

**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF ARTS AND
SOCIAL SCIENCES**

**BIOGENIC BUILDING MATERIAL FORMATION BY THE TREATMENT OF
NATURAL STONE WASTE WITH MICROBIAL INDUCED CALCITE
PRECIPITATION**



M.A. THESIS

Erdem GÜNSÜR

Department of Interior Design

International Master of Interior Architectural Design M.A. Programme

JUNE 2018

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JUNE 2018

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ SOSYAL BİLİMLER ENSTİTÜSÜ

**DOĞAL TAŞ ATIKLARININ MİKROBİYOLOJİK OLARAK UYARILMIŞ
KALSİYUM KARBONAT ÇÖKELMESİ METODU İLE İŞLENMESİ SONUCU
BİYOJENİK YAPI MALZEMESİ ÜRETİMİ**

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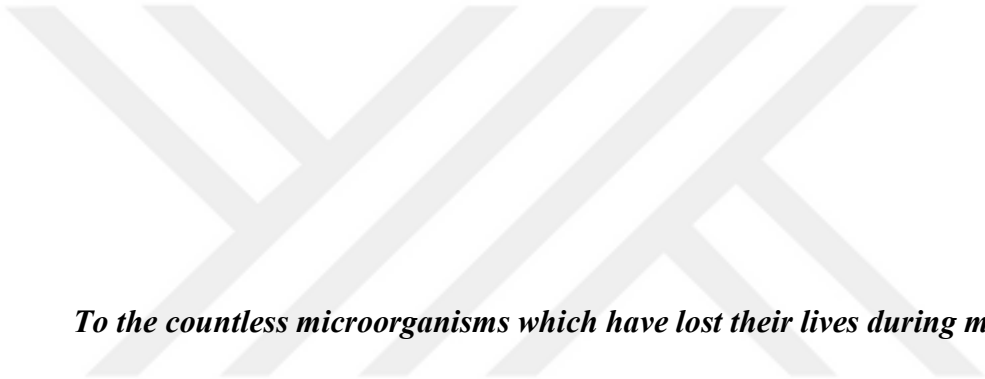
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To the countless microorganisms which have lost their lives during my research,



FOREWORD

This thesis aims to create a potential solution to a specific problem of the natural stone industry through multidisciplinary collaboration and integrative thinking.

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BIOGENIC BUILDING MATERIAL FORMATION BY THE TREATMENT OF NATURAL STONE WASTE WITH MICROBIAL INDUCED CALCITE PRECIPITATION

SUMMARY

The rapid increase in the use of natural resources, uncontrolled waste production and carbon emission levels have triggered the search for new and sustainable solutions throughout various industries across the globe. The rising demand and awareness for natural and sustainable materials have concluded in environmentally friendly products varying in a wide range of functions from simple household objects to sophisticated construction materials. This attitude has mainly affected industries with high carbon footprints such as the global construction industry. Being in direct contact with architecture, design and civil engineering disciplines, the construction industry has always been exposed to rapid change towards technological possibilities and contemporary solutions. Now more than ever, solutions rooted from nature are feeding ideas for this industry, which are not only changing the way for building things but changing the way for building the future.

Nature and its way of work provide a careful eye with solutions which have the potential to change industries. Comprehension and adaptation of natural processes and the study of living forms can open up pathways beyond conventional ways of looking at things. This can be achieved by multidisciplinary collaboration which is the essential peak for innovation. Through multidisciplinary collaboration and integrative thinking, ideas and informations between various fields can be combined together to come up with solutions which leap in new directions. A specific problem of one field can be solved by different solutions provided by others.

Nearly one third of the world's natural stone reserves are located in Turkey, making the local mining industry one of the backbones of the Turkish economy. Being in direct relation with the construction industry, the mining industry has also grown in time to answer the increase in demand. Besides depleting natural reserves, the mining industry has specific problems in terms of energy consumption and high amounts of waste production. Disposal of such wastes produced during the extraction, sawing, polishing and water treatment processes of natural stones is a vital problem when environmental care is in question. Natural stone waste in the form of dust and thin aggregate can mix with water reserves or easily move with the wind causing great harm to the ecosystem. Since there is no sustainable and economic way of reusing or recycling this waste, producers tend to dispose them either by burying or dumping natural stone wastes on rural sites. This problem suggests that a novel way of reusing or reprocessing these wastes must be researched to achieve sustainable end results as well as economic growth.

Natural stones are products of natural processes which last hundreds of years or even millennia. Some of these natural processes involve living forms which contribute to

the formation of such minerals. Calcium carbonate (CaCO_3) is one of the most common minerals on earth and its precipitation via living organisms, varying from unicellular organisms to crustacea, can form various substances such as natural stones to shells of specific marine animals. Calcium carbonate precipitation by living organisms is a widely researched phenomenon and the advancement in science and technology offers researchers a wide spectrum of opportunities to benefit from such phenomena.

Calcium carbonate precipitation as a process offers a solution towards reusing natural stone wastes, since it can be used to fill the voids between loose particles and bind them together. Scientific researches also indicate that calcium carbonate precipitation via unicellular organisms can be induced under certain lab conditions. Biologically induced biomineralization (BIB) offers suitable and sustainable models for binding loose natural stone particles. One of such models; microbially induced calcium carbonate precipitation (MICP) can be achieved through various ways, using various types of microorganisms. Such mechanisms can be listed as, denitrification, sulphate reduction, iron reduction and etc.

Among various mechanisms of biocalcification, urea hydrolysis (ureolysis) is chosen as the mechanism which is to be used for binding loose particles together, since it has proven to be the most energy efficient mechanism among others and since urease activity can be seen in a vast variety of organisms ranging from microorganisms to plants.

MICP through ureolysis is being used for binding loose sand/soil particles together for various purposes such as erosion control, dust mitigation, ground improvement, rehabilitation of ancient monuments and etc. Once the microorganisms are placed inside the solid medium consisting of loose particles and cell retention is achieved, the voids between loose solid particles function as nucleation sites for the microorganisms. Extracellular enzyme secretion is coupled with the controlled flow of required ions and enzyme activity (ureolysis) is triggered, resulting in the filling of the voids between loose solid particles with calcium carbonate crystals.

MICP as a method, offers a sustainable solution for reusing such wastes both in terms of end product, production processes and the lifecycle of the product. The chemical composition of natural stones highly consist of calcium carbonate (CaCO_3), through microbial induced calcite precipitation such loose particles (natural stone wastes) can also be bound together with calcium carbonate (CaCO_3) links, resulting in a natural, inorganic and biogenic product with approximately the same chemical composition with the processed wastes. The process requires no sintering, firing or cooking of any sort which minimizes energy consumption and offers a carbon free production process.

Throughout the masters project, a thorough research about such biological processes (focusing on MICP) and the Turkish mining industry is made to fully comprehend such subjects in detail. A link between the faculty of architecture and the department of molecular biology and genetics is made within İstanbul Technical University to ensure multidisciplinary collaboration which is essential for the project.

The project is conducted in two phases; research and development of the material and its analysis. The research and development phase of the project is conducted at the molecular biology biotechnology and genetics research center. The research and development phase of the project consists of the optimization of the bacteria's cultivation parameters and extracellular enzymatic activity, biomass creation,

controlling the reaction rate and precipitation parameters, optimization of the solid media, small scale cementation experiments, bio-reactor design, upper scale experiments and cell retention improvements. The analysis of the created inorganic and biogenic material is conducted at the construction materials laboratory. The analysis phase of the project consists of granulometry analysis, capillary absorption tests, compression tests and SEM imagings.





DOĞAL TAŞ ATIKLARININ MİKROBİYOLOJİK OLARAK UYARILMIŞ KALSİYUM KARBONAT ÇÖKELMESİ METOTU İLE İŞLENMESİ SONUCU BİYOJENİK YAPI MALZEMESİ ÜRETİMİ

ÖZET

Doğal kaynakların ivmelenerek artan tüketimi, kontrolsüz atık üretimi ve karbon salımı değerlerindeki yükseliş dünya çapında bir çok endüstride yeni ve sürdürülebilir çözümlerin arayışını tetiklemiştir. Gün geçtikçe artan farkındalık seviyesinin yanında doğal ve sürdürülebilir ürünlere karşı olan talep basit ev gereçlerinden kompleks yapı malzemelerine kadar muhtelif amaçlara hizmet eden çevre dostu bir çok ürünün ortaya çıkması ile sonuçlanmıştır. Söz konusu tutum global inşaat endüstrisi başta olmak üzere karbon salımı değeri oldukça yüksek bir çok endüstriyi etkilemiştir. Mimarlık, inşaat ve çevre mühendisliği gibi disiplinler ile doğrudan ilişkili olan inşaat endüstrisi teknolojik gelişmeler ve çağdaş çözümler kapsamında her zaman hızla gelişen yeniliklere ve değişikliklere karşı açık olmuştur. Kaynağını doğadan alan çözümler günümüzde her zamankinden daha güçlü biçimde söz konusu endüstriyi besleyen fikirlerin gelişimine zemin hazırlamaktadır.

Doğa ve doğanın çalışma biçimi dikkatli bir çift göze endüstrileri değiştirme potansiyeline sahip çözümler sunabilmektedir. Doğal süreçlerin ve canlılığın sunduğu olanakların incelenerek anlaşılması ve söz konusu süreçlerin adaptasyonu olayların algılanması noktasında konvansiyonel bakış açılarından sapmayı sağlamaktadır. Bu türden bir farklılık multidisipliner işbirlikleri neticesinde elde edilebilmektedir. Multidisipliner işbirlikleri ve yenilikçi düşünce ile farklı disiplinler arasındaki fikir ve bilgiler bir araya getirilebilmekte ve alışılmışın dışında yeni yollar açan çözümlere ulaşılabilmektedir. Herhangi bir disipline ait spesifik bir sorun farklı disiplinlerin sunacağı çözüm önerileri ile çözülebilmektedir.

Dünyadaki doğal taş rezervinin neredeyse üçte biri Türkiye’de bulunmakta ve bu durum yerel doğal taş endüstrisini Türk ekonomisinin bel kemiklerinden biri haline getirmektedir. İnşaat endüstrisi ile doğrudan ilişkili olan doğal taş endüstrisi zaman içerisinde artan talebe cevap verebilmek adına büyümüştür. Doğal kaynakların tüketiminin yanı sıra, doğal taş endüstrisinin yüzleştiği temel sorunlar arasında yüksek enerji maliyetleri ve atık üretimi gelmektedir. Konu çevrenin korunumuna geldiğinde ise doğal taş endüstrisinin madencilik ve fabrika işlemleri esnasında ürettiği atıkların ortadan kaldırılması ciddi sorun arz etmektedir. Toz ve kum şeklindeki ince doğal taş atıkları rüzgar ile kolayca hareket edebilmekte, yeraltı sularına karışabilmekte ve ekosisteme ciddi biçimde zarar verebilmektedir. Söz konusu atığı geri kazanmanın sürdürülebilir ve ekonomik bir yolu olmadığı için üreticiler genellikle doğal taş atıklarını bakir alanlara dökmekte ve belirli yerlere gömmektedir. Bahsi geçen problem söz konusu atıkların geri kazanımı ve/veya işlenmesi noktasında çevreyi ve yerel ekonomiyi olumlu etkileyecek sürdürülebilir çözümlerin araştırılmasının önemini irdelemektedir.

Doğal taşlar yüzlerce yıl süren doğal süreçler sonucunda oluşmaktadır. Söz konusu süreçlerin bazıları doğal taş oluşumuna etki eden canlıları da içermektedir. Kalsiyum karbonat (CaCO_3)doğada en yaygın biçimde bulunan minerallerden biridir. Tek hücrelilerden kabuklulara kadar bir çok canlının metabolik aktivitesinin sonucu oluşan kalsiyum karbonat doğal taş oluşumundan deniz canlılarının kabuklarına kadar bir çok maddeyi meydana getirmektedir. Kalsiyum karbonatın canlılar tarafından biyolojik süreçler neticesinde oluşturulması yaygın biçimde incelenen bir hadise olmanın yanında bilim ve teknolojiadaki gelişmeler söz konusu süreçlerden fayda sağlama noktasında bir çok olanak sunmaktadır.

Kalsiyum karbonat oluşumu bir süreç olarak doğal taş atıklarının birbirine bağlanarak geri kazanılması noktasında potansiyel bir çözüm sunmaktadır. Bilimsel araştırmalara göre tek hücreli canlılar kullanılarak laboratuvar ortamında kalsiyum karbonat oluşumunu sağlamak mümkündür. Çeşitli biyomineralizasyon modelleri bu noktada serbest parçaların birleştirilmesi kapsamında sürdürülebilir bir çözüm sunmaktadır. Söz konusu modellerden biri olan mikrobiyolojik olarak uyarılmış kalsiyum karbonat çökmesi (microbial induced calcite precipitation)bir çok farklı mikroorganizma ile bir çok farklı mekanizma yoluyla kullanılabilir. Bahsi geçen mekanizmalar arasında üre hidrolizi en enerji verimli mekanizma olmasının yanında tek hücrelilerden çok hücrelilere kadar bir çok farklı canlıda rastlanan bir enzim aktivitesi olduğu için çalışma kapsamında tercih edilen mekanizma olmuştur.

Üre hidrolizi yolu ile mikrobiyolojik olarak uyarılmış kalsiyum karbonat çökmesi sağlanması erozyon kontrolü, toz engelleme, zemin güçlendirme ve tarihi eserlerin korunumu gibi konularda tercih edilen bir metottur. Mikroorganizmalar serbest parçacıklardan oluşan katı ortama yerleştirilmekte ve hücre dışı enzim salgısı tetiklenmektedir. Gerekli iyonların katı ortam içerisine kontrollü akışının sağlanması ile serbest parçacıkların arası çöken kalsiyum karbonat ile dolmaktadır.

Bir metot olarak mikrobiyolojik olarak uyarılmış kalsiyum karbonat çökmesi doğal taş atıklarının geri kazanımı göz önüne alındığında sadece final ürün olarak değil aynı zamanda ürünün yaşam döngüsü noktasında da sürdürülebilir bir çözüm sunmaktadır. Doğal taşların kimyasal yapısı yüksek miktarda kalsiyum karbonat içerdiğinden söz konusu parçacıkların benzer yapıdaki kalsiyum karbonat ile birbirine bağlanması girdi ile oldukça benzer bir kimyasal yapıya sahip doğal, inorganik ve biyojenik bir çıktı elde edilmesini sağlamaktadır. Üretim süreci herhangi bir sinterleme, fırınlama veya pişirme gerektirmediğinden enerji tüketimi oldukça düşük ve çevreye duyarlı bir süreçtir.

Tez çalışması kapsamında ilintili biyolojik süreçler ve Türk doğal taş endüstrisi hakkında kapsamlı bir araştırma yapılmıştır. İstanbul Teknik Üniversitesi bünyesinde Mimarlık Fakültesi ve Moleküler Biyoloji ve Genetik Bölümü arasında bir köprü kurulmuş ve multidisipliner bir çalışma gerçekleştirilmiştir.

Çalışma araştırma ve geliştirme aşaması ve analiz aşaması olmak üzere iki ana aşamada yürütülmüştür. Araştırma ve geliştirme aşaması İTÜ Moleküler Biyoloji Biyoteknoloji ve Genetik Araştırma Merkezi bünyesinde tamamlanmıştır. Söz konusu aşama kapsamında bakterinin yetiştirilme şartlarının ve metabolik aktivitesinin optimizasyonu, biyokütle oluşturulması, üre hidrolizi reaksiyonu parametrelerinin optimizasyonu, küçük ölçekli taşlaşma deneyleri, biyo-reaktör tasarımı ve üretimi, büyük ölçekli taşlaşma deneyleri ve katı ortam içerisinde hücre tutunmasını geliştirmeye yönelik deneyler bulunmaktadır. Analiz aşaması İTÜ Yapı Malzemeleri Laboratuvarında tamamlanmıştır. Söz konusu aşama kapsamında katı ortamın optimizasyonu, granülometri analizi, kapiler su emme deneyleri, basınç testleri ve elektron mikroskobu görüntüleme işlemleri bulunmaktadır.





1. INTRODUCTION

1.1 Turkish Natural Stone Industry

Being one of the most ancient and enduring construction materials human kind has used throughout history, natural stones still are amongst the most favoured construction materials because of their physical and chemical properties alongside their aesthetic value. Therefore, natural stone deposits are amongst the most valuable natural resources in terms of economic and industrial value. Due to its geographical location on the Alpine-Himalayan belt, Turkey has a vast variety of natural stone deposits bearing more than 250 different types of marbles [1].

Natural stones are categorized as igneous, sedimentary and metamorphic rocks according to their formation in nature. One of the most popular and preferred natural stones, marble is a type of metamorphic rock consisting predominantly of calcite and/or dolomite [2]. Due to their beauty marbles are among the most preferred and demanded aesthetic stones. With 3,872,000,000 m³ of valuable and extractable marble reserves [3], Turkey plays a vital role on the global natural stone industry withholding nearly 40% of the world's extractable natural stone reserves [4].

Within the last decade, the acceleration in the growth of the local construction industry in Turkey caused a rise in the demand for natural stone (marble, granite, travertine etc.) construction materials. Only in the year of 2009, the amount of marble produced in Turkey was approximately 2.7 Mm³ [4].

Since certain types of natural stones are unique to certain geographical locations, a vast variety of natural stones are exported from Turkey across the globe, making the local natural stone industry one of the backbones of the Turkish economy. However certain factors such as high prices due to energy consumption and logistics cause economical challenges for the producers. Another aspect which can be described as the most vital problem of the natural stone industry is the high amounts of waste generation during the mining, sawing, polishing and water treatment processes.

1.1.1 Waste generation of the natural stone industry

The production of natural stone materials consists of extraction, mining, sawing, polishing and water treatment procedures. During each procedure a considerable percentage of the stone is lost in the form of dust and/or aggregate, thus the natural stone industry is considered as one of the major waste generating industries. Natural stone wastes produced as the result of such processes are categorized as solid wastes and semi-liquid or slurry wastes [5]. Varying with the process involved in the production of the natural stone end material, the generated slurry waste can be equal to 20-30% of the stone block which is put to the process [6]. Combined with the other processes such as extraction and sawing nearly 70% of the natural stone is lost in the form of dust, aggregate and slurry [7].

Since there is no effective way of reprocessing or recycling such wastes, they are dumped into designated pits, burial sites, pasturelands, agricultural fields or even nearby riverbeds causing environmental pollution [8-9]. Natural stone waste is considered as a highly polluting waste because of its highly alkaline nature and because of the techniques used during its manufacturing process [10]. Fine wastes in the form of dust and powder can easily pollute aquatic resources and move with the wind negatively effecting flora and fauna. Slurry wastes produced during water treatment and polishing procedures can also mix with water resources if not properly filtered or collected, harming the natural environment and destroying plant life.

1.1.1.1 Applied ways of re-using natural stone waste

Waste generation has always been a vital problem for the natural stone industry. In accordance to scientific and technological developments methods for recycling and reusing such wastes have been developed throughout history. Such methods vary in a wide range from using such wastes for primers for painting techniques to formation of certain construction materials. However natural stone waste is primarily considered as a raw material or as a reinforcement material in construction materials such as bricks [11], ceramics [12], cement additives [13], desulphurization processes [14], infiltration [15], polymer based composite materials [16] and fillers in asphalt cement [17].

Although there are a wide range of novel methods and techniques for recycling or reusing natural stone wastes, such methods are insufficient for the recovery of huge

amounts of wastes. Another problem with such methods is that they require chemical adhesives and large amounts of energy consumption because of firing, cooking or sintering processes resulting in synthetic, inert and carbon emitting processes and end products which are not natural or environmentally friendly.

1.2 Chemical Composition of Natural Stone Wastes

Natural stones are products of natural processes. Wastes which are produced during the processing of natural stones are also natural by products. Metamorphic stones such as marbles are highly composed of the calcium carbonate crystal calcite (CaCO_3) and/or dolomite ($\text{CaMg}(\text{CO}_3)_2$) [2]. XRD analysis conducted on various natural stones indicate that a high percentage of the stones composition are of oxides such as calcium oxide (CaO) followed by silicon dioxide (SiO_2) [13].

1.3 Calcium Carbonate Formation as a Natural Process

Geological formations are constantly exposed to long lasting natural cementation processes through mineral precipitations due to physiochemical and microbiological reactions [18]. A large quantity of natural cementation of geological formations occur due to biomineralization, which is a process where living forms produce minerals.

Calcium carbonate (CaCO_3) is one of the most common minerals on earth and its precipitation via living organisms, varying from unicellular organisms such as prokaryotes to multicellular organisms such as plants or crustacea, can form various substances like natural stones to shells of specific marine animals.

Mineral synthesis by prokaryotes can be studied under two major classes: Biologically Controlled Mineralization (BCM) and Biologically Induced Mineralization (BIM) [19]. In the case of biologically controlled mineralization (BCM) synthesis of minerals occur at a specific location inside or on the cell. Whereas, mineral synthesis occurs extracellularly in the case of biologically induced mineralization (BIM) due to the metabolic activity of the organism [20].

Calcium carbonate precipitation by living organisms due to biologically induced biomineralization (BIM) is a widely researched phenomenon and the advancement in science and technology offers researchers a wide spectrum of opportunities to benefit from such phenomena.

1.3.1 Microbially induced calcite precipitation (MICP)

Microbially induced calcium carbonate precipitation (MICCP) also known as microbially induced calcite precipitation (MICP) is one of the most widely studied branches of biologically induced mineralization (BIM) processes. MICP is studied and discussed between multiple fields varying from biotechnology, geotechnology, paleobiology, civil engineering and environmental engineering [20].

MICP is a process where the formation of calcium carbonate crystals (calcite, vaterite, aragonite) occur due to the metabolic activity of the microorganism [21].

Calcite precipitation can occur through various processes including, denitrification [22], sulphate reduction, inducing dolomite precipitation [23], iron reduction, inducing ankerite precipitation [24] and urea hydrolysis [25].

1.3.1.1 MICP by urea hydrolysis

Urea is a common by product of certain biological processes of many living forms, i.e. mammals release urea to their natural environment as a detoxification product. Urease (urea amidohydrolase EC 3.5.1.5) is the enzyme which is apparent in a wide variety of organisms that inhabit aquatic and land environments. Biotic urease activity can be found both in soil and aquatic environments and is apparent in the metabolic activity of bacteria, yeasts, filamentous fungi [28], algae and plants [29].

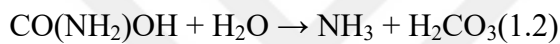
Enzymatic hydrolysis of urea is the most energy efficient process amongst other processes considered in biological calcite precipitation [26] and the required enzyme activity can be found in a vast variety of living forms ranging from microorganisms to plants [27]. The commercial use of purified enzymes are expensive and provide a limited interval of use, which renders the case of using organisms as enzyme production sources as a more plausible and attractive scenario. Once such organisms are cultivated and extracellular enzyme secretion is triggered under optimized conditions, the organisms can be used as sources for enzyme production. Stocking such grown cultures or biomass can provide the needed amount of cells for enzyme production at any given time with any desired quantity. Amongst the unicellular organisms with active urease activity, a Gram-positive prokaryote *Sporosarcina pasteurii* (American Type Culture Collection 6453), formerly known as *Bacillus pasteurii*, is favoured because of its high extracellular enzyme secretion attribute [34].

Certain factors are crucial for MICP through ureolysis to occur. Such factors include temperature, pH levels, the state of the bacteria (phases regarding lifecycle and its positioning on the growth curve), rate of enzyme secretion, enzyme activity, chemical composition of solutions regarding ion feed, the flow rate of ions and solutions and etc.

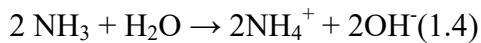
The chemical reactions regarding the hydrolysis of urea and calcium carbonate precipitation in relation with the enzyme activity can be explained in seven major steps as follows;



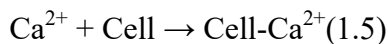
Urea is hydrolyzed by the urease enzyme producing ammonia and carbamate (Eq.1.1).



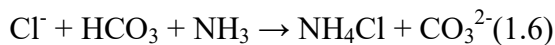
Carbamate instantly decomposes producing another ammonia molecule and carbonic acid (Eq.1.2).



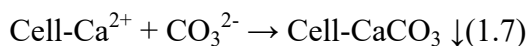
Carbonic acid and ammonia then equilibrate in water, forming bicarbonate, ammonium and hydroxide ions (Eq.1.3-1.4).



Positively charged calcium ions deposit on the cell surface of the microorganism which is negatively charged (Eq.1.5).



The presence of hydroxide ions result in an increase of pH levels which shifts the bicarbonate equilibrium and concludes in the formation of carbonate ions (Eq.1.6).



Free carbonate ions are attracted to the calcium ions which serve as nucleation sites by being deposited on the cell surfaces, resulting in the precipitation of calcium carbonate (Eq.1.7) [36].

Urease activity found in certain unicellular organisms is an extracellular metabolic activity, which results in the secretion of free enzymes into the environment. Calcium ion deposition on the cell surface of such microorganisms function as nucleation sites which trigger the induction of calcium carbonate within a specific range where the microorganism is present. Calcium carbonate crystals tend to grow around such nucleation sites in case optimum ion flow is conducted and the needed conditions are provided regarding temperature, pH levels and flow rate. Such growing calcium carbonate crystals (calcite, vaterite or aragonite) can function as bridges, binding loose particles together.

The morphology and formation of calcium carbonate crystals can differ according to the reaction rate, level of saturation and certain chemical factors such as solution chemistry and external factors like temperature, resulting in the creation of calcite, vaterite and aragonite [35]. Although the chemical formulas are the same, the morphology of such crystals define their attributes regarding stability and endurance. Calcite, having the most stable morphology is considered to be the most durable and strong crystal type of calcium carbonate in contrast to vaterite and aragonite.

1.3.2 MICP ranges of use

MICP is a widely studied phenomenon which has attracted a considerable amount of interest in the past years, since the range of possibilities offered by the method is vast. Various disciplines have implemented and adapted the use of MICP for various purposes. Such uses of MICP as a method include the restoration of natural stone structures and monuments [30], bioremediation [31], waste water treatment [32], strengthening of concrete [33], oil recovery [31] and ground improvement [34].

Multidisciplinary or cross disciplinary collaboration between multiple fields is essential for the acceleration of innovative technological and scientific developments, in the case of MICP various solutions are offered by multiple fields of research which address various problems. Although parallel fields such as biotechnology, geotechnology and paleobiology profit from MICP, certain cross disciplinary fields like civil engineering, environmental engineering and even

architecture benefit from MICP as a method for coming up with innovative solutions [20].

Such solutions include structure strengthening where certain loose particles or voids between solid bodies are filled by calcium carbonate precipitation with the retention of organisms or enzymes on specific locations within or on such solid media. Other solutions include the filtration of liquid media rich in carbonate by attracting the ions and triggering calcium carbonate precipitation within such liquid media.

1.3.2.1 MICP as a method for ground improvement

Among the many uses of MICP, the use for ground improvement is one of the most distinctive uses of the method in question for binding loose solid particles together. MICP is used for soil stabilization, dust prevention and erosion control in various landscapes where loose particles of sand, soil, dust and aggregate cause such environmental problems. Soil stabilization by MICP is conducted in several steps. First the targeted area of loose particles are injected with the liquid media containing grown bacteria with ureolytic activity. Once the injection is completed, precautions are made to ensure that the bacteria is retained within the solid medium and not washed out. Such precautions include the optimum amount of ion flow with the opposite charge in contrast to the cell surface charge of the bacteria and/or in-situ incubation. After cell retention is achieved, controlled flow of specific solutions with the suitable chemical composition is conducted for triggering the hydrolysis of urea and the precipitation of calcium carbonate. Once the reaction is complete and the desired values of cementation are achieved throughout the solid body the process is terminated [34].

Using MICP as a method for ground improvement is one of the most suitable methods for binding loose solid particles together by creating calcium carbonate bridges between such particles. With the correct reaction rates and optimized reaction parameters stable and durable calcium carbonate crystal formations (calcite) can be achieved in theory, concluding in a successful cementation reaction and a stable solid body. Certain factors regarding the solid particles are also crucial for the cementation reaction or MICP through ureolysis to succeed. Such factors include, particle size distribution, particle surface morphology, pore volume of the solid body, pore throat size and the chemical composition of the particles.

Pore volume and pore throat size are directly linked to the particle size distribution of the solid body. Optimum values for pore volume and pore throat size are calculated according to the size of the microorganism (bacteria) which is used for the process. There are two factors which are vital regarding the size of the bacteria; the initial size of the bacteria and its final size after ureolysis which can increase 20 times in volume. If the particle sizes are above optimum values, the voids between such particles would be too large to be filled with precipitated crystals and cell retention may be prevented resulting in the flushing out of the cells. If the particle sizes are below optimum values, the voids between such particles would be too small for the bacteria to pass through resulting in clogging and failure in uniform cementation throughout the entire body. The correct size of uniform particles can result in obtaining the desired pore volume as well as the correct mixture of particles with various sizes.

It is also possible to conduct such experiments under controlled laboratory conditions using customized systems. Solid media can be placed inside specific apparatuses which function similar to filtration systems, keeping the solid media intact but allowing liquid flow throughout the entire body at the same time. This aspect of such specific systems allow control over various parameters like reaction intervals regarding time, liquid flow rate, pressure, temperature and pH levels which are vital for the reaction. Thus allowing the user to experiment and obtain results in relation to specific changes regarding specific parameters. Such systems also allow the cemented solid body to be taken out analyzed. Similar systems have been designed and used by various researchers for such purposes, the most prominent one among such systems is known as the 'calcite in-situ precipitation system'.

The sample obtained from such controlled cementation experiments can then be put to tests to analyze certain chemical and physical characteristics of the cemented body. Such tests include capillary water absorption experiments, compression experiments, scanning electron microscope (SEM) imaging, XRD analysis and etc. Conclusions of these experiments can be used to determine the function of the material obtained from cementation reactions. Whereas the obtained data can also be used to make alterations in the parameters which control the cementation reaction and eventually change the chemical and physical characteristics of the end product according to the desired outcome.

1.4 Purpose of the Thesis

Natural stone industry in Turkey can be considered as one of the backbones of the Turkish economy. Both in terms of national assets and export volume the local natural stone industry is a powerful force which navigates the economy. With nearly 40% of the worlds natural stone reserves, Turkey is one of the leading manufacturers of natural stone products throughout the globe [4]. Considering that the natural stone industry is one of the leading industries in high amounts of waste production, the amount of produced waste is directly proportional with natural stone production. Precautions about recycling or reusing such wastes have proven to be insufficient considering the vast amounts of produced waste. Such precautions can also fall short in reusing or recycling such a waste which is hard to process and which threatens the natural environment.

Microbial induced calcite precipitation (MICP) offers a plausible way of reprocessing such wastes. Through MICP, natural stone wastes which are loose particles rich in calcium carbonate can be bound with induced crystal bridges which are also composed of calcium carbonate. Without the need for sintering, firing or chemical adhesives, MICP mimicks the same process of calcium carbonate formation which can be found in nature. By accellerating the natural process of calcium carbonate formation under laboratory conditions, MICP offers a fast and sustainable solution for reusing a waste which poses a major problem in terms of environmental pollution and sustainability.

The purpose of the thesis is to use MICP as a method for reprocessing natural stone wastes to create an inorganicand biogenic material. Which promotes sustainability not only in terms of waste reuse, but also prevention of environmental pollution and by the creation of a material which can be ground and reprocessed over and over after it has completed its estimated life cycle. The goal is to differentiate from other existing methods for reusing natural stone wastes by a completely natural process which does not require high levels of energy consumption or carbon emission. Thus, producing a natural and biogenic material from a completely natural material which is hard to dispose of.

Regarding the attention and demand for natural and sustainable materials and the rising awareness considering environmental care, creation of such natural and

biogenic materials from a waste product has the potential to offer new solutions both in terms of function and added value creation.



2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strain

The specific strain of Gram positive alkaliphilic bacteria; *Bacillus pasteurii* (ATCC 11859), renamed as *Sporosarcina pasteurii* with biosafety level 1 is used for microbial induced calcite precipitation because of its highly active extracellular urease activity.

The strain is obtained from ATCC in freeze dried (lyophilized) condition, stored at 2 to 8°C. ATCC (American Type Culture Collection) is a non-profit organization which collects and distributes standard reference microorganisms for research and development based studies.

2.1.2 Culture media

2.1.2.1 NH4-YE liquid medium

The specific growth medium suggested for *S.pasteurii* is identified as NH4-YE growth medium (ATCC Medium 1376: *Bacillus pasteurii* NH4-YE medium). For the cultivation of the bacteria in liquid medium the composition of the growth medium is given as follows;

20g/L of Yeast Extract (Acumedia)

10 g/L of Ammonium Sulfate (NH₄)₂SO₄ (Merck)

0.13M Triss Buffer (pH 9.0) (AppliChem)

2.1.2.2 NH4-YE agar

The specific growth medium suggested for *S.pasteurii* is identified as NH4-YE growth medium (ATCC Medium 1376: *Bacillus pasteurii* NH4-YE medium). For the cultivation of the bacteria on agar the composition of the growth medium is given as follows;

20g/L of Yeast Extract (Acumedia)

10g/L of Ammonium Sulfate $(\text{NH}_4)_2\text{SO}_4$ (Merck)

0.13M Triss Buffer (pH 9.0) (AppliChem)

20g/L Agar (Acumedia)

2.1.3 -80°C stock solutions

2.1.3.1 Glycerol

85% sterile glycerol solution is used for the preparation of -80°C stock cultures.

2.1.4 Cementation fluid for ureolysis

The cementation fluid consists of solutions required for ureolysis to occur and calcium carbonate to precipitate as a result of the chemical reaction. The base of the cementation fluid is mainly composed of the specific liquid growth medium $\text{NH}_4\text{-YE}$. The composition of the cementation fluid is as follows;

1M Urea $\text{CO}(\text{NH}_2)_2$ (Sigma-Aldrich)

1M Calcium Chloride dihydrate $\text{CaCl}_2 \cdot 2\text{dH}_2\text{O}$ (Merck)

20g/L Yeast Extract (Acumedia)

10g/L Ammonium Sulfate $(\text{NH}_4)_2\text{SO}_4$ (Merck)

0.13M Tris Buffer (pH 9.0) (AppliChem)

2.1.5 Fixation fluid for cell retention

The fixation fluid is used for the supply of positively charged calcium ions which are used to attract negatively charged cell surfaces resulting in the retention of cells inside the solid medim. The fixation fluid is composed of;

50mM Calcium Chloride dihydrate $\text{CaCl}_2 \cdot 2\text{dH}_2\text{O}$ (Merck)

2.1.6 Natural stone waste

2.1.6.1 Specific type of marble: Agora Beige

The processing wastes (sawing, polishing and milling processes) of Agora Beige, a specific type of marble mined from the region of Manisa, Turkey are collected from the factory of Efendioğlu Marble Industry Trade Inc. located in Bursa, Turkey. The

natural stone waste is composed of solid particles with various sizes ranged between 1000µm-60µm.

The physical and chemical analysis of the specific natural stone is made by Süleyman Demirel Univeristy Mining Engineering Department Natural Stone Technology Laboratory for Efendioğlu Marble Industry Trade Inc. on June 2015. The chemical analysis of the natural stone is given in Table 2.1 below.

Table 2.1: Chemical Analysis of the marble named Agora Beige.

CHEMICAL ANALYSIS (TS EN 15309)	Al ₂ O ₃	CaO	Fe ₂ O ₃	MgO	Na ₂ O	K ₂ O	SiO ₂	SO ₃	LOI
	0.10	43.24	0.45	12.25	0.25	0.01	0.46	0.10	43.29

2.1.6.1 Specific type of marble: Olive Avantgarde

The processing wastes (sawing, polishing and milling processes) of Olive Avantgarde, a specific type of marble mined from the region of Kastamonu, Turkey are collected from the factory of Efendioğlu Marble Industry Trade Inc. located in Bursa, Turkey. The natural stone waste is composed of solid particles with various sizes ranged between 1000µm-60µm.

The physical and chemical analysis of the specific natural stone is made by Süleyman Demirel Univeristy Mining Engineering Department Natural Stone Technology Laboratory for Efendioğlu Marble Industry Trade Inc. on June 2016. The chemical analysis of the natural stone is given in Table 2.2 below.

Table 2.2: Chemical Analysis of the marble named Olive Avantgarde.

CHEMICAL ANALYSIS (TS EN 15309)	Al ₂ O ₃	CaO	Fe ₂ O ₃	MgO	Na ₂ O	K ₂ O	SiO ₂	SO ₃	LOI
	0.56	53.54	0.17	1.15	0.053	0.212	1.59	0.21	43.34

2.1.7 Cementation Experiment Materials

2.1.7.1 Well Plate Cementation Experiment System

For the small scale cementation experiments well plates (Corning® Costar® TC-Treated Multiple 24 Well Plates) are used, with a single well volume of approximately 3.2mL, shown in (Fig.2.1.) Each of the wells are drilled to allow excess fluid flow. Single sheets of filter paper are placed inside each well to prevent solid particles to be flushed out.



Fig.2.1 : 24 well plate example.

2.1.7.2 Falcon cementation experiment system

For the small scale cementation experiments 15ml falcon tubes (Capp) are used, shown in (Fig. 2.2.) Each falcon is drilled at the bottom end to allow excess fluid flow. 1-3ml of sponge filters are placed inside each falcon to prevent solid particles to be flushed out. The falcons are then fixed inside a stand on top of a collection flask where the flushed liquid media are collected.



Fig. 2.2 : 15ml falcon tube example.

2.1.7.3 Calcite in situ precipitation system

For upper scale controlled cementation experiments a ‘calcite in situ precipitation system’ is designed and produced. The system consists of a reactor body and collection cup, a peristaltic pump and tubes, a flask containing the required solutions and a magnetic stirrer.

Reactor Body

A reactor body is designed and produced for controlled liquid flow through the solid media. The reactor is composed of five parts; cylindrical upper cap (PVC) with a

pneumatic fixing for tube connection, cylindrical upper filter body (PVC) for the placement of stainless steel ball filters and sponge filters ensuring a homogenous liquid flow, cylindrical reactor core (teflon) with an inner volume of 24,54ml (radius: 1,25cm, height: 5cm) shown in, cylindrical lower filter body (PVC) for the placement of sponge filters and cylindrical lower cap (PVC).

The reactor core is produced from teflon to ensure that the cemented solid body does not stick inside the core and that it can be removed with ease.

All of the five parts are designed to be screwed together, each having rubber O-rings in between for the prevention of liquid leakage between joints, shown in (Fig. 2.3.)

The reactor body resembles a cylindrical shape when each of the five parts are fixed together. The reactor body is then placed on top of a plastic collection cup where the flushed liquid media are collected.

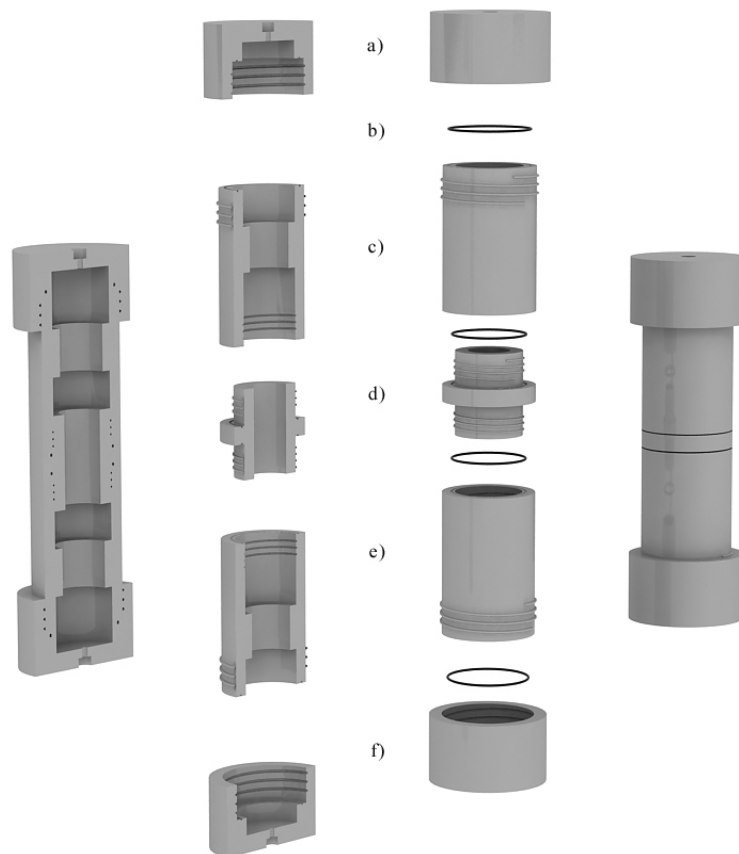


Fig 2.3 : Overall reactor design dismantled and fixed (right) section of the parts (left); upper cap (a), o-rings (b), upper filter body (c), reactor core (d), lower filter body (e), lower cap (f).

Peristaltic Pump and Tubing

A two direction flow rate controlled peristaltic pump (Kamoer KCP3-S04W) with fixed 1.0 mm inner tube circumference and 3.0 mm outer tube circumference is used for controlled liquid flow through the reactor body. The flow rate is proportional with the inner circumference of the tubing and is controlled by the flow rate control switch of the pump. Minimum flow rate of the system is 200 ml/h and maximum flow rate is 875 ml/h.

Additional silicon tubes with inner circumference of 1.0 mm and outer circumference of 4.0 mm are used to transfer liquid media from the flasks into the reactor body, by being fixed to the pneumatic fixings on the reactor caps.

Erlenmeyer Flask and Magnetic Stirrer System

The required liquid media for the cementation reaction are placed inside erlenmeyer flasks with differing volumes. Certain solutions are heterogenous, which requires constant stirring in order for the ingredients to be transferred into the reactor body successfully. Erlenmeyer flasks containing such solutions are placed on top of the magnetic stirrer for constant stirring of the solution throughout the entire cementation experiments.

2.2 Methods

2.2.1 Cultivation and optimization of the bacteria

2.2.1.1 Cultivation in liquid media

Cultivation of the bacteria is conducted under axenic conditions. All of the solutions needed for the composition of NH₄-YE liquid growth medium (ATCC Medium 1376: *Bacillus pasteurii* NH₄-YE medium); 20g/L of Yeast Extract (Acumedia), 10 g/L of Ammonium Sulfate (NH₄)₂SO₄ (Merck), 0.13M Triss Buffer (pH 9.0) (AppliChem) are separately autoclaved at 121°C for 15 minutes. The solutions are mixed after being autoclaved to form the desired amount of NH₄-YE growth medium in volume. Once the sterile growth medium is prepared, inoculation from stock cultures are made in varying volumes according to need.

The inoculated culture is then sealed loosely with aluminium foil or sterile cotton plugs to enable the oxygen to be transferred inside the medium since the microorganism makes aerobic respiration. After being sealed, the inoculated culture

is placed on a shaker inside an incubator or heated room. The culture is incubated for 16-72 hours at 30°C, shaken at 150-170 rpm depending on the level of desired growth.

The desired bacterial growth phase is the early stationary-late exponential phase. During this phase highest values of cell forming units (cfu) are reached, meaning that the number of microorganisms inside the medium is at the peak and the nutrients present in the growth medium start to become insufficient. Extracellular enzyme secretion is also at its maximum rate during this phase regarding the specific strain which is being used. In theory, higher amounts of bacteria in the bacterial batch result in a higher probability of cell retention in the solid media and an increase in the number of reaction sites positively affecting the overall biomineralization process. The optical density values for the early stationary-late exponential phase of the specific strain of bacteria is between 3.0-5.0 under 600nm.

2.2.1.2 Cultivation on agar

Cultivation of the bacteria is conducted under axenic conditions. All of the solutions needed for the composition of NH₄-YE agar growth medium (ATCC Medium 1376: *Bacillus pasteurii* NH₄-YE medium); 20g/L of Yeast Extract (Acumedia), 10g/L of Ammonium Sulfate (NH₄)₂SO₄ (Merck), 0.13M Triss Buffer (pH 9.0) (AppliChem), 20g/L Agar (Acumedia) are separately autoclaved at 121°C for 15 minutes. The solutions are mixed after being autoclaved to form the desired amount of NH₄-YE growth medium in volume. Once the sterile growth medium is prepared, it is poured inside petri dishes and inoculation from stock cultures are made in varying volumes according to need. The petri dishes are then capped and labelled.

The inoculated culture is placed inside an incubator or heated room. The culture is incubated for 16-72 hours at 30°C depending on the level of desired growth and visible colony formation.

2.2.1.3 Gram staining

Gram Staining Method is conducted to understand if the grown colonies are *Sporosarcina pasteurii* colonies.

Colonies are circled with a colored pen and numbered individually on the petri lid. Lamels are numbered with the pen accordingly, a bunsen burner is fired to ensure

sterilization. Each single colony is gently scraped with autoclaved wooden toothpicks. The toothpicks are then smeared on the lamels according to their number then burned and discarded. The backsides (empty faces) of the lamels containing the colony samples are slightly heated to ensure that the bacteria sticks to the glass. The petri containing the colonies and the NH₄-YE growth medium is sealed with parafilm. Gram's Crystal Violet Reagent is dripped on the samples on the lamels for 1 minute. The samples are then rinsed with dH₂O for 5 seconds. Iodine Reagent is dripped on the samples on the lamels and waited for 1 minute. The samples are then rinsed with dH₂O for 5 seconds. Ethyl Alcohol is applied dropwise slowly for 15-20 seconds until the dye stops running from the smears. The samples are then rinsed with dH₂O for 5 seconds. The samples are gently dried by paper towels. Samples are placed under a microscope with 100x magnification for imaging.

Rod shaped stained (purple in color) cells are expected to be observed since *S.pasteurii* is a rod shaped Gram positive bacteria.

2.2.1.4 Optical density measurements

Optical density measurements are conducted by a spectrometer under 600nm wavelength. 1ml of blank and sterile growth medium (same growth medium used for the cultivation of the bacteria) is placed in each of the two plastic cuvettes. For an accurate measurement, the grown culture is diluted by a factor of four, meaning 250µl of grown culture is mixed with 750 µl of blank growth medium inside the cuvette. The two of the former solutions are used as blank media for synchronization, their optical density fixed to zero. Then the grown medium is placed inside the spectrometer and the measured value under 600 nm is noted. The value is then compared with the growth curve of the bacteria and the growth phase (lag phase, log phase/exponential phase, stationary phase and decline/death phase) is determined.

2.2.1.5 Stock

Stock types of the grown cultures are preferred according to various uses with or without the addition of glycerol accordingly. Glycerol acts a cryoprotector by forming stable hydrogen bonds between water molecules which decreases the freezing point of the solution. This aspect inhibits the outflow of intracellular water and prevents shrinkage of the cells as well as preventing ice crystal formation which may harm the cells.

Grown cultures in liquid media are placed inside 50ml falcon tubes (Capp) and centrifuged at 5000 rpm for 15-20 minutes. The liquids in the centrifuged falcons are discarded and the pellets are left in the falcons. The falcons are then sealed with parafilm and stored at -20°C without the addition of glycerol. This type of stock is preferred for short term use.

1ml cryo-tubes are labeled (Date/Name/Content) and samples taken from grown cultures are placed in the tubes with the following concentrations; 765 µl of grown culture and 235 µl %85 pure glycerol (not autoclaved). Thus, making %20 of the total content glycerol. The cryo-tubes are then placed in a -80°C freeze-proof box and stored at -80°C. This type of stock is preferred for long term use.

Grown cultures are also sealed in falcon tubes or erlenmeyer flasks with parafilm and stored at +4°C, without the addition of glycerol, for 2-3 weeks. This type of stock or subculture is preferred for the rapid use of the culture, i.e. during repetitive CIPS experiments.

2.2.2 Urease activity determination

2.2.2.1 Nessler's reagent tests

Nessler's method is used for colorimetric and spectrophotometric determination of ammonia [37]. Ammonia is produced during the first step of the hydrolysis of urea. Urease is secreted into the liquid medium during the cultivation stages of the bacteria. A sample taken from the grown culture is mixed with urea to start ureolysis. 2ml of the sample is mixed with 100µl of Nessler's Reagent and allowed to react for approximately one minute.

Change in color due to the addition of Nessler's Reagent is used to determine the ammonia presence in the liquid medium. Color of the medium is expected to change into yellowish-orange with a slight precipitation if ammonia is present in the medium.

For the spectrophotometric determination of ammonia, the sample is placed in the spectrophotometer and optical density measurements are conducted at the wavelength value of 425 nm. Absorbance values are then compared with NH₄Cl standards measured under same conditions.

2.2.2.2 Precipitation tests

Precipitation of CaCO_3 is used for the determination of urease activity in the liquid medium. Since free urease enzymes are present in the liquid medium due to the extracellular secretion activity of the bacteria, a fast reaction occurs with the addition of urea and CaCl_2 . Immediate visual precipitation occurs with respect to the added urea and CaCl_2 concentration.

2.2.3 Collection and preparation of waste natural stone

2.2.3.1 Collection of factory wastes

Natural stone wastes are collected from the factory of Efendioğlu Marble Industry Trade Inc. located in Bursa, Turkey. Factory wastes from sawing, milling and polishing procedures of natural stone are collected on site. Wastes of two specific local marble types are collected. Agora Beige (mined from Manisa, Turkey) and Olive Avantgarde (mined from Kastamonu, Turkey). The wastes are composed of different particle sizes due to the different processing methods.

Slurry samples are also collected along with solid samples. Slurry samples are composed of very fine ($<63\mu\text{m}$) solid wastes and treatment water.

2.2.3.2 Dry heat sterilization

Sieved samples are dry heat sterilized at 150°C for 30 minutes in an industrial type ceramics oven. This process is crucial for ensuring that the cementation experiments are conducted under sterile conditions. The risks of harming the bacteria or causing potential failures in cell retention and enzymatic activity inside the solid medium are aimed to be minimized by sterilizing the solid particles.

2.2.4 Small scale cementation experiments

2.2.4.1 Well plate cementation experiment system

For the small scale cementation experiments 24 well plates (Corning® Costar® TC-Treated Multiple Well Plates) are used, with a single well volume of 3.4mL. Each of the wells are drilled to allow excess fluid flow. Single sheets of filter paper are placed inside each well to prevent solid particles to be flushed out, shown in (Fig.2.4).

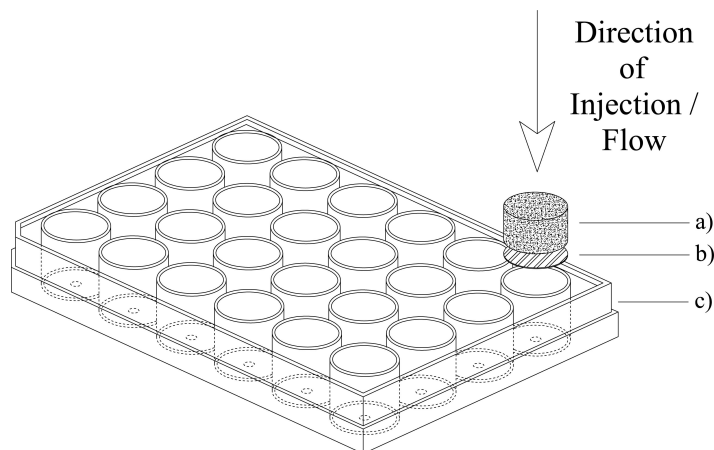


Fig. 2.4 : Well plate experimentation system; solid medium (a), filter paper (b), drilled 24 well plate (c).

Controlled injection of bacterial batches, urea medium and calcium chloride medium are conducted with sterilized pipettes. Wells are filled with solid media with differing particle sizes. dH₂O injection in each well is conducted to analyze the saturation values in accordance with particle sizes (higher absorption values in larger particle sizes, lower absorption values in smaller samples). Injection batches are prepared accordingly.

Urea medium is prepared by mixing 20 ml of fresh NH₄-YE growth medium with 0,6g CO(NH₂)₂ (urea), 0,3g NH₄Cl (ammonium chloride) and 0,0636g NaHCO₃ (sodium bicarbonate). When the dry ingredients are dissolved in the 20 ml blank NH₄-YE growth medium, 10 ml of OD₆₀₀: ~ 1,5 bacterial batch is added to the medium. A separate CaCl₂ Solution is prepared by mixing 7 g CaCl₂ with 50 ml dH₂O. The CaCl₂ solution is not mixed with the bacteria+NH₄-YE+Urea Medium solution.

Injections are conducted in ten steps, with time intervals of 20-24 hours (overnight) and 4-5 hours (during the day). The final step being the rinsing and dH₂O injection step.

After the samples are dried, each sample is removed from the wells and cementation analysis is conducted.

2.2.4.2 Falcon cementation experiment system

For the small scale cementation experiments 15ml falcon tubes (Capp) are used. Each falcon is drilled at the bottom end to allow excess fluid flow. 1-3ml of sponge

filters are placed inside each falcon (top and bottom with the solid medium in between) to prevent solid particles to be flushed out. The falcons are then fixed inside a stand on top of a collection flask where the flushed liquid media are collected, shown in (Fig. 2.5).

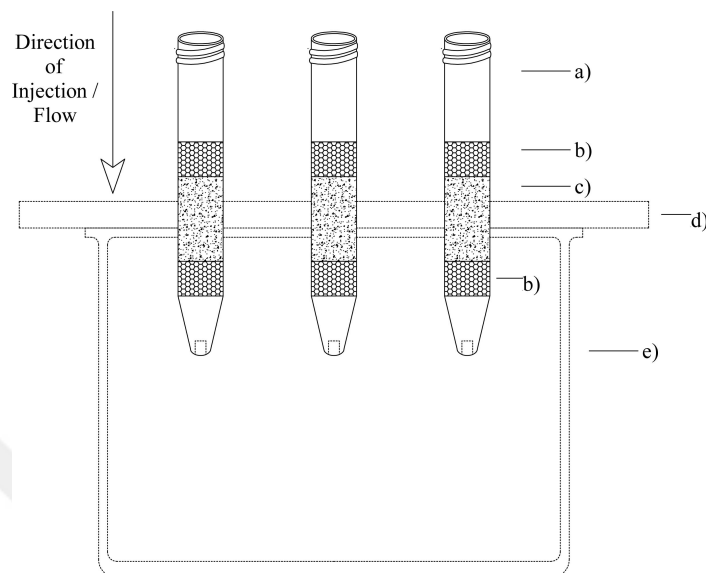


Fig. 2.5 : Falcon experimentation system; drilled 15ml falcon tubes (a), 3ml sponge filter layer (b), 5ml solid media layer (c), fixing stand (d), collection cup (e).

Controlled injection of bacterial batches, fixation fluid batches and cementation fluid batches are conducted with sterilized pipettes. Wells are filled with 3-5ml of solid media with differing particle sizes. dH₂O injection in each well is conducted to analyze the saturation values and pore volume in accordance with particle sizes (higher absorption values in larger particle sizes, lower absorption values in smaller samples). Injection batches are prepared accordingly.

The bacterial batch (one pore volume) of bacteria grown to late exponential/early stationary phase (OD_{600} :3.0-5.0) is injected to the solid medium without being diluted. It is imperative to inject the culture as it is, without diluting it with growth medium/urea medium or a saline solution (CaCl₂ etc.). It is also very important to use the bacteria at late exponential/early stationary phase. Since all the nutrients are depleted in its growth medium, the bacteria tend to stick to the new environment in theory. Injection of the fixation fluid is crucial to ensure cell retention in the solid medium. Fixation fluid of 50mM CaCl₂ (9 pore volume) is injected as soon as the batch of bacteria is fully absorbed by the medium. Once the fixation fluid is fully absorbed by the medium, the cementation fluid (9 pore volume in 3 batches) is

instantly injected in the medium. The cementation fluid consists of equimolar CaCl_2 (1M) and Urea (1M). Once the first batch of cementation fluid (3 pore volume) is fully injected, the system is left at room temperature for approximately 24 hours. Injection of the second cementation fluid batch (3 pore volume) is made after the 24 hour waiting period is complete. After 2 hours the final cementation fluid batch (3 pore volume) is made and the system is left at room temperature until all of the remaining liquid media are fully absorbed by the solid media.

Finally the cemented solid media are taken out of the falcons to be analyzed.

2.2.5 Calcite in situ precipitation system

For upper scale controlled cementation experiments a ‘calcite in situ precipitation system’ is designed and produced. The system consists of a reactor body and collection cup, a peristaltic pump and tubes, a flask containing the required solutions and a magnetic stirrer, shown below in (Fig.2.6).

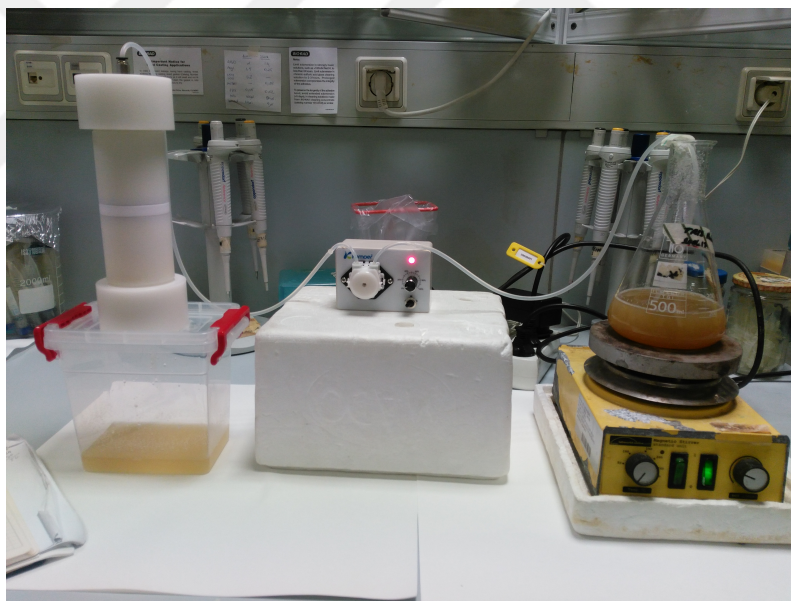


Fig. 2.6 : Reactor body and collection cup (left), peristaltic pump and tubing (middle), magnetic stirrer and flask containing the required solutions (right).

As the first step of the calcite in situ precipitation system or CIPS experiment; the sponge and stainless steel ball filters are placed inside the filter bodies of the bio-reactor. The lower filter body is then screwed in the lower cap. The reactor core is then fixed to the lower filter body with 1-2 filter paper (Whatman) layer in between. The sterilized solid medium with a specific particle size range is placed inside the reactor core. The upper filter body is then screwed to the rest of the reactor body.

2-5 pore volume of dH₂O is directly injected to the solid medium without the use of the peristaltic pump. This step is crucial to ensure that the gaps between the solid particles are minimized and the solid volume is compressed.

2-5 pore volume of bacterial batch grown to late exponential/early stationary phase (OD₆₀₀:3.0-4.0) is injected in the filter body and the reactor is capped. The peristaltic pump and the tubing is connected to the reactor body and air flow is conducted with an approximate flow rate of 200ml/h.

Once the first bacterial batch is fully absorbed by the solid medium, the fixation fluid of 50mM CaCl₂(2-5 pore volume) is injected in the reactor body via the pumping system with the flow rate of 200ml/h.

After the fixation fluid is fully absorbed by the solid medium a second bacterial batch can be injected following the same bacterial batch injection procedure as before, although this step is not mandatory.

After the fixation fluid or the second bacterial batch is fully absorbed by the solid medium, the cementation fluid injection is conducted. The cementation fluid consists of equimolar CaCl₂ (1M) and Urea (1M) dissolved in a sterile NH₄-YE growth medium (2-5 pore volume in 3 separate batches with equal volumes). The first batch is injected immediately after the fixation fluid or the second bacterial batch is fully absorbed by the solid medium, via the peristaltic pump and the tubing system with the flow rate of approximately 200ml/h. The second cementation fluid is injected 16-24 hours after the first cementation fluid injection. Followed by the third cementation fluid injection 2-4 hours after the second one.

Once the liquid media are fully absorbed by the solid medium and the experiment is complete, the tubing is removed and the reactor body is dismantled. The reactor core containing the cemented sample is placed in the heated room at 30°C or 37°C to dry. The remaining reactor parts are cleaned and sterilized and the sponge filters are discarded.

After the cemented sample is dried it is removed from the reactor core and prepared for analyses.

2.2.6 Material specific analyses and experimentations

2.2.6.1 Granulometry experiments and analysis

Waste natural stone particles collected from the factory are sieved to analyze the particle size distribution and to contrast cementation experiment results with solid media having differing particle sizes. Sieves with sizes of 2000 μm , 1000 μm , 500 μm , 250 μm , 212 μm , 200 μm , 150 μm , 125 μm , 100 μm and 63 μm are used during the experiments (U.S.A. Standard Testing Sieve).

According to the data obtained from the sieving experiments, a granulometry curve is graphed in order to understand the particle size distribution of both natural stone wastes (Agora Beige and Olive Avantgarde).

2.2.6.2 Capillary absorption experiments and analysis

Capillary absorption is the phenomenon where water molecules move against gravity inside a material in relation with the materials pore structure. When the materials bottom surface is in direct contact with water it may absorb water through capillarity. The absorbed water results in an increase of the overall mass of the material. The material can be weighed throughout the experimentation process to find the capillarity coefficient, the unit of which can be given as; $\text{cm}/\text{min}^{-2}$ or $\text{cm}/\text{sec}^{-2}$ [38].

The reactor core is designed to produce cylinder shaped samples with the circumference of 2,5cm and the height of 5cm. The samples are dried at 105°C and left at room temperature prior to the capillary water absorption tests. The dry mass of the samples are measured and each sample is placed on a wire grid slightly touching the water underneath. Distilled water is used in the experiments. Time is kept with a chronometer and the samples are periodically weighed.

Data obtained from the experiments is used for determining the capillarity coefficient of the samples by using the following equation (2.1);

$$N = \frac{m_1 - m_2}{A\sqrt{t}} \quad (2.1)$$

N: Capillarity Coefficient ($\text{cm}^3/\text{cm}^2\sqrt{\text{min}}$)

m: Mass of the samples under ambient air humidity (gr)

m_0 : First measured mass of the samples (gr)

m_1 : Mass of the samples after a certain amount of time (gr)

A: Surface area of the samples in contact with water (cm^2)

t: Time of contact with water (min)

2.2.6.3 Water absorption tests

After the experiments are completed for determining capillarity, water absorption experiments are conducted under atmospheric pressure. The samples are placed inside distilled water in a manner where all the surfaces of the samples are covered in water. The samples are left inside distilled water until no change in their mass is observed. The mass of the samples are then measured in water by an archimedes scale. The samples are then taken out of water and their wet surfaces gently wiped by a cloth and their mass is measured.

The water absorption percentages, weight per unit volume and specific weight of the samples are measured according to the results of the experiments and by using the formulae below;

$$S_a = \frac{P_1 - P_0}{P_0} \times 100 \quad (2.3)$$

$$\Delta = \frac{P_0}{\pi r^2 h}$$

$$(2.4)$$

$$\delta = \frac{P_1}{P_1 - P_2}$$

$$(2.5)$$

S_a : Percentage of water absorption (%)

Δ : Weight per unit volume (gr/cm^3)

δ : Specific weight (gr/cm^3)

P_0 : Ambient weight (gr)

P_1 : Saturated weight (gr)

P_2 : Weight inside water (gr)

2.2.6.4 Compression tests and analysis

Compression tests are conducted in order to determine how the material reacts when it is compressed. Fundamental parameters are measured which determine the behaviour of the material under a specific compressive load. The durability of the material is measured by dividing the maximum load which a sample can bear with the samples initial cross sectional area [39].

Cylinder shaped samples obtained from the results of the CIPS experiments are put to the tests. Each sample has a radius of approximately 12.5mm and a height of approximately 50.0mm (height the twice of its diameter). Both ends of the samples are first covered in plaster footings to stabilize the samples during compression experiments. Once the plasters are dried the samples are placed inside the compression testing machine and the the parameters are measured (force and readings). The compression test machine MFL Prüf-und Meßsysteme GmbH D-6800 in the ITU construction materials lab is used for the experiments. The data obtained from the experiments are then used to plot a stress (N/mm^2) / strain (mm/mm) graph and to determine the durability characteristics of the material.

The maximum compressive strength of the material is measured by using the following formula;

$$\sigma = \frac{P}{A} \quad (2.6)$$

σ : Compressive Strength (N/mm^2)

P : Breaking Load (N)

A : Surface Area (mm^2)

The modulus of elasticity also known as Young's modulus (numerical constant) is used to determine the ability of a material to resist changes in length due to applied tension or compression. Modulus of elasticity for each sample is calculated from the stress/strain graph of the relevant sample. Tangent α or the slope of the linear stress/strain graph is calculated for the determination of the modulus of elasticity.

2.2.6.5 Scanning electron microscope (SEM) imagings

Microscopic pore structures and crystal morphologies are viewed by using a scanning electron microscope.

Samples are coated with platinum via plasma radiation (for approximately 135 seconds). The coated samples are then scanned under the electron microscope (Jeol JSM-5410). The electron microscope is located at the department of materials engineering at Istanbul Technical University.

2.2.7 Precautions for strength improvement according to sample analyses

According to the data obtained from analyzing the CIPS experiment samples certain precautions are made for overall strength improvement regarding the end product. Strength improvement is directly linked to certain parameters such as reaction rate which results in various calcium carbonate crystal morphologies (certain calcium carbonate crystals are more stable than others such as calcite), bacterial cell retention inside the solid media and pore throat size and particle size distribution of the solid media.

2.2.7.1 Biological and chemical precautions for cell retention improvement

In-Situ Incubation

Since the fixation fluid and the cementation fluids are injected right after bacterial batch injections during the cementation experiments (CIPS experiments), there is a risk of flushing the bacteria out with the excess fluids and preventing successful cell retention inside the solid medium. In-situ incubation of the bacteria within the solid medium is taken as a precaution to prevent such failures, allowing time for bacterial cell retention.

Once the bacterial batch or batches are injected inside the solid medium, the reactor is sealed and the bacteria is incubated at optimum conditions (30°C) for 24 to 48 hours.

CaCl₂ Concentration in the Growth Medium

Researches indicate that CaCl₂ concentrations present in the growth medium during the cultivation of the bacteria contribute to bacterial cell retention in the solid medium and increase the overall urease activity [40].

NH₄-YE liquid growth media with CaCl₂.2H₂O concentrations varying between 3mM-6mM-9mM are prepared and inoculated to understand the effects of CaCl₂ on cellular growth and retention inside the solid media. Optical density measurements are also conducted to understand the effect of CaCl₂ on bacterial growth.

2.2.7.3 Mechanical precautions for minimizing pore throat sizes

Particle Size Admixtures

According to the particle size distribution analysis and SEM images, new admixtures of free solid particles with various particle sizes are prepared. Calculations are made to ensure that the pore throats and voids between large particles are minimized by the addition of smaller particles with optimum sizes regards to the larger ones.

This method is used to increase the number of reaction sites inside the solid medium and to enable effective bridging of calcium carbonate crystals between solid particles.



3. RESULTS AND DISCUSSIONS

This study aims to understand the effect of biomineralization and microbial induced calcite precipitation on certain natural stone wastes and to offer a solution in recycling such wastes by the formation of a sustainable and biogenic material.

The overall research is conducted in two major phases regarding the creation of the material (formerly referred as the research and development phase) and the analysis phase of the material.

The experiments are conducted in small scales at first to understand certain parameters which affect the biomineralization process. The scale of the experiments are then increased to obtain larger samples in specific sizes suitable for certain analyses. The samples are then analyzed to determine the physical and chemical characteristics of the material as well as to testify calcium carbonate formation within the solid media.

The results of the experiments are given with.

3.1 Research and Development Phase

The research and development phase of the project is conducted at the molecular biology biotechnology and genetics research center of İstanbul Technical University. This phase of the thesis consists of the optimization of the bacteria's cultivation parameters and extracellular enzymatic activity, biomass creation, controlling the reaction rate and precipitation parameters, optimization of the solid media, small and upper scale cementation experiments and cell retention improvements.

3.1.1 Cultivation of the bacteria and optimization of ureolytic activity

3.1.1.1 Cultivation in liquid and agar growth media

Cultivation of the alkaliphilic bacteria is conducted under aerobic batch conditions. Stock cultures are used for the inoculation of the specific growth medium NH₄-YE.

Inoculated liquid growth media are incubated at 30°C, shaken at 150-170 rpm for time intervals varying from 48 to 96 hours according to the inoculation ratio (volume/volume).

Cultivation on agar have been problematic due to the long incubation periods which result in the dehydration of the agar media and contamination.

Once visible growth is observed in both liquid and agar growth media, Gram staining is conducted to determine the type of grown microorganisms. Rod shaped Gram positive bacteria with the exact physcial characteristics of the specific strain have been observed under 100x magnification, shown in (Fig. 3.1).

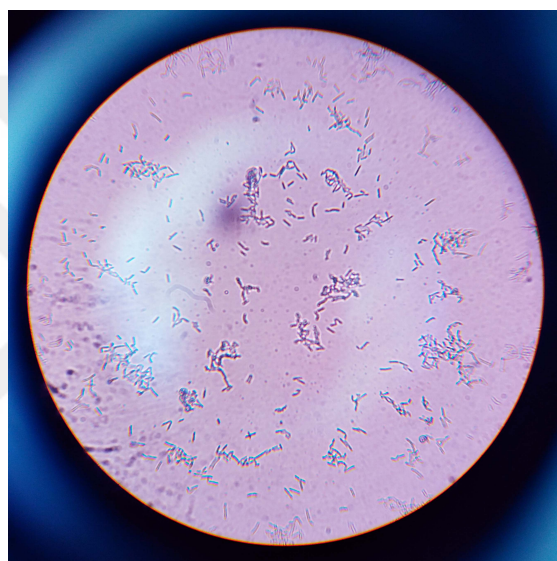


Fig. 3.1 : Gram stained grown culture.

3.1.1.2 Urease activity determination

Once the grown bacteria is verified to be *S.pasteurii*, experimentations are conducted to determine active urease activity in the growth media. The bacteria release certain enzymes including urease extracellularly during the cultivation period. This aspect suggests that there is free enzyme presence inside the growth medium in which the bacteria is cultivated. To ensure that there is urease presence and active urease enzymes inside the bacterial batch, certain experiments such as Nessler's reagents tests for the spectrophotometric determination of ammonia are conducted.

A 500µl sample taken from the grown culture (OD_{600} : 1.5) is diluted by deionized water (Fig. 3.2) and 100µl of Nessler's reagent is added to the solution (Fig. 3.3).

Instant change in color and a slight precipitation is observed indicating the ammonia presence.

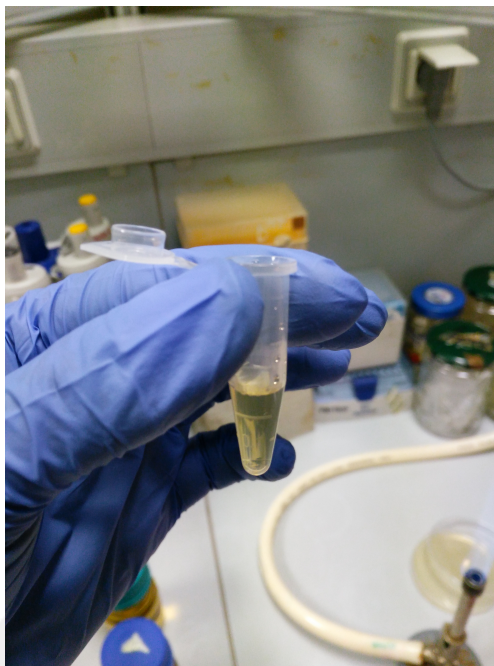


Fig. 3.2 : Diluted grown culture without the addition of Nessler's reagent.



Fig. 3.3 :Observed color change and precipitation after the addition of Nessler's reagent.

The sample is then placed in a cuvette for the optical density reading at 425nm. Absorbance readings are then compared with standard NH_4Cl readings under same conditions.

3.1.1.3 Precipitation tests

Once the spectrophotometric determination of ammonia is made and active ureolytic activity is observed, certain solutions are added to the grown culture to observe visible CaCO_3 precipitation. Various solutions are added to batches of grown cultures to understand the effect of molar concentrations of urea and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ on precipitation rates and amounts.

Amongst the precipitation tests made with equimolar urea and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ concentrations (0.5M – 1M – 2M), the optimum values for saturation and precipitation rates / amounts is observed at a molar concentration of 1M (Fig. 3.4 and Fig. 3.5).



Fig. 3.4 : Addition of <0.5M urea+ $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to the grown batch and observed precipitation.

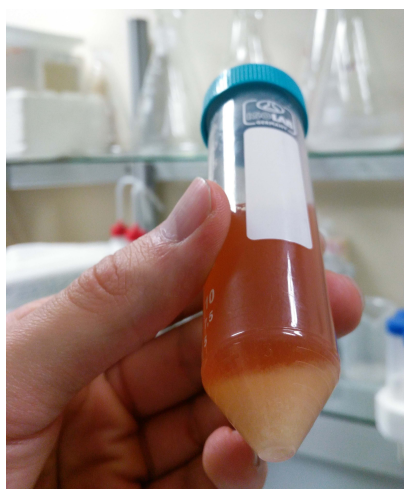


Fig. 3.5 : Addition of ~ 1M urea+ $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to the same grown batch and observed precipitation.

The effect of bacterial growth on the precipitation values are also tested. Grown cultures with OD₆₀₀ values of 0.6, 1.0 and 1.5 are tested with the addition of 1M urea+CaCl₂·2H₂O to understand the effects. Results indicate that precipitation amounts increase directly proportional with OD₆₀₀ values as shown in (Fig. 3.6).

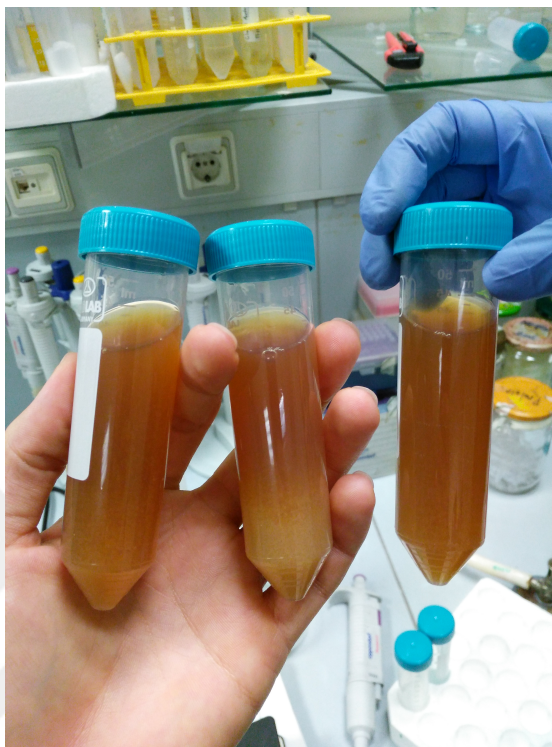


Fig. 3.6 : Precipitation observations made with the addition of 1M urea+CaCl₂·2H₂O to grown cultures with OD₆₀₀ values of 1.0 (left), 1.5 (middle) and 0.6 (right).

3.1.2 Biocementation experiments

Biocementation (or biomineralization) experiments are conducted starting from small scale for understanding vital parameters affecting the biocementation process. The scale of the experiments are then increased in order to obtain larger samples suitable for certain analyses.

3.1.2.1 Well plate cementation experiments

Two identical well plates (2x24 wells) are used for the experiment. Each well is drilled at the bottom end to ensure liquid flow outside the system and a filter paper is placed in each well prior to the placement of the solid media to prevent the leakage of any solid particles.

Approxiamtely 3 ml of heat sterilized natural stone wasteswith various particle sizes are placed in the well plates (2x24 wells) according to their particle size distribution;

- Column 1 – Wells A1-B1-C1-D1: 200-150 μm Olive Avantgarde
- Column 2 – Wells A2-B2-C2-D2: 150-100 μm Olive Avantgarde
- Column 3 – Wells A3-B3-C3-D3: 100-63 μm Olive Avantgarde
- Column 4 – Wells A4-B4-C4-D4: < 63 μm Olive Avantgarde
- Column 5 – Wells A5-B5-C5-D5: 1000-500 μm Agora Beige
- Column 6 – Wells A6-B6-C6-D6: 1000-500 μm Agora Beige

The pore volumes of the dry solid media are measured by injecting sterile dH_2O in the control group (Plate No.1, Wells: D1-D2-D3-D4-D5-D6). The absorbed volume of dH_2O for each sample is measured as follows;

- Well D1: 200-150 μm Olive Avantgarde; $\sim 2\text{ml}$
- Well D2: 150-100 μm Olive Avantgarde; $\sim 2\text{ml}$
- Well D3: 100-63 μm Olive Avantgarde; $\sim 1,2\text{ ml}$
- Well D4: < 63 μm Olive Avantgarde; $\sim 2\text{ml}$
- Wells D5-D6: > 200 μm Agora; $\sim 5\text{ ml}$

The urea medium ($\text{NH}_4\text{-YE}$ growth medium with $\text{CO}(\text{NH}_2)_2$ (urea), NH_4Cl (ammonium chloride) and NaHCO_3 (sodium bicarbonate) and OD_{600} : $\sim 1,5$ bacterial batch) is injected, followed by the CaCl_2 Solution (7g CaCl_2 + 50 ml dH_2O) as shown in (Fig. 3.7). The CaCl_2 solution is not mixed with the bacteria+ $\text{NH}_4\text{-YE}$ +Urea Medium solution.

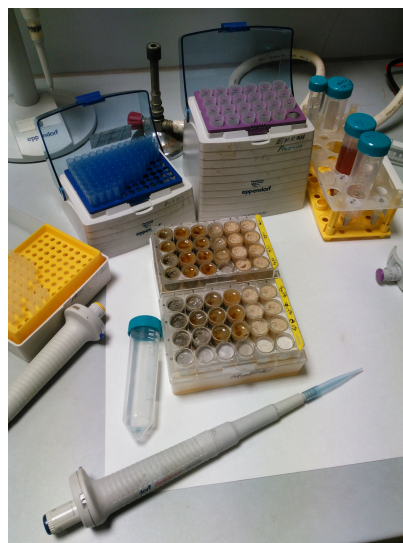


Fig. 3.7 : Well plate cementation system experiment between injection intervals.

Injections are conducted in ten steps, with time intervals of 20-24 hours (overnight) and 4-5 hours (during the day). The final step being the rinsing and dH₂O injection step.

An overall decrease in liquid absorption and clogging is observed progressively. Liquid absorption rates are observed to be decreasing according to the decrease in particle size as well.

After the samples are rinsed with dH₂O they are left to dry for approximately 72 hours under room temperature. Dried samples are shown in (Fig. 3.8).

The samples in the control group disintegrated immediately after they are removed from the wells. Varying amounts of calcification is observed on the samples from the wells of the 1st plate; well numbers: A5-B5-C5-A6-B6-C6. The volumes of calcification is as follows; A5=A6>B5=B6>C5=C6.

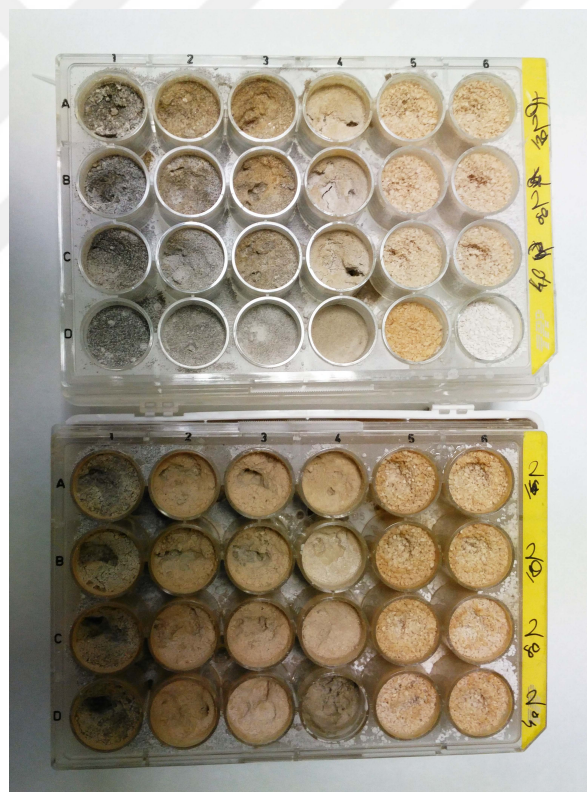


Fig. 3.8 : Dried well plate cementation experiment samples.

All of the cemented samples have disintegrated under vertical pressure. It is also observed that the hardest parts of most of the samples are the top layers. This may indicate that a quick reaction might have happened on the top layer immediately after

the cementation fluid injection, resulting in the cementation of the first layer and rapid clogging.

The cementation in certain samples are observed to be considerably weak. One of the main reasons for such failure is thought to be because of uncontrolled flow rates resulting in uncontrolled reaction rates. Other potential reasons for failure may be linked to high CaCl_2 concentrations which might have caused osmotic shock for the bacteria resulting in the loss of cell bound extracellular enzymes [34], disturbance of the solid media during injections, injection volumes and intervals.

Diluting the bacterial batch with the urea medium may have caused potential problems leading to the termination of the bacteria due to instant change in medium, pH and free ions present in the liquid medium. Uncontrolled rapid reactions and very high reaction rates are also observed during the addition of CaCl_2 solutions to the urea medium. This aspect hints that separation of the bacterial batch and the urea content may prove fruitful both in terms of reaction rates and the overall condition of the bacteria. The chemical composition of the urea medium is also revised in manner which only includes the needed components (mainly urea) and discards the additional chemicals which may agitate and/or affect the bacteria negatively.

Liquid flow out of the wells are observed to be considerably fast which may prevent the required amount of optimum time for biocementation to occur inside the solid media. Slowing down the flow rate and increasing the volume of treated solid media are thought to be useful regarding the matter.

3.1.2.2 Falcon cementation experiments

Results and observations obtained from the well plate experiments are used for the design of a new system for a more successful small scale biocementation experiment. The experiment mimics the closed upper scale precipitation system. 15ml falcon tubes function as main structures holding the filters and the solid media together. Once the falcon tubes are drilled from the conic bottom end (for the discharge of the excess liquid media) sponge filters (each 3ml in volume) are placed inside the tubes to prevent the solid media from being flushed out. Approximately 5ml of solid media are placed in the tubes on top of the sponge filters. Another layer of sponge filters (each 3ml in volume) are placed on top of the solid media to prevent the solid media

from being disturbed during injections and to ensure a homogenous and slower liquid flow.

Injection of one pore volume of dH₂O is conducted at first to stack the free solid particles together and rinse the solid media. After the dH₂O injection, 1-2 pore volumes of bacterial batch grown to early stationary-late exponential phase (OD₆₀₀: 3,08) is made directly on the solid media, followed by 9 pore volumes of fixation fluid consisting of 50mM calcium chloride (CaCl₂.2H₂O). After the fixation fluid injection 3x9 pore volumes of cementation fluid consisting of equimolar (1M) urea and calcium chloride (CaCl₂.2H₂O) is conducted.

The time intervals between the cementation fluid injections vary from 24 to 4 hours accordingly. The system is left at room temperature between injection intervals.

For the first falcon cementation experiments, 2x5ml of 1000-500µm sized Agora Beige samples and 2x5ml of 200-150µm sized Olive Avantgarde samples are used due to the observations and results of the small scale well plate cementation experiments as shown in (Fig. 3.9).

It is observed that the liquid absorption rate is directly proportional with the particle size of the samples. i.e.: 200-150µm sized samples have a rate of absorption around 20ml/hour where as the 1000-500µm samples have a higher absorption rate. A considerable decrease in the absorption rate is observed in each sample after the second cementation fluid batch injection (10ml/hour).

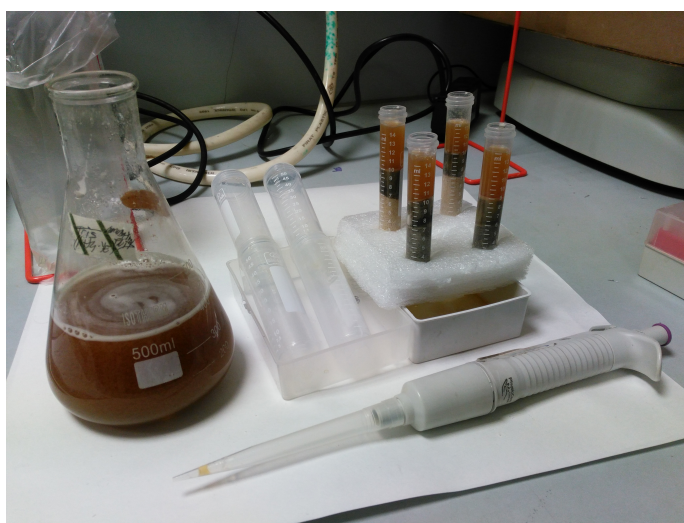


Fig. 3.9 : Falcon cementation system during injection intervals.

Once the experiment is completed the samples are left at 37°C to dry. Successful cementation is observed in all three samples except one of the 200-150µm sized sample which disintegrated during the removal from the falcon tube. Results are shown in (Fig. 3.10 and Fig. 3.11) accordingly.



Fig. 3.10 : Successful cementation of a 5ml 1000-500µm sized Agora Beige sample.



Fig. 3.11 : Successful cementation of a 5ml 200-150µm sized Olive Avantgarde sample.

This small scale experimentation system is used throughout the research and development process for observing the results of certain changes in various parameters affecting the biocementation process such as particle / pore throat sizes, in-situ incubation and for testing new solutions because of its effectiveness and savings in raw materials. The samples obtained from such experiments are also suitable in size for SEM imagings.

3.1.2.3 Calcite in situ precipitation system (CIPS) experiments

According to the results and observations obtained from the falcon experiments, upper scale CIPS experiments are conducted to obtain larger samples suitable for certain material specific analyses. The calcite in situ precipitation system consisting of a main reactor body and a peristaltic pump allows a more controlled reaction.

Waste samples within the particle size ranges of 500-1000 μm , 250-500 μm , 212-250 μm , 150-212 μm , 125-212 μm , 125-150 μm , 100-125 μm are treated during the CIPS experiments as shown in (Fig. 3.12).



Fig. 3.12 : Calcite in situ precipitation system during cementation fluid injection intervals.

Parameters of the successful CIPS experiments are compared. Results indicate that slow reaction rates tend to conclude in more stable calcium carbonate crystal formations [34], therefore stronger samples are obtained at lower flow rates (~200-250ml/h).

Start-stop injections also concluded in more successful results in contrast to continuous flow injections where no waiting time is apparent between the injection of each separate batch. The injection intervals between cementation fluid batches have been proven to play a vital role as well. Leaving the system overnight after the first cementation fluid injection concluded in more successful results in contrast with continuing the second injection after 2-6 hours after the first one. This may be due to allowing the optimum amount of time required for a stable cementation reaction to occur, in contrast with continuing liquid flow which may terminate the process and result in the flushing out of the bacteria.

The condition of the bacteria (growth phase, stock conditions, metabolic activity) is also critical for successful biocementation. Premature batches (lag phase) may result in weaker biocementation values due to lesser amounts of cells per unit volume whereas late stationary phase batches may result in instant clogging due to excess amounts of free enzymes preventing a homogenous cementation to occur across the solid body.

Certain aspects are observed to be problematic in terms of the sustainability of the reaction. One major issue is due to the flushing out of large quantities of free enzymes present in the bacterial batches grown to late exponential-early stationary phase. During every experiment dense precipitations are observed inside the collection cup where the excess fluids disposed from the reactor are collected. This aspect may be linked to the flushing out of free enzymes and/or bacteria which vitally affects the overall biocementation process and the strength of the end product.

Certain changes in vital parameters affecting the biomineralization process are made throughout the research and development phase of the thesis which are put to test via CIPS experiments.

Successfully biocemented samples (shown in Table 3.1) are labeled according to experiment parameters and prepared for material specific analyses such as capillary absorption experiments, water absorption experiments, compression experiments and SEM imagings as shown in (Fig. 3.13) and (Fig. 3.14).



Fig. 3.13 :250-500µm sized Olive A. sample after treatment (left), reactor core (right).

Table 3.1 : List of successful CIPS experiment samples.

Successful CIPS Experiment Samples														
Sample No.	AG1	AG2	AG3	OA1	OA2	OA3	EAG2	EAG3	XOA2	XOA3	XOA4	XOA5	XOA6	XOA7
Waste Type	Agora B.	Agora B.	Agora B.	Olive A.	Olive A.	Olive A.	Agora B.	Agora B.	Olive A.	Olive A.	Olive A.	Olive A.	Olive A.	Olive A.
Particle Size Range (µm)	250-500	250-500	250-500	250-500	250-500	250-500	500-1000	500-1000	125-212	%70 250-500 %30 <63	250-500	250-500	250-500	250-500
Cell Retention Experiment	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Diameter (mm)	25,41	25,11	25,20	25,11	25,12	25,18	25,16	25,12	25,13	25,12	25,18	25,18	25,21	25,11
Height (mm)	47,35	47,94	48,89	41,88	46,99	46,58	49,23	45,84	46,43	43,17	44,85	44,74	44,95	45,37
Ambient Weight (P0,gr)	34,41	36,07	37,40	31,35	33,22	35,24	36,91	35,59	37,50	34,29	32,13	34,00	32,89	33,65

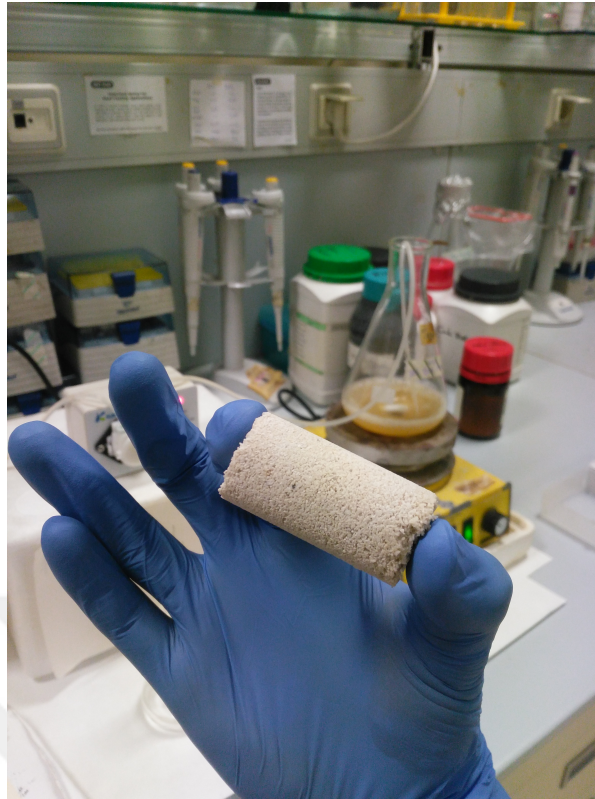


Fig. 3.14: 500-1000 μ m sized Agora B. sample after treatment.

3.1.3 Material specific analyses and experimentations

3.1.3.1 Granulometry experiments and analysis

Waste natural stone particles (factory processing wastes) are sieved to analyze the particle size distribution and to contrast cementation experiment results with solid media in differing particle size ranges shown in Tables 3.2-3.7.

Granulometry curves of both types of natural stone wastes are plotted from the obtained data as shown in Figure 3.15 and Figure 3.16.

Table 3.2 : Granulometry analysis of Agora Beige wastes.

Granulometry Analysis of Agora Beige Waste Sample 1				
Sieve Diameter	Retained Mass	Retained Cumulative Mass	Passed Cumulative Mass	Passed % by mass
(mm)	(g)	(g)	(g)	%
2.00	0,10	0,10	297,91	99,97
1.00	0,80	0,90	297,11	99,70
0.5	231,17	232,07	65,34	21,93
0.25	61,87	293,94	4,07	1,37
0.212	0,80	294,74	3,27	1,10
0.125	1,40	296,14	1,87	0,63
0.063	2,39	298,53	0,38	0,13
Collection Cup	0,38	298,91		
Total Mass (g)	298,91			

Table 3.3 : Granulometry analysis of Agora Beige wastes.

Granulometry Analysis of Agora Beige Waste Sample 2				
Sieve Diameter	Retained Mass	Retained Cumulative Mass	Passed Cumulative Mass	Passed % by mass
(mm)	(g)	(g)	(g)	%
2.00	0,10	0,10	99,28	99,90
1.00	0,94	1,04	98,34	98,35
0.5	72,17	73,21	26,17	26,33
0.25	24,46	97,67	1,71	1,72
0.212	0,09	97,76	1,62	1,63
0.125	0,57	98,33	1,05	1,06
0.063	0,83	99,16	0,22	0,22
Collection Cup	0,22	99,38		
Total Mass (g)	99,38			

Table 3.4 : Granulometry analysis of Agora Beige wastes.

Granulometry Analysis of Agora Beige Waste Sample 3				
Sieve Diameter	Retained Mass	Retained Cumulative Mass	Passed Cumulative Mass	Passed % by mass
(mm)	(g)	(g)	(g)	%
2.00	0,05	0,05	200,18	99,98
1.00	7,05	7,10	193,13	96,45
0.5	142,53	149,63	50,60	25,27
0.25	45,52	195,15	5,08	2,54
0.212	0,80	195,95	4,28	2,14
0.125	0,96	196,91	3,32	1,66
0.063	2,54	199,45	0,78	0,39
Collection Cup	0,78	200,23		
Total Mass (g)	200,23			

Table 3.5 : Granulometry analysis of Olive Avantgarde wastes.

Granulometry Analysis of Olive Avantgarde Waste Sample 1				
Sieve Diameter	Retained Mass	Retained Cumulative Mass	Passed Cumulative Mass	Passed % by mass
(mm)	(g)	(g)	(g)	%
2.00	39,23	39,23	160,86	80,39
1.00	64,57	103,80	96,29	48,09
0.5	36,18	139,98	60,11	30,02
0.25	27,04	167,02	33,07	16,52
0.212	7,54	174,56	25,53	12,75
0.125	19,94	194,50	5,59	2,79
0.063	5,30	199,80	0,29	0,15
Collection Cup	0,29	200,09		
Total Mass (g)	200,09			

Table 3.6 : Granulometry analysis of Olive Avantgarde wastes.

Granulometry Analysis of Olive Avantgarde Waste Sample 2				
Sieve Diameter	Retained Mass	Retained Cumulative Mass	Passed Cumulative Mass	Passed % by mass
(mm)	(g)	(g)	(g)	%
2.00	48,26	48,26	251,29	83,89
1.00	88,57	136,83	162,72	54,32
0.5	60,36	197,19	102,36	34,17
0.25	49,32	246,51	53,04	17,71
0.212	18,86	265,37	34,18	11,41
0.125	27,40	292,77	6,78	2,26
0.063	6,41	299,18	0,37	0,12
Collection Cup	0,37	299,55		
Total Mass (g)	299,55			

Table 3.7 : Granulometry analysis of Olive Avantgarde wastes.

Granulometry Analysis of Olive Avantgarde Waste Sample 3				
Sieve Diameter	Retained Mass	Retained Cumulative Mass	Passed Cumulative Mass	Passed % by mass
(mm)	(g)	(g)	(g)	%
2.00	24,67	24,67	75,44	75,36
1.00	31,20	55,87	44,24	44,19
0.5	16,58	72,45	27,66	27,63
0.25	13,12	85,57	14,54	14,52
0.212	2,09	87,66	12,45	12,44
0.125	7,96	95,62	4,49	4,49
0.063	4,29	99,91	0,20	0,20
Collection Cup	0,20	100,11		
Total Mass (g)	100,11			

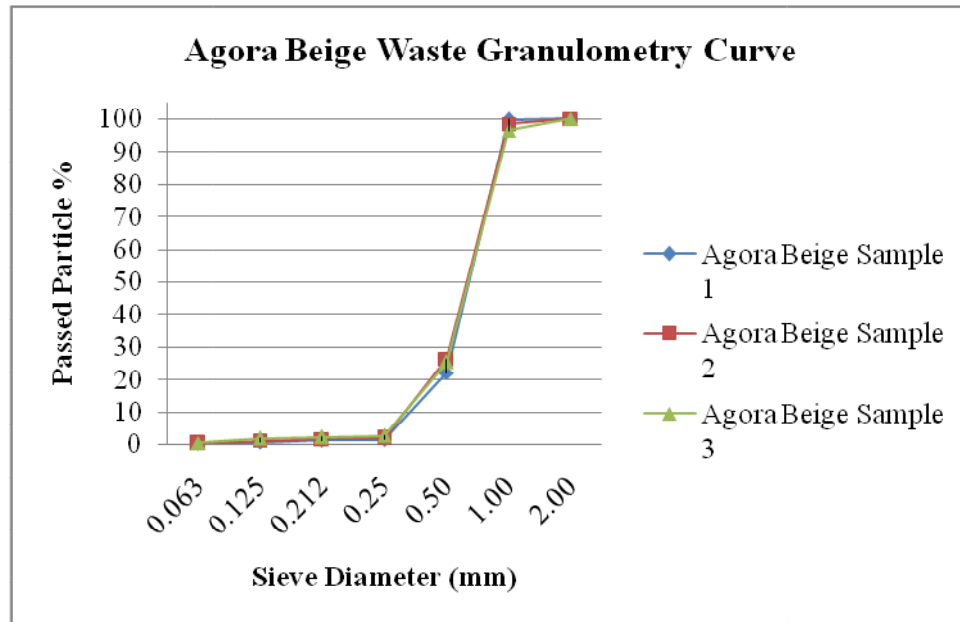


Fig. 3.15 : Granulometry curve of analyzed Agora Beige wastes.

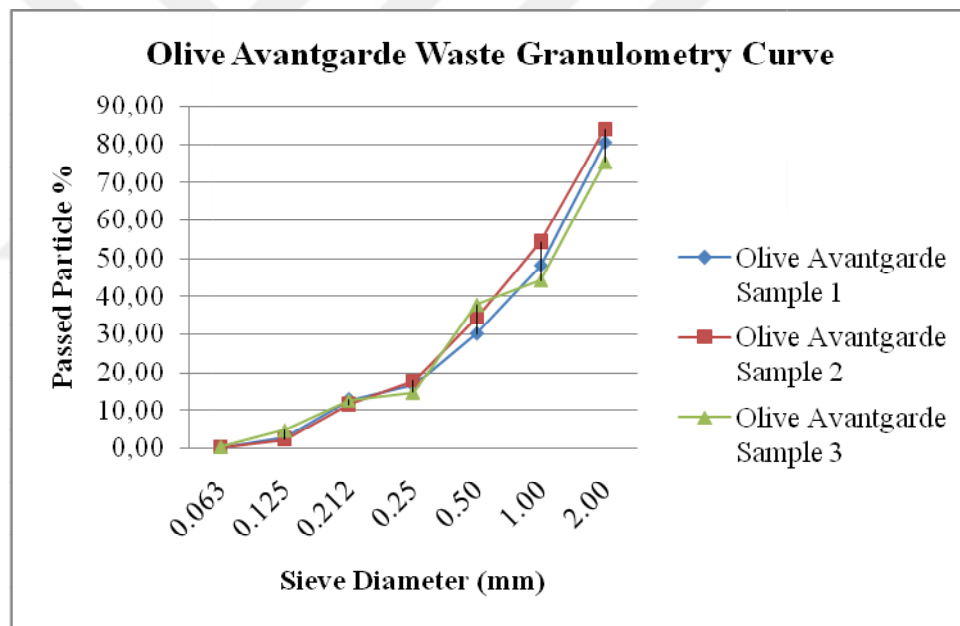


Fig. 3.16 : Granulometry curve of analyzed Olive Avantgarde wastes.

Results indicate that a wider particle size distribution is apparent amongst the Olive Avantgarde wastes ranging from 2000-125 μ m, where as the Agora Beige wastes are mainly composed of particles within the size range of 1000-250 μ m. This is due to the fact that the collected Agora Begie wastes have been previously sieved in the factory, resulting in a more unifrom particle size range. Olive Avantgarde wastes are used as the primary source for solid media in biocementation experiments due to their variety in particle size ranges, providing the opportunity to observe and contrast the effect of

particle size distribution and pore throat size on the overall biocementation process. Agora Beige samples are used to understand the effect of larger and uniform particle sizes on the overall biocementation process as well.

3.1.3.2 Capillary absorption experiments and analysis

The setting for capillary absorption experiments consists of a tray filled with dH_2O , a wire grid placed in the tray with its upper surface slightly in water, a precision scales for weight measurement and a chronometer for keeping the time.

Samples obtained from the successful CIPS experiments are placed on a wire grid slightly touching the water underneath as shown in (Fig. 3.17). Time is kept with a chronometer and the samples are periodically weighed.

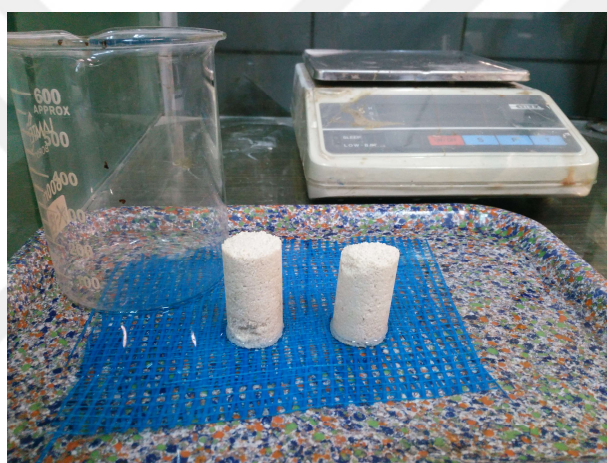


Fig. 3.17: Capillary water absorption experiments conducted with two 500-1000 μm sized treated Agora B. samples obtained from CIPS experiments.

Very fast capillary water absorption rates are observed among most of the samples. The rate of capillary water absorption is observed to increase directly proportional with particle size. Increase in particle size results in an increase in overall porosity of the material which directly affects capillary water absorption parameters. Although the capillary water rise occurs rapidly, the samples do not disintegrate when left in water.

Data obtained from the experiments is used for determining the capillarity coefficient and plotting a capillary water absorption curve for each sample given in Fig. 3.18-3.25 and Table 3.8.

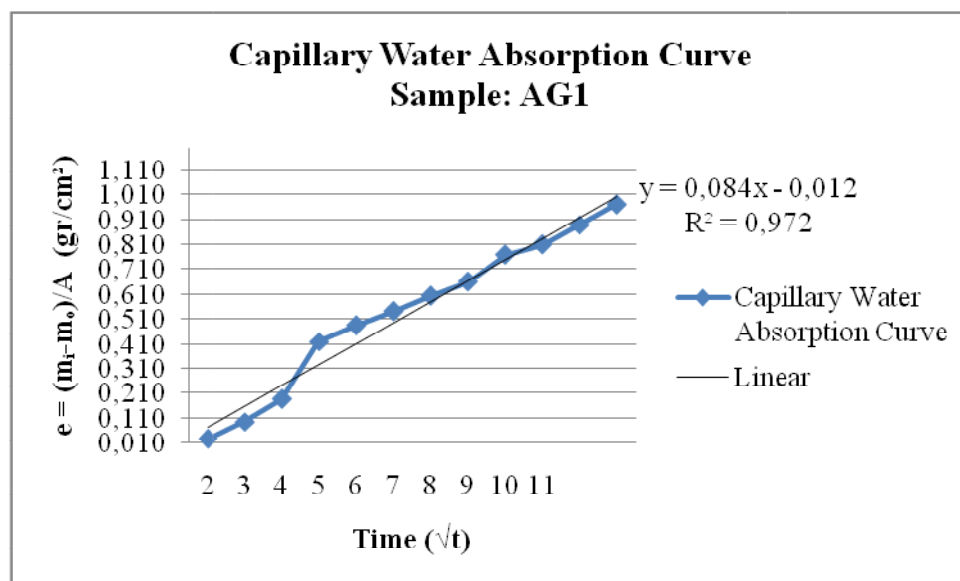


Fig. 3.18 :Capillary water absorption curve of sample no. AG1 plotted from the analysis data.

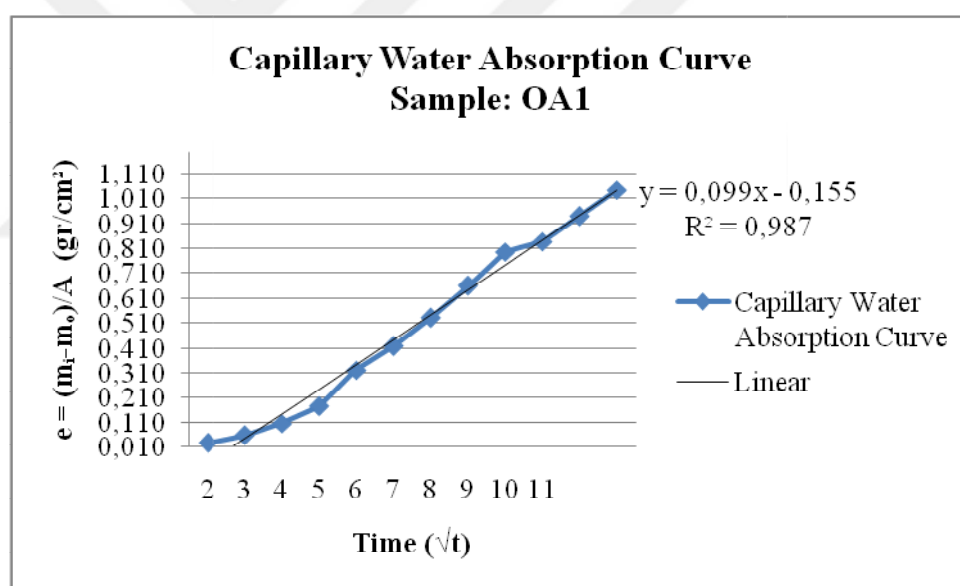


Fig. 3.19 : Capillary water absorption curve of sample no. OA1 plotted from the analysis data.

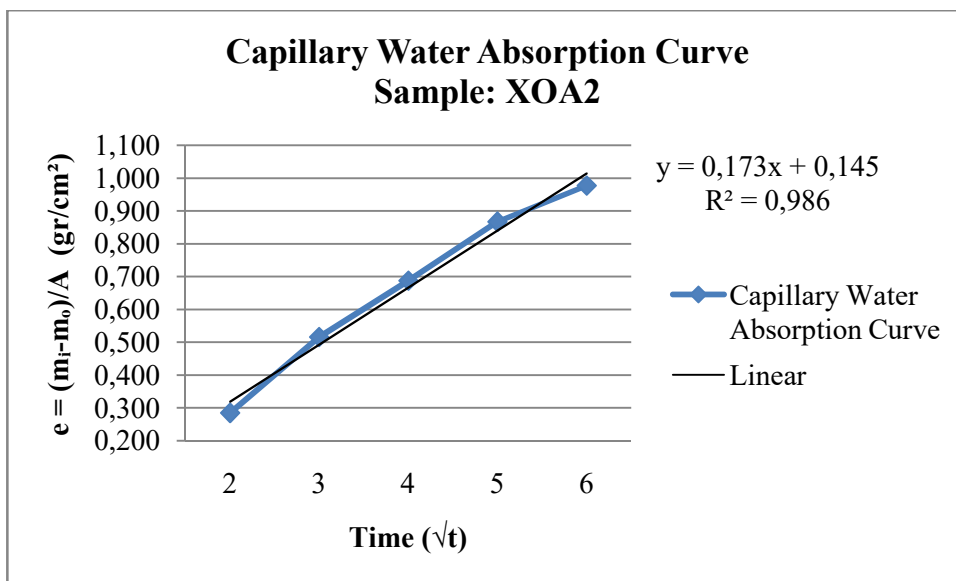


Fig. 3.20 : Capillary water absorption curve of sample no. XOA2 plotted from the analysis data.

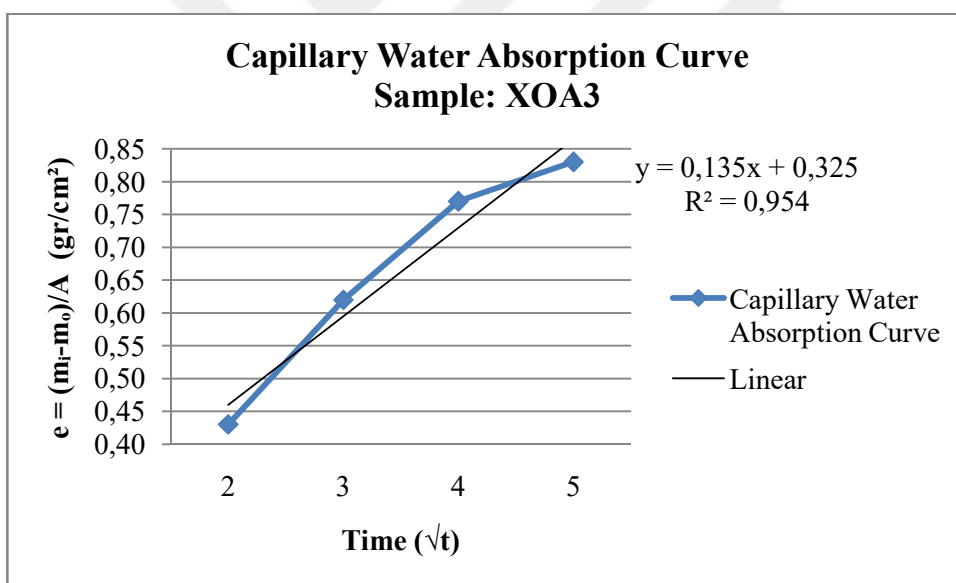


Fig. 3.21 : Capillary water absorption curve of sample no. XOA3 plotted from the analysis data.

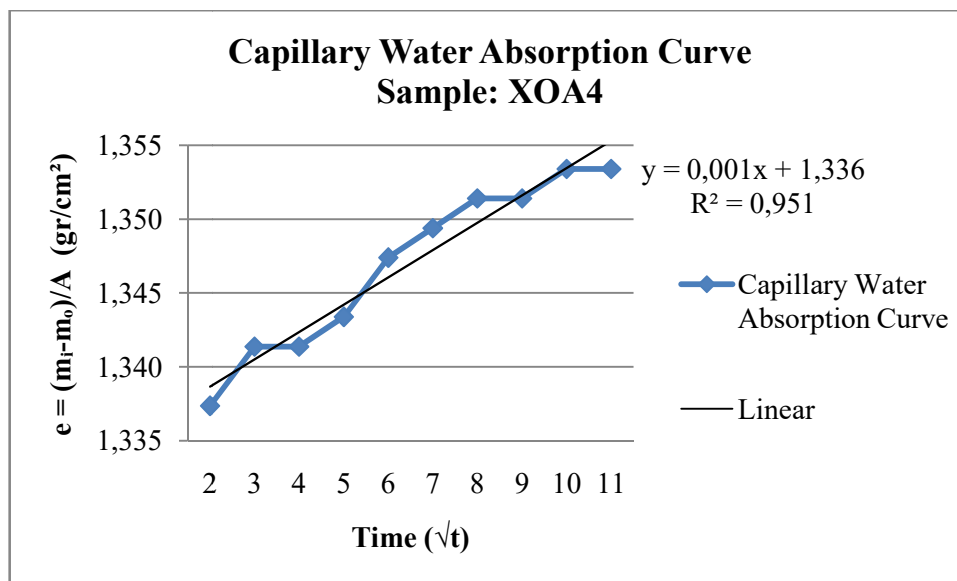


Fig. 3.22: Capillary water absorption curve of sample no. XOA4 plotted from the analysis data.

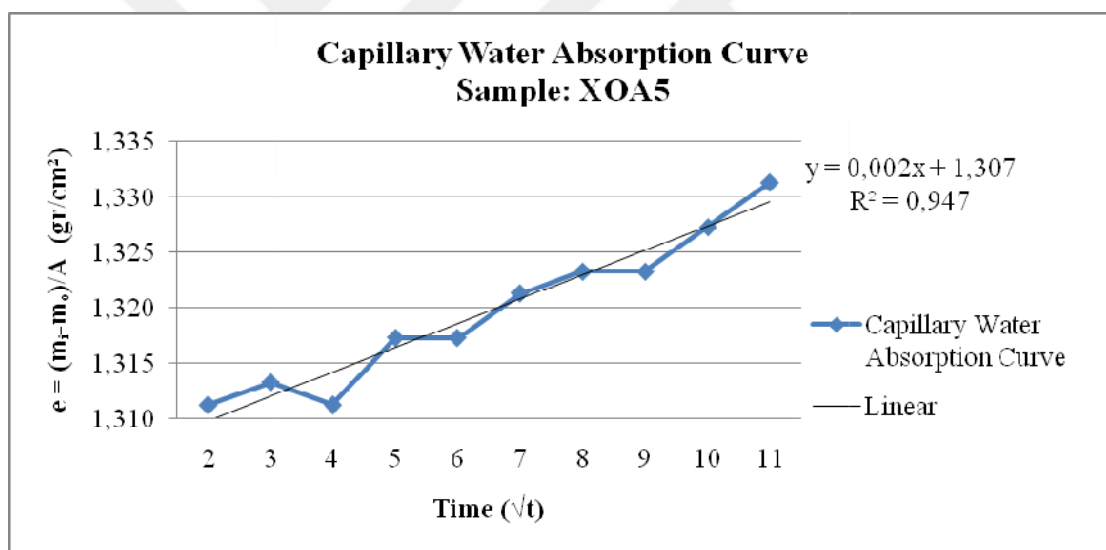


Fig. 3.23 : Capillary water absorption curve of sample no. XOA5 plotted from the analysis data.

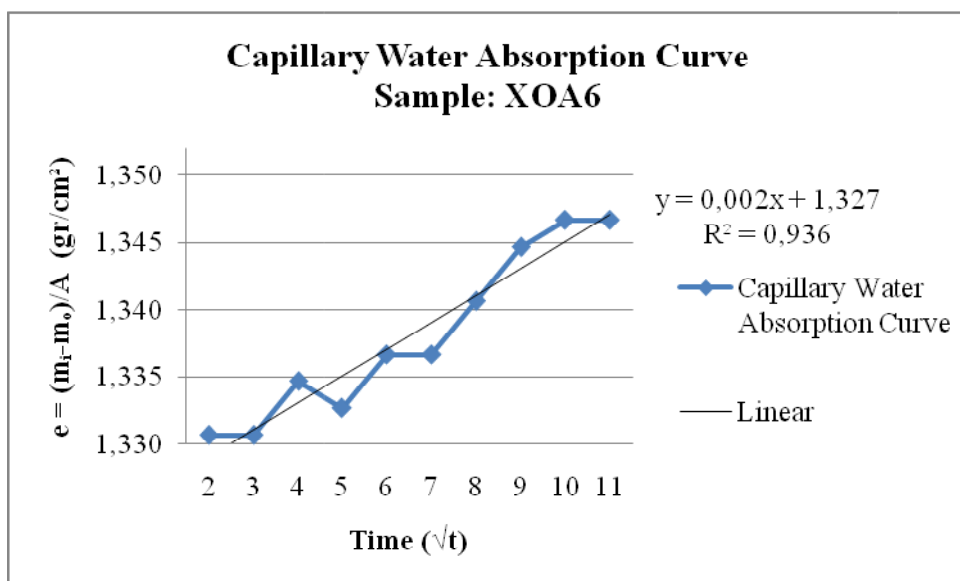


Fig. 3.24 : Capillary water absorption curve of sample no. XOA6 plotted from the analysis data.

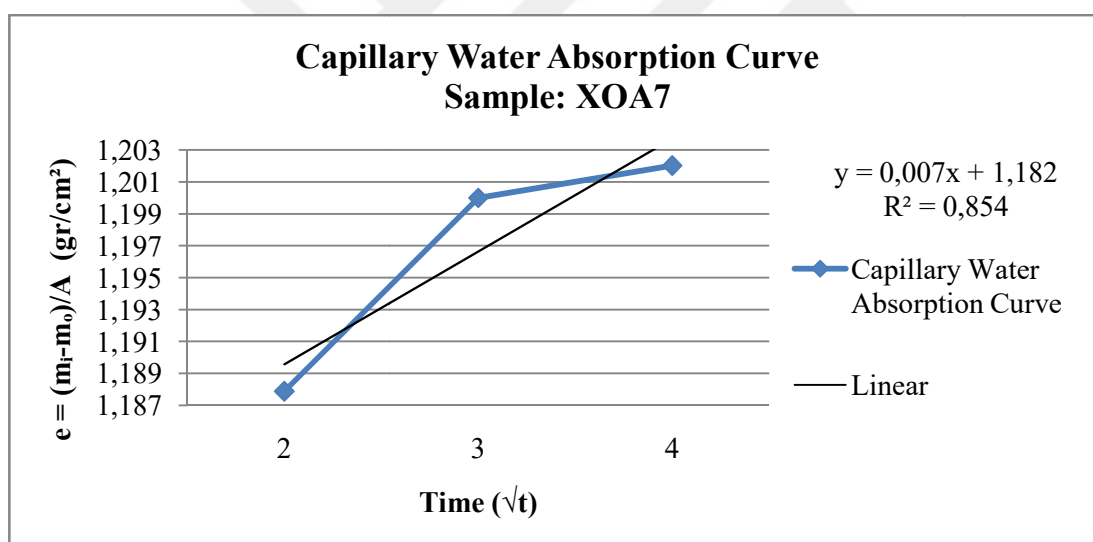


Fig. 3.25: Capillary water absorption curve of sample no. XOA7 plotted from the analysis data.

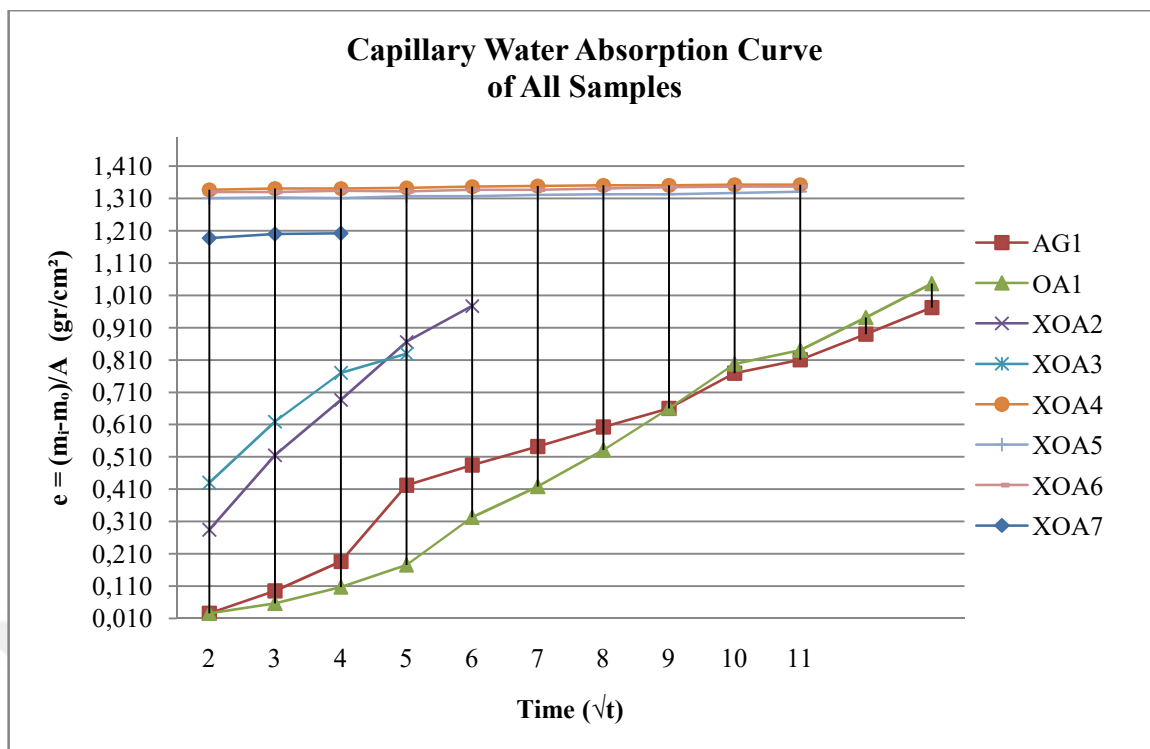


Fig. 3.26 : Capillary water absorption curve of all samples.

Table 3.8 : Capillary coefficient of analyzed samples.

Capillarity Coefficient Data of Analyzed Samples								
Sample No.	AG1	OA1	XOA2	XOA3	XOA4	XOA5	XOA6	XOA7
Capillarity Coefficient ($\text{cm}^3/\text{cm}^2\text{min}^{-2}$)	84×10^{-3}	99×10^{-3}	173×10^{-3}	135×10^{-3}	1.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}	7.0×10^{-3}

3.1.3.3 Water absorption tests

After the experiments are completed for determining capillarity, water absorption experiments are conducted under atmospheric pressure.

The samples are placed inside distilled water in a manner where all the surfaces of the samples are covered in water as shown in (Fig. 3.26). The samples are left inside distilled water until no change in their mass is observed (24 hours to 2 weeks).

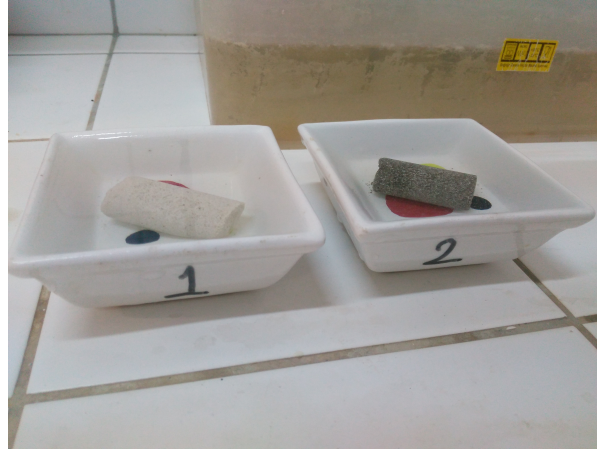


Fig. 3.27 : CIPS experiment samples left in dH₂O for water absorption experiments.

The mass of the samples are then measured in water by an archimedes scale. The samples are then taken out of water and their wet surfaces gently wiped by a cloth and their mass is measured. The data is used for the determination of the percentage of water absorption for each sample as shown in Table 3.9.

No disintegration is observed on the samples during water absorption tests which indicate successful cementation has occurred between free solid particles.

Table 3.9 : Water absorption data of analyzed samples.

Water Absorption Data of Analyzed Samples							
Sample No.	AG1	OA1	XOA3	XOA4	XOA5	XOA6	XOA7
Diameter (mm)	25,41	25,11	25,12	25,18	25,18	25,21	25,11
Height (mm)	47,35	41,88	43,17	44,85	44,74	44,95	45,37
Ambient Weight (P0,gr)	34,41	31,35	34,29	32,13	34,00	32,89	33,65
Saturated Weight (P1,gr)	43,46	39,05	39,37	39,68	41,43	40,33	40,35
Weight inside Water (P2,gr)	21,02	18,74	20,12	18,76	20,03	19,29	19,68
Percentage of Water Absorption (Sa)	26,30	24,56	14,82	23,50	20,29	22,62	19,91
Weight per Unit Volume (Δ ,gr/cm ³)	1,43	1,51	1,60	1,44	1,53	1,47	1,50
Specific Weight (δ ,gr/cm ³)	1,94	1,92	2,05	1,90	1,96	1,92	1,95

3.1.3.4 Compression tests and analysis

Compression tests are conducted in order to determine how the material reacts when it is compressed. Plaster footings are prepared for each cylinder sample obtained from CIPS experiments to ensure that the samples are properly placed between the surfaces of the compression test machine (Fig.3.27).



Fig. 3.28 : Plaster footings for preparing the samples for compression tests.

Compression tests are applied to all prepared samples and readings are observed accordingly as shown in Figures 3.28-3.30.



Fig. 3.29 : Compression test for sample no. XOA2.



Fig. 3.30 : Compression test close-up for sample no. EAG2.



Fig. 3.31 : Deformation of sample no. XOA3 after the compression test.

Results and observations from the experiments are used to plot strain/stress graphs and to calculate the modulus of elasticity for each tested sample shown in Figures 3.31-3.40 and Table 3.10.

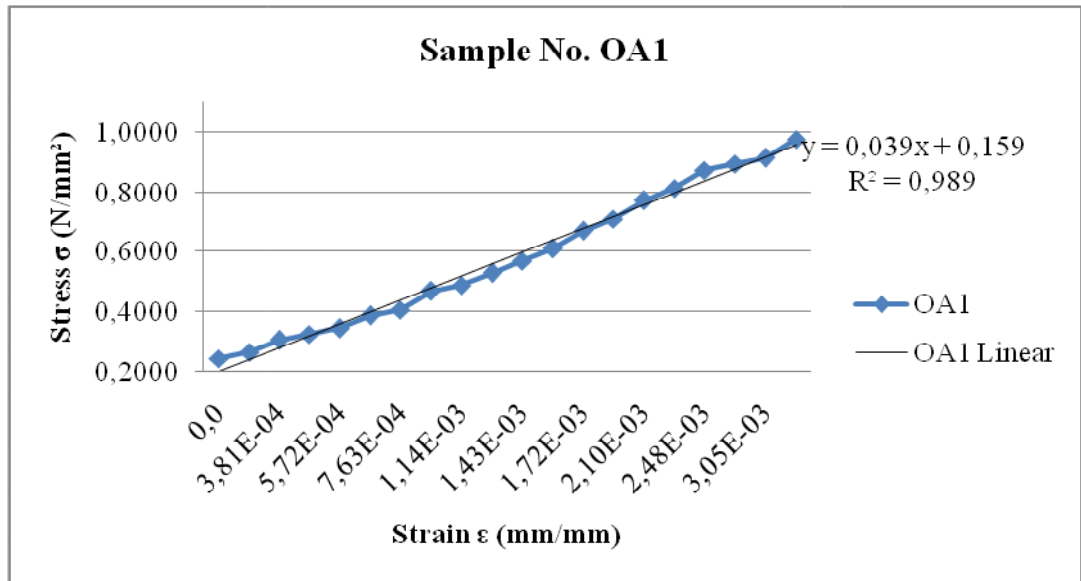


Fig. 3.32 : Stress / Strain curve of sample no. OA1.

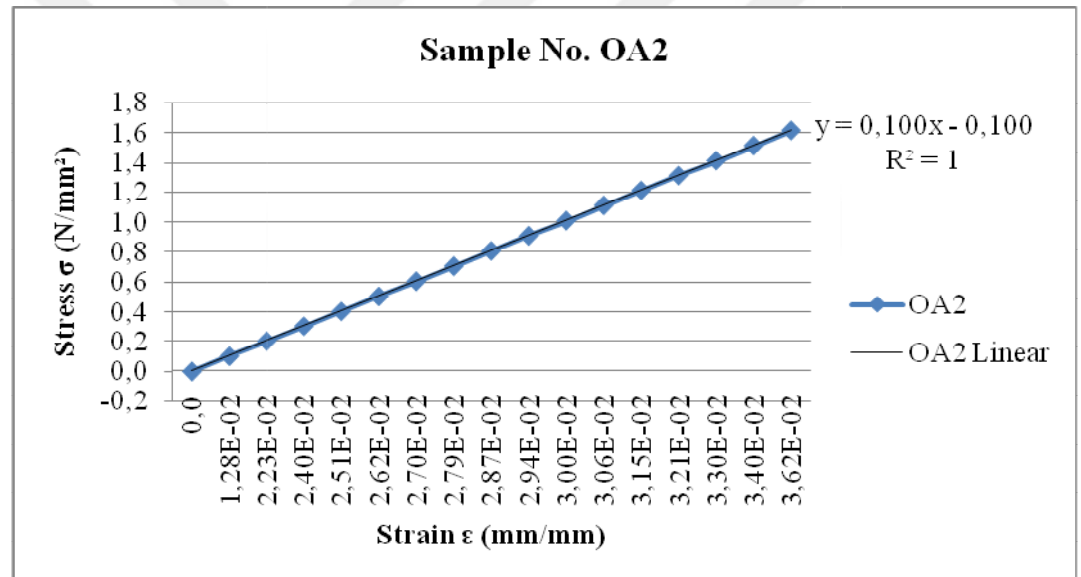


Fig. 3.33 : Stress / Strain curve of sample no. OA2.

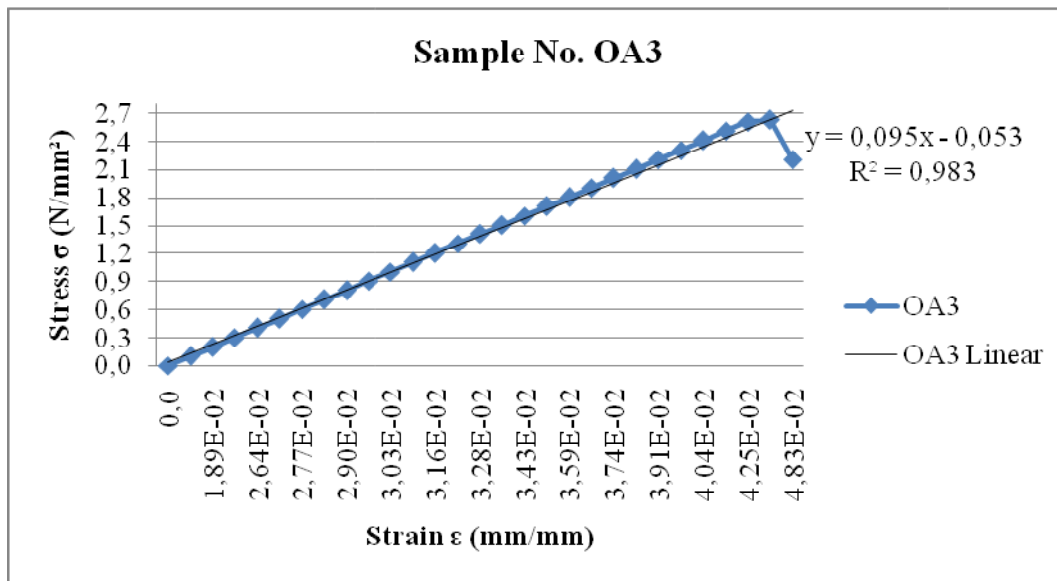


Fig. 3.34 :Stress / Strain curve of sample no. OA3.

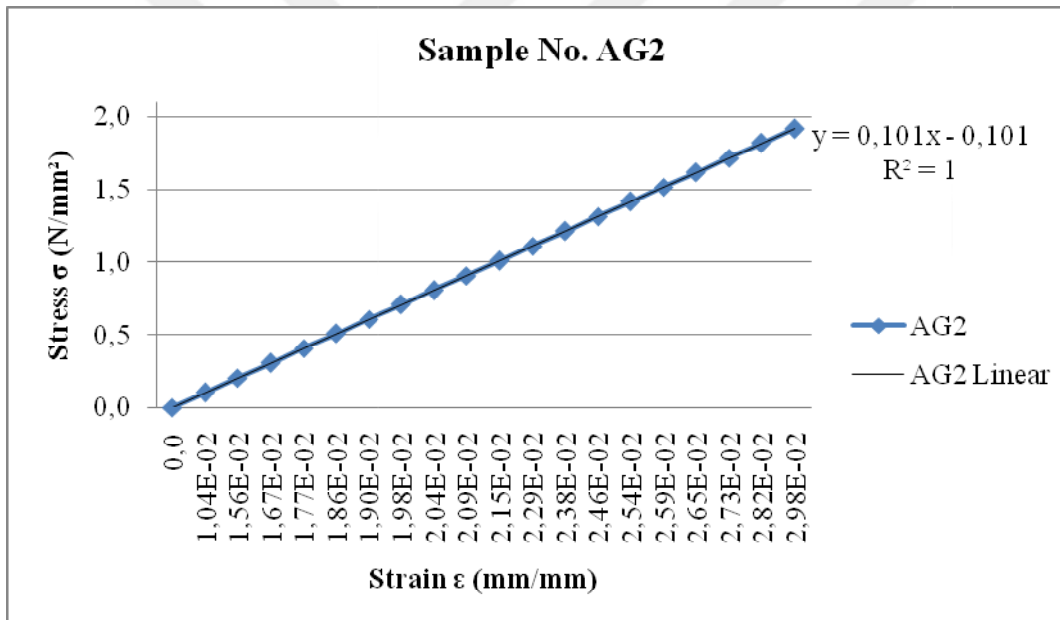


Fig. 3.35 :Stress / Strain curve of sample no. AG2.

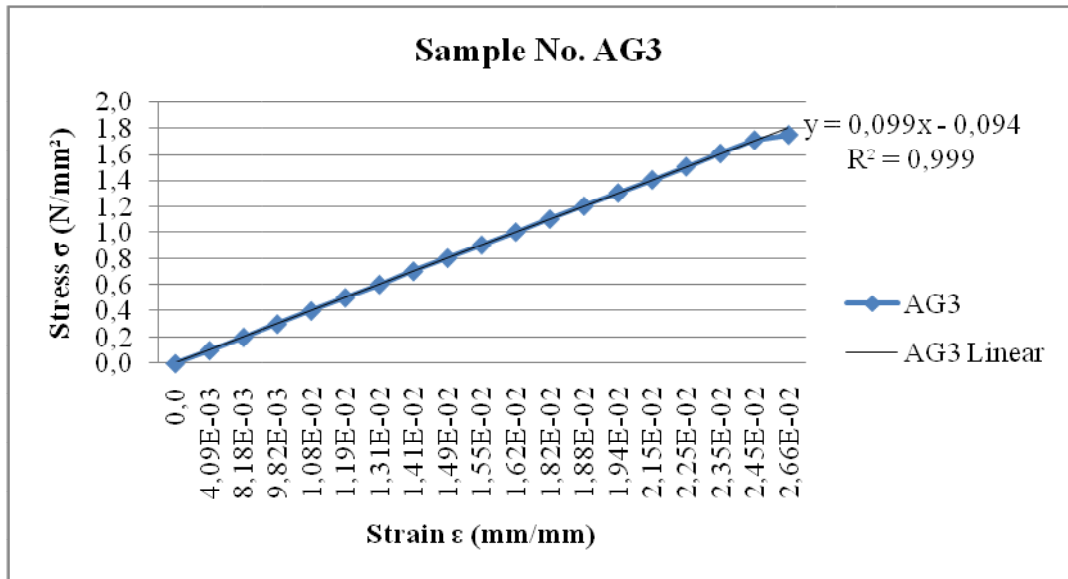


Fig. 3.36 : Stress / Strain curve of sample no. AG3.

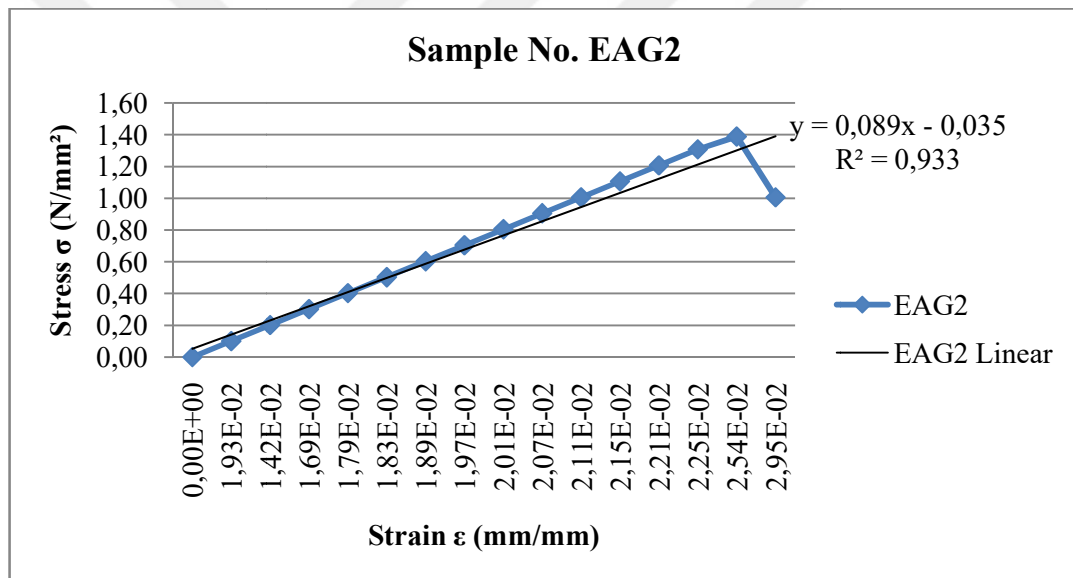


Fig. 3.37 : Stress / Strain curve of sample no. EAG2.

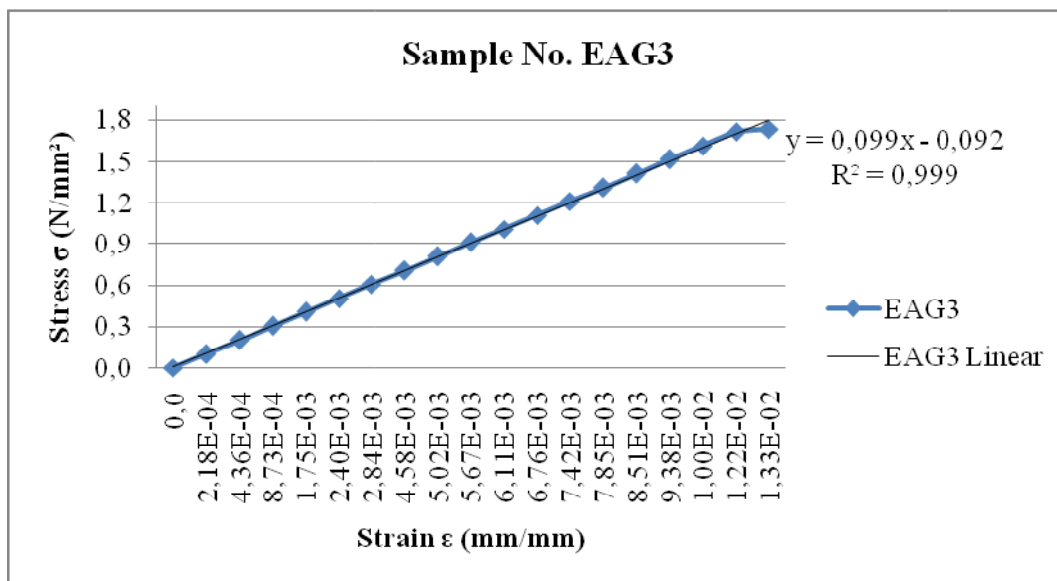


Fig. 3.38 : Stress / Strain curve of sample no. EAG3.

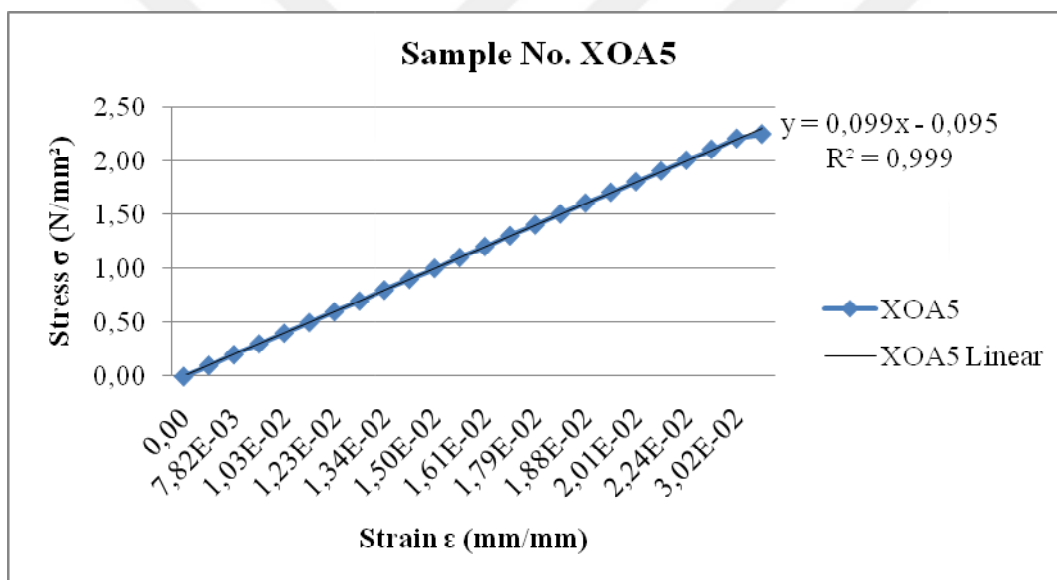


Fig. 3.39 : Stress / Strain curve of sample no. XOA5.

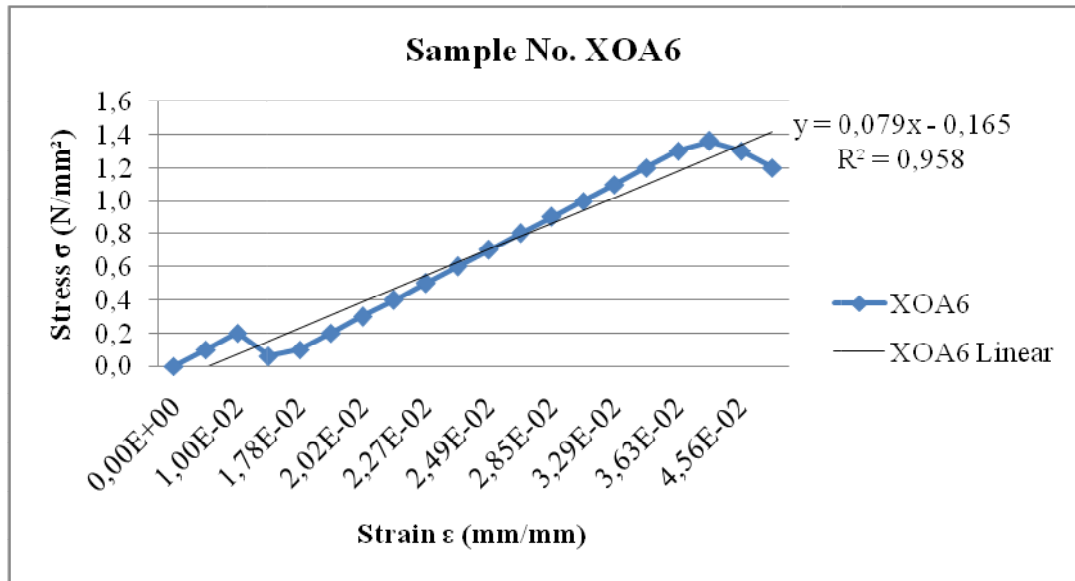


Fig. 3.40 : Stress / Strain curve of sample no. XOA6.

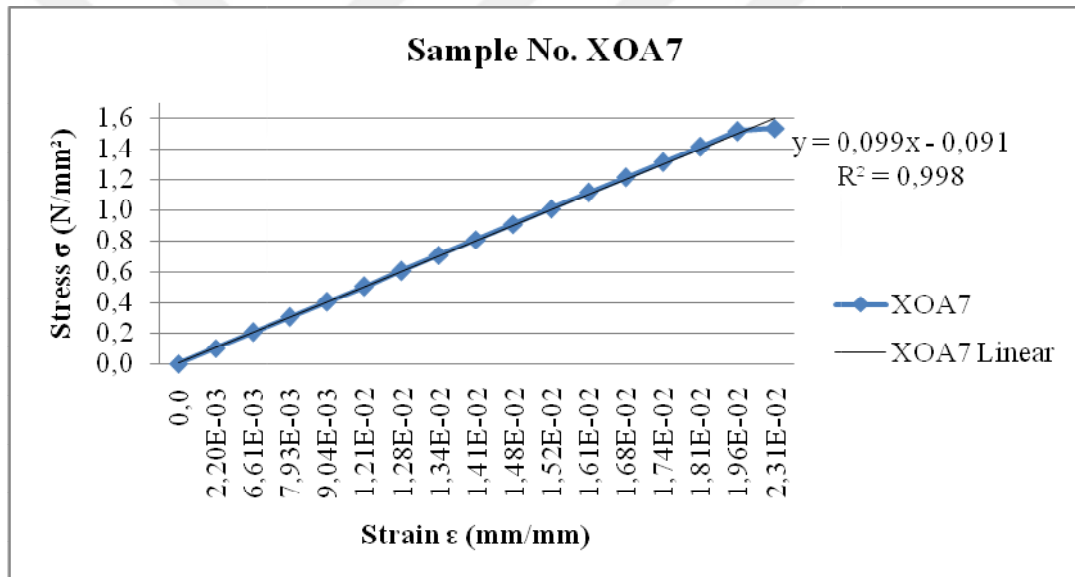


Fig. 3.41 : Stress / Strain curve of sample no. XOA7.

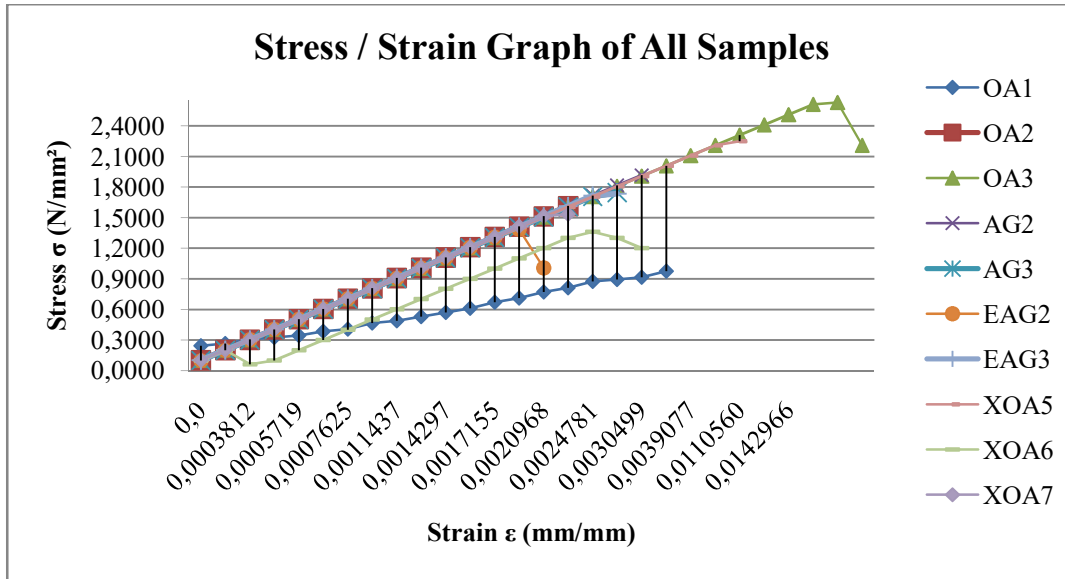


Fig. 3.42 : Stress / Strain curve of all samples.

Table 3.10 : Modulus of elasticity for each sample.

Sample No.	Modulus of Elasticity ($E=\sigma/\epsilon$) Data of Analyzed Samples									
	OA1	OA2	OA3	AG2	AG3	EAG2	EAG3	XOA5	XOA6	XOA7
Modulus of Elasticity (N/m^2)	39.0×10^3	100.0×10^3	95.0×10^3	101.0×10^3	99.0×10^3	89.0×10^3	99.0×10^3	99.0×10^3	79.0×10^3	99.0×10^3

Results indicate that the durability of the samples are considerably low. However observations point that with potential improvements regarding the optimization of particle size distribution and cell retention the overall strength of the material can be increased.

3.1.3.5 Scanning electron microscope (SEM) imagings

Scanning electron microscope images of specific samples obtained from small scale falcon cementation experiments and CIPS experiments are taken to observe the crystal morphology inside the solid media and the overall pore structure of the samples.

SEM images of 500-1000 μ m sized Agora B. sample show the gaps between free particles are considerably large and visible local calcium carbonate precipitations indicate that the formations are metastable vaterite crystals as shown in (Fig. 3.41) below.

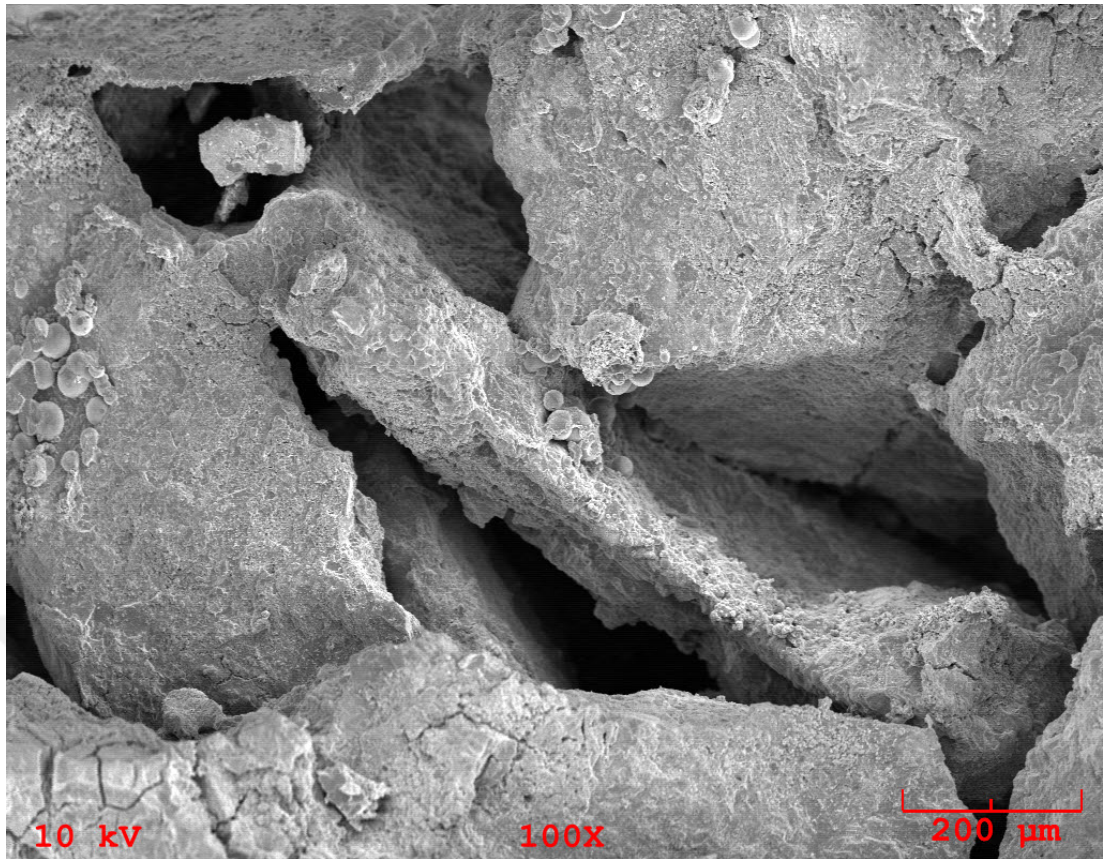


Fig. 3.43 : SEM image of treated 500-1000µm sized Agora B. sample under 100x magnification.

High amounts of calcium carbonate precipitation is observed between free solid particles however the voids between the particles are too large for bridging to occur as shown in (Fig. 3.42) below.

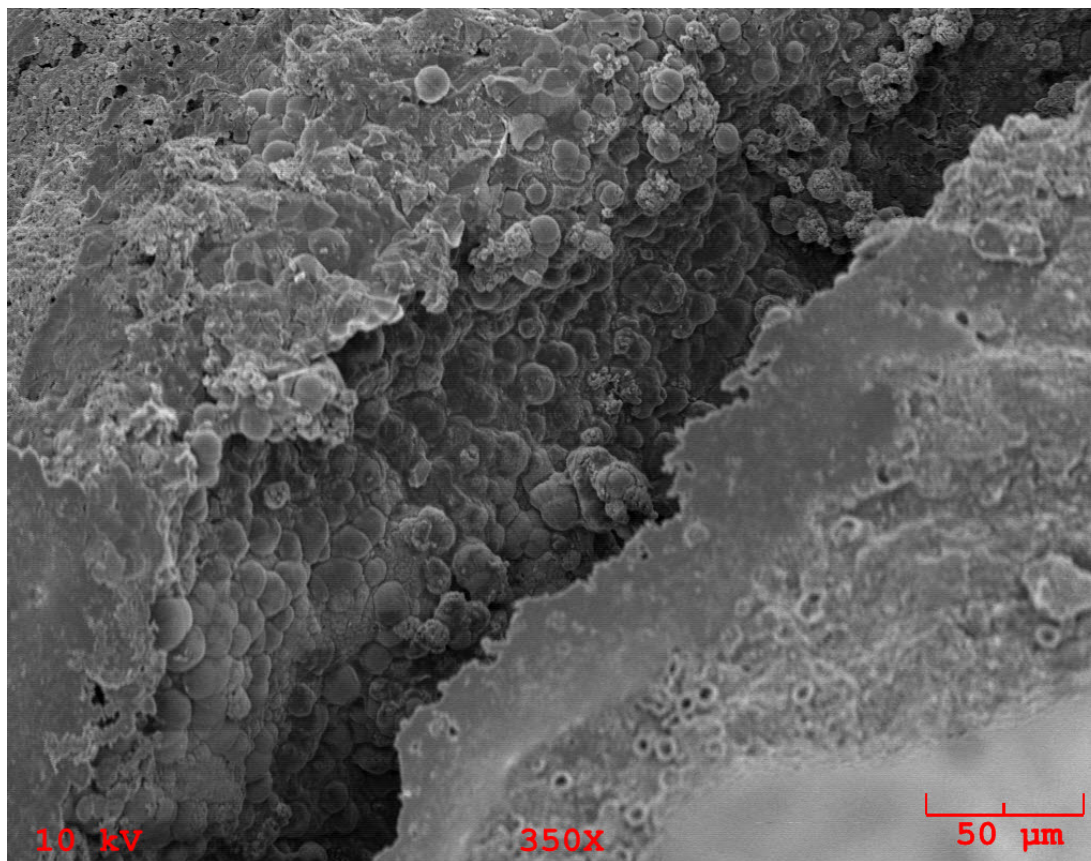


Fig. 3.44 : SEM image of treated 500-1000µm sized Agora B. sample under 350x magnification.

Calcium carbonate bridges have been observed where loose particles touch each other, which hints the importance of void minimization as shown in (Fig. 3.43). Spherical vaterite crystal formation (Fig. 3.44) also hints that the reaction rates are not suitable for the formation of the most stable calcium carbonate crystal formerly referred as calcite.

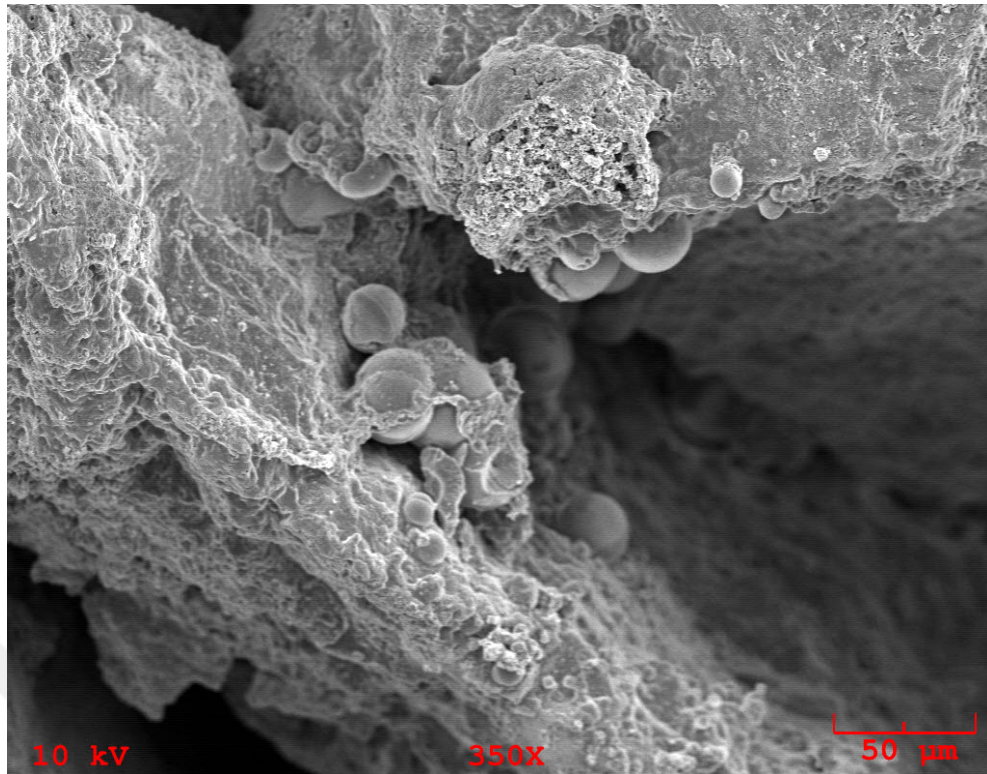


Fig. 3.45:SEM image of treated 500-1000µm sized Agora B. sample under 350x magnification and visible vaterite crystal formation.

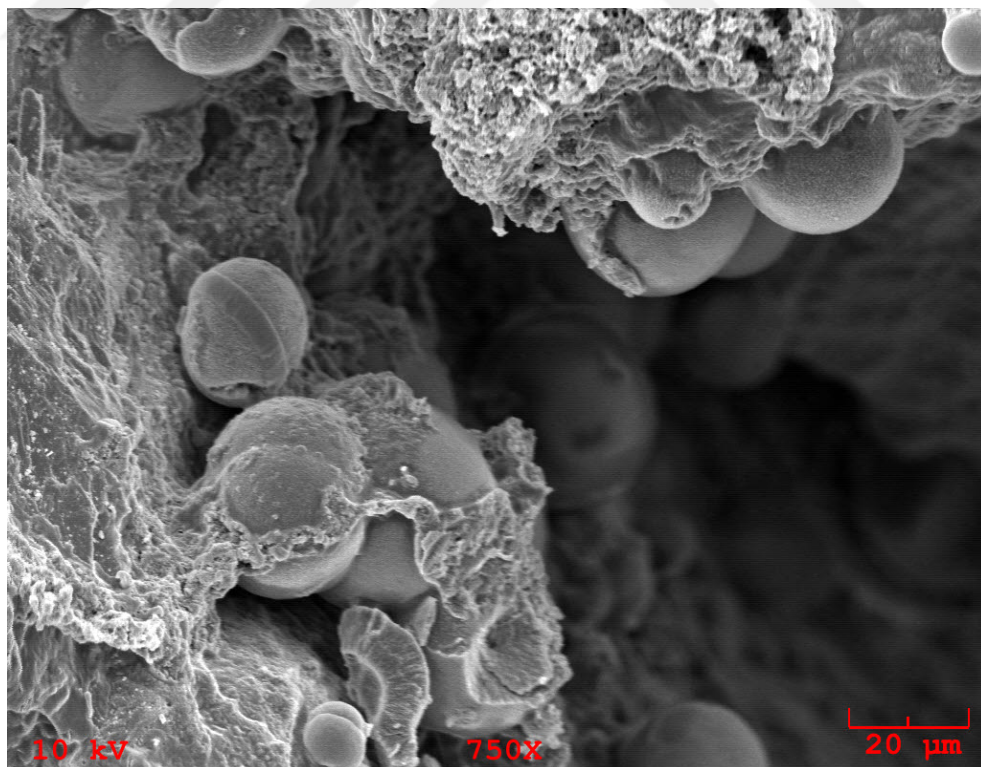


Fig. 3.46:SEM image of treated 500-1000µm sized Agora B. sample under 750x magnification.

Sharp edges observed in certain crystal morphologies may hint a failure in calcite formation as seen in (Fig. 3.45) and (Fig. 3.46) below.

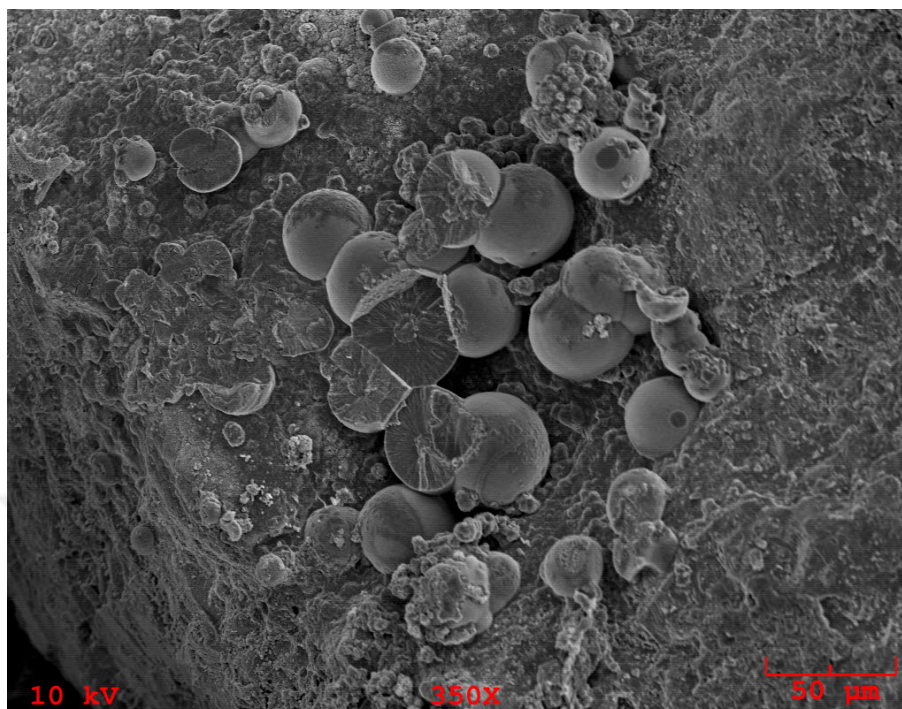


Fig. 3.47 : SEM image of treated 500-1000µm sized Agora B. sample under 350x magnification and visible vaterite crystal formation.

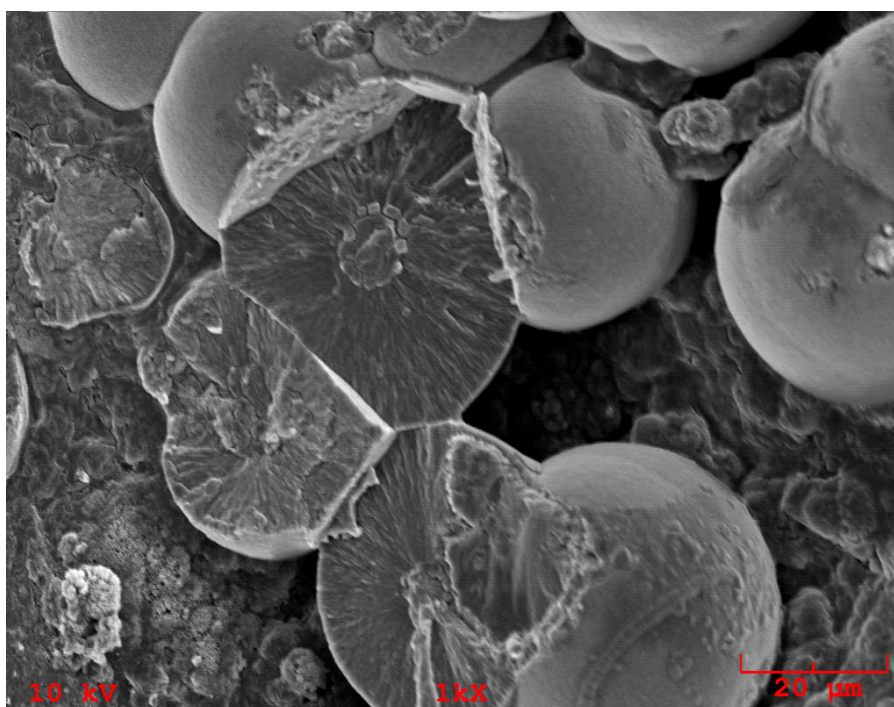


Fig. 3.48 : SEM image of treated 500-1000µm sized Agora B. sample under 1kx magnification and visible vaterite crystal formation.

3.1.4 Precautions for strength improvement according to sample analyses

3.1.4.1 In situ incubation and CaCl₂ concentration in the growth medium

Bacteria are cultivated under optimum conditions in three NH₄-YE growth media with differing CaCl₂.2H₂O concentrations; 3mM, 6mM, 9mM respectively. A rapid increase in cellular growth is observed during incubation periods in contrast to normal NH₄-YE cultivation parameters even though the same volume of inoculation is made and the same conditions for incubation are provided. Optical density values which are observed during previous cultivation attempts are observed with approximately one-third of the required amount of time. The optical density measurements of the cultures under 600nm after approximately 25 hours is as follows;

- 200ml NH₄-YE+3mM CaCl₂.2H₂O - ¼ OD₆₀₀: 0,8206 – OD₆₀₀: 3,2824

- 200ml NH₄-YE+6mM CaCl₂.2H₂O - ¼ OD₆₀₀: 0,8276 – OD₆₀₀: 3,3104

- 200ml NH₄-YE+9mM CaCl₂.2H₂O - ¼ OD₆₀₀: 0,7473 – OD₆₀₀: 2,9892

Results indicate that the optimum molar concentration of calcium chloride content inside the growth medium accelerates the bacterias metabolic activity and decreases the required amount of time for cultivation.

Parallel small scale falcon experiments and a CIPS experiment are conducted with the cultures grown in the presence of CaCl₂.2H₂O. 6 falcons are prepared each having approximately 5ml of 250-500µm sized Olive A. wastes to be injected with the three of the grown cultures with different CaCl₂.2H₂O concentrations. The parallel CIPS experiment is conducted with the solid medium composed of 500-1000µm sized Agora B. wastes which is treated with the culture grown in the presence of 6mM CaCl₂.2H₂O (OD₆₀₀: 3,3104).

Successful cementation results are obtained both from the falcon experiments and the CIPS experiment. SEM images of the treated sample with 250-500µm sized Olive A. solid medium indicate a thorough filling of voids between free particles as shown in (Fig. 3.47).

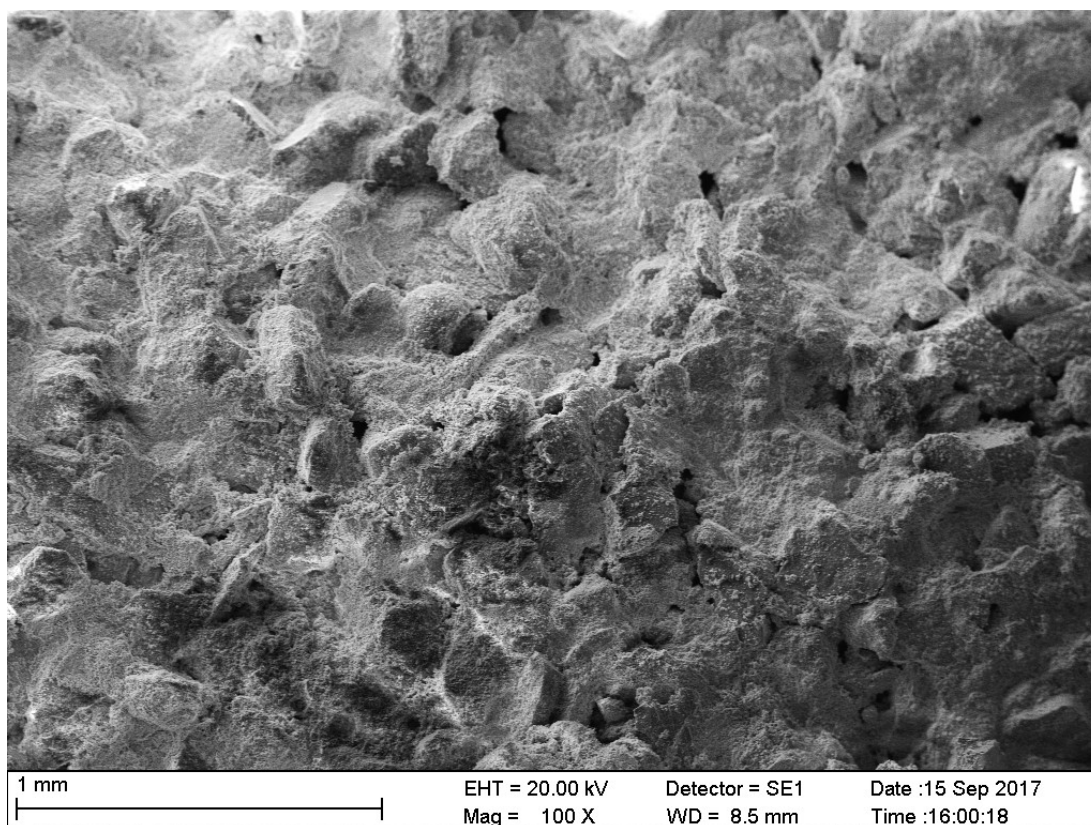


Fig. 3.49 :SEM image of treated 250-500µm sized Olive A. sample under 100x magnification.

Scanning electron microscope images of the sample also show a more successful calcium carbonate crystal formation on the surfaces of free solid particles in contrast with the earlier experiment results conducted without bacteria grown in the presence of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Fig. 3.48). This observation indicates that $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in the growth medium may support cell retention inside the solid medium when the bacterial batch is injected.

Very dense calcium carbonate precipitation is also observed locally on the same sample (Fig. 3.49).

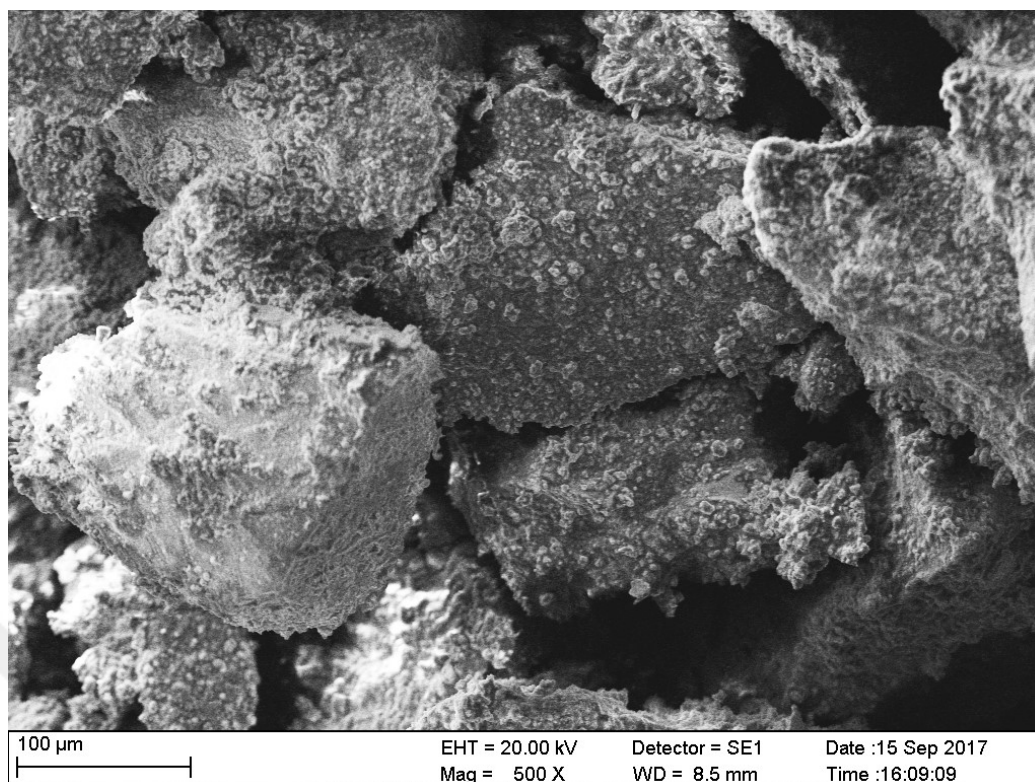


Fig. 3.50 :SEM image of treated 250-500µm sized Olive A. sample under 500x magnification

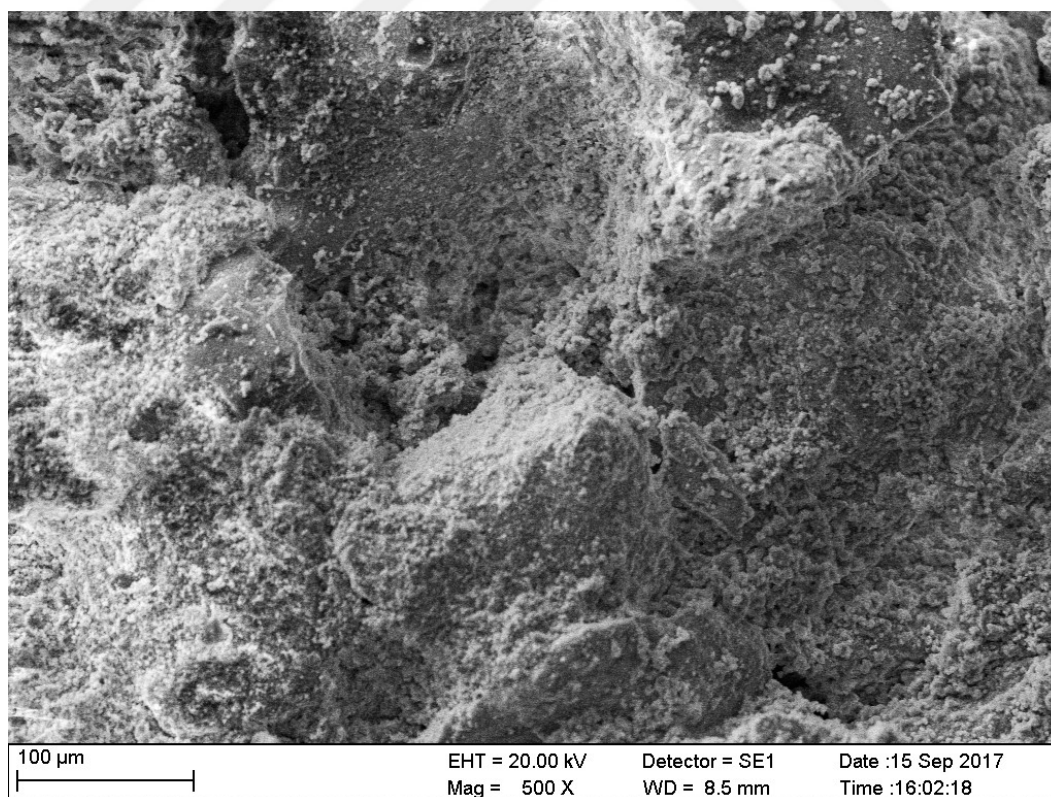


Fig. 3.51 :SEM image of treated 250-500µm sized Olive A. sample under 500x magnification.

Following the cementation experiments, in situ incubation is conducted to compare the results in terms of cell retention. 250-500 μ m sized Olive A. wastes are used for the CIPS experiment. The solid medium is injected with the bacterial batch taken from the culture grown in the presence of 6mM CaCl₂.2H₂O (OD₆₀₀: 3,3104). Once the batch is fully absorbed by the solid medium, the reactor is sealed and placed in the heated room at 30°C and incubated for approximately 48 hours. After the incubation period is complete, the rest of the CIPS experiment procedure is conducted (excluding the fixation fluid injection). The sample completely disintegrated at the end of the experiment. The potential reason for the failure may be linked to the depletion of nutrients and agitation of the bacteria during the in situ incubation period.

3.1.4.2 Particle size admixtures

Particle size admixtures are made according to the calculations made to ensure that the smaller particles fit between the voids between larger particles enabling a more successful calcium carbonate bridging between free solid particles. The most effective particle admixture is calculated to be composed of %70 250-500 μ m sized particles having a pore throat size range of approximately 77.4-38.7 μ m, with %30 100-63 μ m or <63 μ m sized particles. A CIPS experiment is conducted for the treatment of a solid medium with the indicated admixture. The bacterial batch used for the experiment is the culture grown in the presence of 6mM CaCl₂.2H₂O (OD₆₀₀: 3,3104). The result of the experiment formerly referred as sample no. XOA3 is put to analyses including water absorption and compression tests.

3.1.4.3 XRD analyses and measurements

XRD analyses are conducted in order to understand the chemical composition of the treated samples. Since the targeted crystal morphology for the precipitated calcium carbonate is calcite rather than vaterite and aragonite, a final composition with high calcite percentage is expected.

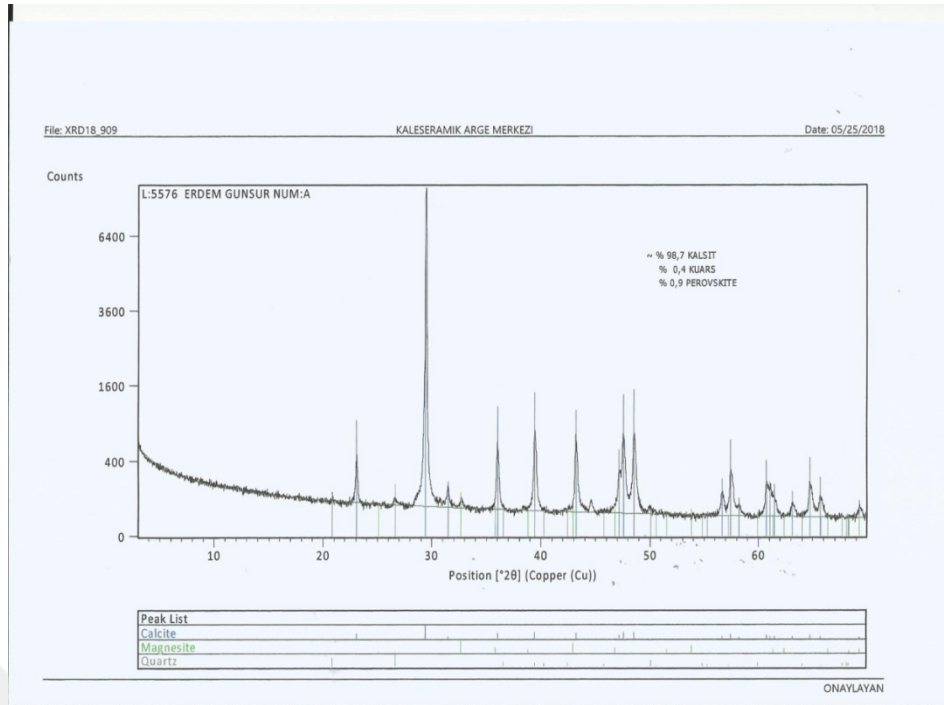


Fig. 3.52 :XRD analyses result of 250-500 μ m sized Olive Avantgarde waste treated with in-situ incubation for 48 hours at 30°C with the bacterial culture grown in the presence of 3mM CaCl₂.2H₂O during cultivation (Falcon Experiment Sample).

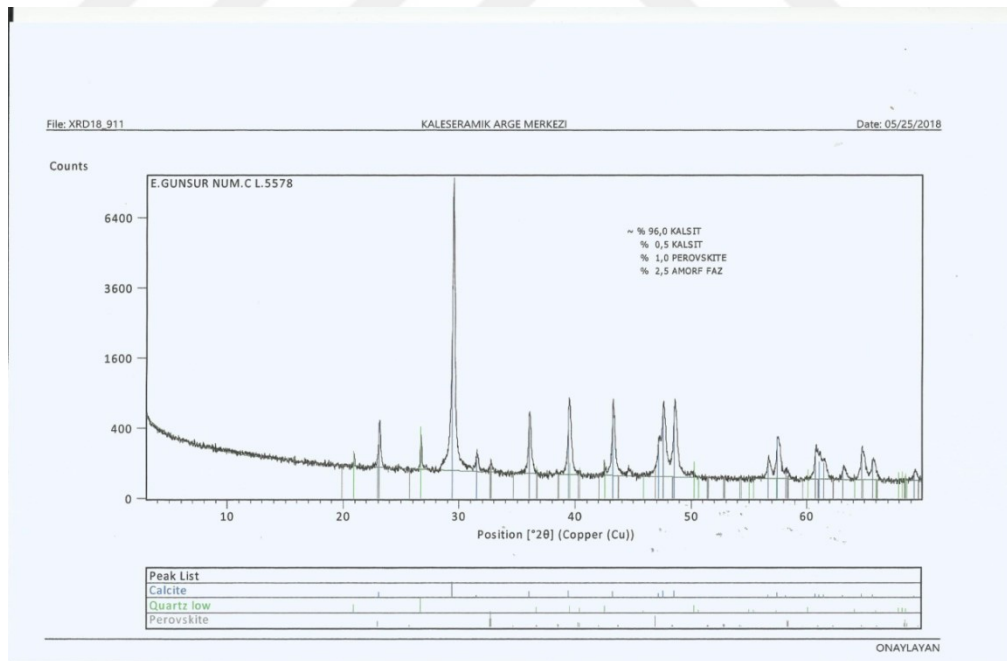


Fig. 3.53 :XRD analyses result of 250-500 μ m sized Olive Avantgarde waste treated with in-situ incubation for 48 hours at 30°C with the bacterial culture grown in the presence of 6mM CaCl₂.2H₂O during cultivation (Falcon Experiment Sample).

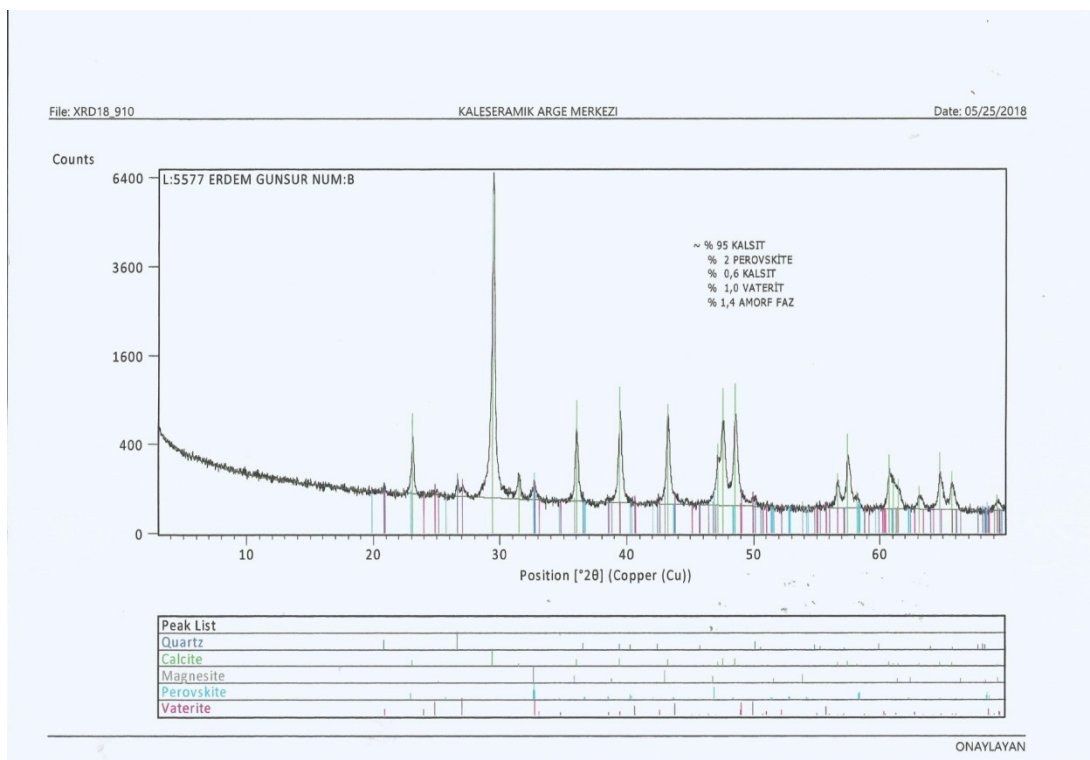


Fig. 3.54 :XRD analyses result of 250-500µm sized Olive Avantgarde waste treated with in-situ incubation for 48 hours at 30°C with the bacterial culture grown in the presence of 9mM CaCl₂.2H₂O during cultivation (Falcon Experiment Sample).

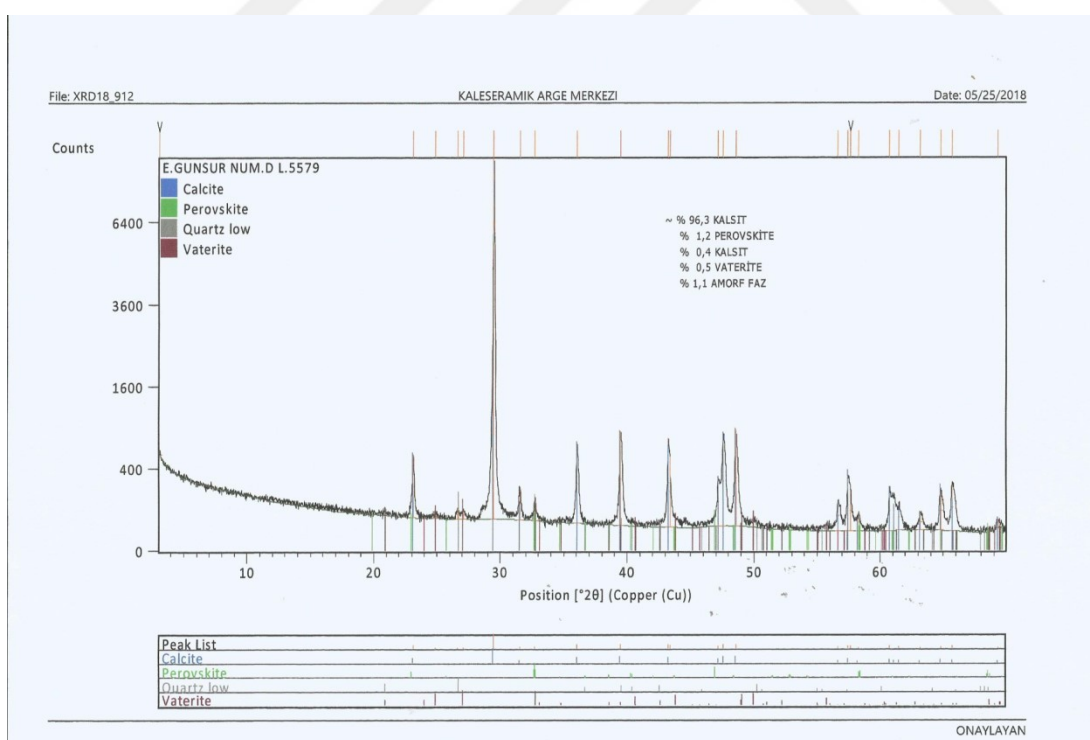


Fig. 3.55 :XRD analyses result of 250-500µm sized Olive Avantgarde waste treated with in-situ incubation for 48 hours at 30°C with the bacterial culture grown in the presence of 9mM CaCl₂.2H₂O during cultivation (CIPS Sample).

XRD analyses have proven successful calcite precipitation within the samples which are treated both with in-situ incubation and particle size admixtures. Results indicate that the presence of calcium chloride during the cultivation of the bacteria have a positive effect on calcite precipitation. However this effect is relative according to the concentration of calcium chloride. According to the results the optimum value for calcium chloride concentration is 3mM which has resulted in approximately 98,7% calcite. The percentage of precipitated calcite decreases with increasing calcium chloride concentration in the growth medium.





4. CONCLUSION

Waste generation is a major issue regarding the natural stone industry. During the processing of limited quantity of natural reserves, a considerable amount of the precious mineral is lost in the form of aggregate and dust. Although there are numerous attempts for recycling and revitalizing such wastes, most of them do not offer a sustainable solution.

Within the scope of the thesis, collected natural stone wastes in various particle size ranges have been biologically treated to offer a potential sustainable solution to the referred problem. The treatment and the method in question is microbial induced calcite precipitation.

Experimentations have been conducted starting from small scales for understanding vital parameters affecting the biocementation process. After optimizations are made the scale of the experiments have been increased, resulting in the design and production of a reactor and a closed cementation (CIPS) system.

The samples obtained from the upper scale cementation experiments have been put to various analyses to understand the physical and chemical characteristics of the materials.

Results of the analyses and observations indicate that the produced material is not a durable material however improvements can be made to increase this aspect. The capillary absorption experiment results have shown that the produced materials absorb water with a considerably fast rate. The results of the water absorption experiments also hint that the material can endure in liquid media without being disintegrated. Aforementioned results and observations suggest that the material is suitable for being used as a filtering material.

Scanning electron microscope imagings have shown that the voids between free particles or pore throats are not successfully bridged or filled with calcium carbonate crystals after the treatments except for the sample composed of the admixture of particles in various sizes which has been treated with the CaCl_2 grown culture. It is also observed that the crystal formation is the metastable calcium carbonate crystal

referred as vaterite. The failure in calcite formation hints that further research is needed to be conducted in order to ensure a successful reaction rate.

As conclusion, biomineralization and the specific method of microbial induced calcite precipitation through ureolysis is a suitable way of recycling and/or revitalizing natural stone wastes in the form of aggregate and dust by turning such wastes into sustainable materials. Certain physical characteristics of the materials such as endurance and porosity can be improved by altering certain parameters which are affecting the overall biomineralization process.



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