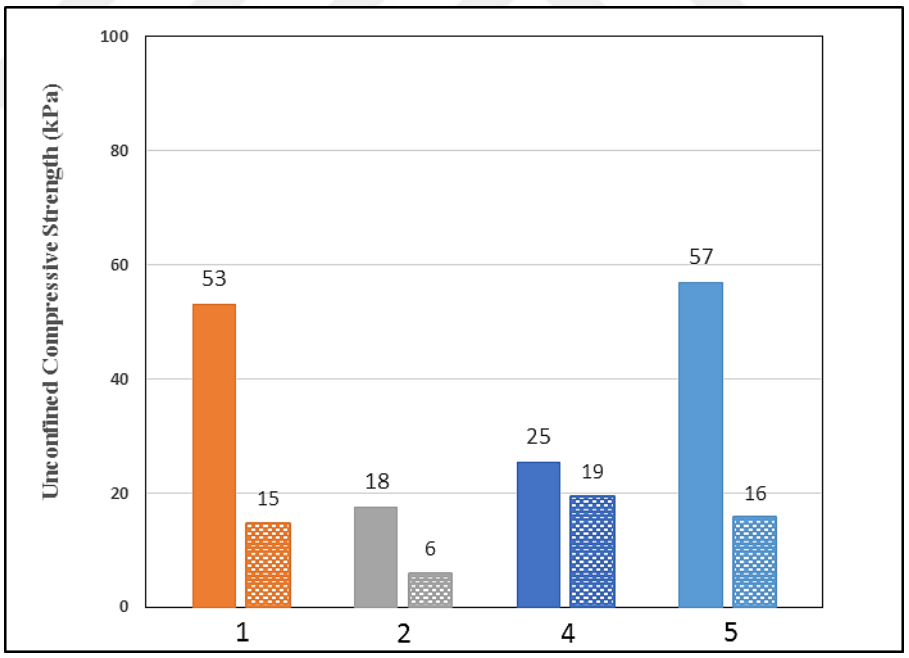


Figure 4.14 Comparison of UCS values of same samples in Set 1 and Set 2 (solid fills denote Set 1 and pattern fills denote Set 2, sample numbers are written on horizontal axis)

The observations and experiment results indicated the importance of more homogenous samples. Necessary precautions were taken (injection rates, injection strategy etc.) against this situation, yet heterogeneity problem was obvious especially in some samples. Early precipitations in *S. pasteurii* samples could have happened and this could lead to clogging at certain locations. Also, there were examples of samples, treated with *S. pasteurii*, with stronger bottom parts compared to weaker upper parts (Figure 4.10-d).

The unconfined compressive strength values are within the range of 6 to 57 kPa. The strain values corresponding to the peak strength values are between 1.0 and 3.6 % for Set 1. In Set 2, peak strengths were reached at lower strain values changing between 0.2 and 1.4 %. For samples treated with *S. pasteurii*, in Set 1, the unconfined compressive strength values varied between 50 and 57 kPa, whereas in Set 2, they were in between 15 and 55 kPa. The samples treated with *B. licheniformis* have strength values of 18 and 25 kPa in Set 1 and values changing between 6 and 23 kPa in Set 2. The strength value of Sample 7 in Set 2 is remarkable compared to other samples in the same set. Unfortunately, there is not a corresponding sample tested in Set 1 for comparing the behavior of this sample for different number of treatments. Still, in Set 1, the same sample was observed to have signs of considerable strength (Figure 4.9-f). Stress-strain curves for samples in both sets are provided in Appendix.

In Set 1, the tested samples treated with *S. pasteurii* had greater strength values than the ones treated with *B. licheniformis.* In Set 2, except the pair of samples 5 and 6, the same trend occurred. Overall, the success of *S. pasteurii* over *B. licheniformis* can be deduced from the results (Figure 4.13). The stronger smell during the tests, some possible signs of early precipitations, the visual inspections following the shearing of samples also support this claim.

For comparing the effect of treatment numbers and durations, 4 pairs, with members from each set, were considered. Both in the samples treated with *S. pasteurii* and *B. licheniformis,* there was a clear indication of stronger samples with increasing treatment numbers and durations (Figure 4.14). This finding is similar to what Feng and Montoya (2016) suggested that increasing cementation levels happened with increasing number of treatments.

In the literature, studies related to sand type and its mineralogy existed which were mentioned in Chapter 2. To make a comparison in this study about the sand type, samples 1 & 5 in Set 1 and samples 1 & 5, 2 & 6, 4 & 8 in Set 2 can be used. Sand 1 (Sample 1) and Sand 2 (Sample 5) treated with *S. pasteurii* with target relative density of 40% had similar unconfined compressive strength values in both sets: In Set 1, the strength values were 53 and 57 kPa, in Set 2 values were 15 and 16 kPa for Sands 1 and 2, respectively. For samples treated with *B. licheniformis*, in Set 2, in sample pair 2 & 6, sample with Sand 2 had higher strength value. However, for sample pair 4 & 8, Sand 1 had higher unconfined compressive strength value than Sand 2. This study does not indicate any certain deduction related to sand type, mineralogy.

In the research by Rowshanbakht et al. (2016), it was stated that increasing relative density resulted in higher unconfined compressive strength and lower calcium carbonate precipitation. However, in this study no such overall trend was observed. The main difference was observed in Set 2 between samples 5 and 7. Sand 2 treated with *S. pasteurii* had unconfined compressive strength values of 16 and 55 kPa with relative density values of 40% and 70% respectively.

CHAPTER 5

SUMMARY, CONCLUSIONS AND FUTURE WORK

5.1 Summary

In this study, the engineering properties of sands were improved by modifying physical characteristics through the employment of biological agents, i.e., bacteria, which was achieved due to the process called Microbially Induced Calcium Carbonate Precipitation (MICP). As one of the most recent and interesting ground improvement methods, bacteria were preferred in this study to be able to handle the issues related to sustainability and economy.

With the aim of understanding the general scheme of MICP and therefore producing an environmentally friendly improvement technique, the experiments were accomplished incrementally at two stages in this study: (i) small scale experiments in syringes and (ii) performing conventional unconfined compression tests. In the first stage, the focus of the small scale experiments in syringes was on the usage and comparison of two species of *Bacillus*, namely *S. pasteurii* and *B. licheniformis* for MICP. The main reason behind the selection of these bacteria was to compare the calcium carbonate precipitation ability of a widely studied bacteria (*S. pasteurii*) and relatively an under-recognized one (*B. licheniformis*) on two different sand (named Sand 1 and Sand 2) specimens. Sand 1 was obtained from the vicinity of Ankara, Turkey and Sand 2 is Quartz Sand. In this scope, optimization of some variables for the nutrients were covered. The interesting findings of the analytical methods such as X-ray diffraction (XRD), scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) for the verification of calcium carbonate precipitation were the complementary parts of this work.

In the second part of this study, the main objective was to demonstrate and measure the strength increase in lab specimens and quantify this enhancement in engineering terms. Unconfined compression tests were carried out using two sets of specimens to understand the contribution of the cementation to improvement on the strength properties of Sand 1 and Sand 2, prepared at two different target relative densities, 40% and 70%, respectively. The bacteria same as experiments in syringes were used on sands. The investigations were focused on using different treatment duration and number of treatments.

5.2 Conclusions

Understanding the concepts of MICP and drawing conclusions accordingly was a challenge, requiring digestion of combined information at the intersection of different fields such as Biology, Chemistry and Civil Engineering. Within this framework, the main concept of this work was the application of MICP on the sands. Investigation of treatment behavior of Sand 1 was performed for the first time in the literature. Experiments lead to draw following conclusions in this study:

- Experiments in syringes indicated microbially induced calcium carbonate precipitation in both type of sands which were confirmed with SEM and XRD monitoring techniques.
- The behavior of *B. licheniformis* needs to be further examined to ensure its effects on calcium carbonate precipitations as chemical precipitation is possible whenever the required conditions are satisfied.
- In general, the success of *S. pasteurii* over *B. licheniformis* was deduced from the results of unconfined compression tests and experiments in syringes.
- The unconfined compressive strength values of treated sands changed between 6 and 57 kPa. The corresponding strain values are between 1.0 and 3.6 % for the first set. In the second set, however, peak strengths were reached at lower strains, changing between 0.2 and 1.4 %.
- It was concluded that the more number of treatments and durations were applied, the stronger samples were obtained.

- The post-test inspections of the samples and results of the experiments showed the importance of having homogenously improved laboratory soil samples. Especially with samples treated with *S. pasteurii* and tested using unconfined compression test, stronger bottom parts compared to weaker upper parts were observed.
- Although this study inherently intended to investigate also the effects of use of different mineralogy of sands prepared at different relative densities, the results did not produce meaningful relationships and therefore further studies are needed.

5.3 Future Work

With two different approaches taken for accurately comprehending the engineering behavior of microbiologically improved soils, the study ended in opening new horizons for future works, the incomplete list of which are outlined below:

- This study focused on the use of two sands for understanding improvement behavior of bacteria. The variability in the type of sands as well as using different relative densities are the immediate next step of this work.
- Similarly, the use of other types of bacteria for more effective improvement is a further study subject.
- Although small scale experiments are intended in this study, in order to understand the field behavior of microbiologically treated soils, transitions to large scale experiments are necessary, which will also enlighten practical aspects of this improvement technique.
- In treatment studies, the use of different bacteria, changing the amount of calcium chloride for treatment need to be investigated further to see their corresponding effects, whether or not they increase the success rate of improvement either qualitatively or quantitatively.
- Not only specifically for this study, but also almost all the scientific work performed with bacteria indicate that the treated soils are mostly sand

because of its inter-granular and molecular structure. However, the use of silty soils and clayey soils needs to be further investigated using modified views of MICP ground improvement philosophy.

- The effects of migration of ammonium or adjusting pH of the media used in treatments need to be investigated as such studies have the potential to produce better remediated soil samples.
- Instead of using bacteria as additive materials, other ways of improving the soils such as bio-stimulation needs to be further examined as it makes the use of existing microbial populations in soils.

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APPENDIX

STRESS-STRAIN CURVES OF SAMPLES

In this part of the thesis, stress-strain curves for the samples tested in unconfined compression test are provided.

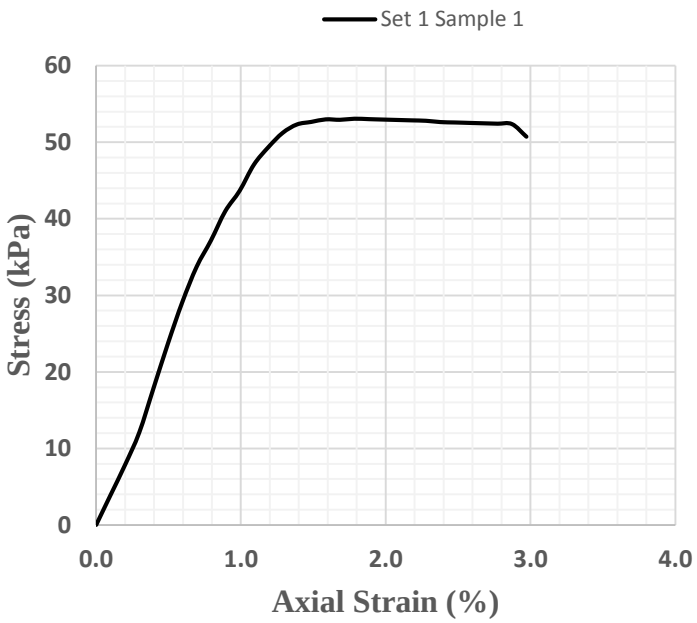

Figure A.1 Stress-Strain curve for Set 1 Sample 1

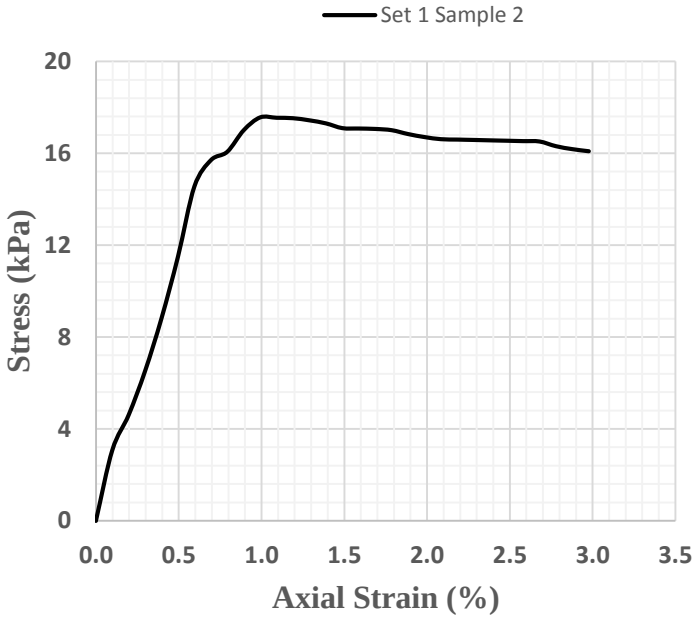

Figure A.2 Stress-Strain curve for Set 1 Sample 2

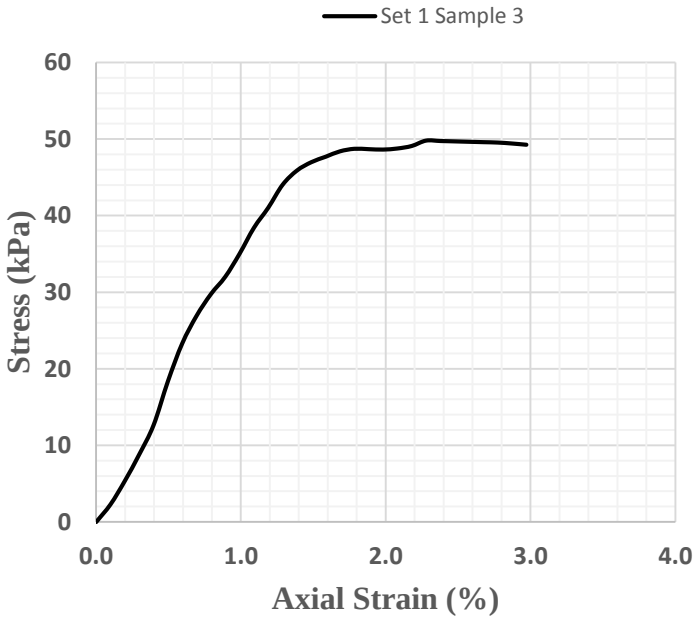

Figure A.3 Stress-Strain curve for Set 1 Sample 3

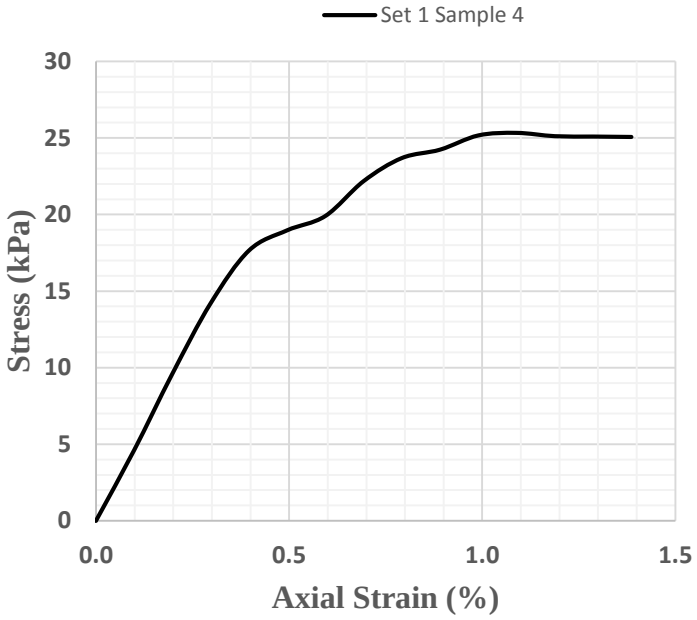

Figure A.4 Stress-Strain curve for Set 1 Sample 4

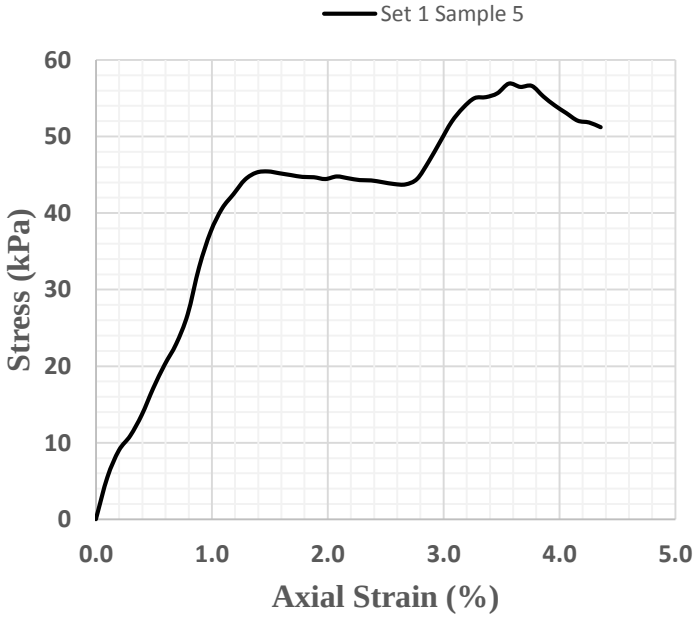

Figure A.5 Stress-Strain curve for Set 1 Sample 5

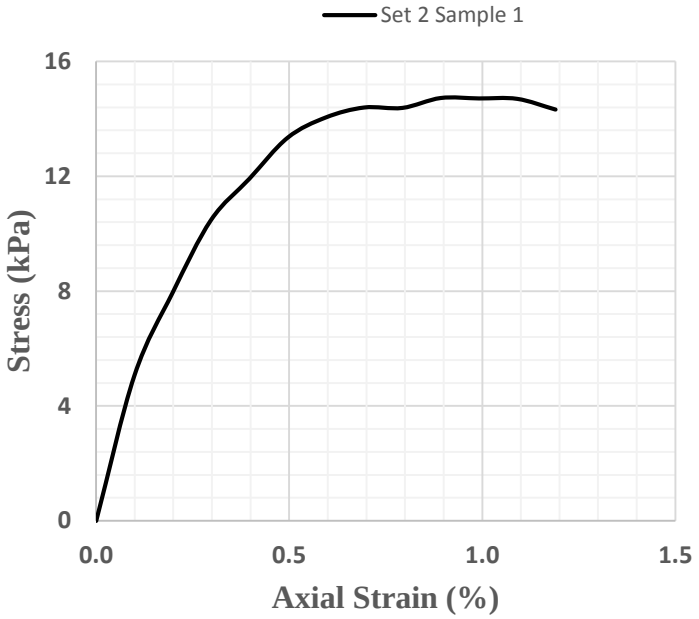

Figure A.6 Stress-Strain curve for Set 2 Sample 1

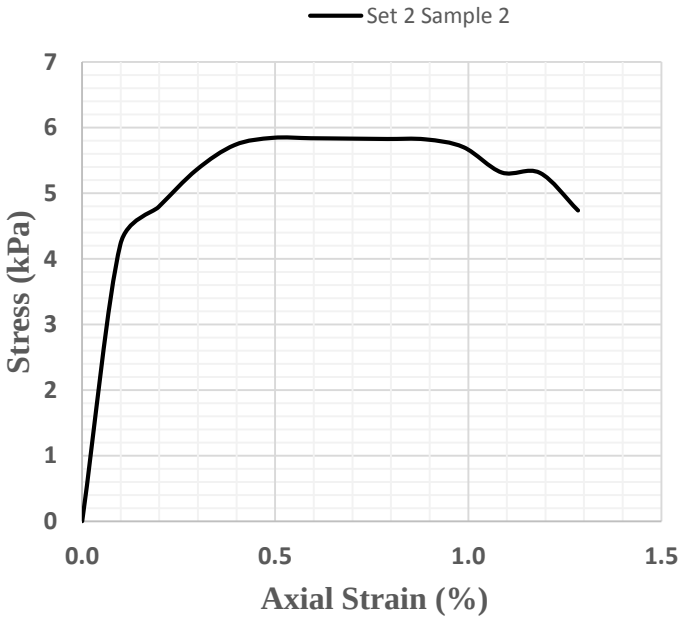

Figure A.7 Stress-Strain curve for Set 2 Sample 2

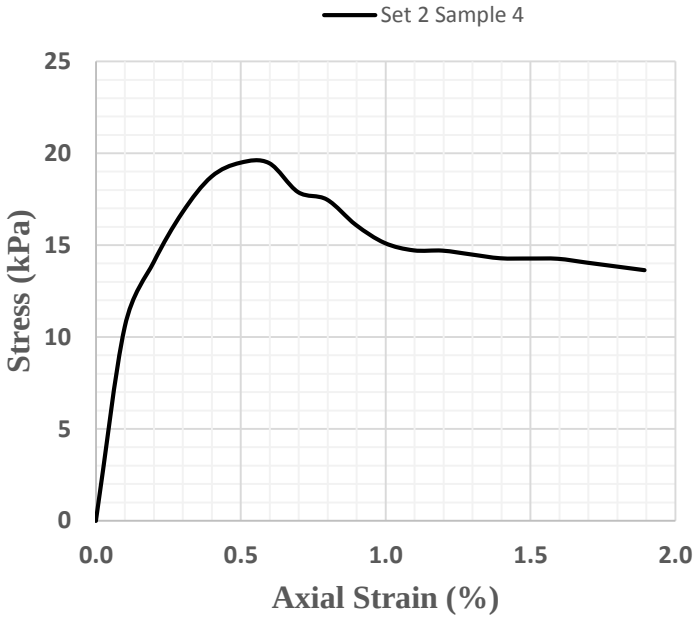

Figure A.8 Stress-Strain curve for Set 2 Sample 4

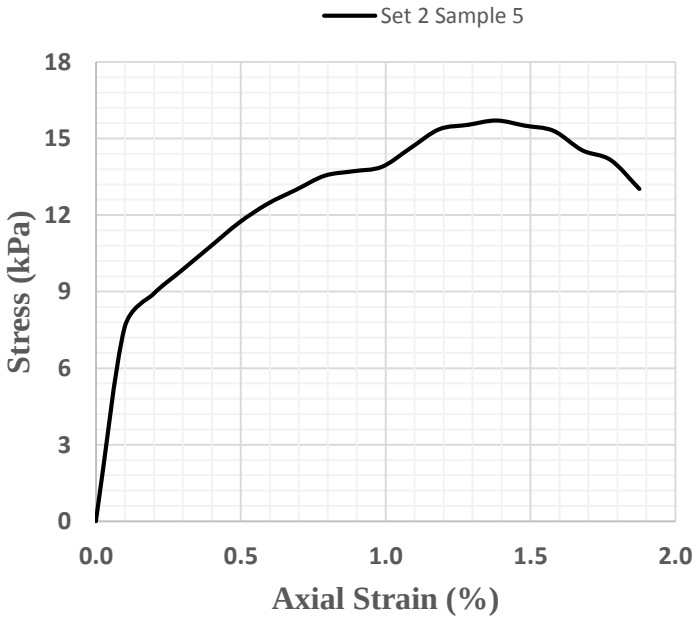

Figure A.9 Stress-Strain curve for Set 2 Sample 5

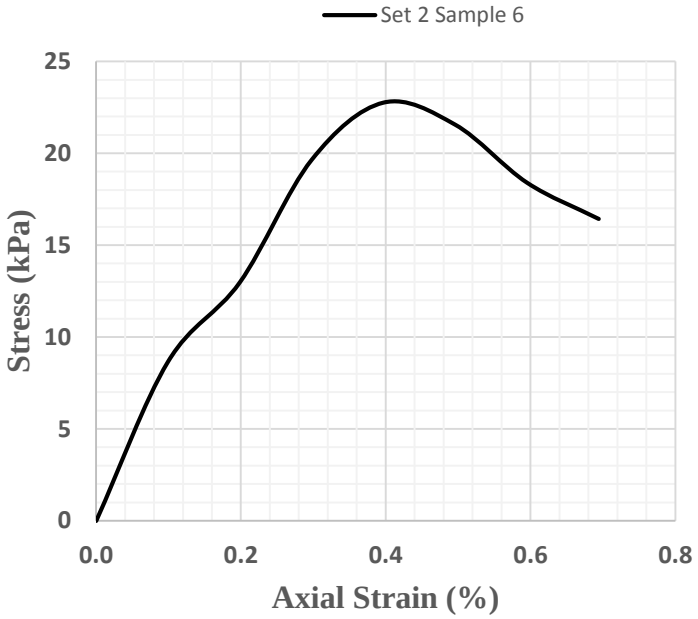

Figure A.10 Stress-Strain curve for Set 2 Sample 6

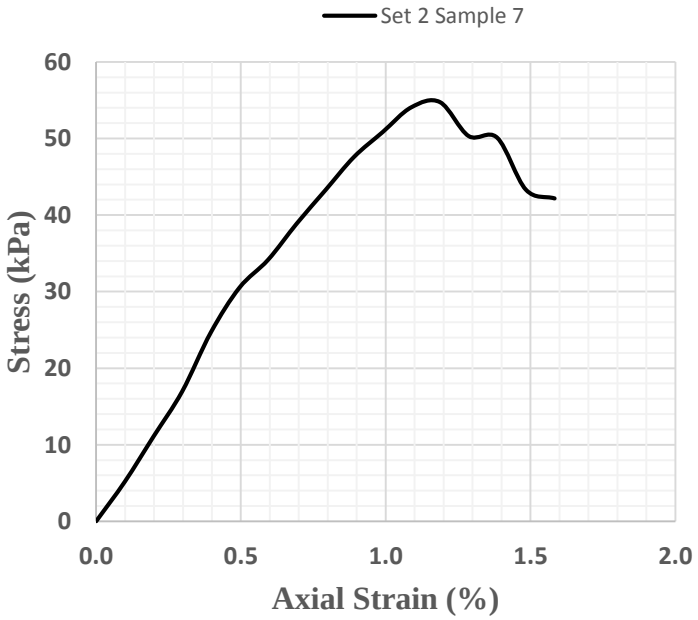

Figure A.11 Stress-Strain curve for Set 2 Sample 7

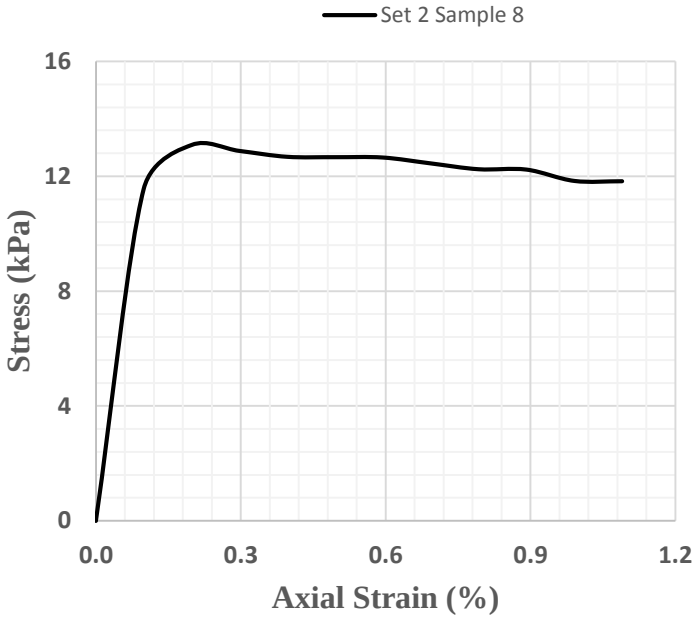

Figure A.12 Stress-Strain curve for Set 2 Sample 8