BIOLOGICAL MORTAR APPLICATION FOR MICRO-CRACK REMEDIATION IN STONES OF TRAVERTINE MONUMENTS

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ABSTRACT

BIOLOGICAL MORTAR APPLICATION FOR MICRO-CRACK REMEDIATION IN STONES OF TRAVERTINE MONUMENTS

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Traditional conservation methods for the preservation of historical stones including applications with inorganic and organic polymers are often inadequate and results in introduction of new deterioration problems to historic structures. At this point, consolidating a stone by re-creating a structure similar to original microstructure of stone is a new approach developed to ensure the maximum compatibility by comparison to the traditional conservation techniques.

In this study it was aimed to develop a sustainable and an eco-friendly repair material, biological mortar (BM)/infill, to be used in conservation interventions for healing micro-cracks (<1 mm) in travertines of historical monuments. This new repair material contains an environmental strain of a known bacteria species.

In this context, bacterial isolation and identification were carried out from thermal spring water resources in Pamukkale Travertines (Denizli). *Bacillus cereus*, already known to have high calcite production capacity, was selected for BM development studies within isolated strains. Upon specifying all components of BM in details, mortar set up was performed in defined proportions and applied to micro-cracks of

artificially aged test stones. Performance of this repair material was examined through physical, physico-mechanical, microstructural analyses, and other evaluation criteria.

Consequently, a strong bond between the grains and matrix of BM was determined in relation with the calcite production activities of *B. cereus.* Moreover, in all samples where BM application was performed, interface of biological mortar and original material showed continuous and coherent structure. Hence, biological mortar and production substructure developed in this study, could be used for remediation of micro-cracks and micro-voids in historical travertine structures such as sculptures, ornaments, capitals, and elements.

In future studies, to increase the rate and concentration of bacterial calcite precipitation in mortar, some parameters in the experimental procedure could be improved by adding specific enzymes or macromolecules having potential to induce nitrogen cycle pathways to the nutritive medium, using large fermenters that could yield high quantities of bacteria, using multiple bacterial populations that might also increase the amount of calcite production and identifying genes related with the mineral production process and with the adjustment of these genes, bringing out large quantities of macromolecules that might induce calcite precipitation.

Keywords: calcite, bacteria, micro-crack, biological mortar, conservation, travertine

ÖZ

TARİHİ ESERLERİN TRAVERTENLERİNDEKİ MİKRO-ÇATLAKLARIN İYİLEŞTİRİLMESİ İÇİN BİYOLOJİK HARÇ UYGULAMASI

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Bozunma gözlemlendiği tarihi taşların korunmasında inorganik ve organik polimerlerle yapılan geleneksel koruma yöntemleri çoğunlukla yetersiz olup zaman içerisinde farklı bozunma sorunlarına yol açmaktadır. Bu noktada, bir taşın orijinal mikroyapısına benzer bir yapıyı yeniden oluşturarak sağlamlaştırılması, maksimum uyumluluğu sağlamak için geliştirilmiş yeni bir yaklaşımdır.

Bu çalışmada, tarihi travertenlerde oluşan mikro-çatlakların (< 1 mm) iyileştirilmesi için yürütülen koruma müdahalelerinde kullanılmak üzere, sürdürülebilir ve çevre dostu bir biyolojik harç/dolgu malzemesi geliştirilmesi amaçlanmıştır. Bu yeni onarım malzemesi bilinen bir çevre suşunu içermektedir.

Bu kapsamda, Pamukkale Travertenlerindeki (Denizli) termal su kaynaklarından bakteri izolasyonu ve identifikasyonu yapılmıştır. Elde edilen izolatların arasından, halihazırda kalsit üretme kapasitesi yüksek olduğu bilinen *Bacillus cereus* suşu biyolojik harç geliştirilmesi için seçilmiştir. Biyolojik harç bileşenlerinin detaylı olarak tanımlanması yapıldıktan sonra, bu bileşenlerin uygun oranlarda bir araya getirilmesi ile biyolojik harç üretilmiş ve yapay olarak yaşlandırılmış test taşlarındaki

mikro-çatlaklara uygulanmıştır. Sonrasında ise bu onarım malzemesinin başarımı fiziksel, fizikomekanik, mikro-yapı ve morfolojik analizler ile değerlendirilmiştir.

Sonuç olarak, *B. cereus*'un kalsit üretmesine bağlı olarak, biyolojik harcın matrisi ile tanecikleri arasında güçlü bir bağ kurulduğu belirlenmiştir. Ayrıca, harç uygulaması yapılan tüm örneklerde, biyolojik harç ile orijinal malzeme yüzeyinin devamlı ve uyumlu bir yapı gösterdiği tespit edilmiştir. Dolayısıyla, bu çalışma ile geliştirilen biyolojik harç ve üretim altyapısının heykeller, bezemeler, sütun başları ve yapı elemanları gibi tarihi traverten eserlerde oluşan mikro-çatlak ve mikro-boşlukların onarımında kullanılabileceği belirlenmiştir.

Gelecek çalışmalarda, harç içindeki bakteriyel kalsit üretim hızının ve konsantrasyonunun arttırılabilmesi için, besleyici ortama azot döngüsü yollarını indükleme potansiyeline sahip spesifik enzimler veya makromoleküllerin eklenmesi, yüksek miktarda bakteri üretebilen büyük fermentörler kullanılması, kalsit üretim miktarını arttırabilecek çoklu bakteri popülasyonları kullanılması, mineral üretim süreci ile ilgili genlerin tanımlanması ve bu genlerdeki düzenlemeler ile kalsit presipitasyonunu indükleyebilecek fazla miktarda makromoleküllerin üretilmesi gibi bazı deneysel parametreler üzerine çalışabilir.

Anahtar kelimeler: kalsit, bakteri, mikro-çatlak, biyolojik harç, koruma, traverten

To my beloved daughter Defne

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CHAPTER 1

INTRODUCTION

As a microbiologist working in the field of conservation of cultural heritage, author defines her main role as contributing to the development of bio-based methods, come forward with the incompatibilities of existing conventional treatments, to protect cultural property for future generations. It is expected that this study contributes to the field by stimulating the development of the relationship between cultural heritage and different disciplines and also such multidisciplinary studies as a source of inspiration for the creation of new potentials.

1.1. Aim and Scope of the Study

The aim of this study is to produce an environmentally friendly biological mortar or bio-mortar (BM) that is compatible with the original material by using bacteria capable of producing calcium carbonate. The produced BM was used to heal micro-cracks of travertines in our experimental setup and showed promising results to be used in the field of historical travertines. This repair material is thought to make a significant contribution to the existing medium-high porous stone repair problems.

In this context following studies were carried out, respectively:

I. Isolation and identification of bacteria from Pamukkale Travertines (Denizli).
II. Determination of calcite production capacity of target bacteria *(Bacillus cereus)*

III. Production of the biological mortar by using the most efficient calcite precipitating bacteria isolated from Pamukkale Travertines (Denizli), stone powder of travertine from Denizli/Kocabaş, and nutritive solution prepared in laboratory.

IV. Application of biological mortars to artificially weathered travertine samples and determining the performance of the applications both in laboratory and open-air conditions.

V. Defining the BM application methodology for historical travertines with the help of experimental results

The production and application process developed in this study have also a potential to be used for the development of new bioinspired building materials.

Moreover, this type of biotechnology application is currently not available in any domestic company and therefore produced repair material namely bio-mortar will have an added value.

1.2. Stone Conservation

Heritage conservation is defined as "all the processes of looking after a place so as to retain its cultural significance. It includes maintenance and may according to circumstances include preservation, restoration, reconstruction and adaptation, and will be commonly a combination of more than one of these" in the Burra Charter (Burra Charter, 1979).

Conserving a monument helps providing historical matter as a document and enlightening historical construction techniques. Hence, conservation has to safeguard historical matter and the remains of its production and construction techniques. Eventually, it is obvious that both preventive and remedial types of interventions, conducted for the sustainability and maintenance of historical monuments must satisfy those demands.

Since the primary objective of conservation is to protect historical buildings & objects from further deteriorations, developed material in accordance with this purpose is described by a commonly accepted approach: "…to do minimum intervention that is to try to keep the interventions at the minimum level; only to do what is strictly necessary and to try and to ensure the long term compatibility and durability of the treatments" (Sasse and Snethlage, 1997)

Before deciding to do anything to reduce or prevent of deterioration of historical monuments, any conservation study should start with the *diagnosis* as a first step in. In this stage a number of information is to be collected step by step in order to make correct decisions to increase the lifetime of studied monument.

Diagnosis process should start with the documentation of the current state of the building which is the principal need of any conservation study to comprehend the historical structure and collect information regarding its physical condition before making any intervention (Hassani, 2015). More specifically, documentation comprises physical description, historical background, a summary of heritage value (aesthetic, historic, scientific, cultural, and etc.), list of elements (façade, doors, windows, buried or concealed features, deforming or moving components etc.). In addition, information regarding treatment history, seasonal weather conditions, and geological and physical properties of the construction materials should also be included in the documentation report.

On the other hand, documenting the weathering problems is also the important part of a diagnostic study. It includes analyzing of extent, severity and rate of decay to reach an accurate and consistent outcome. Besides, considering particularly stone deterioration, different types of decay forms led researchers to use a common language in discussions related with stone decay. Even if sources as "The ICOMOS-ISCS Illustrated Glossary on Stone Deterioration Patterns" acts as a bridge for definition and clarification across languages, determination of the extent and severity or rate of decay still appears as difficult and complex (Anson Cartwright et al., 2008).

Nevertheless, quantifying decay is also significant to make comprehensive conclusions while enlightening its causes. Therefore, rather than using a single technique to turn qualitative data to quantitative, several techniques such as 3D laser scanning, ultrasonic measurements, infrared thermography (IRT) can be used to analyze the type, form and behavior of decay over time (Doehne et al., 2010a).

Characterization of stone is determined by its physical properties such as surface-hardness, porosity, capillarity, hygric and thermal expansion, pore size distribution, mechanical strength, sonic velocity, resistance to salt crystallization etc. In addition to those, scanning electron microscope (SEM) coupled with energy dispersive X-ray (EDX), and X-ray diffraction (XRD) analyses make significant contribution to the stone characterization and its weathering related problems. All those experimental analyses help to move beyond the basic characterization and enables researchers to understand "material behavior" and "maintenance necessary to sustain long-term performance" (Doehne et al., 2010a).

With the help of diagnostic studies, the causes and mechanisms of decay can be revealed in any conservation project. Decay phenomena appertaining to historical stone is generally explained in three subtitle; physical, chemical and/or biological (Doehne et al., 2010b).

1.2.1. Weathering of Natural Stones and Crack Formations

While looking attentively at a stone of a historical building or monument, even very few stones seem as sound and little affected against daily and seasonal changing weather conditions, biological organisms, pollutant materials and soluble salts, through centuries, one can easily observe undergoing gradual deterioration in the majority of stones as crack & deformation, detachment, material loss, discoloration & deposit, biological colonization (Anson Cartwright et al., 2008).

Among these deterioration types, cracks are the ones endangering survival of monuments for many years. And they are defined as "individual fissure, clearly visible

by the naked eye, resulting from separation of one part from another" in the studies of Anson et al. (2008). There are several types of cracks such as: fracture, star crack, hair crack, craquele, splitting, and definitions of them are as below (Anson Cartwright et al., 2008) :

-Fracture: stone piece is completely crossed by a crack formation.
-Star crack: resembles star form, generally caused by rusted iron and mechanical effect.
-Hair crack: width dimension is < 0.1 mm.
-Craquele: network of hair cracks.
-Splitting: planes of stone are cracked through their weakness zones

The critical level and extent of cracks are changing based on their properties and causes of occurrence. Micro-cracks and fissures may not be seen as important, but they can propagate and lead to surface detachments and larger cracks in the long run mostly due to clay swelling. Calcite thermal expansion, freeze/thaw damage, salt crystallization and biological activities are other important weathering factors that can create stress in stone either by initiation or propagation of cracks (McNabb, 2012). In addition, weak zones or cavities near the surface as karstic veins are the places for initial fissure formations as the materials carried away and these openings turn into larger cracks with the continuation of the dissolving process on the carbonate rocks (Abd El Aal, 2017).

1.2.2. Present Techniques Used in Remediation of Micro-cracks

As one of the most harming type of weathering that threatens the survival of monuments, cracks have been the subject of numerous conservation research and treatments for the stones of historic monuments. Repair materials used in these treatments should be "compatible" with historic materials in terms of physical, chemical and mechanical properties in order to assure the durability of the historic stone monuments on the long term. Criteria for compatibility are defined based on the

characteristic of original material and the performance of the repair material after its application to the original material.

In brief, materials used in protection and remediation of micro-cracks are divided mainly into two main groups as consolidants and repair mortars More specifically, epoxies, silanes & tetrasilanes, lime-based treatments (nano-lime technology) and biomineralization are the types of consolidants, whereas cement-based mortars, lime-based mortars and biological mortar are the types of repair mortars used in conservation treatments.

From the aforementioned materials, epoxy resins, silanes & tetra-silanes have several disadvantages as pore blocking effect and poor penetration (Doehne et al., 2010a). Furthermore, they reduce aesthetic appearance such as color and texture incompatibility and need constant maintenance (Annamalai et al., 2013). In addition, those costly treatments commonly result as incompatible, harmful surface films after a while (Jagadeesha Kumar et al., 2013).

On the other hand, cement-based mortars have different thermal expansion coefficient compared to original material – stone (Palomo et al., 2002) and forms soluble salts (Pacheco-Torgal et al., 2012). Moreover, dangerous gases release during curing of this material which causes environmental health hazards (Rodriguez-Navarro et al., 2003a).

Nano lime technology, derived from lime wash applications, is available after some years of development. Even the technique represented better results than any other consolidants, long-term testing and further development of nano-lime materials is necessary because it has still some disadvantages as; formation of white haze on the treated surface, quick evaporation or over saturation of the solvent, insufficient strength (Borsoi, 2017), and its efficiency in the presence of soluble salts has not yet been studied in detail.

It is widely accepted that the lime-based repair materials, designed for the needs of the historic monuments, are more preferable than any cement-based product. Since lime based repair mortars show significant compatibility with the original material, a large number of studies are conducted in the field of lime mortars and lime based conservation materials (Böke et al., 2008; Veiga et al., 2009; Klisińska-Kopacz et al., 2010; Schueremans et al., 2011). However, even the capacity of lime-based mortars for filling voids, gaps and micro-cracks are not satisfying, they are still preferred in conservation treatments to prevent further and more serious deteriorations.

On the other hand, there are bio-based techniques referred as biomineralization and biological mortar used in consolidation and crack repair treatments for historical stone conservation. Since our main issue focuses on biological mortar derived from biomineralisation concept, biomineralization and biological mortar techniques are handled in more detail below in terms of why they are more desirable and preferred than the aforementioned consolidation and repair mortars.

1.3. Biomineralisation & Biological Mortar

During the last century, use of global materials (construction materials) has increased 8-fold. (Krausmann et al., 2009). In parallel with this, there is an environmental threat related to the depletion of non- renewable raw materials (Allwood et al., 2011). Therefore, it is obvious that as the demand for material increase in the forthcoming years, its impact on environment will also increase substantially (Krausmann et al., 2009). In relation to this issue, researchers have turned their interest on "sustainability" and "environmentally friendly green technology" with novel repair materials including agents of biological origin that can mimic nature. Since none of the materials used in remediation treatments has proven to be quite satisfactory (De Muynck et al., 2010a; Annamalai et al., 2013), biomineralisation and biological mortar has been proposed as an alternative and environmentally friendly crack remediation technique for the conservation of deteriorated carbonate stones (Le Metayer-Levrel et al., 1999a; Orial et al., 2003a). This application includes calcite-producing bacterial sediment as a binder, nutritive medium as feeding solution and stone powder as aggregate.

The application is developed to response the difficulty of interventions as filling up cracks and fixing of scales or flaking for soft stones (Orial et al., 2003a). Moreover, biomineralization-based techniques has been highly desirable since bacterially induced calcite precipitation provides calcite in high purity in turn enabling compatible repair material production with low cost (Lee, 2003).

1.3.1. Biomineralisation Process and the Metabolic Pathways

In nature, Ca-carbonate (calcite - $CaCO_3$) precipitation may occur through two different mechanisms: biologically controlled or biologically induced mineralization.

Biologically controlled mineralization, is a controlled process by organism in terms of nucleation and growth of the mineral particles, to a large extent and, synthesized minerals are specific to the species involved but independent from environmental conditions. There are several mineral examples formed with this type of mechanisms as magnetite and silica, by the activity of magnetotactic bacteria (Bazylinski et al., 2007) and diatoms, unicellular algae and coccolithophores (Perito et al., 2014), respectively.

On the other hand, $CaCO_3$ production by bacteria is usually considered to occur as a result of biologically induced mineralisation in which produced mineral is highly dependent on the environmental conditions, to a high degree (Rivadeneyra et al., 1994) and there is no specific mechanisms thought to be connected with this process (Perito et al., 2014). But, according to several studies, bacteria types and abiotic factors as salinity and pH are critically involved in calcium carbonate precipitation in different ways for changing environments (Knorre and Krumbein, 2000; Rivadeneyra et al., 2004).

There are four interrelated key factors involved in calcium carbonate precipitation through biologically induced mineralization (Castanier et al., 1999; Hammes et al., 2003)

(1) the calcium concentration (necessary to bond with carbonate ions in production of calcite mineral)
(2) the concentration of dissolved inorganic carbon (DIC) (availability of HCO_3^-, CO_3^2 is crucial to establish carbonate–bicarbonate equilibria)
(3) the pH (best at 8 to 9)
(4) the availability of nucleation sites (necessary as a binding zone (in the cell of microorganisms) with divalent cations as Ca^{2+})

Bacterially mediated calcium carbonate precipitation follows mainly two different metabolic ways: Autotrophic pathways and Heterotrophic pathways.

In autotrophy process, there are 3 different metabolic pathways: non-methylotrophic methanogenesis, oxygenic photosynthesis and anoxygenic photosynthesis (Figure 1). In all these pathways the main process uses CO_2 as a carbon source while producing organic matter as an end product. As a result of this usage, CO_2 depletion is observed in the medium or the environment that caused a shift in the equilibrium toward production of calcium-carbonate and CO_2, as well (Castanier et al., 1999).

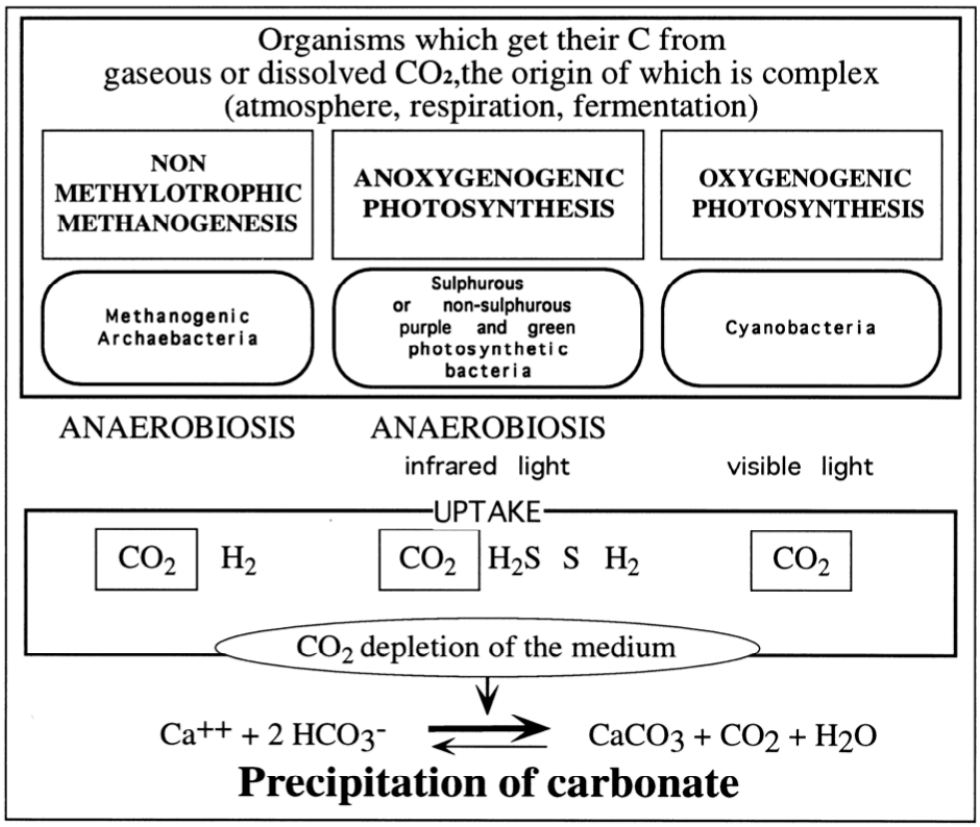


Figure 1: Precipitation of carbonate in Autotrophy (Castanier et al., 1999)

In autotrophy process, there are two bacterial processes as passive and active and these can take place simultaneously.

In passive precipitation, calcium carbonate precipitation takes place with the production of carbonate and bicarbonate ions and induction of several chemical alterations in the medium. There are two metabolic cycles involved in passive precipitation: the nitrogen cycle and the sulphur cycle.

The nitrogen cycle might follow three different pathways followed by bacterial precipitation (Castanier et al., 1999):

(i) the ammonification of amino-acids in aerobiosis: occurs in the presence of gaseous or dissolved oxygen, organic matter and calcium

(ii) the dissimilatory reduction of nitrate: occurs in the presence of nitrate, calcium and organic matter in abiotic or microaerophilic conditions

(iii) the degradation of urea or uric acid: occurs in the presence of urea or uric acid, calcium and organic matter in abiotic conditions.

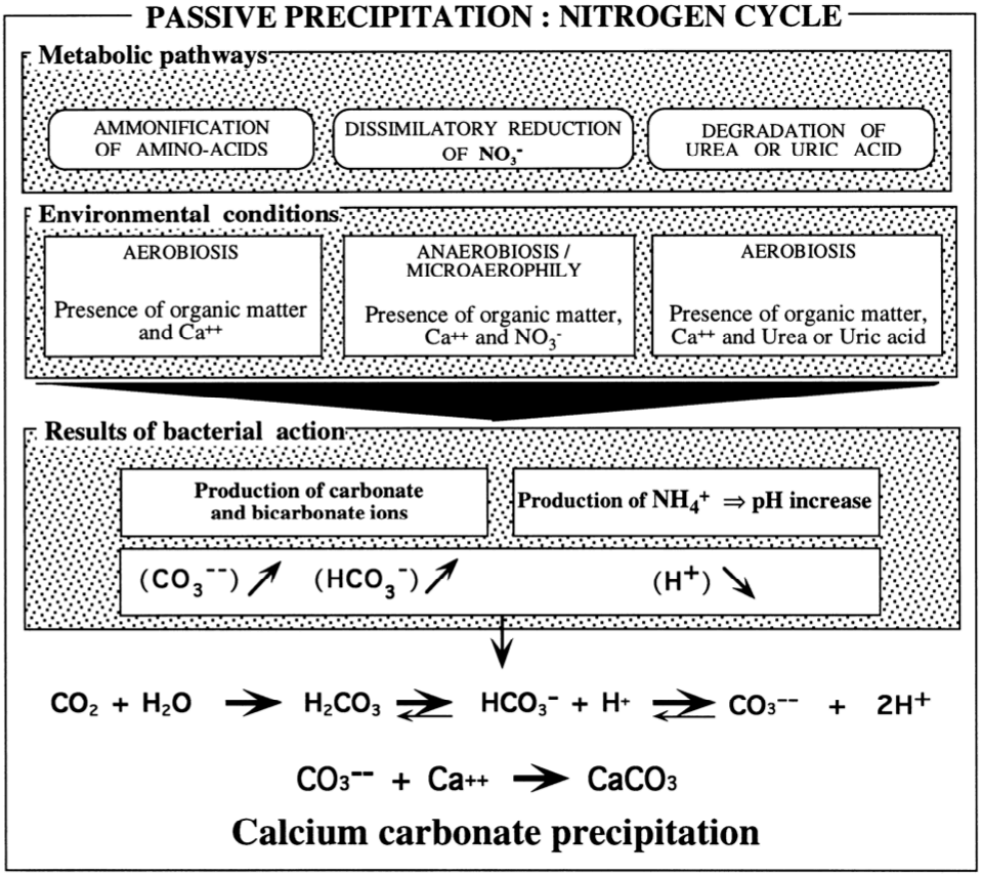


Figure 2: Passive carbonate precipitation by bacteria with the nitrogen cycle (Castanier et al., 1999)

Bacterial action in these three pathways induce several metabolic end-products as carbonate ions, bicarbonate ions and ammonia as well, that is well known for their effect on pH increase (Figure 2). Thus, decrease in H+ concentration turns the carbonate–bicarbonate equilibria to the direction of CO_3^{--} ion production, which leads to calcium carbonate precipitation in the presence of Ca^{2+}. On the other hand, the absence of Ca^{2+} in the medium leads to accumulation of carbonate and bicarbonate ions, and increase of pH, which in turn catalyzes zeolite formation by the bacterial activity as seen in soda lakes (Castanier et al.).

The sulphur cycle follows only one metabolic pathway as the dissimilatory reduction of sulfate (Figure 3). The presence of organic matter, calcium and sulfate is necessary

together with anoxic conditions for reaction chains to be active for this pathway. As a metabolic end-product of this pathway, carbonate, bicarbonate ions and hydrogen sulfide are produced by bacteria. In presence of calcium ions, calcium carbonate precipitation occurs based on the behavior of hydrogen sulfide. In other words, following to degassing of hydrogen sulfide, pH increases and calcium carbonate precipitation takes place. But this hydrogen sulfide can be utilized by other bacteria as anoxygenogenic sulfide phototrophic bacteria and oxygenogenic sulfide oxidizing autotrophic bacteria. Depending on the reaction chains, calcium carbonate precipitation is occurred following to increase in pH or no solid calcium carbonate appears due to decrease in pH with anoxygenogenic and oxygenogenic sulfide bacteria, respectively (Castanier et al., 1999).

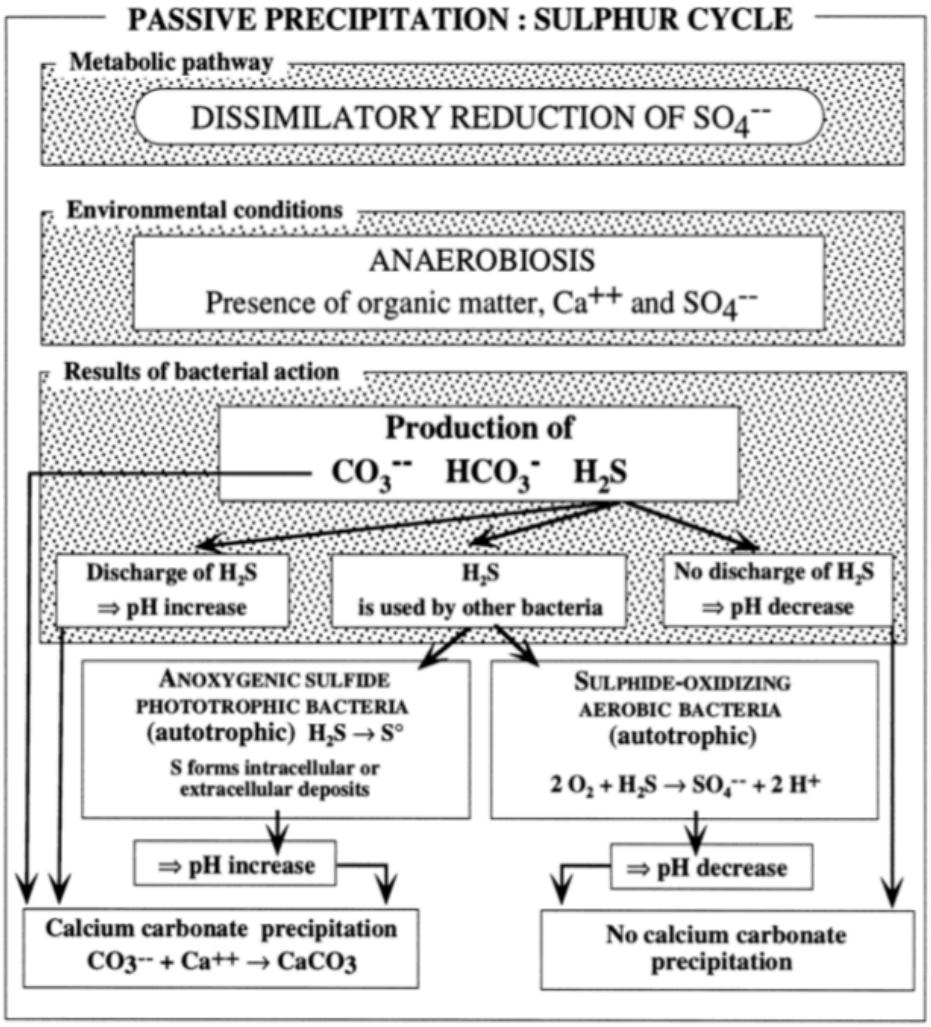


Figure 3:Passive carbonate precipitation by bacteria with the sulphur cycle (Castanier et al., 1999)

In active precipitation, the carbonate precipitation process is rather related with ion exchanges through the cell membrane which is induced by the stimulation of calcium and/or magnesium ionic pumps or channels, likely related with carbonate ion production. Several bacterial species are capable of operating this process (Castanier et al., 1999).

Based on numerous experiments conducted to understand the principal mechanism for the occurrence of calcium carbonate precipitation or carbonatogenesis could be concluded as this phenomenon is the response of heterotrophic bacterial communities to the organic matter increase in environment. The increase of bacterial growth takes place concurrently with the accumulation of several metabolic end-products after a phase of latency which leads to an accumulation of carbonate and hydrogenocarbonate ions in the medium. Thus, carbonate precipitation occurs as a response to increase in pH of the medium. The latency phase turns to a steady state since large portion of initial content of medium is used up by bacteria. Thus, amount of calcium carbonate particles increases during the exponential phase and ends around the beginning of the steady state. On the other hand, most of the time, passive carbonatogenesis follows the active one that starts first, and promotes the shape and growth alterations of preliminary mineral particles (Castanier et al., 1999).

1.3.2. Common microorganisms used in biomineralisation and biological mortar treatments

Since the capability of some bacterial populations to produce calcium carbonate in a biologically induced way had been proven in the past (Adolphe and Billy, 1974a; Boquet et al., 1973a), calcite forming bacteria used both in conservation of contemporary buildings and historical monuments are summarized in Table 1. To clearly show the present situation regarding successful use of bacteria in conservation studies, applications of bio-based treatments especially for crack remediation of stones are classified according to the objective of the treatment, used microorganism, environments that microorganisms sampled from, type of crystal precipitated, type of specimen, research group and research date in Table 1 & Table 2.

In Table 1, the studies on the applications of calcite precipitating bacteria for the strength enhancement and conservation of contemporary building materials are also included to provide an in-depth comparison. A timeline which demonstrates the advancement of bio-based methods, namely biomineralisation and biological mortar, for the conservation of stones of historical monuments is also given (Figure 4)

Table 1: Overview of the different methodologies used for the deposition of a layer of calcium carbonate on stone and concrete.

** Methodologies used for the deposition of a layer of calcium carbonate for conservation of historical monuments.

	Application	Microorganism	Specimen Type/Porosity or Content	Reference
BIOMINERALISATION	Surface consolidation	10 different isolates of *Bacillus sphaericus*	Mortar Cubes/Portland cement	(Heirman et al., 2003)
		Bacillus cereus	Plaster/Composed of gypsum crystals	(Anne et al., 2010)
		Bacillus cereus'	Miniature walls Fine grained limestone (40%) Coarse grained limestone (30%)	**(Le Metayer-Levrel et al., 1999)
		Micrococcus sp *Bacillus subtilis*	Bioclastic limestone cubes (40%)	**(Tiano et al., 1999)
		Myxococcus xanthus	Bioclastic calcarenite cubes (24-32%)	**(Rodriguez-Navarro et al., 2003)
		Bacillus sphaericus Bacillus lentus	Euville , crinoidal limestone cubes (16%)	(Dick et al., 2006)
	Crack remediation	*Sporosarcina pasteurii* NCIMB 8841	Mortar Cubes/Cement	(Abo-El-Enein et al., 2012)
		Bacillus pasteurii ATCC 11859 Two recombinant *Escherichia coli*	Concrete /Cement	(Bang and Ramakrsihnan, 2007)
		Bacillus sphaericus	Mortar cubes/Cement	(Annamalai et al., 2013)
		Bacillus sphaericus	Concrete cubes/Portland cement	(Belie and Muynck, 2009)
	Strength enhancement & crack remediation	*Bacillus pseudofirmus* *Bacillus halodurans* *Bacillus pseudoformis*	Concrete cubes/Clay	(Jonkers and Schlangen, 2009)
		Bacillus subtilis *Bacillus pasteurii* *Salinicoccus sp* *Escherichia coli*	Mortar cubes/Cement	(Vempada et al.)
		Bacillus flexus *Bacillus pasteurii* *Bacillus sphaericus*	Mortar cubes/Portland cement	(Jagadeesha Kumar et al.)
	Surface consolidation &Crack remediation	Five different strains of *Bacillus sphaericus* One strain of *Bacillus lentus*	Laboratory conditons	(Chahal et al., 2011)
BIOLOGICAL MORTAR	Crack remediation	*Bacillus cereıs*	Tuffeau limestone cube	**(Le Metayer-Levrel et al., 1999)

Table 2: Overview of different bacteria species and their calcite forming capacity

Type of microorganism	Production capability	System	Crystal type	Reference
Sporosarcina pasteurii	(++)	National Collection of Industrial and Marine Bacteria	Calcite	(Abo-El-Enein et al., 2012)
Bacillus pseudofirmus	(+)	Natural soda lake German collection of microorganisms and cell cultures (DSMZ)	Calcite	(Jonkers and Schlangen, 2009)
Five different strains of *Bacillus sphaericus* One strain of *Bacillus lentus*	(+)	Rhizopheric soil(tulsi plant) and alkaline soil	Calcite	(Chahal et al., 2011)
Bacillus sphaericus	(++)	Search from their previous researh	Calcite	(Annamalai et al., 2013)
Bacillus sphaericus	(++)	Calcareous sludge from a biocatalytic ureolytic calcification reactor and had been deposited at the BCCM culture collection in Ghent	Calcite	(Belie and Muynck, 2009)
Bacillus flexus *Bacillus pasteurii* *Bacillus sphaericus*	(++) (-) (-)	Concrete environment Natural Chemical Lab. (NCL) Natural Chemical Lab. (NCL)	Calcite	(Jagadeesha Kumar et al.)
Bacillus subtilis *Bacillus pasteurii* *Salinicoccus sp* *Escherichia coli*	(++) (+) (+) (-)	JNTUH Bioteck Laboratory	Calcite	(Vempada et al.)
Bacillus cereus	(++)	Bioconcept firm	Calcite	(Anne et al., 2010)
10 different isolates of *Bacillus sphaericus*	(+)	-	Calcite	(Heirman et al., 2003)
Bacillus pasteurii	(++)	-	Calcite	(Bang and Ramakrsihnan, 2007)
Bacillus sphaericus Bacillus lentus	(++) (+)	Calcareous sludge from a biocatalytic ureolytic calcification reactor	Calcite	(Dick et al., 2006)
Myxococcus xanthus	(++)	Provided by the Spanish Type Culture Collection, Burjasot, Valencia, Spain.	Calcite	**(Rodriguez-Navarro et al., 2003a)
Micrococcus sp *Bacillus subtilis*	(-)	Isolated from a monument (wild strain) Culture type collection (Phototrophic strain)	Calcite	**(Tiano et al., 1999)
Bacillus cereus	(++)	Karstic deposits, lagunas of the Bolivian Altiplano and Pamukkale calcareous falls	Calcite	**(Le Metayer-Levrel et al., 1999a)

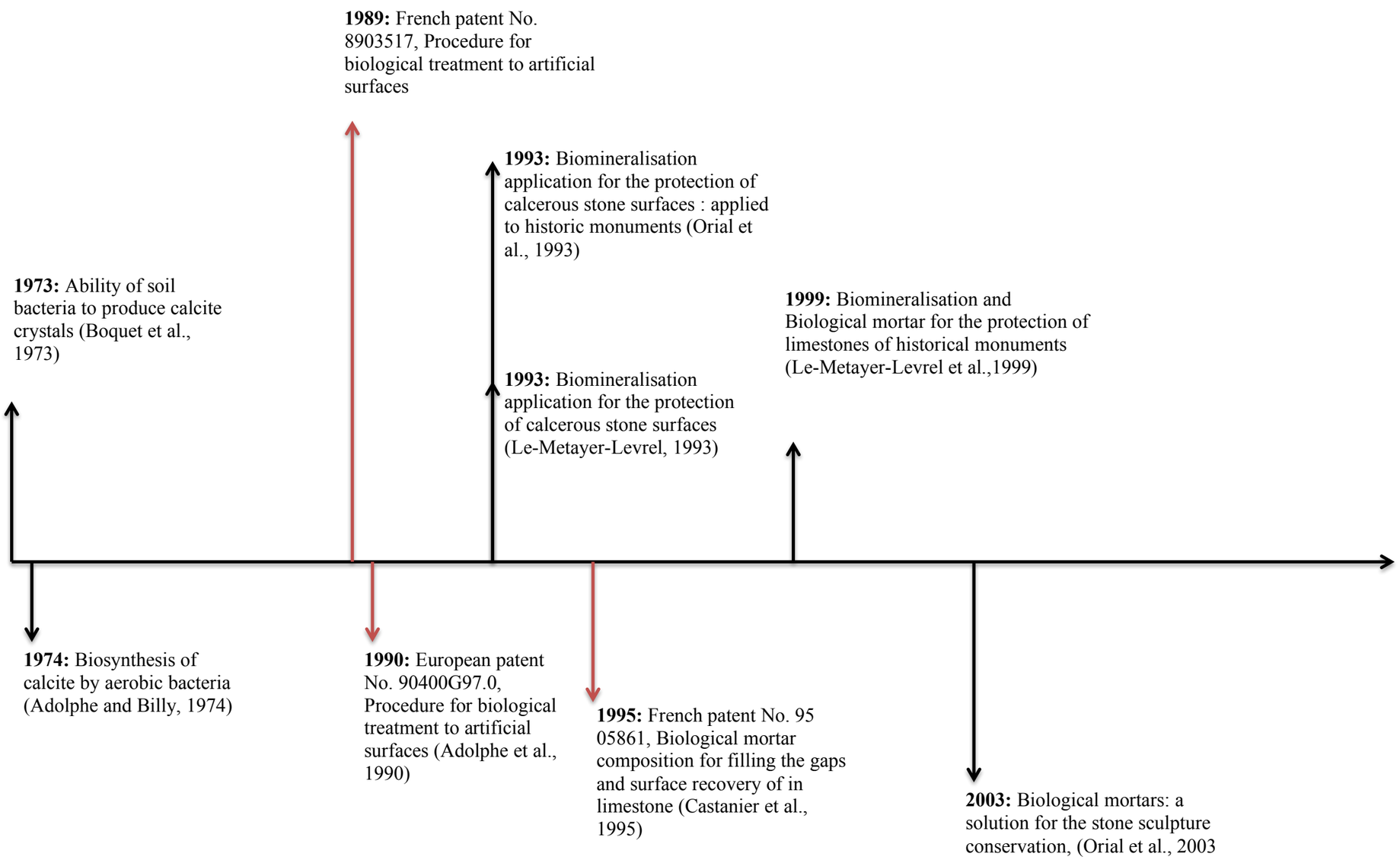


Figure 4: A timeline demonstrating the improvement of biomineralisation & biological mortar technique.

The microorganism induced mineral precipitation was investigated and the ability of soil bacteria to precipitate calcium carbonate was determined in the earlier studies of Boquet et al. (1973). While previous research had only concerned marine bacteria in liquid media (Shinano, 1972), the studies of Boquet investigated crystal formation by soil bacteria on solid media (Adolphe and Billy, 1974b; Boquet et al., 1973b).

Since the bacterially precipitated crystal is in the form of calcite, the use of calcite precipitating bacteria has been comprehensively investigated (Table 1) and basically two interrelated treatments using calcite precipitating bacteria have been determined for different kind of applications: biomineralisation and biological mortar. (Table 1).

Even though biomineralisation technique was firstly used in studies related with the conservation of historical monuments, today it is also widely used for the strength enhancement and conservation of materials in contemporary buildings. On the other hand, it can be seen that studies related with biological mortar has been conducted only to confront the problems of soft stone repair for conservation of historical monuments up to this time (Table 1). In other words, even the point of origin of the biomineralisation studies was aroused from the idea to conserve historical stones in an eco-friendly manner, it has been widely used for different kind of applications in materials of contemporary buildings since the beginnings of 2000s**.**

When biomineralisation treatment is evaluated according to the type of applications, the major difference is found in the type of specimens used in the experiments. Because research groups studying on materials of contemporary buildings selected mortar and concrete specimens based on cement whereas others selected limestone cubes for their experiments. This situation actually explains the reason for choosing different bacterial species in different type of biomineralization applications.

In more detail, choice of microorganism and proposed metabolic pathways, summarized in Table 1, may be an evidence of an adaptation effort for microorganism to survive in different type of specimens having extreme alkaline environment. Even though researchers use microorganisms that are isolated from calcareous

environments, polyurethane polymer or silica sol gels are needed in some treatments for the immobilization of bacteria in order to protect them from the strong alkaline environment in concrete (at pH values above 11, the bacterial activity is stopped) (Bang and Ramakrsihnan, 2001; De Muynck et al., 2010b).

After mentioning the application field of biomineralisation in a comparative manner, its success in the application for conservation studies, especially crack remediation for stones of historical monuments is discussed below in a detailed manner together with a timeline to demonstrate the advancement of biomineralisation technique (Figure 4).

Various research groups, studied biomineralisation technique for the conservation of historical monuments. In Italy, Tiano et al., (1999), used *Micrococcus* spp. and *Bacillus subtilis* strains on Pietra di Lecce bioclastic limestone for their experiments. According to the author the treatment has negative consequences on monumental stones, such as; a) presence of products of new formation; b) formation of stained patches. Therefore, due to obtained results and side effects, treatment is commented as inapplicable for this kind of intervention (Tiano et al., 1999).

In Spain, (Rodriguez-Navarro et al., 2003a), used *Myxococcus xanthus* for the consolidation of limestone surfaces. In their study, noteworthy results had been achieved when the *Myxococcus xanthus* was applied to bioclastic calcarenite cubes. It was reported that bacteria induced calcium carbonate precipitation protected and consolidated porous ornamental limestone by depositing on the walls of pores without plugging them (Rodriguez-Navarro et al., 2003a). In Spain, (Rodriguez-Navarro et al., 2003a), used *Myxococcus xanthus* for the consolidation of limestone surfaces. In their study, noteworthy results have been achieved when the *Myxococcus xanthus* is applied to bioclastic calcarenite cubes. It is reported that bacteria induced calcium carbonate precipitation protects and consolidates porous ornamental limestone by depositing on the walls of pores without plugging them (Rodriguez-Navarro et al., 2003a).

Lastly, in France, a large group of researchers including Castanier, Orial, Loubiere, Le Metayer-Levrel and Perthuisot commented on their biomineralisation studies,

which have been continuing since the 1980s. Their biomineralisation technique is reported as appropriate and effective for the consolidation of historical stone surfaces. Since biological mortar is derived from the mentioned biomineralisation technique and the only bio-based technique applied for the crack remediation of historical stones, it would be better to go in detail with a timeline created to demonstrate the advancement and the current situation of biological mortar as a material for consolidation of scales and flakes, as well as remediation of cracks in historical stones.

According to previous studies (Figure 1), it is determined that the treatment is based on the ability of bacteria to promote the precipitation of carbonate (Boquet et al., 1973b) (Adolphe and Billy, 1974b; Boquet et al., 1973b)**.** Ongoing studies concerning utilization of carbonate precipitating bacteria for the protection of surfaces and remediation of cracks of stones led them to develop two patents; "Procedure for the biological treatment to artificial surfaces" is taken in 1990 (Adolphe et al., 1990) and "Biological mortar composition for filling the gaps and the surface recovery of limestone" is taken in 1995 (Castanier, 1995)**.**

In 1999, Le Metayer-Levrel, Castanier, Orial, Loubiere and Perthuisot, made a significance progress in biomineralisation concept such as; the creation of patinas and the manufacturing of biological mortars and cements which were also enhanced in the laboratory (Le Metayer-Levrel et al., 1999a).

Furthermore, following the same year, Orial and her colleagues, conducted an actual restoration of western portal of the 12th century Argenton-Cahteau church, in western France. Church is built with a soft limestone, Tuffeau. Because the sculpture of this portal had been badly damaged due to severe climatic conditions, a conservation and restoration project was needed for the consolidation of the scales and flakes in addition to filling up cracks. The study was started with feasibility tests and continued through the optimization process of the three main components of mortar:

- The aggregate, i.e, the stone powder (Tuffeau powder, constituent of the Argenton-Chateau)

- Water, for feeding solution of bacteria
- The binder, as a bacterial sediment which is composed of *Bacillus cereus*

The most critical optimization is conducted during the preparation of mortar since the balance between weight of bacteria and weight of stone powder is critical to obtain cohesive mortar. After examination of prepared mortars by using light microscope and SEM, it is reported that mortar/stone interface showed a good and continuous contact. Therefore, the product is applied to the problematic zones, including scales, flakes and cracks, by using spatula, a brush, or a syringe, being careful not to break the bacterial bodies still alive. This conservation and restoration project was finished in 2000. Two years after completion, in 2002, the zones, where the application is carried out, showed that everything was in good condition. At the end of the study a scientific group is created to observe the condition of application in the following years (Orial et al., 2003a).

1.3.3. Biomineralisation technique and its application

Biomineralisation is defined as the production of surficial protecting coatings with the ability of bacteria to precipitate calcium carbonate on limestone buildings, monuments and statuary (Le Metayer-Levrel et al., 1999a). This technique produces a material that is remarkably similar to the limestone substrate, as it uses the same natural process that formed many limestones (Le Metayer-Levrel et al., 1999a).

In Figure 5, illustration of the biomineralization application is given as step by step.

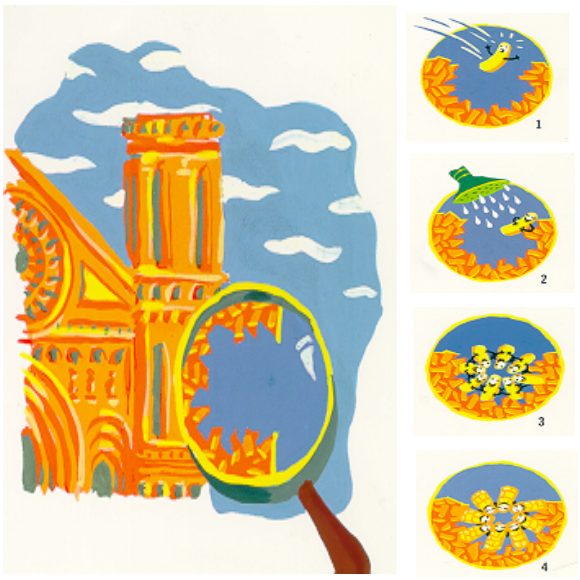


Figure 5: Biomineralisation illustration (1) Spraying of bacteria onto the surface, (2) Spraying of the nutritional medium, (3) Bacterial multiplication, (4) Formation of calcite crystals

1.3.4. Biological mortar technique and its application

Biological mortar, which is derived from biomineralisation, is a mixture of aggregate (stone powder), binder (bacterial sediment), the necessary water to form the paste (feeding solution). The treatment is referred as “biological” mortar because of the bacterial origin of its binder.

Biological mortar components are optimized separately in order to obtain a compatible biological mortar with the original material; aggregate optimization is done based on granulometry of stone powder, feeding solution optimization is made based on concentration of ingredients, and bacteria optimization is performed based on preparation, storage and efficiency during mortar preparation process.

After mortar ingredient optimization, preparation mode of mortar is also optimized by varying weights of the three main components. Then samples are poured in molds and left to dry in room temperature. After curing period, mortar mixtures are evaluated

based on aspect, color, presence of micro-cracks or excess of liquid on the surface and disintegration to components.

In theory, feeding solution provides bacteria with necessary elements for the production of calcium carbonate and by the help of this biochemical process, bacteria succeed in binding the grains of stone. Therefore, by mineral precipitation similar with the minerals in materials to be restored, biological mortar works in harmony with the treated material, with no difference in color and texture (Orial et al., 2003a).

1.4. Structure of the thesis

The thesis is composed of five chapters. The first chapter is the introduction chapter including the aim and scope of the study, literature review and structure of the thesis.

The second chapter comprises adjustment of biological mortar components, establishing mortar by defined proportions, application to micro-cracks of artificially aged test stones, performance measurement of this application and application conducted test stones through physical and physico-mechanical, microstructural analyses, and other evaluation criteria.

The third chapter starts with selecting target bacteria from microbial library and gives information about its metabolic features and growth characteristics. Upon defining the compositional and proportional features of biological mortar, results of above-mentioned examination methods regarding the BM application and the test stone from which this application was made are presented in detail.

The fourth chapter focuses on the milestones of the study such as the process of biological mortar development, the characteristics of test stones, and the performance of the repair material and its application and criticize these points comprehensively.

The fifth chapter is the conclusion part of the study which states major findings, contribution of the present study to the existing field, constraints and research area/recommendations for future studies.

CHAPTER 2

MATERIALS AND METHODS

As a first step, study was started with obtaining and selection of calcifying bacteria from environmental samples. Secondly, upon defining all components of biological mortar in detail, mortar was set up in defined specification/composition or proportions after several trials. Thirdly, biological mortar was applied to micro-cracks of test stones and left for 28 days in open air and laboratory conditions. Eventually, together with test stones, performance of this repair material was examined through several physical, physico-mechanical, microstructural analyses and other evaluation criteria as well.

A TUBITAK (Turkish Scientific and Technical Research Council) project (Project number: 115M188) prepared by the author together with Dr. Göze Akoğlu, Assoc. Prof. Dr. Kıvanç Bilecen, Prof. Dr. Neriman Şahin Güçhan, following to thesis proposal jury, based on the aim and the outline of this study and applied to 1001-The Support Program for Scientific and Technological Research Projects. In June of 2015, acceptance of the project proposal was announced with a budget 616,500.00 TL. In the November of the same year, this project, which will last for 3 years has started. This PhD thesis is the main outcome of this TUBİTAK project which will be completed very soon. Within the context of the TUBİTAK project, until September 2018, 3 progress reports, submitted and approved successfully. The final TUBİTAK, which is already completed parallel to this thesis, will be submitted in 1st of November, 2018.

2.1. Materials

The instruments used in this study are listed in Table 3.

Table 3:List of the instruments.

Instrument	**Model**	**Producer**
Shaking incubator	KS 4000i control	IKA, Germany
Low temperature incubator	BK800	Thermo SCIENTIFIC, Germany
Biological safety cabinet	KS9	Thermo SCIENTIFIC, Germany
Medical Refrigerator (+4)	MPR-426	ANTECH, China
UV/VIS Spectrophotometer	SP-3000 plus	OPTIMA INC., Japan
Centrifuge	Rotofix 32A	Hettich Zentrifugen, Germany
Ultrasonic pulse velocity tester	PUNDIT Plus	Germann Instruments A/S, Germany
Light microscope	DM4500P	Leica, Germany
Stereomicroscope	Z16 APO A	Leica, Germany
Vortex	Reax top	Heidolph Instruments, Germany
Orbital shaker	Reax top	Heidolph Instruments, Germany
Magnetic stirrer	MR Hei-Standard	Heidolph Instruments, Germany

Instrument	Model	Producer
Scanning electron microscope	QUANTA,400F Field Emission SEM	Thermo SCIENTIFIC, Germany
X-ray diffraction instrument	Ultima IV X-Ray diffractometer	Rigaku, Japan
Autoclave	CL-32L	ALP Co. Ltd, Japan
Weighing scale	FA2204N	DAIHAN, Korea
Weighing scale	XY6002C	DAIHAN, Korea

The reagents used in this study are listed in Table 4.

Table 4: List of reagents.

Reagents	Producer
Natamycin	Alfasol, Turkey
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, German
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, German
Ammonium chloride (NH_4Cl)	Merck, German
Calcium chloride ($CaCl_2$)	Merck, German
D (+) Glucose monohydrate	Merck, German
Peptone, Bacteriological	HIMEDIA, India
Potassium nitrate (KNO_3)	Merck, German
di-Potassium hydrogen phosphate (K_2HPO_4)	Merck, German
Sodium chloride (NaCl)	Merck, German
Calcium acetate hydrate ($Ca(CH_3COO)_{2*}H_2O$	Merck, German
Urea ($CO(NH_2)_2$	Merck, German

The culture mediums used in this study are listed in Table 5.

Table 5: List of culture mediums.

Media	**Producer**
Nutrient broth	Merck, German
Bacteriological agar	CONDA, Spain
Luria Broth	CONDA, Spain
Yeast extract granulated	Merck, German

2.2. Sampling Site - Pamukkale Travertines (Denizli, Turkey)

Pamukkale travertines were selected for sampling site depending on their nature of being a quite large active travertine deposition area including numerous hot water springs which increases the probability of obtaining of calcite precipitating bacteria strains from collected water samples.

As being one of the UNESCO World Heritage Site, with its spectacular natural geothermal resources in the world, Pamukkale travertines are located 17 km north from Denizli and has been attracting the interests of scientist for long years (Figure 6). The site includes thermal water springs, displaying high level of carbon dioxide concentrations, situated on a range of faults. The calcareous water of this thermal spring discharges from approximately 100 m high and it appears as about 3 km white travertine, namely “cotton castle” (Pentecost et al., 1997).

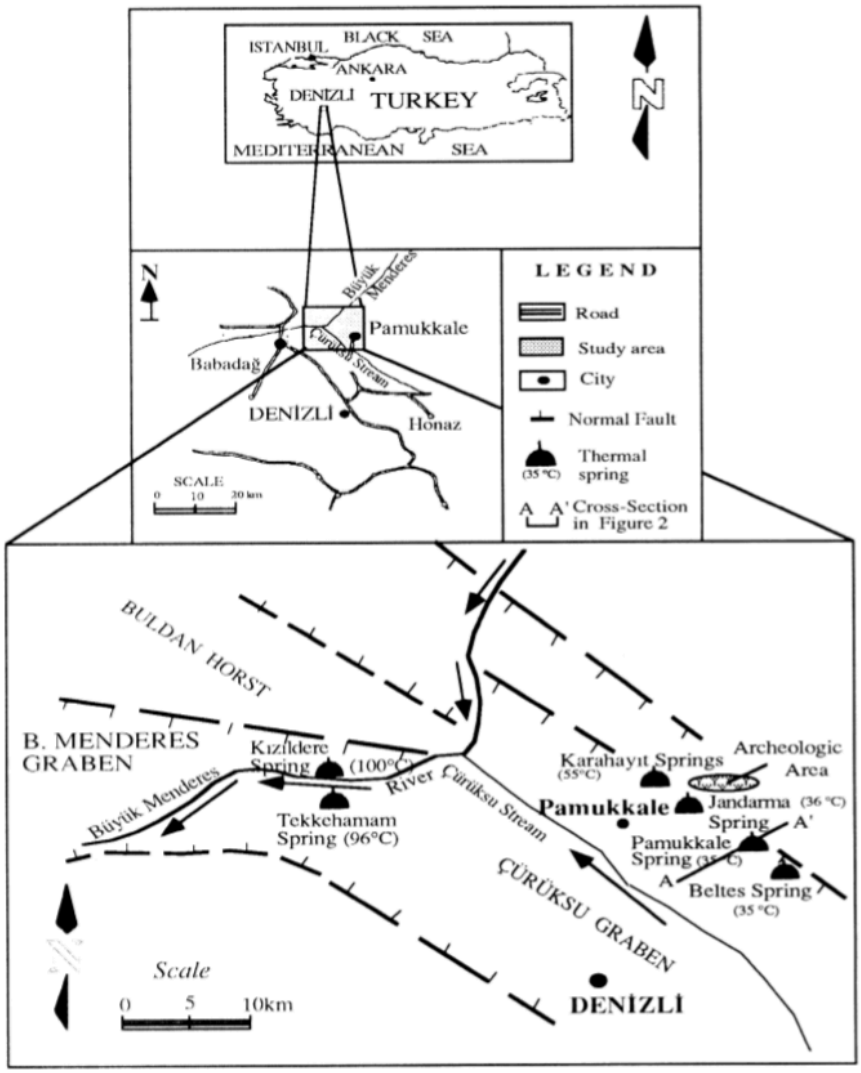


Figure 6: Map of Pamukkale springs (Simsek et al., 2000).

In addition to these calciferous springs and terraces, there are remains of Roman city of Hierapolis established in the 2nd century and became famous with Roman baths built in vicinity with hot springs in the 2nd and 3rd centuries (Simsek et al., 2000).

There are four major springs located in the travertine area along with a major fault line from northwest-southeast, namely Pamukkale, Jandarma, Karahayit and Beltes (Figure 6). The temperature of spring waters is almost 36 °C and the pH value is in the range of 5.85- 6.01 (Simsek et al., 2000).

2.3. On-Site Sampling Locations

Field works at Pamukkale were carried out on 3 April 2016 (Figure 7) by collecting water samples aseptically from thermal spring water resources mentioned in Tables 6, 7 and 8.

Detailed description of sampling procedure, sampling zones, sampling equipment and a brief list of experiments conducted are given in Table 6.

Figure 7: Bird's eye photograph of the area where the field study was conducted.

Table 6: Details of samplings performed in Pamukkale.

1st field study				
Sampling zone		**Description of sampling**	**Equipment**	**Experiments to be conducted**
Thermal spring water	Bridal bath (main source and closed channel in continuation)	Spring water was collected into sterile disposable bottles (500 ml)	*Sterile, disposable bottles	Isolation, identification, bacteria library establishment (+4°C) & stock preparation (-80°C)
	Beltes (main source and closed channel in continuation)			
	Ploutonion			
	Gendarme pool			
	Ancient pool (water pump)			

Table 7:Wide-angle photographs of thermal spring water resources

Bridal bath		**Beltes**	
Plutonium		**Gendarme pool**	
Ancient pool (closed area)		**Ancient pool (in use)**	

Table 8: Sampling zones and their features.

1st field study			
Sample Name	**Date**	**Location**	**Photograph**
1.1-1.2	03/04/2016	Bridal bath (main source)/ 37,924356° -29,127620°	
2.1-2.2-2.3-2.4	03/04/2016	Bridal bath (continuation)/ 37,924046°-29,126407°	
3.1-3.2	03/04/2016	Beltes (main source)/ 37,923257°-29,126719°	
4.1-4.2-4.3-4.4	03/04/2016	Beltes (continuation)/ 37,922200°-29,1255665°	

5.1-5.2-5.3-5.4-5.5	03/04/2016	Ploutonion N 37°55′34″ E 29°73′80″	
6.1-6.2-6.3-6.4-6.5	03/04/2016	Gendarme pool N 37°55′40″ E 29°72′90″	
7.1	03/04/2016	Ancient pool N 37°55′28″ E 29°72′40″	
8.1	03/04/2016	Last thermal spring water outlet point to the travertine whitening zone.37,924437°-29,123864°	

2.4. Adjustment of Biological Mortar Components

As mentioned in Chapter 1; bacterial sediment, stone powder and nutrient medium are the three components of biological mortar. Therefore, first of all, a bacteria strain that has been proven to precipitate calcite among identified isolates, was selected and investigated in detail. Based on the gathered information, bacterial sediment optimization was performed.

Besides bacterial sediment preparation and optimization, other components as aggregate and nutritive medium were defined in terms of their grain size and content, respectively.

2.4.1. Obtaining and Selecting Bacteria

Upon collecting samples from Pamukkale, they were transferred to laboratory and stored at +4°C until use.

Study with field samples was started with obtaining all culturable microorganisms in Luria Bertani (LB) agar plates, continued with the identification process and storage was made at different temperatures as +4°C and -80°C depending on the purpose of the use.

Moreover, selected isolate was investigated regarding their mineral precipitation ability (type and capacity/efficiency), which is the determining factor during bacteria selection for the bacterial sediment component of the biological mortar.

In fact, based on well-known properties and success in conservation studies, *Bacillus cereus,* was expected to be isolated from collected thermal spring waters.

2.4.1.1. Isolation of Bacteria from Different Type of Samplings

Collected liquid samples were filtrated using a vacuum filter setup with a sterile 0.45µm pore size cellulose nitrate (CN) membrane filter and placed in LB agar plates. These plates were incubated at 35°C for 2 days (Figure 8). The temperature of incubation conditions was adjusted based on the temperature of thermal spring water determined in place during sampling.

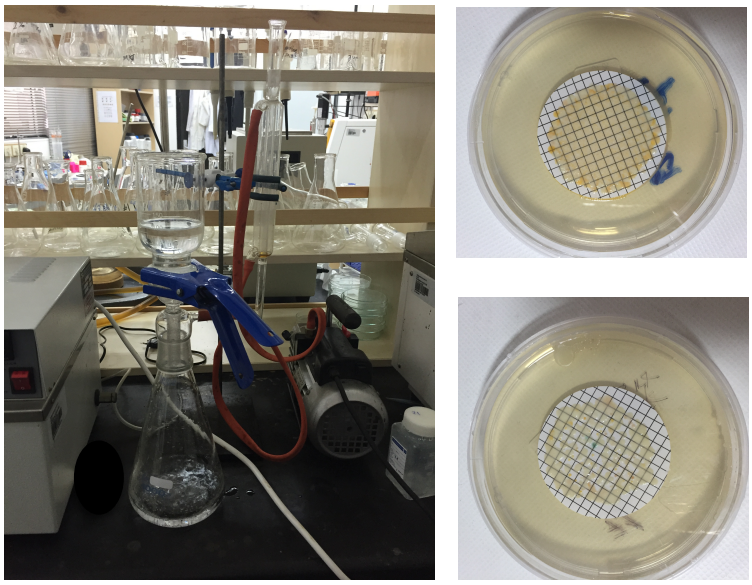


Figure 8:(a) Filtration system, (b) and (c) membrane filters on LB agar plates.

After incubation period, membrane filters were removed from plates with sterile pens and placed into 15 ml NaCl solution including glass beads (1mm) in order to allow microorganisms passing from membrane filter to the isotonic solution during vortexing. This solution was assumed as stock (10^0) and serial dilutions were made by 0.9% NaCl solution until 10^{-6}. Aliquots (1 ml) from the 10^{-5} and 10^{-6} were plated as triplicates onto LB agar plates and incubated at 35°C for 1 day. Pure cultures of isolates were obtained just after one streaking.

2.4.1.2. Establishment of Bacteria Library and Creating Stocks

Isolated microorganisms numbered from 1 to 97 were plated onto each grid drew in LB agar plates and incubated at 35°C for 18 hours in order to create a microbial library.

Following to incubation period, overnight culture of each microorganism was prepared in LB broth and 500 µL of each of these cultures was added to 500 µL of 50% glycerol in 2 ml eppendorf tubes and gently mixed. Then each stock was stored at -80°C (Figure 9).

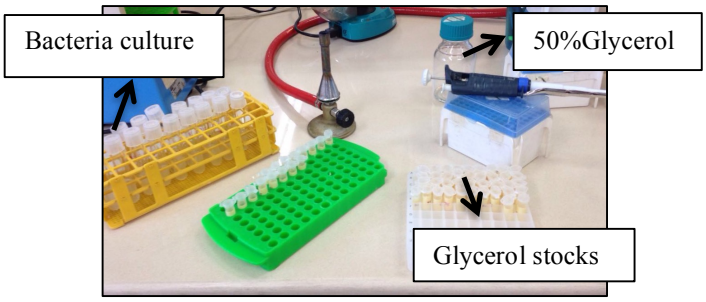


Figure 9: Components of glycerol stock method

2.4.1.3. Molecular Identification of Isolates

Characterization of isolated bacterial colonies was done using a matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) system (Vitek MS). To this end, prepared bacterial libraries were refreshed on a new LB agar plate and were incubated at 35°C for at least 18 hours to obtain visible colonies. Then portion of each colony was smeared onto the instrument's slide and α-cyano-4-hidroxycinnamic acid (CHCA) matrix solution was added on each of the spotted colonies. Slides were air dried and inserted into the device for taking measurements. Generated ribosomal spectra were analyzed by the instrument's software using local database. Any result with a percentage score of 99 or above was accepted as a correct identification (Bilecen et al., 2015)

2.4.1.4. Characterization of Precipitated Crystal by Selected Isolate

To find out the type of precipitated crystal, 50 ml of B-4 liquid medium (2.5 g of calcium acetate, 4 g of yeast extract, 10 g of glucose per liter) (Boquet et al., 1973b) adjusted to pH 8 with 1 M sodium hydroxide (NaOH), was inoculated with overnight culture of selected bacteria. Inoculated B-4 medium was incubated aerobically at 35°C for 3 weeks and in a 180-rpm shaking incubator. The experiment was carried out with a control of the medium that was non-inoculated.

After incubation period, B-4 medium were filtrated by Whatman No:1 filter paper, washed with sterile distilled water (dH_2O), and air dried at room temperature for 48 h. The crystal structure of the mineral collected from filter paper was identified by scanning electron microscopy (SEM)-coupled with energy dispersive X- ray (EDX) Analyzer.

2.4.1.5. Determination of Calcite Production Potential of Selected Isolate

Pure culture of selected isolate was plated on 2 different solid mediums namely, Urea-$CaCl_2$ (3 g of nutrient broth, 10 g of ammonium chloride, 2.12 g of sodium bicarbonate, 20 g of urea, 3.70 g of calcium chloride, 15 g agar powder per liter), and B-4 (2.5 g of calcium acetate, 4 g of yeast extract, 10 g of glucose, 18 g agar powder per liter) (Boquet et al., 1973b). The pH of these mediums was adjusted to 8 with 1 M sodium hydroxide (NaOH).

After incubation at 35°C for 3 weeks, individual colonies arose and $CaCO_3$ precipitates in the surrounding of these colonies were analyzed using an optical microscope.

2.4.2. Preparation of Bacterial Sediment

Upon determination of calcite production potential of identified strains, *Bacillus cereus,* environmental spore forming organism, was selected as a binder microorganism of biological mortar depending on their ability to promote carbonate precipitation under favorable conditions. Moreover, other reasons of selecting *B. cereus* strain was the biosafety level of its reference strain (14579) being 1 according to the American Type Culture Collection (ATCC) for working in a standard microbiology lab, and their efficiency & capability for being used industrially.

Bacterial preparation process was started with recovering of frozen *B. cereus* (-80°C stock) by scraping of the top using a sterile pipette tip and streaking the bacteria onto

an LB agar plate. This step is necessary to make sure that experiments will begin with a single bacterial cell. After 16 hours of incubation at 35°C, single colonies appear and for practical purpose, bacteria library for +4 °C storage was made by plating pure cultures of *B.cereus* to LB agar plate in triplicates and left for another 16-18 hours of incubation at 35°C. In this way, fresh bacteria were made ready to use for bacterial sediment preparation.

To determine the characteristic growth pattern of *B.cereus,* 100 ml fresh LB broth was inoculated with 1 ml overnight bacteria culture and incubated at 37°C for 105 hours in a 230-rpm shaking incubator. During incubation period bacterial population (absorbance) was measured periodically by a UV/VIS Spectrophotometer. Thus, an exponential growth curve, including viable bacteria number against time was obtained, which is also known as "growth cycle" (Figure 10).

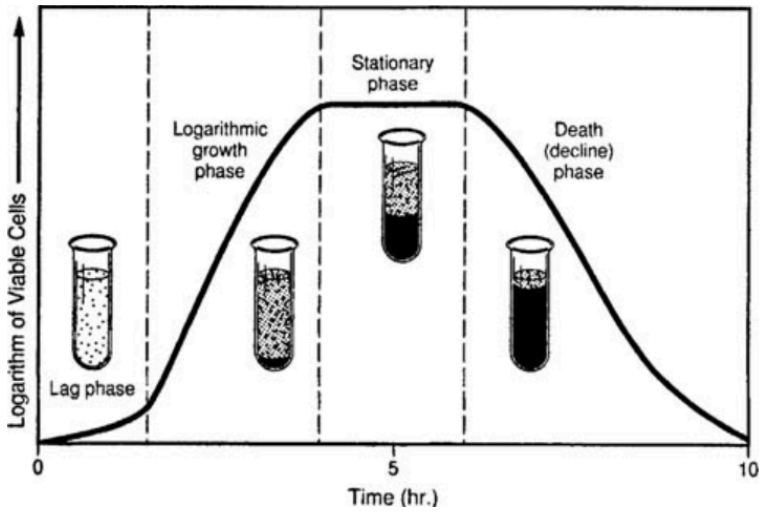


Figure 10: An example of a growth curve of a bacterial population demonstrating four main phases: lag phase, logarithmic growth phase, stationary phase, death.

Moreover, in parallel, colony forming unit (CFU)/ml, known as estimated number of viable cells grown in agar plates, was also counted concurrently with OD600 measurements in order to have an estimation regarding CFU/ml corresponds to 1 OD600. In this way, this correlation give insight into the cell concentration of bacterial sediment prior to being added to the mortar mixture.

To prepare bacterial sediment component of biological mortar, very small amount of a single bacteria colony was taken by sterile pipette tip from LB agar plate and dropped into 10 ml LB broth. Then, loosely covered with a cap and incubated at 35°C for 16-18 hours in a 180-rpm shaking incubator. Upon incubation period, two 500 ml LB broth was inoculated with 5ml of bacteria culture in order to increase the amount of bacterial pellet in grams and incubated at 35°C for 32-36 hours in a 180-rpm shaking incubator.

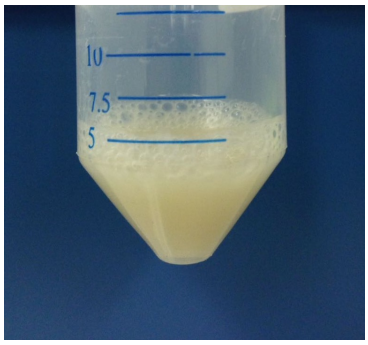


Figure 11: Bacterial sediment obtained from harvested cells.

Upon incubation period, 1 L of LB broth was transferred into falcon tubes (50 ml) and centrifuged at room temperature, 5-minute, 4000 rpm in order to harvest bacterial cells. In each centrifuging process, supernatant was removed and pellets were collected in a falcon tube to be used in mortar mixture (Figure 11).

2.4.3. Preparation of Nutritive Medium

Calcium carbonate precipitation takes place in *B. cereus* with two specific processes, namely the ammonification of amino acids in aerobiosis and the reduction of nitrates in microaerophilic conditions. Therefore, nutritive medium must contain; organic elements, a specific protein, a calcium and a nitrate source (Orial et al., 2003a).

Considering the mentioned ingredient types, nutritive medium was prepared with the below mentioned chemicals and autoclaved before mixing with other biological mortar components (Orial et al., 2003a).

- Peptone: 36g/l
- Yeast extract: 36g/l
- Calcium acetate: 5,7g
- Potassium nitrate: 4g/l
- Sodium chloride: 7g/l
- Natamycine (fungicide): 0,1g/l

2.4.4. Preparation of Aggregate

Since bacterial component of biological mortar was isolated from Pamukkale travertines, test stones to be used in mortar applications were selected from a close location, the quarries in Denizli/Kocabaş. Stone powder, which is the aggregate component of the biological mortar was prepared by grinding of these selected stones.

Several trials with granulometry between 38-150 μm were performed and tested to develop a consistent biological mortar.

2.5. Biological Mortar Set Up

As mentioned in the introductory chapter, mortar mixture is composed of bacterial sediment as binder, nutritive solution as necessary water and stone powder as aggregate (Figure 12).

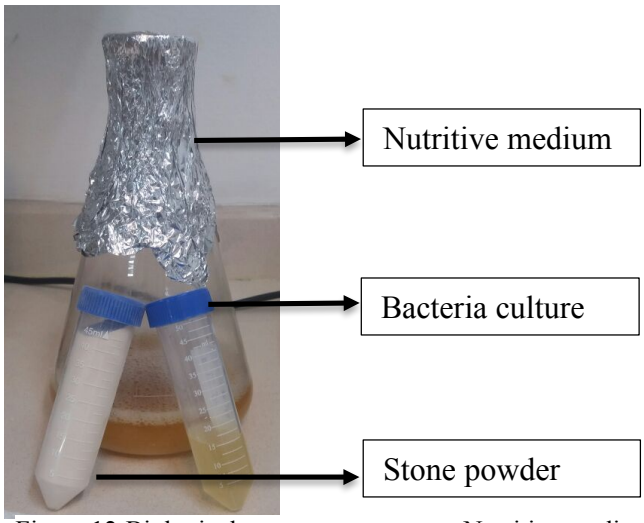


Figure 12:Biological mortar components: Nutritive medium, bacteria culture (before centrifuging), stone powder

During optimization of these components, the weight of bacteria and the nutritive solution kept as the same amount and varieties in the weight of the bacterial sediment and the stone powder proportions (one part of bacterial sediment for 4, 5, 6 and 7 parts of stone powder) applied while setting up mortar mixture.

While doing these optimization works, the mixture was either poured into small molds (2.2 cm x 2.2 cm x 2.2 cm) or hand-shaped and left to dry at room temperature (Figure 13).

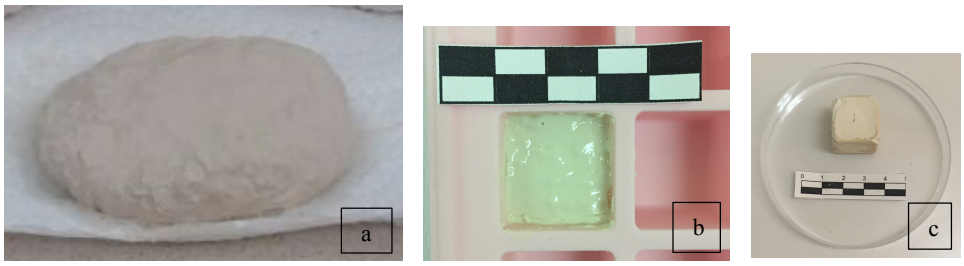


Figure 13: Biological mortar moulding (b) & (c) and shaping (a) for further analyses.

After 10 days of curing, biological mortar mixture was examined through several microstructural analyses and evaluation criteria.

2.6. Application of Biological Mortar to Micro-Cracks of Test Stones

After designing an optimum proportion for biological mortar, application was performed generally artificially generated cracks of test stones (Table 9) by a sterile syringe or small angled spatula (Figure 14) and left for 28 days in open air (T11, T15, T20, T37, T39) and laboratory conditions (T2, T5, T6, T14, T33, T34, T35, T36, T38). Temperature and relative humidity of these conditions were recorded by a data logger during this curing period. Biological mortar applied crack width was generally less than 1 mm but some of them were wider (max. 2mm) than this to determine the appropriate crack widths to which mortar can be applied successfully.

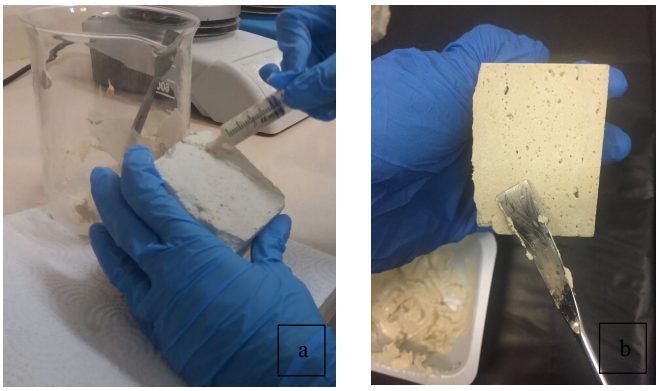
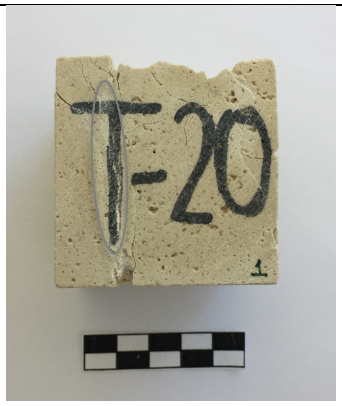
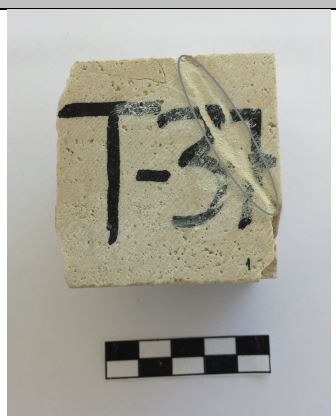


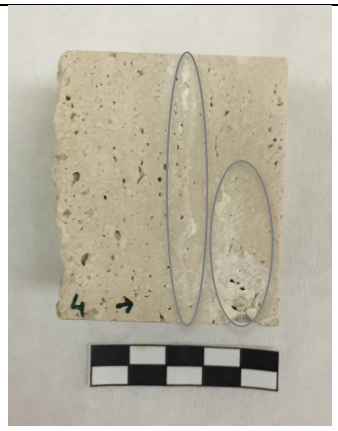
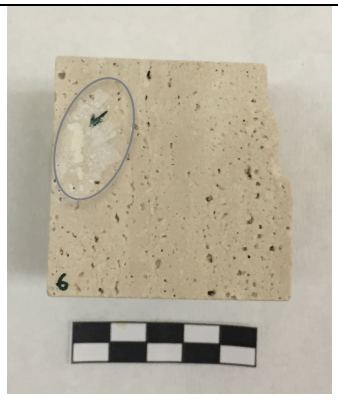
Figure 14: Biogical mortar application either by (a) syringe or (b) spatula

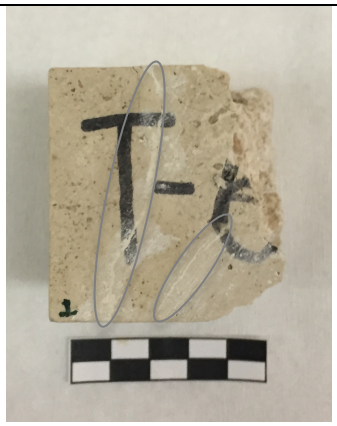
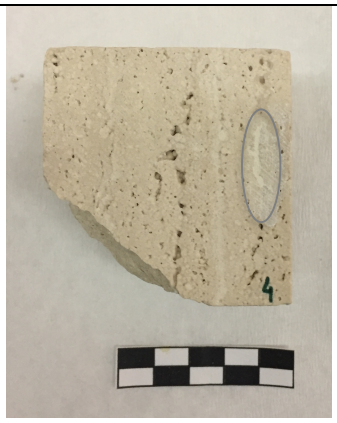
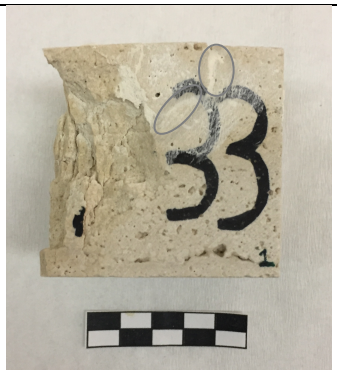
Table 9: Biological mortar application to cracks of test stones

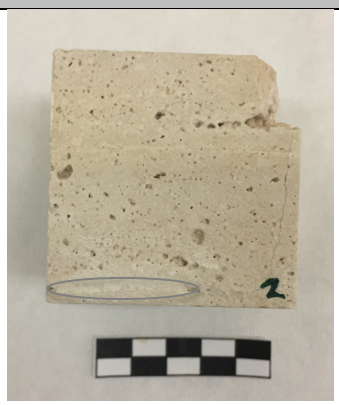
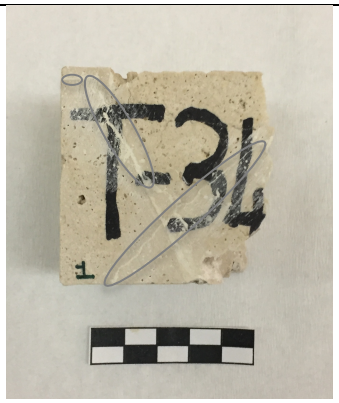
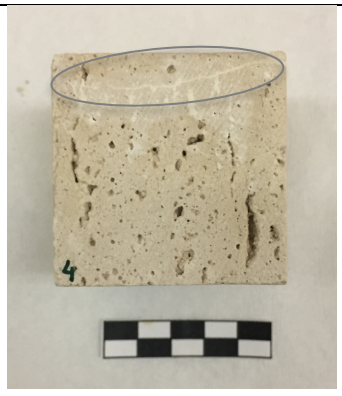
	Sample code	Biological mortar application
Open air conditions	**T15**	
	T11	
	T20	

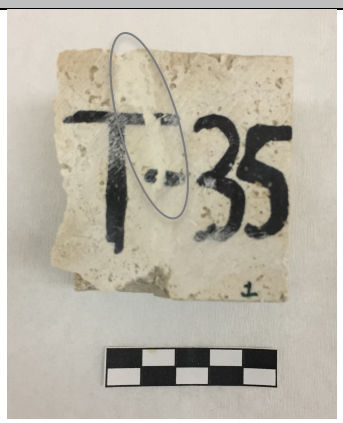
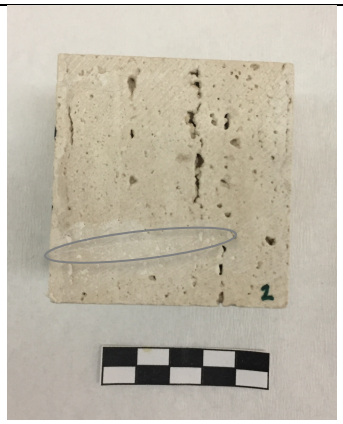
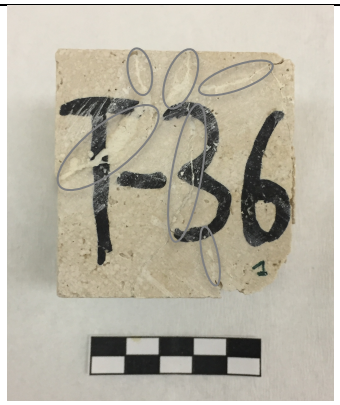
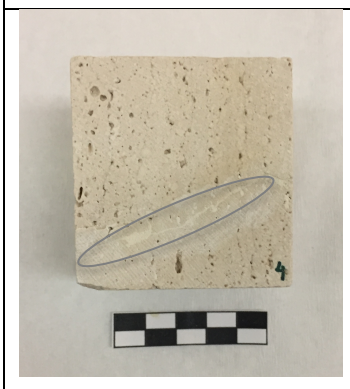
	Sample code	Biological mortar application
	T20	
Open air conditions	T20	
		

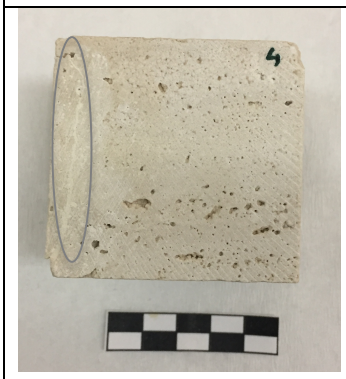
	Sample code	Biological mortar application
	T37	
	T37	
Open air conditions		

	Sample code	Biological mortar application
	T39	
		
Laboratory conditions	T2	
Laboratory conditions	T2	

	Sample code	Biological mortar application
	T5	
	T6	
	T14	
Laboratory conditions	**T33**	

	Sample code	Biological mortar application
		
		
	T34	
Laboratory conditions	T34	

	Sample code	Biological mortar application
	T35	
	T36	
	T36	
Laboratory conditions		

	Sample code	Biological mortar application
	T38	
		

2.7. Examination Methods of Test Stones, Repair Material and Its Application

As a terrestrial sedimentary rock, travertine, was selected for the biological mortar applications since they are formed by the precipitation of calcium carbonate in mineral springs and/or hot springs (American Geological Institute, 1962; Monroe, 1970). Therefore, cubic samples (5cm x 5cm x 5cm) were prepared from block samples of travertine stone from Kocabaş quarry (Denizli/Turkey).

To determine the index properties of travertine stone samples, several laboratory tests and measurements were conducted before and after artificial salt crystallization cycles. In addition, thin section and XRD analyses were also conducted to understand the microstructural properties of those stone samples.

Investigation of biological mortar properties and its performance measurement for application to test stones were conducted by physical, physico-mechanical tests and microstructural analyses. Besides, mortars were investigated based on other evaluation criteria in order to reach a holistic interpretation while evaluating the repair material at the end.

The laboratory tests and measurements were performed according to ASTM (2010), ASTM (2011) and ASTM (2001). On the other hand, the specifications of stone material and its characteristics were made based on Anon (1977) and ISRM (1981). In general, ASTM standards were preferred since tests were carried out on stones that were not historical and at the same time the procedure give faster results since adapted for more harsh conditions than RILEM standards. It would be appropriate to point out that the difference of ASTM standards than others are based on the material type used and the terminology.

2.7.1. Determination of Physical and Physico-Mechanical Properties

To understand the physical and physico-mechanical behavior of cubic test stones and follow changes in these properties before and after artificial salt crystallization cycles: bulk density and effective porosity, ultrasonic pulse velocity (UPV), uniaxial compressive strength and colorimetric measurements were conducted by the standard test methods.

Artificial salt crystallization tests were performed in cubic test stones according to "standard test method for evaluation of durability of rock for erosion control using sodium sulfate" (ASTM, 2013).

At the same time, physical and physico-mechanical tests were also conducted for biological mortar and its applications such as UPV, point load, color together with a series of microstructural analyses by XRD, SEM-EDX, thin section analyses by optical microscopy and cross section analyses by stereomicroscopy. In addition, physical appearance of mortar was also examined through some parameters.

2.7.1.1. Artificial Salt Crystallization by Sodium Sulfate (Na_2SO_4)

As an artificial aging method, exposing stone samples to sodium sulfate salt cyclically is widely used to determine the changes in physical and physico-mechanical behavior of the stones. This accelerated weathering method can be used in observation of crystallization effects of salt on the rock samples at laboratory conditions (ASTM D5240 / D5240M, 2013).

This method was applied to cubic travertine samples (5cm x 5cm x 5cm) essentially for generating micro-cracks on these stones for biological mortar applications. However, the changes in bulk density, effective porosity, UPV, UCS and color were also determined after those cyclic salt crystallization tests.

Figure 15: Cubic travertine stone samples (numbered from 1 to 30) in sodium sulfate solution

In the procedure of this method, sodium sulfate solution was prepared with a density changing between 1,151 and 1,174 (200 g of sodium sulfate/1 L of water). Firstly, cubic stone samples were put in an oven at 60 °C for around 12-16 hours (Yavuz and Topal, 2016). After then, those dried cubes were soaked into salt solution for 16-18 hours (Figure 15) and dried at atmospheric conditions for 4-6 hours. This is the first cycle of aging with salt crystallization and these cycles continued until fractures were observed in the stone samples.

In this study, 30 cubic travertine samples were subjected to sodium sulfate test that had lasted for 30 cycles. 14 travertine samples (T2, T5, T7, T10, T11, T13, T14, T15, T16, T21, T25, T27, T28, T29) were started to crack and degrade due to fractures and 16 travertine samples (T1, T3, T4, T6, T8, T9, T12, T17, T18, T19, T20, T22, T23, T24, T26, T30) were stayed intact without changing the shape. Those 16 travertine samples were washed for 1 week to get rid of the salt. In the salinity measurement of the last wash, it was determined that the washing water came to the pure water salinity level and therefore, washing process was ended.

2.7.1.2. Effective Porosity and Density

Density and effective porosity are the two basic and significant index properties of the intact rocks. Porosity can be defined as the ratio between the pore volume inside the rock to volume of the entire rock specimen and it is expressed as percent ratio whereas density is the weight of a unit volume of a rock (ASTM-D653, 2011)

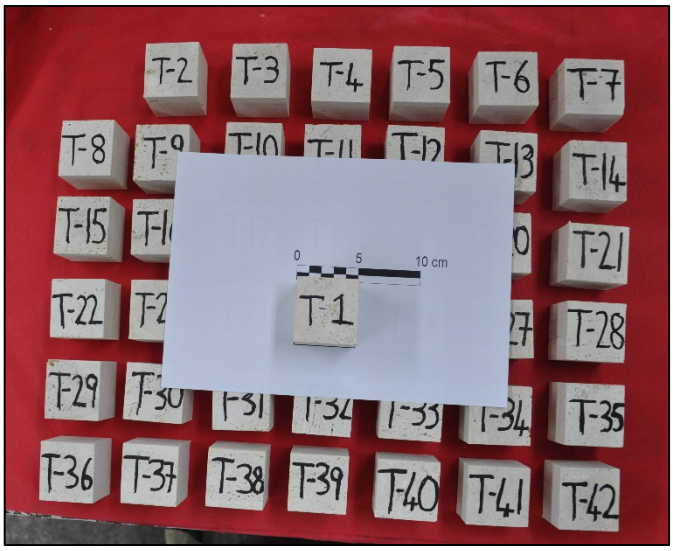


Figure 16:Cubic travertine samples (5 cm x 5 cm x 5 cm) used in physical and physico-mechanical tests

Density and porosity measurements were conducted on 42 test stones (Figure 16). These measurements were repeated for 30 test cubes (numbered from 1 to 30) that were subjected to salt test (sodium sulfate) lasted for 30 cycles.

These test samples were saturated with distilled water at least 1 hour in a vacuum chamber which is necessary for penetration of water to the finest pores (Figure 17). Then, each sample was weighed while immersed in water (M_{sub}) (Figure 18) and saturated with water (M_{sat}). In addition, the samples were left in oven at 105°C for 24 hours and their dry weight (M_{dry}) was recorded.

Figure 17:Vacuum chamber used in porosity measurement experiment

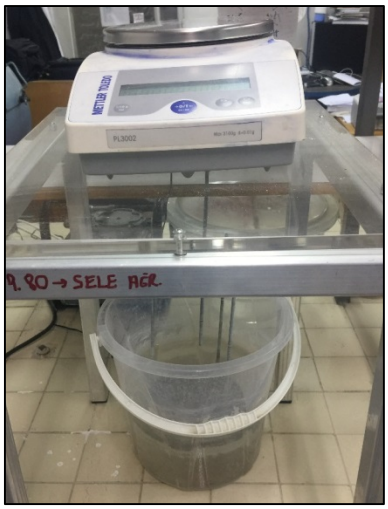


Figure 18: Weighing instrument both for dry/saturated and submerged weighings.

Density and porosity measurements were calculated by following equations and related definitions;

$V_v = M_{sat} - M_{dry}$

$V = M_{sat} - M_{sub}$

$n = (V_v / V) * 100$

$\rho_d = M_{dry} / V$

where;

Vv is void volume, V is total volume, Msat is saturated mass, Msub is submerged mass, Mdry is dry mass, n is porosity and ρd is dry density.

To determine changes in test stones (T1, T3, T4, T6, T8, T9, T12, T17, T18, T19, T20, T22, T23, T24, T26, T30) density and porosity measurements were also conducted after artificial salt crystallization cycles.

2.7.1.3. Ultrasonic Pulse Velocity (UPV)

Ultrasonic pulse velocity (UPV) is a kind of non-destructive test method which is related with density and porosity properties of the stone (Akoğlu, 2011) and commonly used for observing the softening properties of rocks (Lama and Vutukuri, 1978). Since ultrasonic velocity measurements can detect changes as deterioration in stone material (Calcaterra et al., 2004; Grinzato et al., 2004), this test was conducted on 42 test stones before and after artificial salt crystallization cycles (Figure 16). Moreover, this test was performed in biological mortar cubes (Figure 19) and biological mortar applied test stones and left in laboratory and open-air conditions for 28 days.

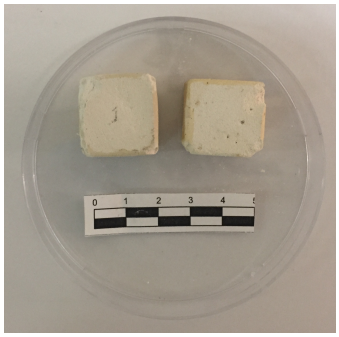


Figure 19: UPV measurement conducted biological mortar cubes

On the other hand, even ultrasonic velocity decreases in stones subjected to weathering, it doesn't always give the same results in the same conditions (Akoğlu, 2011). In other words, velocity value can be affected by other factors such as water content, anisotropy and etc. (Kandemir, 2010).

The methodology of this test is designed to determine the velocity propagation of elastic waves through rocks in laboratory (ASTM-E494–95, 2001). Sonic velocities and frequency properties are directly related to physical, mechanical and microstructural features of the materials.

UPV measurements were performed in laboratory by using PUNDIT Plus test equipment with its transmitter and a receiver probes of 220 kHz (Figure 20). This device measures the time elapsed between the generation and reception of the waves from the transmitter probe to the receiver probe. In order to increase the effectiveness of the test, the probes were coated with a thin film of vaseline.

Figure 20: UPV measurement instrument

The sonic velocity waves was calculated by the following formula (ASTM-E494–95, 2001);

$$V = x/t$$

Where,
V is velocity (m/s), x is the distance traversed by the wave (m) and t is travel time (s).

There are three ways for conducting UPV measurements: direct, semidirect and indirect transmission modes that transducers are placed at the opposite surfaces, at 90° angle, and on the same surface of tested element, respectively (ASTM-E494–95,

2001). In this study, direct transmission mode was preferred for performing ultrasonic measurements. In addition to UPV measurements in dry state of travertine stones, UPV values for saturated state were also noted in order to observe changes in UPV values during drying period.

2.7.1.4. Uniaxial Compressive Strength (UCS)

Uniaxial compressive strength (UCS) test is designed to determine the strength classification and characterization of rocks having uniform geometry (ASTM-D7012–10, 2010).
In UCS test, 2 loaders placed on top and bottom apply pressures directly to the entire surface of a specimen (Figure 21).

This test was carried out on 6 dry and 6 saturated travertine samples (12 in total) in 5cm x 5cm x 5cm size. Firstly, the surface areas of the samples were calculated and then inserted into the test device to expose axial loading. The maximum load was recorded by a computer program at the time of failure. Finally, the load was calculated for a unit area.

$\sigma u = P / A$
where;
σu is uniaxial compressive strength, P is failure load and A is cross-sectional area.

Length to diameter ratio (D/L) of the samples was around 1. During the tests, a motorized hydraulic compression machine with a loading capacity of 1500 kN was used. The pace rate of hydraulic compression machine was so adjusted that failure takes place in about 5 minutes.

Figure 21:Uniaxial Compressive Strength (UCS) test device

2.7.1.5. Point Load Strength Index (Is)

As an indirect measurement strength measurement, point load index test is similar to UCS in terms of obtaining information on strength properties of the rocks. The difference of these tests is based on their procedures. There are 2 loaders placed on top and bottom part as in UCS test but there are thin tips in these loaders which apply pressure from these pointed plates to the specimen. This test can be applied also to irregular shaped specimen (ISRM, 1985; Topal, 2000). To convert Is values to UCS values, Is values are multiplied by a "k" value which is calculated by dividing average UCS values to average Is values.

Since most of the biological mortar applied test stones were the ones aged with salt crystallization test, their cubic form became deformed. Therefore, point load test was applied to these samples in order to obtain information about impact of mortar on rock in terms of strength. 7 samples were selected for the point load test and where 2 of them were biological mortar applied travertine stones.

2.7.1.6. Colorimetric Measurements

Color measurements were performed on biological mortar cubes (2.2 cm x 2.2 cm x 2.2 cm) and biological mortar applied stone fragment by taking sound and artificially deteriorated test stones as a reference, respectively, in order to see the color uniformity between biological mortar and test stones before and after artificial salt crystallization cycles.

Color measurements were conducted by a spectrophotometer, Konica Minolta 2600Cmd, and based on CIELAB system (Figure 22). This system is often used in instrumental color measurements. Three coordinates are defined in the LAB system: CIE L* represents lightness, CIE a* represents greenness and CIE b* represents yellowness (Wyszecki and Stiles, 1982). Measurement of the color differences between the repair material and the original surface is also possible with this system.

Figure 22: Color measurement device-Konica Minolta 2600Cmd

2.7.2. Determination of Microstructural Properties

Mineralogical properties of biological mortar, biological mortar applied samples and test stones before and after artificial salt crystallization were examined using XRD, SEM-EDX, optical microscope and stereomicroscope.

2.7.2.1. X-ray Diffraction (XRD) Analysis

XRD analysis is based on the determination of crystalline structure and element type of the rocks (Herz and Garrison, 1998).

X-Ray diffraction (XRD) analyses were carried out on around 10 g powdered samples prepared by crushing and grinding them to powder in an agate mortar. Powders were prepared from travertine stone before and after artificial salt crystallization cycles and biological mortar (cured 28 days), separately.

XRD analyses were performed in Rigaku Ultima-IV X-ray diffraction instrument in Central Laboratory of METU. Analyses were conducted using CuKα radiation, adjusted to 40 kV and 30 mA. The XRD traces were recorded in the 10° - 70° 2θ range for 1 deg/minute scan speed.

2.7.2.2. Thin Sections Analyses by Optical Microscopy

Thin section analyses were conducted for determination of mineralogical and petrographical properties of the test stones before and after artificial salt crystallization and biological mortar applied test stones after 28-day curing. Those thin sections were prepared by the thin section laboratory of Department of Geological Engineering in METU and examined by Nikon E200 polarizing microscope.

2.7.2.3. Cross Section Analyses by Stereomicroscopy

To observe the biological mortar-stone contact zones, travertine stone fragments were selected and mortars were applied to these stones in two different ways: in one mortar molded with a fragment of stone (< 2.2 cm x 2.2 cm) and in the other one mortar applied onto a fragment of stone (<1 cm x 1cm) (Figure 23). The sample codes and definitions of these applications are given in Table 10 with brief explanations. These samples were left in laboratory and open-air conditions about 28 days.

After 28 days, samples were molded with epoxy resin together with its hardener component in silicon ice trays (~2.2 cm x 2.2 cm) and left for hardening at least 24 hours in ambient temperature. Then, these samples were taken from trays and cut in 1 mm thickness with diamond blade ((Buehler-Isomet Low Speed Saw). This cut section was coated again with epoxy resin and polished with silicon carbide waterproof abrasive paper before observation with stereomicroscope.

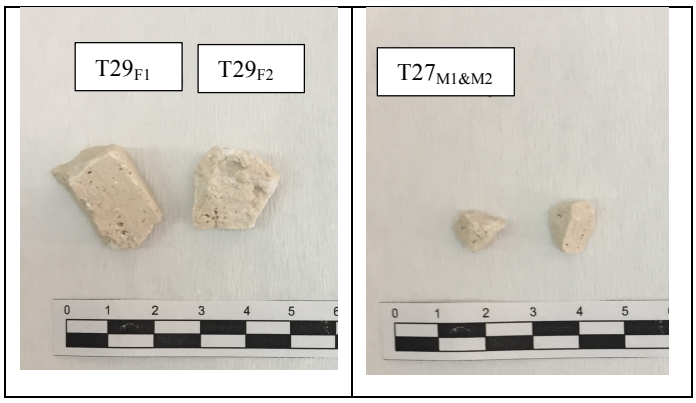
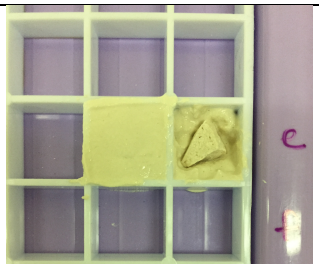


Figure 23:Stone samples selected for biological mortar application

Table 10: Sample codes and its definitions for cross section analyses.

Sample code	Definitions	Applications
$T27_{M1}$	T27: Test stone number M: Mortar molded with a fragment of stone 1: Open air conditions	
$T27_{M2}$	T27: Test stone number M: Mortar molded with a fragment of stone 1: In laboratory conditions	
$T29_{F1}$	T29: Test stone number F Mortar applied onto a fragment of stone 1: Open air conditions	
$T29_{F2}$	T29: Test stone number F: Mortar applied onto a fragment of stone 2: In laboratory conditions	

Prepared samples were examined by a Z16 APO A model Leica stereomicroscope and the photographs of the sections were taken with a Leica Application Suite (LAS).

2.7.2.4. Scanning Electron Microscopy (SEM) Analyses Coupled with Energy Dispersive X-Ray (EDX) Analyzer

Biological mortar cubes (1 cm x 1 cm x 1 cm), particles from B4 medium, TFC (open air conditions), TC and TF (in lab conditions), non-applied test stone & mortar mixture without bacteria as a control, were examined by QUANTA 400F Field Emission SEM Samples to be analyzed in SEM-EDX were coated with Au-Pd.

2.7.3. Other Evaluation Criteria

Biological mortar and its application were also examined based on several evaluation criteria such as crack formation, smell, fungal growth, excess of liquid, material integrity, cohesive consistency and resistance to disintegration.

To conduct this examination in an efficient and practical way, a yes/no table was generated including these criteria. In below, there is an empty example of this table.

Table 11: Yes/no survey table

Parameters	**Biological mortar cubes**		**Biological mortar application**	
	Yes/No	**Additional comment if any**	**Yes/No**	**Additional comment if any**
Cohesive consistency				
Crack formation				
Smell				
Fungal growth				
Excess of liquid				
Material integrity				
Disintegration				

2.8. Feasibility Study

To evaluate the study's potential for success, a feasibility study was conducted on three types of area such as technical feasibility, economic feasibility, and timing feasibility.

More specifically, in technical feasibility, the assessment deals with the technical capacity and the facilities of the organization that will conduct the study. In this case, Material Conservation Laboratory (METU/Faculty of Architecture) was the institution

that study performed, therefore this institute was evaluated based on major instruments required for the whole process.

In economic feasibility, the assessment involves the cost of the product and staff. In more detail, product is composed of bacterial sediment, nutritive medium and stone powder and the cost of each of these components was calculated approximately. Then, the quantity of biological mortar applied to micro-cracks of 14 test stones was calculated considering the measured crack widths, length and assumed depths based on the fluidity of mortar. Thus, total cost of the product was calculated based on the unit cost of the biological mortar.

On the other hand, restorer is the staff who will conduct the application and control the quality of the final product. Therefore, s/he must be specialist for conducting such applications. Furthermore, s/he must be highly qualified which brings the issue of training by a conservation scientist in relation with the material specifications and instructions for application. In addition, spent time in restoration work is another parameter to be involved in costs analysis.

In timing feasibility, the assessment focuses on the time and period to complete the conservation intervention. This feasibility area directly affects the results of financial analysis. Because, when the application is performed in a wrong season, biological mortar doesn’t work properly and requires repetition of the application which leads to a doubling of the total cost.

CHAPTER 3

EXPERIMENTAL RESULTS

Components of biological mortar were optimized and their proportions in mortar mixture were defined after several trials. Following to optimization, biological mortar was applied to micro-cracks of test stones and this application was examined through several physical, physico-mechanical, microstructural analyses, and other evaluation criteria. The results of these analyses are presented in this chapter.

3.1. Adjustment of Biological Mortar Components

A bacterial isolate, *B. cereus*, was selected from microbial library for bacterial sediment preparation and depending on its metabolic features and growth characteristics, the specifications of this component was obtained clearly.

At the same time, optimal grain size of stone powder and nutritive content are defined in the related sections.

3.1.1. Obtaining and Selecting Bacteria

Upon generating +4°C and -80°C stocks of pure cultures, identification of these isolates was performed by MALDI-TOF technique. Among these isolates, *Bacillus cereus* (# 81), Gram-positive and facultative anaerobe bacterium, was selected for the development of biological mortar based on its calcite mineral precipitation ability determined by conducted experiments and also well-known properties and success in conservation studies.

On the other hand, ATCC catalogue strain (10876) of *B. cereus* was investigated in terms of calcite production capacity in comparison to its environmental isolate

3.1.1.1. Isolation of Bacteria from Different Type of Samplings

A total of 97 pure culture were obtained by streak plate method from different thermal spring water sources during field studies (Figure 24).

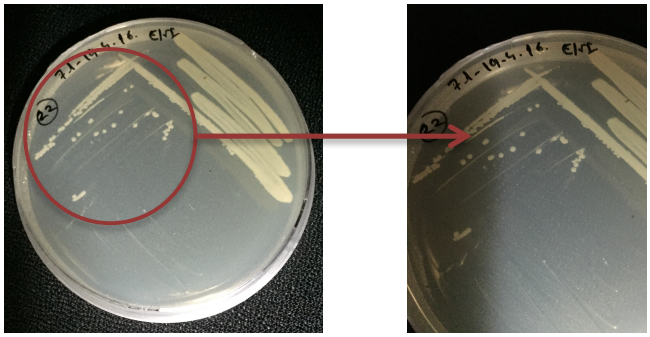


Figure 24:Obtaining pure cultures by streak plate method.

3.1.1.2. Establishment of Isolate Library and Creating Stocks

Pure culture of each plated isolate appeared within the boundaries of drew grids after incubation period (Figure 25). Then, -80°C stocks were prepared from each isolate for long term storage.

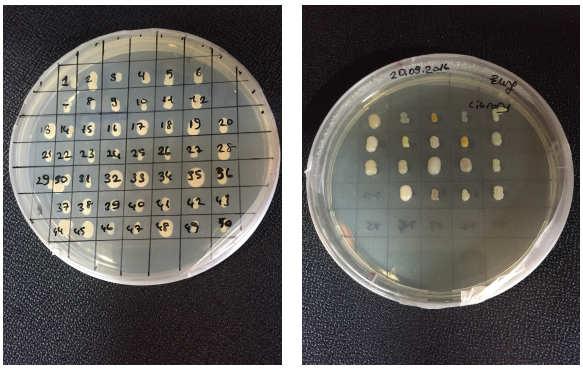


Figure 25:Microbial library prepared on LB agar plates

3.1.1.3. Molecular Identification of Isolates

Pure cultures (numbered from 1 to 97) in microbial library were analyzed by MALDI-TOF technique and species corresponding to each culture was numbered as in Table 12.

Based on well-known properties and success in conservation studies, isolate # 81, *Bacillus cereus*, was selected as a target-bacteria for the development of biological mortar.

Table 12:MALDI-TOF identification results.

Sample number	Isolate
1	*Aeromonas veronii*
2	*Aeromonas veronii*
3	*Staphylococcus epidermidis*
4	*Pseudomonas fluorescens*
5	*Staphylococcus hominis*
6	*Enterococcus faecium*
7	*Aeromonas veronii*
8	*Aeromonas veronii*
9	*Aeromonas veronii*
10	*Acinetobacter baumannii*
11	Unidentified bacterium
12	*Pseudomonas fluorescens*
13	*Pseudomonas fluorescens*
14	*Aeromonas veronii*
15	*Aeromonas veronii*
16	*Aeromonas veronii*
17	*Aeromonas sp.*
18	*Staphylococcus epidermidis*
19	*Aeromonas salmonicida*

Sample number	Isolate
20	*Serratia marcescens*
21	*Enterobacter asburiae*
22	*Aeromonas sp.*
23	*Enterobacter asburiae*
24	*Acinetobacter baumannii*
25	*Acinetobacter baumannii*
26	*Acinetobacter baumannii*
27	*Acinetobacter baumannii*
28	*Aeromonas sp.*
29	*Staphylococcus epidermidis*
30	*Aeromonas veronii*
31	*Acinetobacter baumannii*
32	*Aeromonas salmonicida*
33	*Enterobacter asburiae*
34	*Staphylococcus epidermidis*
35	*Staphylococcus haemolyticus*
36	*Aeromonas hydrophila*
37	*Acinetobacter johnsonii*
38	*Aeromonas sp.*
39	*Aeromonas sp.*
40	*Acinetobacter baumannii*
41	*Hafnia alvei*
42	*Hafnia alvei*
43	*Hafnia alvei*
44	*Aeromonas sp.*
45	*Aeromonas salmonicida*
46	*Acinetobacter baumannii*
47	*Aeromonas veronii*
48	*Aeromonas salmonicida*
49	*Acinetobacter baumannii*

Sample number	Isolate
50	*Acinetobacter baumannii*
51	*Acinetobacter baumannii*
52	*Aeromonas punctata*
53	Unidentified bacterium
54	*Aeromonas punctata*
55	*Aeromonas urinae*
56	*Acinetobacter baumannii*
57	*Acinetobacter baumannii*
58	*Pseudomonas fluorescens*
59	*Acinetobacter baumannii*
60	*Aeromonas urinae*
61	*Acinetobacter baumannii*
62	*Acinetobacter baumannii*
63	*Aeromonas urinae*
64	*Aeromonas urinae*
65	*Pseudomonas fluorescens*
66	*Aeromonas urinae*
67	*Acinetobacter baumannii*
68	*Acinetobacter baumannii*
69	*Aeromonas urinae*
70	Unidentified bacterium
71	Unidentified bacterium
72	Unidentified bacterium
73	*Pseudomonas fluorescens*
74	Unidentified bacterium
75	*Aeromonas salmonicida*
76	Unidentified bacterium
77	Unidentified bacterium
78	Unidentified bacterium
79	Unidentified bacterium

Sample number	Isolate
80	Unidentified bacterium
81	*Bacillus cereus*
82	Unidentified bacterium
83	Unidentified bacterium
84	Unidentified bacterium
85	*Aeromonas sp.*
86	*Aeromonas sp.*
87	Unidentified bacterium
88	Unidentified bacterium
89	Unidentified bacterium
90	Unidentified bacterium
91	Unidentified bacterium
92	Unidentified bacterium
93	Unidentified bacterium
94	Unidentified bacterium
95	Unidentified bacterium
96	Unidentified bacterium
97	Unidentified bacterium

Most of the isolates, obtained from thermal spring water samples, could be identified by MALDI-TOF MS. As MALDI-TOF MS instruments libraries' mostly involve clinical references, it is known that samples from environmental sources can be problematic due to the lack of reference strains, and in our case 24% of all isolates could not be identified.

In Table 13 identified bacteria isolates are linked with sampling locations in order to see their diversity among different thermal spring water resources. This data could also be used as a reference in further studies in relation with the role of microorganisms in calcite precipitation process.

Table 13: Environmental isolates and their sampling locations.

Isolates	Sampling locations	T/pH
Aeromonas veronii *Aeromonas sp.* *Acinetobacter baumannii* *Hafnia alvei* *Pseudomonas fluorescens*	Bridal bath (main source)	33.9°C/6.5
Staphylococcus hominis *Enterococcus faecium* *Acinetobacter baumannii* *Aeromonas veronii* *Staphylococcus epidermidis* *Serratia marcescens* *Aeromonas salmonicida* *Bacillus cereus*	Bridal bath (continuation)	34.2°C/6.5
Aeromonas veronii *Staphylococcus epidermidis* *Pseudomonas fluorescens* *Aeromonas urinae* *Aeromonas sp.*	Beltes (main source)	33.7°C/6.5
Pseudomonas fluorescens *Acinetobacter baumannii* *Aeromonas veronii*	Beltes (continuation)	33.5°C/6
Aeromonas sp. *Aeromonas salmonicida* *Acinetobacter baumannii* *Aeromonas veronii* *Aeromonas punctata* *Aeromonas urinae*	Ploutonion	34.6°C/6.5

Isolates	Sampling locations	T/pH
Acinetobacter baumannii *Aeromonas hydrophila* *Acinetobacter johnsonii* *Aeromonas urinae*	Gendarme pool	34°C/6.5
Enterobacter asburiae *Aeromonas sp.* *Acinetobacter baumannii* *Staphylococcus epidermidis*	Ancient pool	33.7°C/6.5
Aeromonas salmonicida *Enterobacter asburiae* *Staphylococcus epidermidis* *Staphylococcus haemolyticus*	Last thermal spring water outlet point to the travertine whitening zone.	30.6°C/6.5

Moreover, general characteristics of isolates and their calcite precipitation potential are given in Table 14 and Table 15 in a detailed manner.

Table 14:General characteristics of identified isolates.

Isolates	Phylum	Morphology		Motility	Oxygen requirement	Optimum growth temperature	Habitat	Pathogenicity
Acinetobacter baumannii	Proteobacteria	Rod	Gram negative	Non-motile	Aerobe	20-37°C	Nature and hospital environment, both in humid and dry conditions	Pneumonia, wound infection, urinary tract infection, bacteremia and meningitis
Acinetobacter johnsonii	Proteobacteria	Coccus	Gram negative	Non-motile	Aerobe	20-37°C	Skin flora	Bacteremia, urinary tract infections, secondary meningitis
Bacillus cereus	Firmicutes	Rod	Gram positive	Motile	Aerobe Facultative anaerobe	30°C	Soil, water, animals	Opportunistic pathogen. Foodborne illness
Enterococcus faecium	Firmicutes	Coccus	Gram positive	Non-motile	Facultative anaerobe	35°C	Gut commensals of humans and animals	Commensal in the gastrointestinal tract of humans and animals but as a pathogen can cause meningitis or endocarditis
Serratia marcescens	Proteobacteria	Rod	Gram negative	Motile	Facultative anaerobe	5-40°C	Bathroom, dirty environments, subgingival biofilm of teeth	catheter-associated bacteremia, urinary tract infections and wound infections
Aeromonas (hydrophila, punctata, salmonicida, veronii, urinae)	Proteobacteria	Rod	Gram negative	Both motile and Non-motile	Facultative anaerobe	28-35°C	Fresh and salt water	Infections such as gastroenteritis, septicemia, pneumonia and meningitis
Enterobacter asburiae	Proteobacteria	Rod	Gram negative	Motile	Facultative anaerobe	35-40°C	Humid places in nature envrionment	Skin and soft tissue infections
Hafnia alvei	Proteobacteria	Rod	Gram negative	Motile	Facultative anaerobe	22°C	Soil, water, milk, dairy products, sewage water, human and animal excrement	Urinary tract, upper and lower respiratory tract and wound infections
Pseudomonas fluorescens	Proteobacteria	Rod	Gram negative	Motile	Aerobe	25-30°C	Soil and water	Not pathogenic under normal conditions
Staphylococcus haemolyticus	Firmicutes	Coccus	Gram positive	Non-motile	Facultative anaerobe	30-37°C	Skin flora	Infections due to intravenous catheters
Staphylococcus epidermidis	Firmicutes	Coccus	Gram positive	Non-motile	Facultative anaerobe	37°C	Skin flora	Not pathogenic under normal conditions
Staphylococcus hominis	Firmicutes	Coccus	Gram positive	Non-motile	Facultative anaerobe	30-40°C	Skin flora	Not pathogenic under normal conditions

Table 15:Usage of identified strains in conservation works and their calcite production potential.

Isolates	**Calcite production capacity**	**Usage in conservation studies**
Acinetobacter baumannii	X	X
Acinetobacter johnsonii	✓	X
Bacillus cereus	✓	✓
Serratia marcescens	X	X
Enterococcus faecium	X	X
Aeromonas hydrophila	✓	X
Aeromonas punctata	X	X
Aeromonas salmonicida	✓	X
Aeromonas veronii	X	X
Aeromonas urinae	X	X
Enterobacter asburiae	X	X
Hafnia alvei	X	X
Pseudomonas fluorescens	✓	✓
Staphylococcus haemolyticus	✓	X
Staphylococcus hominis	X	X
Staphylococcus epidermidis	✓	X

3.1.1.4. Characterization of Precipitated Crystal by Selected Isolate

Environmental isolate formed crystals after one-week incubation in B-4 liquid medium. The morphology of crystals was observed in a cubic when a drop of this medium was observed by optical microscope (Figure 26).

Moreover, rhombohedral crystals with an elemental composition "CaCO3" were determined in the precipitates, obtained from B-4 liquid medium, by scanning electron microscope (SEM)-coupled with energy dispersive X- ray (EDX) Analyzer (Figure 27).

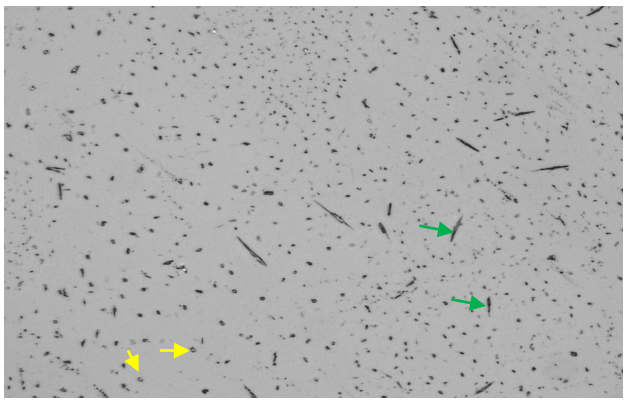


Figure 26:Optical microscope images of cubic crystals labeled with yellow is calcite and needle like structures labelled with green is aragonite (2.5X)

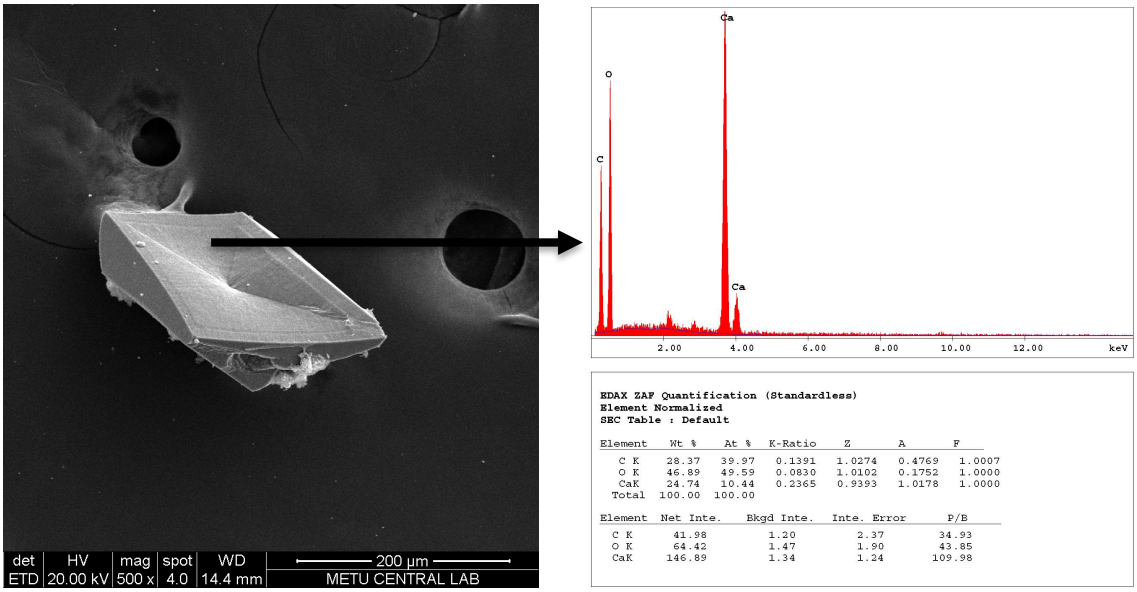


Figure 27: As a bacterial precipitate, calcite crystal in SEM-EDX analyses

3.1.1.5. Determination and Comparison of Calcite Production Potential of Selected Isolate

$CaCO_3$ precipitation was observed in the surrounding of environmental strain of *B. cereus* after 3 weeks of incubation in B-4 and Urea-$CaCl_2$ agar medium (Figure 28, Figure 29). Color of the precipitation was brownish when observed under optical microscope.

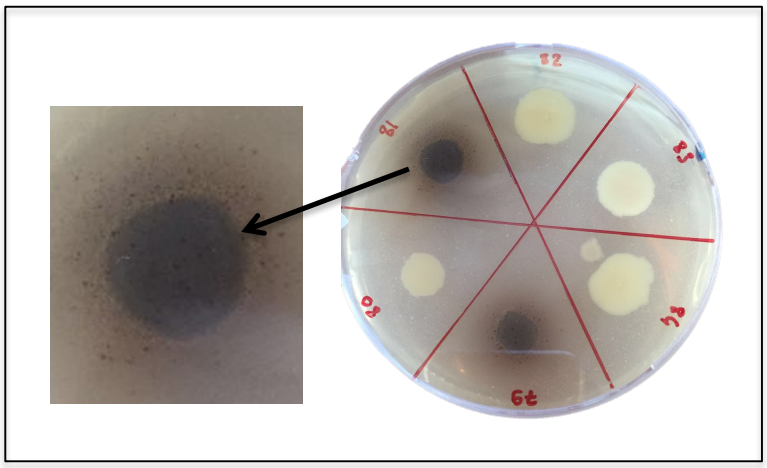


Figure 28:Calcite precipitation by *B. cereus* in Urea-$CaCl_2$ agar medium

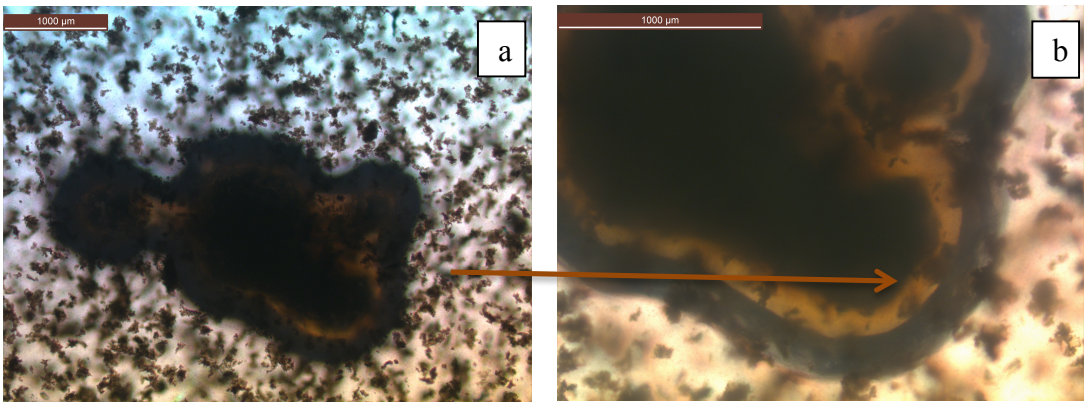


Figure 29: Calcite precipitation inside and around the colony of *B. cereus* in B4 agar medium. (a) 2.5X magnification, (b) 5X magnification

3.1.2. Preparation of Bacterial Sediment

Upon preparing +4 °C library of *B. cereus*, growth curve of this isolate was generated by optical density measurements over 105 hours. In addition, as a parallel study, viable colony forming unit (CFU) was also counted with the same time intervals of OD measurements in that period (Table 16). Thus, based on the below table, it was determined that 1 OD corresponds to 3.14×10^8 CFU.

Table 16: OD and CFU values at changing intervals during 105 hours of incubation of *B. cereus.*

Time	OD	CFU/ml
12	0.073	2.1×10^7
20	0.110	4.3×10^7
24	0.159	5.5×10^7
32	0.391	2.5×10^8
44	0.933	5.4×10^8
48	1.111	2.39×10^8
71	1.835	7.2×10^7
86	1.899	4×10^7

When Figure 30 and Table 16 was evaluated together, it was seen that optimum incubation time for *B. cereus* in 1 L LB liquid medium was between 32nd and 44th hours for the preparation of bacterial sediment since growth curve plateaued starting from 60th hour.

It was determined that approximately 3 g of bacteria was yielded from 1 L of LB liquid medium inoculated with 10 ml bacteria culture after incubation at 35°C for 32-44 hours.

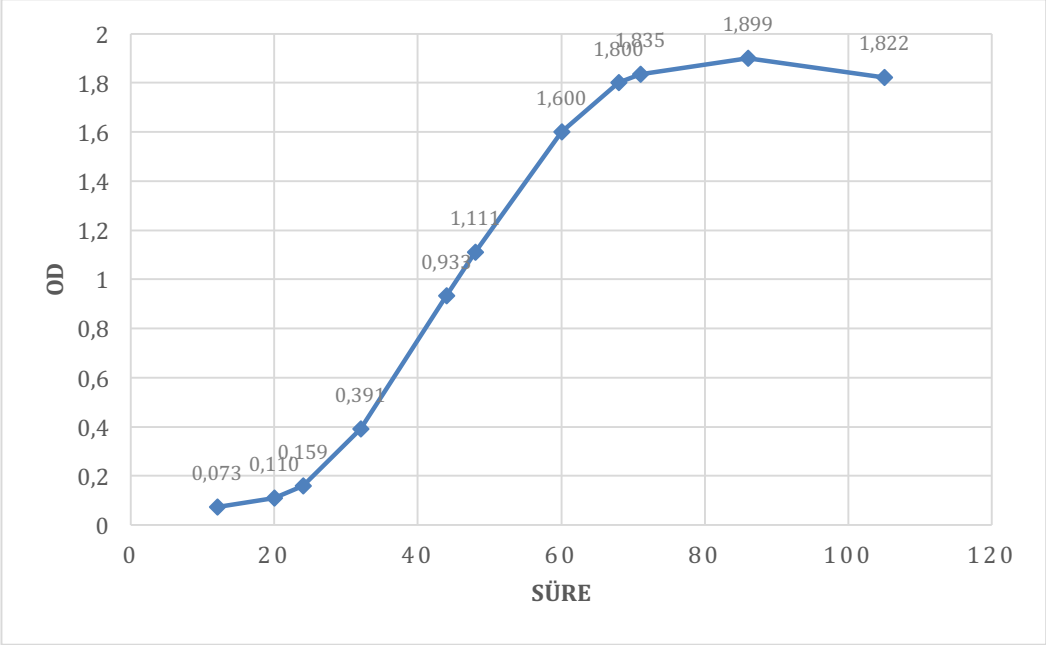


Figure 30: Growth curve of B. cereus generated by OD600 measurements

3.1.3. Preparation of Nutritive Medium

Nutritive medium was prepared with the defined ingredients in Section 2.2.3. Contamination like fungal growth and effluvium wasn't observed either in mortar mixture or +4 °C stocks in long term storage.

3.1.4. Preparation of Aggregate

Optimum granulometry of stone powder was confirmed to be in the range of 38-150 µm after conducting several trials in laboratory and open-air conditions.

3.2. Biological Mortar Set Up

Optimization of mortar components was indeed performed simultaneously with biological mortar preparation process.

Generally, 1L of *B. cereus* inoculated LB liquid medium, provided 3 g of dense bacterial sediment having about 4×10^8 CFU/ml. The CFU of bacterial is sediment is

important since bacteria couldn't bind stone aggregates with their precipitates when CFU value is below 10^7 CFU/ml.

First of all, bacterial sediment was mixed with nutritive solution in order to be sure that all nutrient is accessible for bacteria and the amount of nutritive solution (X g) was kept as the same amount with bacterial sediment (X g) since it is necessary principally for bacterial survival even though critical for mortar consistency.

On the other hand, stone powder with the defined granulometry was added to bacterial sediment-nutritive medium mixture in 4X g, 5X g, 6X g, and 7X g proportions. Molded and hand shaped biological mortars presented their best at X g bacterial sediment, X g nutritive solution, and 5X g stone powder based on other evaluation criteria after drying at room temperature which was about five days. Moreover, after 10 days, mortar mixture also analyzed through microstructural analyses.

3.3. Examination Methods for Test stones, Repair Material and Its Application

Results of laboratory tests, measurements and analyses conducted on travertine samples before and after artificial salt crystallization test are evaluated in detail.

In addition, performance of biological mortar and its application are evaluated through results of physical, physico-mechanical tests, microstructural analyses and other evaluation criteria.

3.3.1. Determination of Physical and Physico-mechanical Properties

Physical and physico-mechanical behavior of cubic test stones and changes in these properties before and after artificial salt crystallization test were evaluated through results of effective porosity and density measurements, ultrasonic pulse velocity (UPV) measurements, uniaxial compressive strength experiments and colorimetric measurements.

At the same time, performance of biological mortar and its applications were evaluated with the results of physical and physico-mechanical tests such as UPV test, point load test, and colorimetric measurements together with a series of microstructural analyses by XRD, SEM-EDX, thin section analyses by optical microscopy and cross section analyses by stereomicroscopy. In addition, mortar performance was also determined by other evaluation criteria.

3.3.1.1. Artificial Salt Crystallization by Sodium Sulfate (Na_2SO_4)

30 test stones were subjected to salt test by sodium sulfate that had lasted for 30 cycles. 14 specimens (T2, T5, T7, T10, T11, T13, T14, T15, T16, T21, T25, T27, T28, T29) were started to crack and degrade due to the fractures and 16 test stones (T1, T3, T4, T6, T8, T9, T12, T17, T18, T19, T20, T22, T23, T24, T26, T30) were stayed intact without changing the shape. These stones were washed to get rid of salt until the salt composition of the wash water of stone and distilled water found to be equal.

To observe the physical and physico-mechanical changes; effective porosity & density measurements, ultrasonic pulse velocity (UPV) tests, uniaxial compressive strength (UCS) tests were applied again to 16 test stones after artificial salt crystallization test.

3.3.1.2. Effective Porosity and Density

Values of effective porosity of the travertine cubes were found to be changing between 6,49 % to 10,65 % with an average value 8,31 ± 0.95 %. On the other hand, values of density of the travertine cubes were found to be changing between 2,34 g/cm^3 to 2,45 g/cm^3 with an average value 2,41 ± 0.02 g/cm^3 (Table 17, Figure 31).

Table 17: Effective porosity and density values of the cubic test samples.

Sample No	M_{sat} (g)	M_{sub}(g)	M_{dry} (g)	Porosity (%)	Density (g/cm^3)
T-1	331,61	198,10	319,52	9,06	2,39
T-2	330,21	196,19	317,83	9,24	2,37
T-3	335,20	201,00	324,92	7,66	2,42
T-4	336,67	201,86	326,28	7,71	2,42
T-5	332,60	198,62	320,43	9,08	2,39
T-6	335,34	200,87	324,94	7,73	2,42
T-7	337,83	203,74	328,36	7,06	2,45
T-8	336,63	202,40	326,64	7,44	2,43
T-9	339,42	205,09	330,70	6,49	2,46
T-10	325,82	193,07	312,07	10,36	2,35
T-11	324,09	191,62	309,98	10,65	2,34
T-12	331,86	198,59	320,32	8,66	2,40
T-13	332,09	198,61	320,06	9,01	2,40
T-14	331,49	197,96	318,91	9,42	2,39
T-15	331,35	197,87	318,73	9,45	2,39
T-16	335,20	200,92	324,85	7,71	2,42
T-17	334,37	200,07	323,30	8,24	2,41
T-18	336,34	201,91	325,78	7,86	2,42
T-19	334,32	200,76	323,99	7,73	2,43
T-20	334,33	200,73	324,19	7,59	2,43
T-21	333,75	200,85	322,65	8,35	2,43
T-22	334,70	200,52	324,94	7,27	2,42
T-23	332,38	198,76	321,81	7,91	2,41
T-24	332,41	198,97	323,10	6,98	2,42
T-25	334,30	200,08	323,83	7,80	2,41
T-26	326,57	194,39	314,75	8,94	2,38
T-27	331,97	197,91	319,84	9,05	2,39
T-28	331,98	198,41	319,14	9,61	2,39
T-29	336,86	203,14	327,96	6,66	2,45
T-30	332,57	199,20	321,57	8,25	2,41
T-31	333,48	200,38	323,72	7,33	2,43
T-32	330,66	197,48	318,03	9,48	2,39
T-33	332,67	198,59	320,42	9,14	2,39
T-34	332,01	198,00	319,56	9,29	2,38
T-35	333,63	200,13	323,14	7,86	2,42
T-36	333,60	200,02	322,15	8,57	2,41
T-37	334,94	200,42	324,30	7,91	2,41
T-38	334,57	200,87	324,32	7,67	2,43
T-39	333,88	200,33	323,56	7,73	2,42
T-40	336,04	201,89	325,93	7,54	2,43
T-41	333,70	200,08	322,53	8,36	2,41
T-42	332,30	198,50	320,02	9,18	2,39
AVERAGE				**8,31**	**2,41**
STANDARD DEVIATION				**0,95**	**0,02**

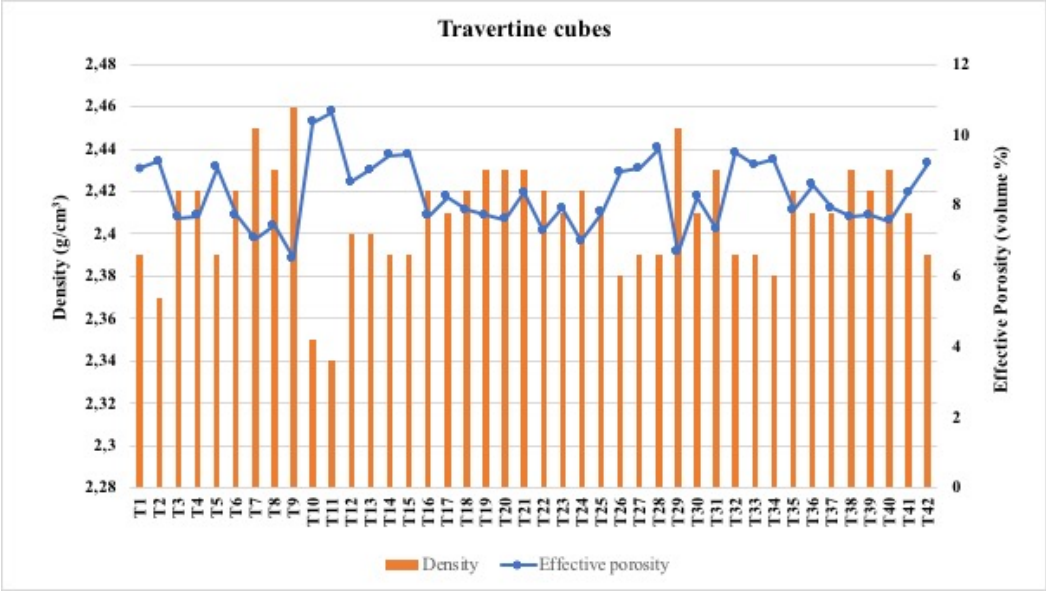


Figure 31: Effective porosity and density of travertine cubes.

Considering the average density (2,41 ± 0.02 g/cm^3) and porosity (8,31 ± 0.95 %) values, travertine stones from Kocabaş quarry fall in the "Moderate" class introduced by (Anon, 1977) (Table 18).

Porosity and density values of the rock specimens which are used in this study showed coherent relationship.

Table 18:Effective porosity and density values and classes by Anon (1977).

Class	Density (g/cm^3)	Description	Porosity (%)	Description
1	< 1.8	Very low	> 30	Very high
2	1.8 – 2.2	Low	30 – 15	Low
3	**2.2 – 2.55**	**Moderate**	**15 – 5**	**Moderate**
4	2.55 – 2.75	High	5 – 1	High
5	> 2.75	Very high	< 1	Very low

After artificial salt crystallization cycles, effective porosity and density measurements were conducted again in test stones. After 30 cycles, porosity and density values of the samples were decreased in a slight manner. The average values of effective porosity of the test stones were observed as 7,26 ± 0.69 % and the average values of density were observed as 2,38 ± 0.02 g/cm^3.

In other words, average of effective porosity and density decreased 7.5% and 1.7%, respectively (Figure 32, Table 19).

Table 19: Comparison of effective porosity and density values before and after aging cycles with sodium sulfate

	Before Sodium Sulfate Test		After Sodium Sulfate Test	
Sample No	**Porosity (%)**	**Density (g/cm^3)**	**Porosity (%)**	**Density (g/cm^3)**
T-1	9,06	2,39	8,92	2,32
T-3	7,66	2,42	7,24	2,39
T-4	7,71	2,42	7,05	2,39
T-6	7,73	2,42	7,07	2,39
T-8	7,44	2,43	7,06	2,40
T-9	6,49	2,46	6,32	2,42
T-12	8,66	2,40	8,04	2,37
T-17	8,24	2,41	7,37	2,38
T-18	7,86	2,42	6,80	2,40
T-19	7,73	2,43	7,16	2,39
T-20	7,59	2,43	6,75	2,40
T-22	7,27	2,42	6,92	2,37
T-23	7,91	2,41	7,12	2,37
T-24	6,98	2,42	6,42	2,40
T-26	8,94	2,38	8,43	2,35
T-30	8,25	2,41	7,55	2,38
AVERAGE	**7,85**	**2,42**	**7,26**	**2,38**
STANDARD DEVIATION	**0,68**	**0,02**	**0,69**	**0,02**

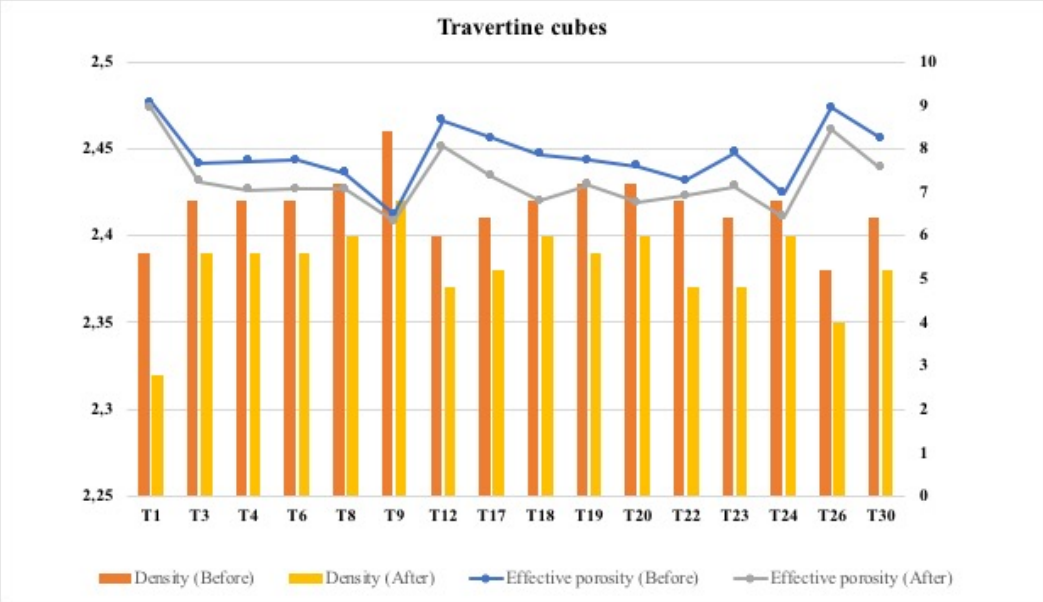


Figure 32: Changes in effective porosity and density values after artificial salt crystallization test

3.3.1.3. Ultrasonic Pulse Velocity (UPV)

Indirect UPV measurements were conducted on dry and saturated travertine cubes. When the sonic velocities of dry and saturated test stones are compared, it is found that dry sonic velocities (3774,68 ± 481,63 m/s) are lower than the saturated ones (4896,37 ± 136,29 m/s) (Table 20, Table 21, Figure 33).

Table 20:UPV measurement values (dry)

Sample No	Length (mm)	Time (msec)	Velocity (m/s)
T-1	51,31	11,43	4490,74
T-2	51,38	11,10	4638,78
T-3	51,46	12,97	3977,67
T-4	51,56	18,20	3248,35
T-5	51,32	11,13	4622,63
T-6	51,77	13,30	3918,92
T-7	51,51	15,13	3514,92
T-8	51,50	14,87	3610,42
T-9	51,21	12,53	4086,89

Sample No	Length (mm)	Time (msec)	Velocity (m/s)
T-10	51,40	13,73	3806,45
T-11	51,19	15,37	3372,86
T-12	51,15	13,47	3825,30
T-13	51,27	13,50	3844,78
T-14	51,26	14,87	3449,75
T-15	51,17	12,00	4285,89
T-16	51,41	12,80	4021,62
T-17	51,51	13,57	3798,25
T-18	51,45	13,33	3858,78
T-19	51,25	12,17	4218,91
T-20	51,40	13,07	3939,03
T-21	51,21	12,23	4193,12
T-22	51,33	11,70	4390,15
T-23	51,31	12,47	4119,26
T-24	51,34	13,63	3774,19
T-25	51,48	12,00	4291,18
T-26	51,25	12,93	3977,21
T-27	51,44	12,27	4199,52
T-28	51,29	14,27	3650,66
T-29	51,63	18,23	2925,90
T-30	51,37	14,40	3597,84
T-31	51,42	16,67	3115,26
T-32	51,30	19,63	2635,03
T-33	51,45	14,33	3600,29
T-34	51,43	20,47	2521,10
T-35	51,55	15,97	3269,13
T-36	51,22	15,23	3373,29
T-37	51,44	11,93	4324,75
T-38	51,25	14,97	3495,65
T-39	51,16	16,00	3208,15
T-40	51,51	14,10	3662,96
T-41	51,35	13,37	3861,35
T-42	51,42	13,50	3819,72
		AVERAGE	**3774,68**
		STANDARD DEVIATION	**481,63**

Table 21:UPV measurement values (saturated)

Sample No	Length (mm)	Time (msec)	Velocity (m/s)
T-1	51,31	10,73	4781,10
T-2	51,38	10,27	5004,37
T-3	51,46	10,43	4932,50
T-4	51,56	10,37	4974,81
T-5	51,32	10,30	4984,60
T-6	51,77	10,30	5027,49
T-7	51,51	10,33	4987,69
T-8	51,50	10,43	4945,76
T-9	51,21	10,90	4706,36
T-10	51,40	11,07	4650,47
T-11	51,19	11,40	4490,28
T-12	51,15	10,43	4903,54
T-13	51,27	10,37	4945,39
T-14	51,26	10,63	4821,14
T-15	51,17	10,97	4686,66
T-16	51,41	10,53	4883,23
T-17	51,51	10,30	5001,01
T-18	51,45	10,23	5027,96
T-19	51,25	10,50	4880,19
T-20	51,40	10,30	4990,35
T-21	51,21	10,17	5038,73
T-22	51,33	10,77	4778,68
T-23	51,31	10,50	4888,65
T-24	51,34	11,23	4590,56
T-25	51,48	10,87	4748,32
T-26	51,25	10,50	4881,39
T-27	51,44	10,43	4944,42
T-28	51,29	10,33	4967,96
T-29	51,63	10,30	5012,64
T-30	51,37	10,50	4901,40
T-31	51,42	10,33	4977,93
T-32	51,30	11,07	4649,61
T-33	51,45	10,47	4915,25
T-34	51,43	10,83	4757,45
T-35	51,55	10,23	5039,76
T-36	51,22	10,23	5005,78

Sample No	Length (mm)	Time (msec)	Velocity (m/s)
T-37	51,44	10,33	4978,96
T-38	51,25	10,40	4929,55
T-39	51,16	10,40	4920,87
T-40	51,51	10,10	5099,57
T-41	51,35	10,27	5002,50
T-42	51,42	10,30	4992,67
		AVERAGE	**4896,37**
		STANDARD DEVIATION	**136,29**

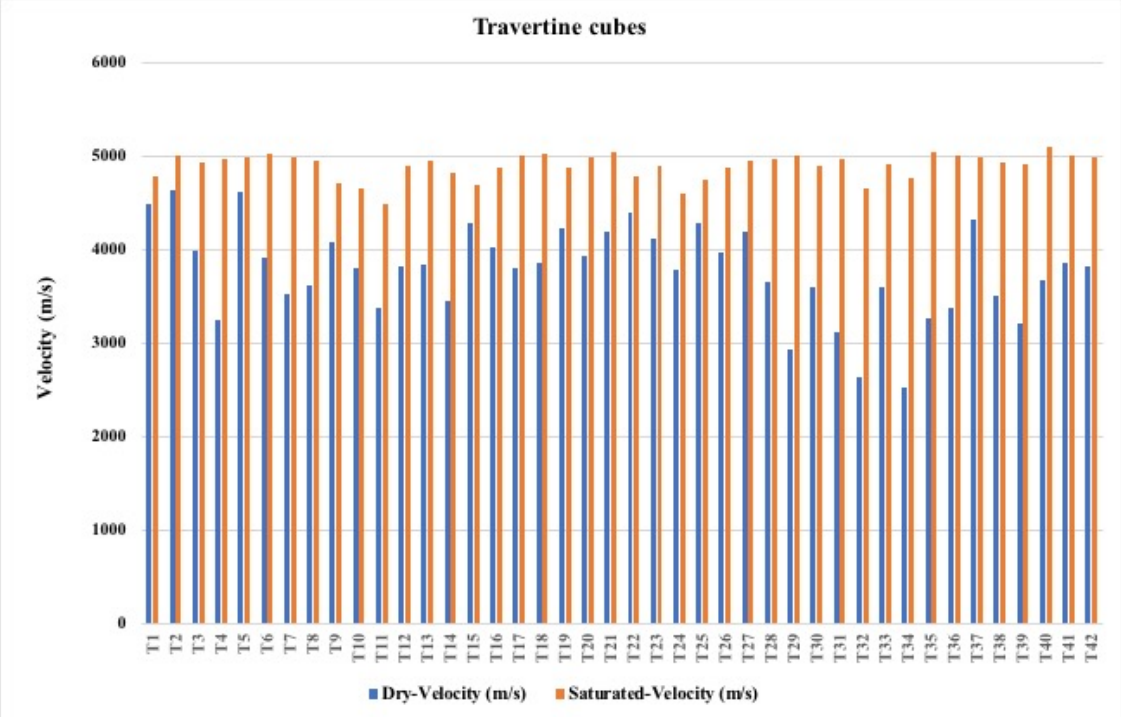


Figure 33: Changes in UPV values for dry and saturated travertine cubes.

After artificial salt crystallization cycles, UPV measurements were conducted again in test stones (T1, T3, T4, T6, T8, T9, T12, T17, T18, T19, T20, T22, T23, T24, T26, T30). The average values of UPV measurements were observed as 2514,07 ± 220,808 m/s and 3651,97 ± 210,493 m/s for dry and saturated test stones, respectively (Table 22, Table 23, Figure 34, Figure 35). In other words, average value of UPV

measurements for dry test stones is decreased 35,98% while 25.29% decrease is observed for saturated test stones after artificial aging cycles by sodium sulfate.

Table 22:UPV values before and after artificial aging test by sodium sulfate for dry travertine cubes.

DRY	Before Sodium Sulfate Test			After Sodium Sulfate Test		
Sample No	Length (mm)	Time (msec)	Velocity (m/s)	Length (mm)	Time (msec)	Velocity (m/s)
T-1	51,31	11,43	4490,74	51,31	20,93	2489,06
T-3	51,46	12,97	3977,67	51,46	20,77	2492,97
T-4	51,56	18,20	3248,35	51,56	20,23	2572,48
T-6	51,77	13,30	3918,92	51,77	20,53	2662,74
T-8	51,50	14,87	3610,42	51,50	20,77	2575,16
T-9	51,21	12,53	4086,89	51,21	25,47	2016,32
T-12	51,15	13,47	3825,30	51,15	19,53	2724,43
T-17	51,51	13,57	3798,25	51,51	20,27	2555,62
T-18	51,45	13,33	3858,78	51,45	23,73	2228,39
T-19	51,25	12,17	4218,91	51,25	19,27	2672,04
T-20	51,40	13,07	3939,03	51,40	20,43	2543,48
T-22	51,33	11,70	4390,15	51,33	19,07	2693,60
T-23	51,31	12,47	4119,26	51,31	23,07	2278,30
T-24	51,34	13,63	3774,19	51,34	18,27	2856,77
T-26	51,25	12,93	3977,21	51,25	23,60	2230,54
T-30	51,37	14,40	3597,84	51,37	19,67	2633,26
AVERAGE	**51,39**	**13,38**	**3927.00**	**51,39**	**20,98**	**2514,07**
STANDARD DEVIATION			**306,53**			**220,808**

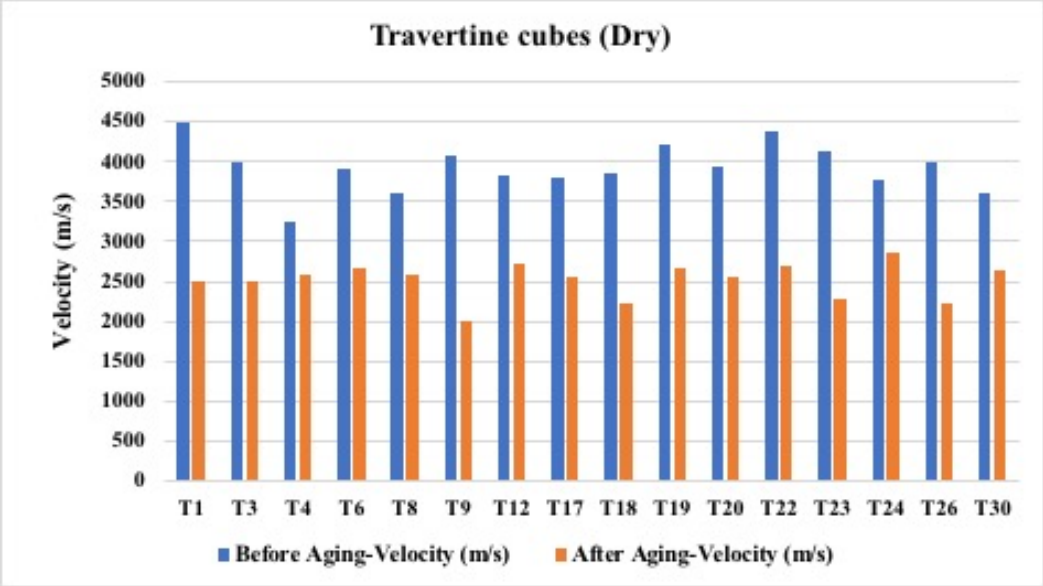


Figure 34: Changes in UPV values before and after artificial aging test by sodium sulfate for dry travertine cubes.

Table 23:UPV values before and after artificial aging test by sodium sulfate for saturated specimens.

SATURATED	Before Sodium Sulfate Test			After Sodium Sulfate Test		
Sample No	**Length (mm)**	**Time (msec)**	**Velocity (m/s)**	**Length (mm)**	**Time (msec)**	**Velocity (m/s)**
T-1	51,31	10,73	4781,10	51,31	14,93	3513,55
T-3	51,46	10,43	4932,50	51,46	14,93	3452,27
T-4	51,56	10,37	4974,81	51,56	13,80	3745,40
T-6	51,77	10,30	5027,49	51,77	14,83	3541,10
T-8	51,50	10,43	4945,76	51,50	14,70	3519,82
T-9	51,21	10,90	4706,36	51,21	15,73	3265,61
T-12	51,15	10,43	4903,54	51,15	13,47	3815,06
T-17	51,51	10,30	5001,01	51,51	14,17	3649,15
T-18	51,45	10,23	5027,96	51,45	12,97	3967,75
T-19	51,25	10,50	4880,19	51,25	15,30	3383,49
T-20	51,40	10,30	4990,35	51,40	13,23	3883,50
T-22	51,33	10,77	4778,68	51,33	13,30	3863,16
T-23	51,31	10,50	4888,65	51,31	14,30	3592,86
T-24	51,34	11,23	4590,56	51,34	13,13	3909,68
T-26	51,25	10,50	4881,39	51,25	14,63	3504,90
T-30	51,37	10,50	4901,40	51,37	13,43	3824,19
AVERAGE			**4888,24**	**AVERAGE**		**3651,97**
STANDARD DEVIATION			**121,247**	**STANDARD DEVIATION**		**210,493**

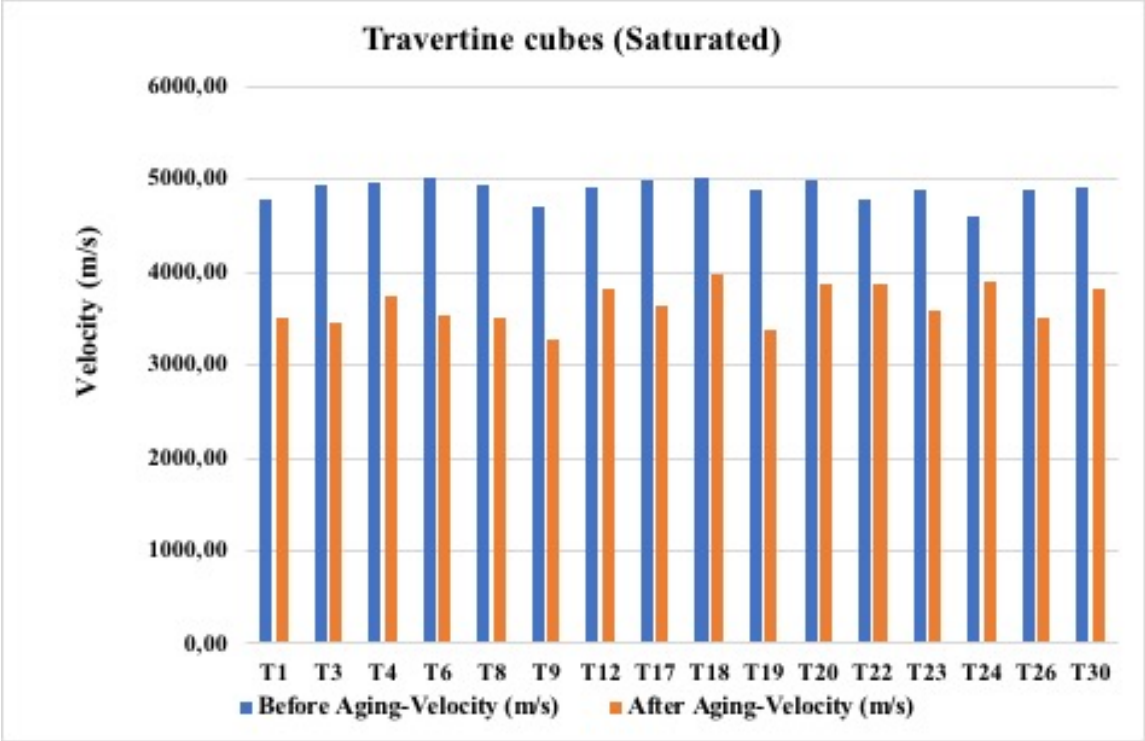


Figure 35: Changes in UPV values before and after artificial aging test by sodium sulfate for saturated travertine cubes.

On the other hand, UPV test was applied to 2 small mortar cubes (2.2 cm x 2.2 cm x 2.2 cm) to observe their physical changes in 7^{th}, 14^{th} and 28^{th} days after molding. The detailed results belong these 2 mortar cubes are shown in Table 24.

According to the test results, it is determined that overall UPV values of biological mortar cubes are increasing throughout the weeks except minor differences.

Table 24: UPV test results conducted on mortar cubes

Cube 1								
7th day			**14th day**			**28th day**		
L (mm)	t (msec)	V (m/s)	L (mm)	t (msec)	V (m/s)	L (mm)	t (msec)	V (m/s)
20,5	20,7	991,30	20,5	19,4	1057,22	20,5	19,4	1057,22
22,1	14,5	1524,83	21,8	13,9	1566,19	21,8	13,8	1577,54
23,2	16,1	1439,13	22,9	15,5	1476,13	22,9	15,8	1448,10
	Av.	**1318,42**		**Av.**	**1366,51**		**Av.**	**1360,95**
Cube 2								
7th day			**14th day**			**14th day**		
L (mm)	t (msec)	V (m/s)	L (mm)	t (msec)	V (m/s)	L (mm)	t (msec)	V (m/s)
21,6	19,2	1123,96	21,6	22,0	980,91	21,6	18,4	1172,83
22,7	28,1	806,41	22,7	23,3	972,53	22,7	26,0	871,54
16,4	15,7	1041,40	16,4	14,3	1143,36	16,4	14,6	1119,86
	Av.	**990,59**		**Av.**	**1032,27**		**Av.**	**1054,74**
	Std. Dev.	**164,76**		**Std. Dev.**	**96,3008**		**Std. Dev.**	**160,854**

3.3.1.4. Uniaxial Compressive Strength (UCS)

UCS test was conducted on dry and water saturated test stones. Durability properties of these stones were evaluated by dry and saturated UCS values. The strength values of dry test stones (T43, T44, T45, T46, T47, T48, T49, T50) were in the range of 43,55-81,06 MPa while water saturated test stones (T51, T52, T53, T54, T55, T56, T57, T58) were in the range of 39,74-71,48 MPa.

In addition, the average strengths of dry and saturated test stones were determined as 60,41 MPa and 55,61 MPa, respectively (Table 25).

Table 25:UCS test results (dry and saturated)

DRY		SATURATED	
Sample No	**F/A (MPa)**	**Sample No**	**F/A (MPa)**
T43	48.99	T51	45.45
T44	64.96	T52	63.01
T45	56.11	T53	47.10
T46	81.06	T54	71.48
T47	69.47	T55	62.52
T48	60.29	T56	59.87
T49	43.55	T57	39.74
T50	58.89	T58	55.70
F/A AVERAGE	**60.41**	**F/A AVERAGE**	**55.61**
STANDARD DEVIATION	**11,74**	**STANDARD DEVIATION**	**10,69**

According to ISRM (1981) classification, the strength values of dry and water saturated test stones show that tested samples in this study are in the category of "strong rock" (Table 26).

Table 26:UCS strength values and classes (ISRM, 1981)

Description	**Average Uniaxial Compressive Strength Value (MPa)**
Extremely Weak Rock	0,25 – 1
Very Weak Rock	1 – 5
Weak Rock	5 – 25
Medium Strong Rock	25 – 50
Strong Rock	50 – 100
Very Strong Rock	100 - 250
Extremely Strong Rock	> 250

After artificial salt crystallization cycles, UCS tests were applied again for dry (T1, T3, T4, T6, T8, T9, T12, T17) and saturated (T18, T19, T20, T22, T23, T24, T26, T30) test stones. Average UCS values of dry and saturated test stones were determined as as 48.57 MPa and 44.66 MPa, respectively (Table 27).

Table 27:UCS values for dry and saturated test stones after artificial aging test by sodium sulfate

DRY		SATURATED	
Sample No	**F/A (MPa)**	**Sample No**	**F/A (MPa)**
T1	33,36	T18	35,28
T3	61,09	T19	15,51
T4	48,95	T20	51,52
T6	72,20	T22	48,97
T8	52,24	T23	52,54
T9	52,41	T24	52,56
T12	26,22	T26	37,99
T17	42,13	T30	62,89
F/A AVERAGE	**48,57**	**F/A AVERAGE**	**44,66**
STANDARD DEVIATION	**14,72**	**STANDARD DEVIATION**	**14,66**

After 30 cycles, strength values of test stones were decreased due to effects of sodium sulfate. In relation with this, UCS values are still indicating “Strong Rock” (ISRM, 1981) for dry and saturated test stones after artificial aging tests (Table 26).

3.3.1.5. Point Load Strength Index (Is)

Point load strength index test was performed on seven test stones: T9, T33, T6, T11, T15, T21, T39. Besides, two of them (T9, T33) includes biological mortar application within their cracks. This test was conducted in order to obtain information about impact of mortar on rock in terms of strength.

Based on the results of point load strength index test, the average Is (50) value of mortar strength and rock strength was found to be 0,06 MPa and 2,11 ± 1,06 MPa, respectively (Table 28). Considering the average UCS (48.57) and Is values (2,4) of aged test stones, the biological mortar strength (UCS) was calculated as 1,2 MPa when the average Is of biological mortar was multiplied by 20 (k value 48,57/2,4=20), which is very weak according to ISRM (1981) and doesn't make a significant contribution to the rock strength.

Table 28: Point load test results for mortar and rock strength

Sample No	W (mm)	D (mm)	D' (mm)	P (kN)	De^2 (mm^2)	De (mm)	Is (MPa)	F	Is(50) (MPa)
T9	52,73	52,18	51,60	0,2	3466,07	58,87	0,0577	1,0851	0,0626
T33	49,47	51,93	51,62	0,2	3253,05	57,04	0,1537	1,0680	0,0657
								AVERAGE	**0,06**
								STD. DEV.	**0,00**
T6	25,73	35,08	35,08	2,8	1149,82	33,91	2,4352	0,8235	2,0054
T11	50,62	50,03	50,03	3,9	3226,14	56,80	1,2089	1,0658	1,2884
T15	19,94	50,39	50,39	5,3	1279,97	35,78	4,1407	0,8459	3,5026
T21	20,35	52,85	52,85	4,5	1370,06	37,01	3,2845	0,8604	2,8260
T39	42,00	45,05	45,05	2,3	2410,32	49,09	0,9542	0,9909	0,9456
								AVERAGE	**2,11**
								STD. DEV.	**1,06**

3.3.1.6. Colorimetric measurements

The color difference between the biological mortar and test stones (sound and artificially aged) were investigated in order to assess the color similarity of repair mortar with the applied stone material.

According to the Italian guidelines prepared for conservation of stone buildings, ΔE value must be smaller than 5 to interpret the color of repair material as aesthetically compatible with the original surface (Uni Normal-43/93).

The results in Table 29 showed that when sound test stone was taken as reference (Ref 1), ΔE* value, which represents the total color change, being smaller than 5, was

negligible and in acceptable limit. However, when artificially aged test stones were taken as reference (Ref 2), it was observed that ΔE* value became higher as compared to the Ref 1 measurements.

Table 29:Color measurements of biological mortar in respect of sound and artificially aged test stones.

	Reference							
	Sound test stone (Ref 1)				Aged test stone (Ref 2)			
	ΔL*	Δa*	Δb*	ΔE*	ΔL*	Δa*	Δb*	ΔE*
BM cubes	3.03	-1.15	1.79.	3.70	6.98	-2.00	-5.92	9.37
BM applied stone fragments (mortar surface)	2.21	-1.19	4.21	4.90	6.15	-1.89	-2.82.	7.06

3.3.2. Determination of Microstructural Properties

Results of XRD, SEM-EDX, thin section and cross section analysis are evaluated for biological mortar, biological mortar applied samples and test stones before and after artificial salt crystallization tests in this section.

While curing period of biological mortar and biological mortar applied samples, the climatic conditions, i.e., relative humidity, temperature were recorded both for laboratory and open-air conditions. In laboratory conditions, temperature and relative humidity were in the range of 24-33.2°C and 24-62%, respectively, while these values were determined as 22-30.9°C and 26-62% for laboratory conditions.

3.3.2.1. X-Ray Diffraction (XRD) Analysis

XRD analysis were conducted on powders of travertine stone before (T1) and after (T33) artificial salt crystallization test and biological mortar (cured 28 days).

Based on the results of XRD analysis, powders of sound & aged travertine stone and biological mortar are found to be homogenous since just main calcite (CaCO3) peak (d value: 3.02^{o}A and 3.03^{o}A) was observed in XRD trace (Figure 36, Figure 37, Figure 38).

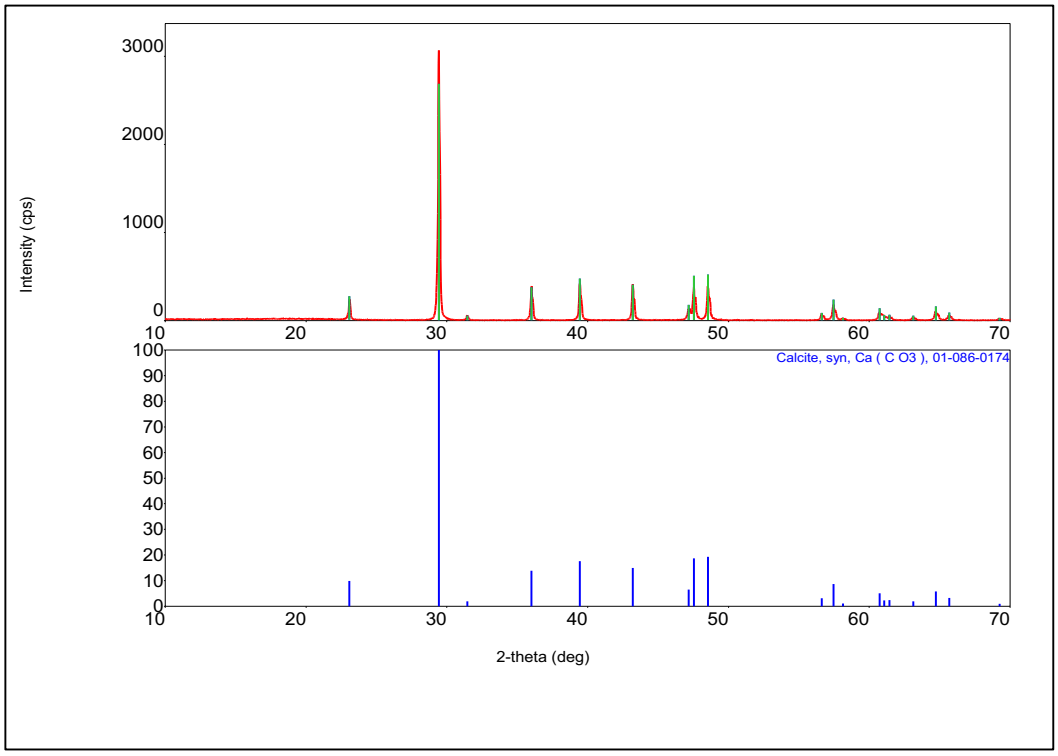


Figure 36: XRD trace of fine grained test stone (T33) powder before artificial salt crystallization cycle

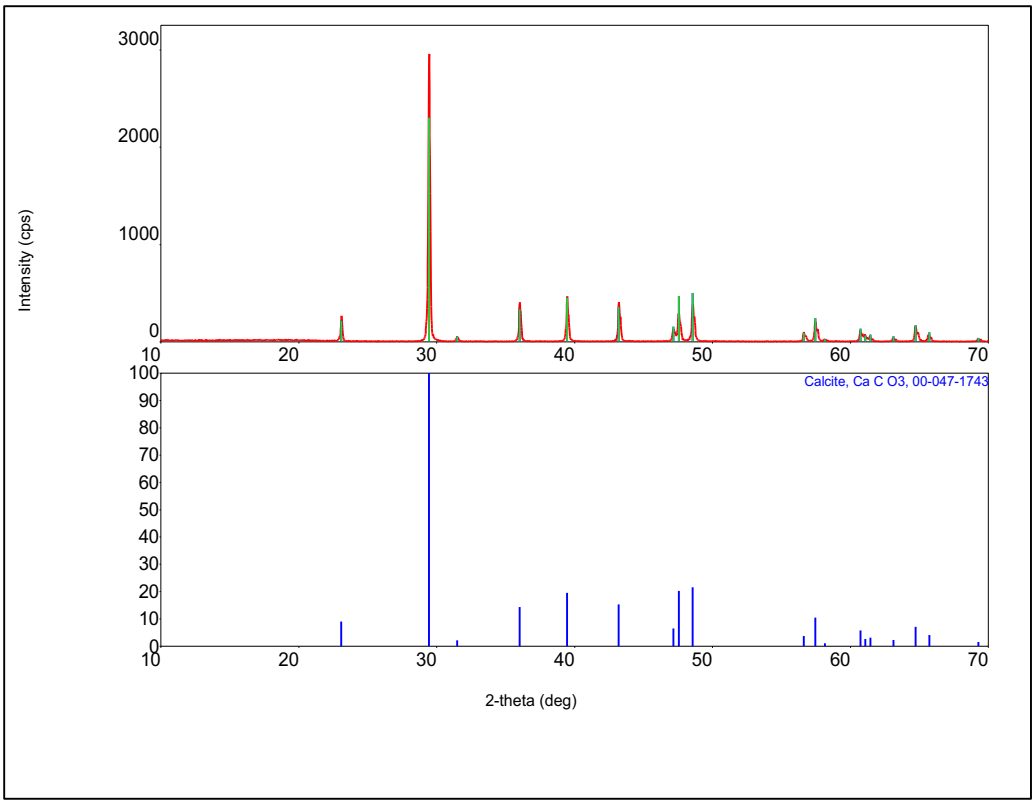


Figure 37:XRD trace of fine grained test stone (T1) powder after artificial salt crystallization cycle.

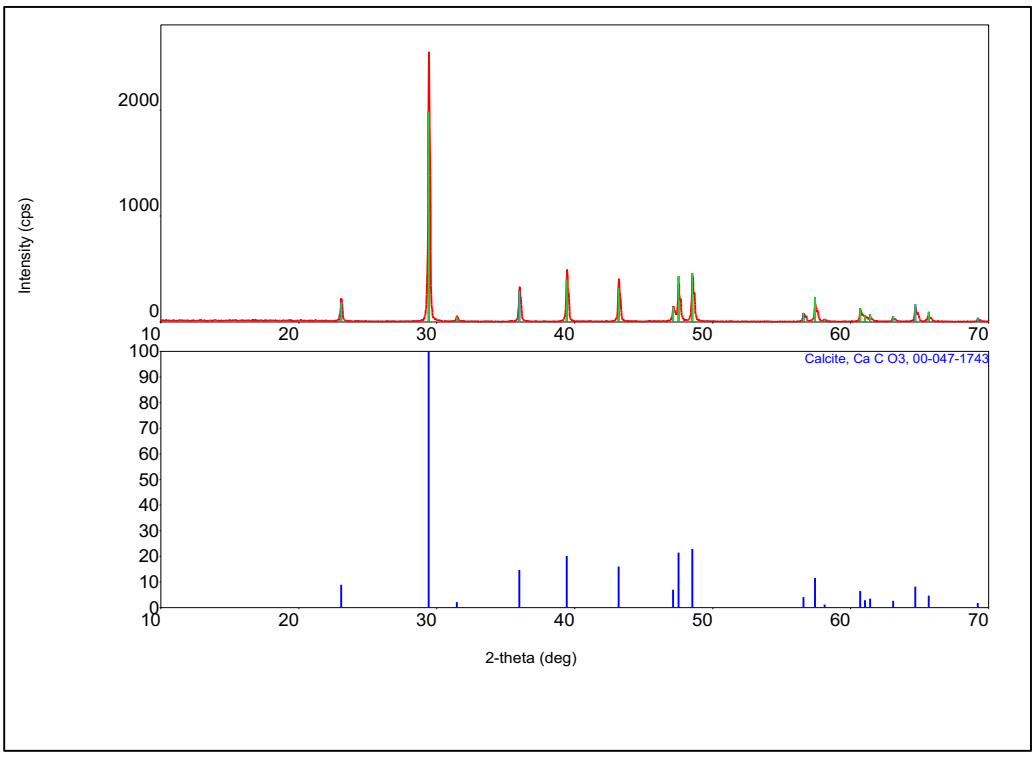


Figure 38:XRD trace of fine grained biological mortar powder (cured 28 days).

3.3.2.2. Thin section Analyses by Optical Microscopy

Thin section analyses were conducted on travertine stone before (T7) and after (T42) artificial salt crystallization test and biological mortar applied test stones cured for 28 days in laboratory (T38) and open-air conditions (T15).

When thin sections of sound and artificially aged test stones are observed under optical microscope, no difference is observed between these two samples in terms of mineralogy. As seen from Figure 39, both of the samples are completely consisting of calcite minerals in addition to some organic materials. Pore spaces can also be observed in both of the samples.

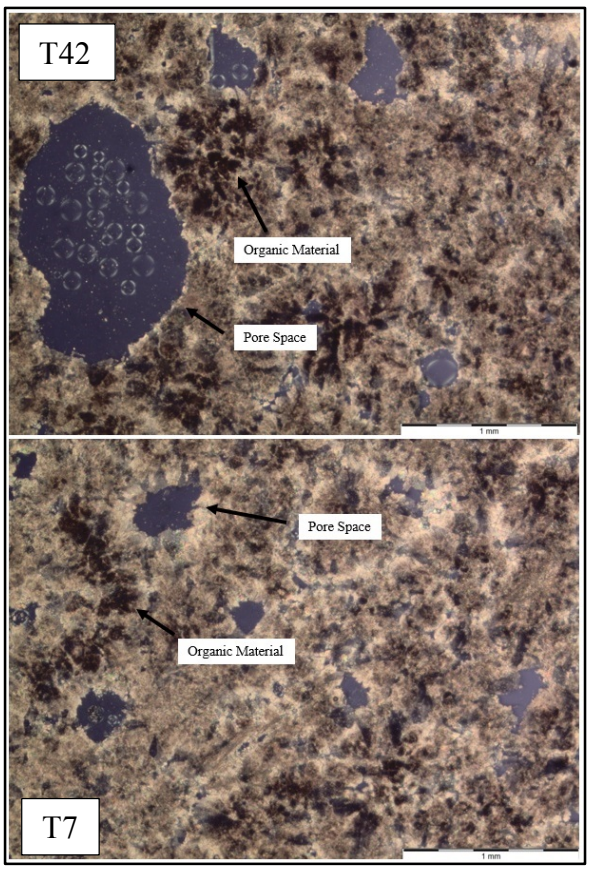


Figure 39:Optical microscope images (4X magnification) of T42 and T7 test stones

Thin sections of biological mortar applied test stones (T15&T38) were also examined under optical microscope.

During thin section preparation it was observed that most of the biological mortar in cracks had been washed out by high pressurized water of cutting blade (Figure 40). The blue lines shown in Figure 40 indicate already washed out biological mortar material where only small amount of it was remained even cutting process hadn't been finished.

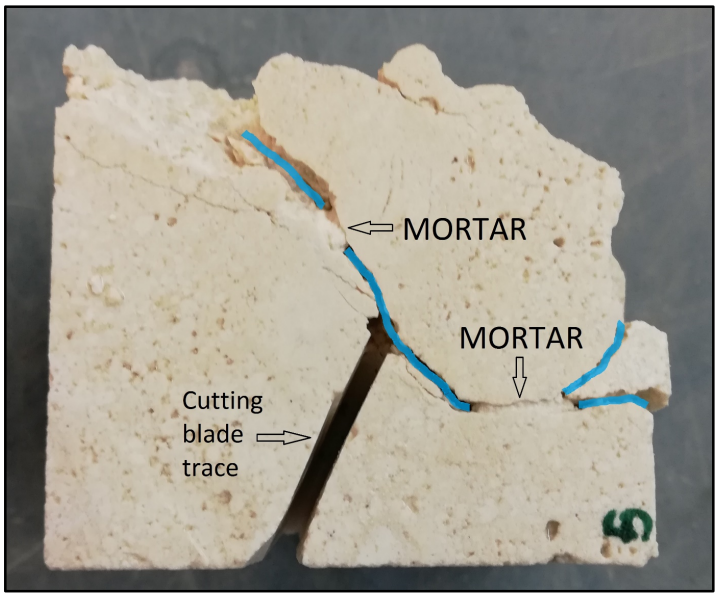


Figure 40: Rock specimen with biological mortar exposed to pressurized water

Nevertheless, thin section samples prepared from mortar applied test stones were investigated under optical microscope (Figure 41). As it seen from Figure 41, some secondary calcite minerals have been formed throughout the micro-cracks in samples cured in laboratory (T38) and open-air conditions (T15).

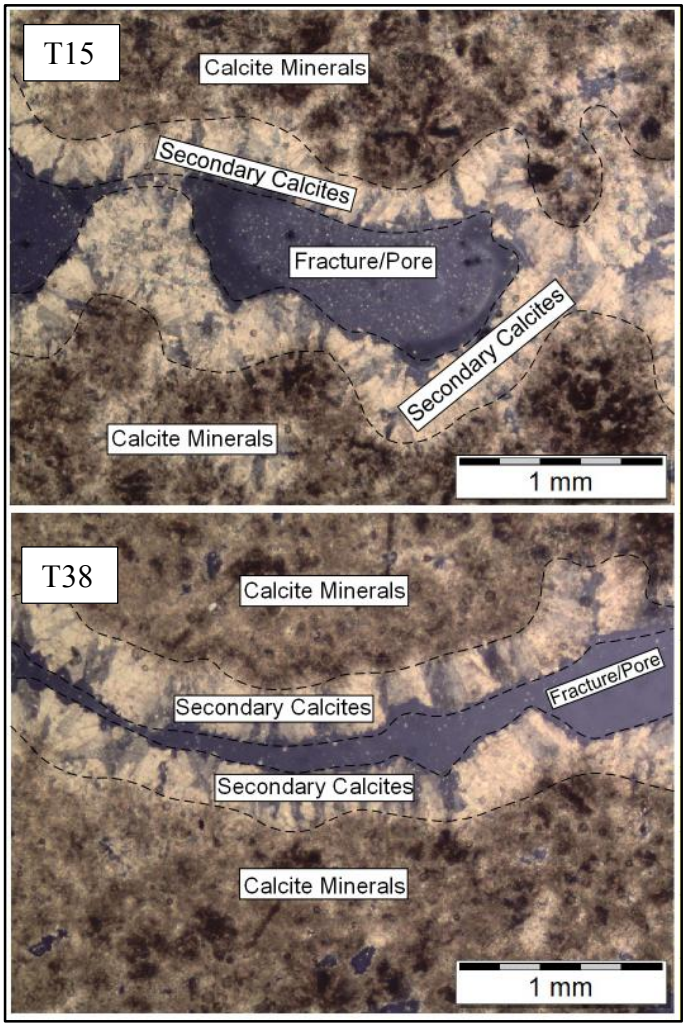


Figure 41: Optical microscope images (4X magnification) of biological mortar applied test stones, T15 and T38

3.3.2.3. Cross Section Analyses by Stereomicroscopy

To observe the contact relations and physical properties of the biological mortar and travertine rock, two types of samples were prepared for examination under stereomicroscope. $T27_{M1}$ and $T27_{M2}$ samples were prepared by molding mortar with a fragment of stone and $T29_{F1}$ and $T29_{F2}$ samples were prepared by applying mortar onto a fragment of stone.

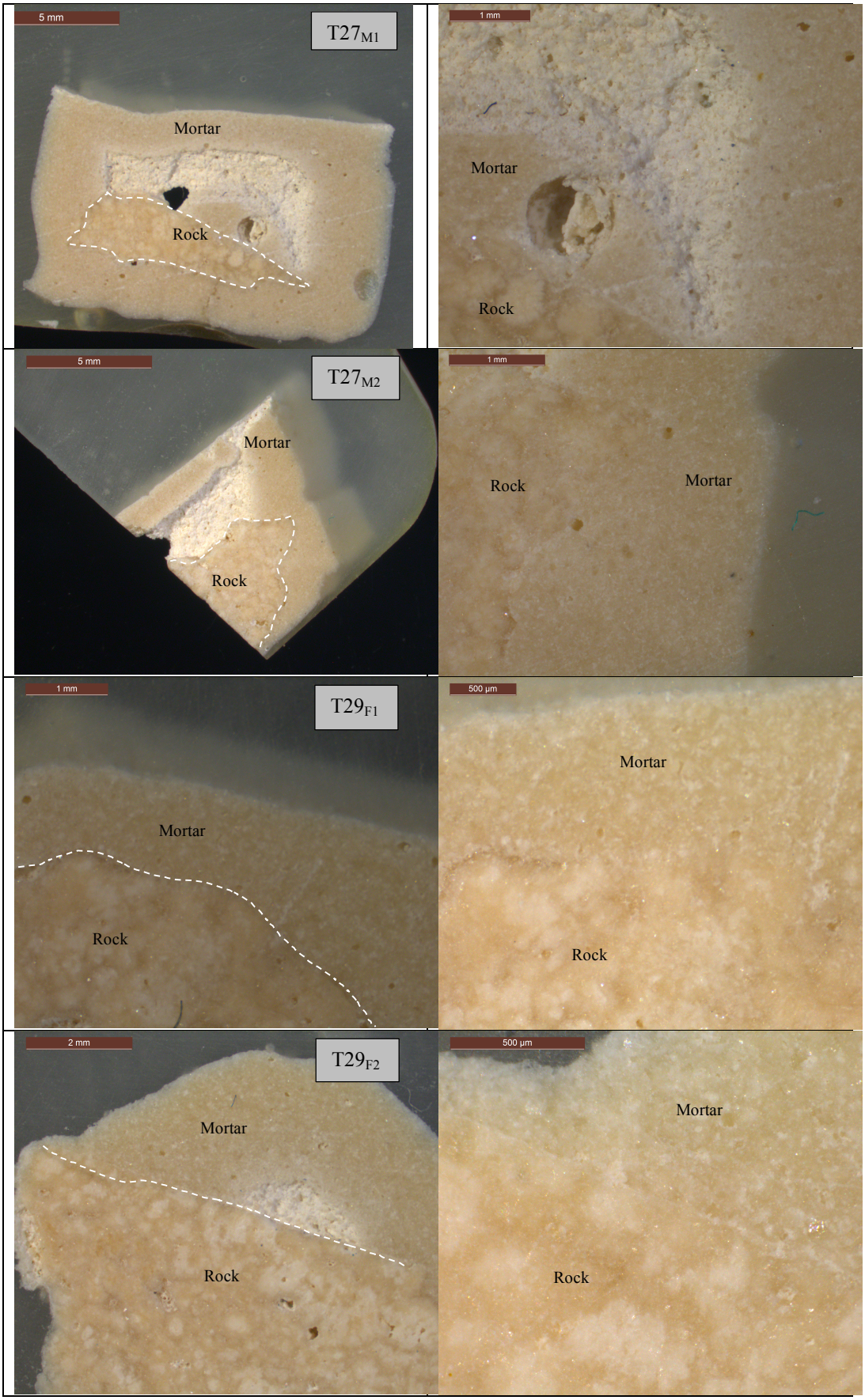


Figure 42:Stereomicroscope images of $T27_{M1}$, $T27_{M2}$, $T29_{F1}$, $T29_{F2}$

After curing for 28 days in room temperature and open-air conditions, cross sections of samples were prepared and observed with stereomicroscope.

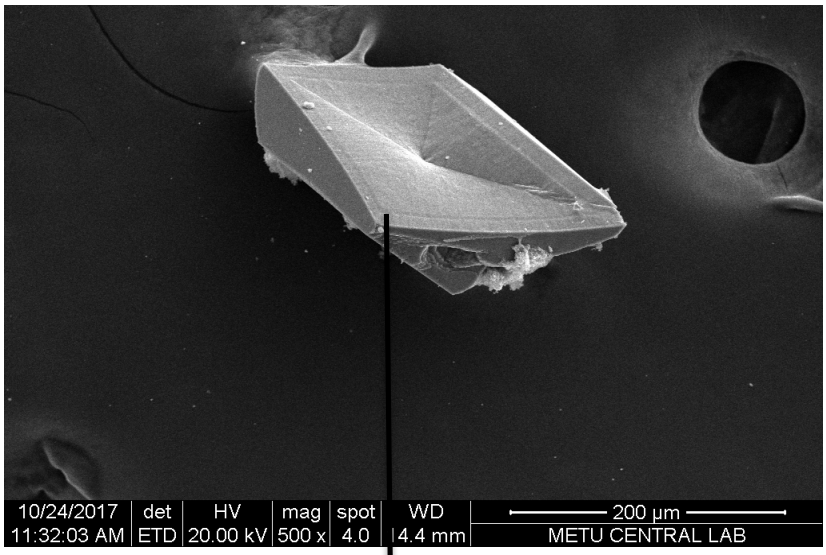
Based on the images, independent of where it was cured, it is seen that there is a continuous and efficient contact between the rock and biological mortar throughout the contact area in all samples. (Figure 42).

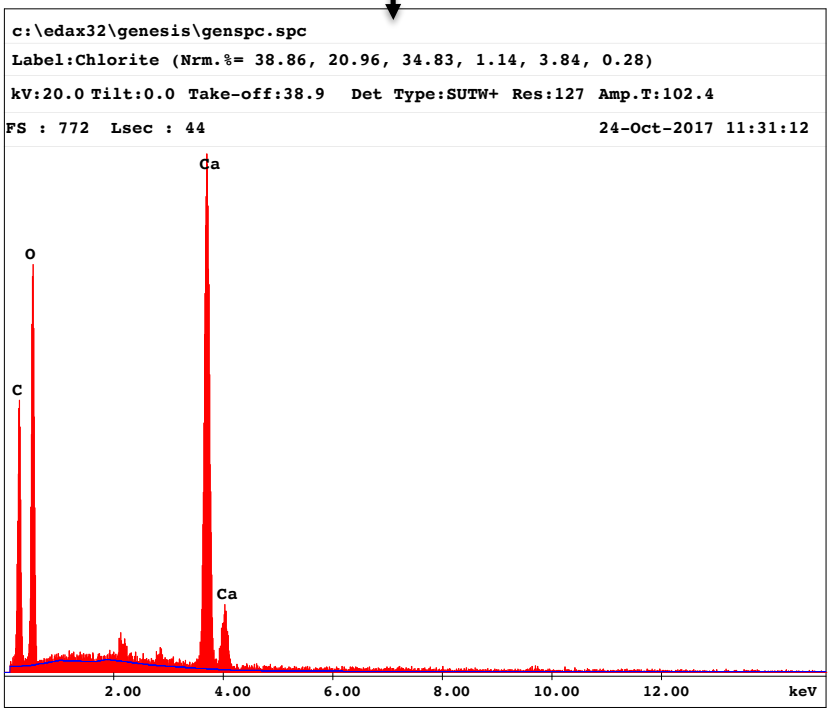
3.3.2.4. Scanning Electron Microscopy (SEM) Analyses Coupled with Energy Dispersive X-Ray (EDX) Analyzer

Precipitates in B-4 liquid medium were analyzed by SEM-EDX. It was found that crystals had a chemical formula as "$CaCO_3$" and were in rhombohedral form (Figure 43).

SEM-EDX analysis of biological mortar both as cubes and in cracks of stones revealed the calcified embedded bodies of *B. cereus* in mortar material. When EDX analyses was conducted from the zone where dead bacterial bodies (endospore) densely existing, chemical composition was determined as "$CaCO_3$" (Figure 45 and Figure 47).

On the other hand, mortar mixture without bacterial sediment and non-applied stone surface were examined by SEM-EDX analysis as a control. It was found that there wasn't any secondary calcite formation due to bacterial activities as expected (Figure 44 and Figure 46).



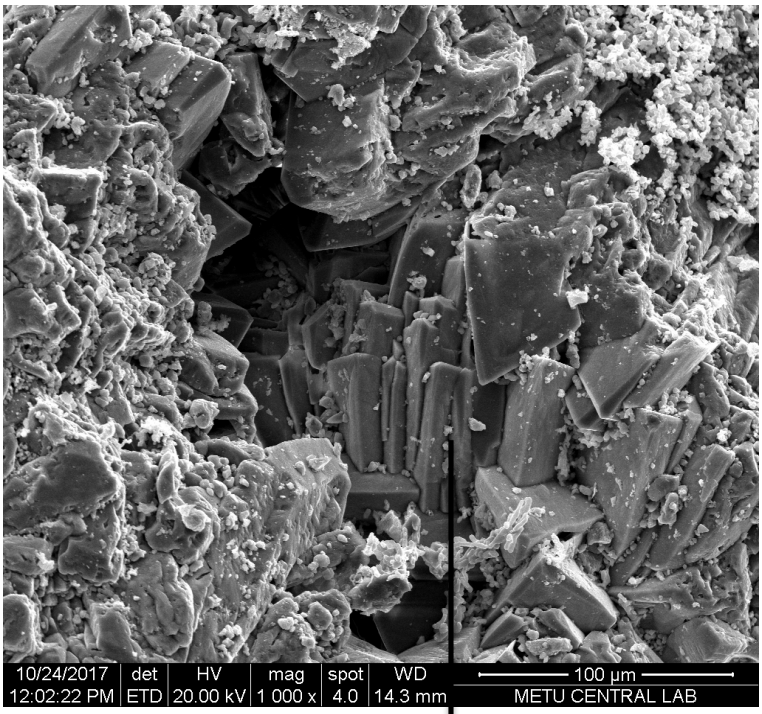


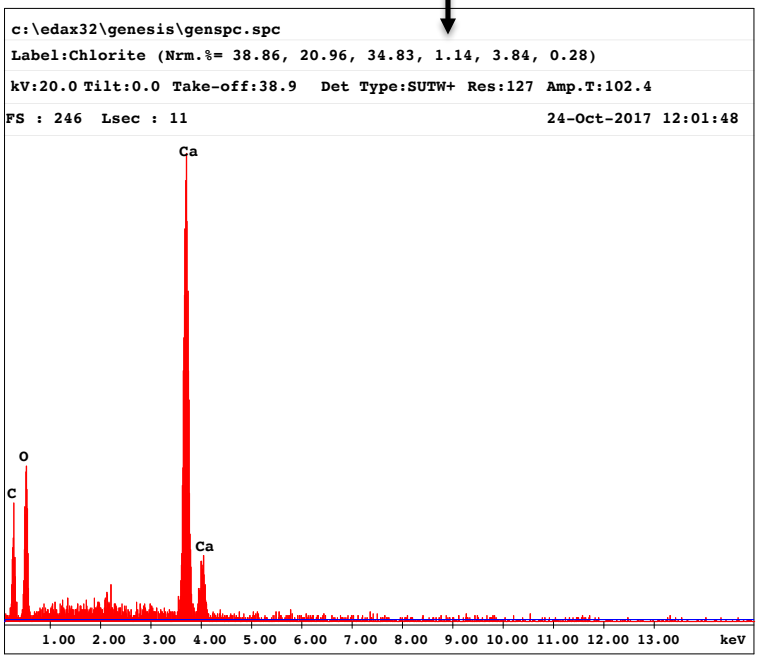
EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	28.37	39.97	0.1391	1.0274	0.4769	1.0007
O K	46.89	49.59	0.0830	1.0102	0.1752	1.0000
CaK	24.74	10.44	0.2365	0.9393	1.0178	1.0000
Total	100.00	100.00				

Element	Net Inte.	Bkgd Inte.	Inte. Error	P/B
C K	41.98	1.20	2.37	34.93
O K	64.42	1.47	1.90	43.85
CaK	146.89	1.34	1.24	109.98

Figure 43:SEM-EDX analysis of calcite crystal formed by B. cereus in B-4 liquid medium



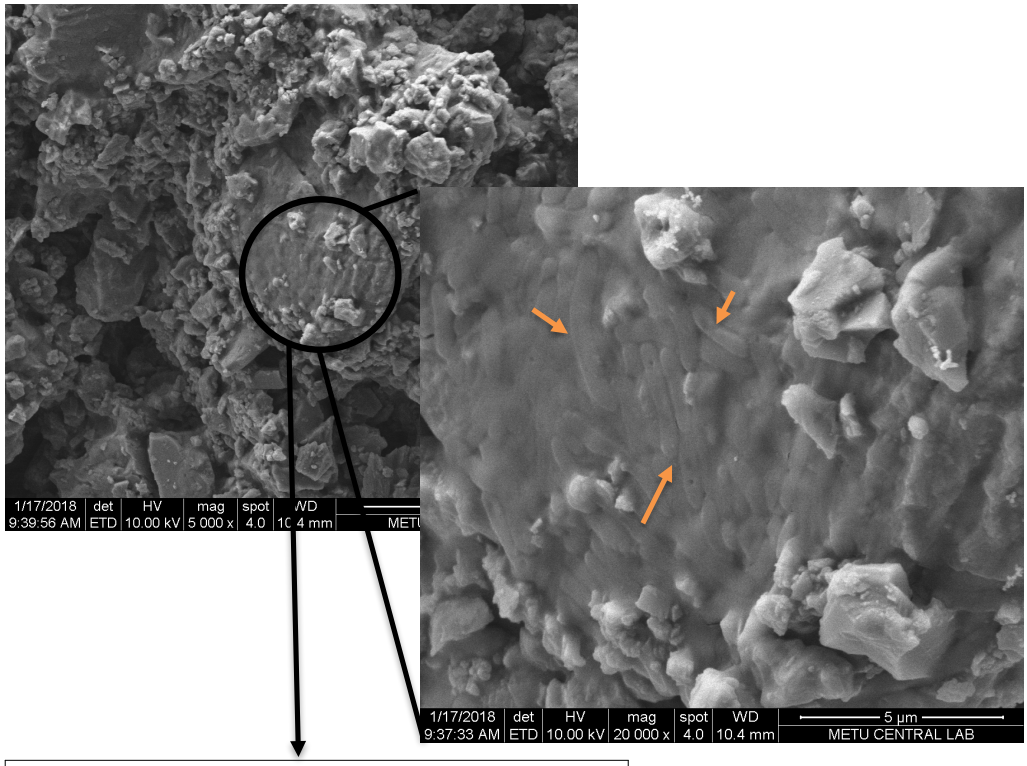


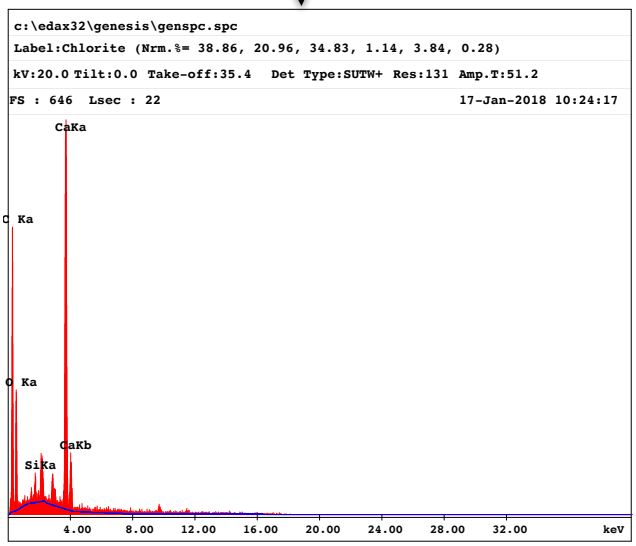
EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	19.97	31.97	0.0927	1.0385	0.4469	1.0008
O K	41.03	49.32	0.0602	1.0210	0.1437	1.0001
CaK	39.00	18.71	0.3756	0.9502	1.0133	1.0000
Total	100.00	100.00				

Element	Net Inte.	Bkgd Inte.	Inte. Error	P/B
C K	20.99	0.54	6.70	39.00
O K	35.08	0.54	5.13	65.17
CaK	175.20	0.99	2.28	177.55

Figure 44: SEM-EDX analysis of non-applied test stone (T14)



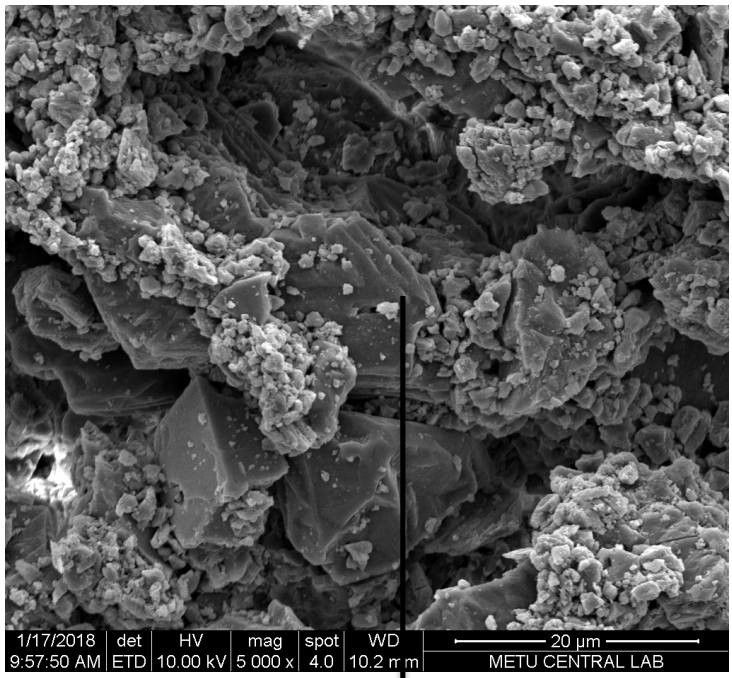


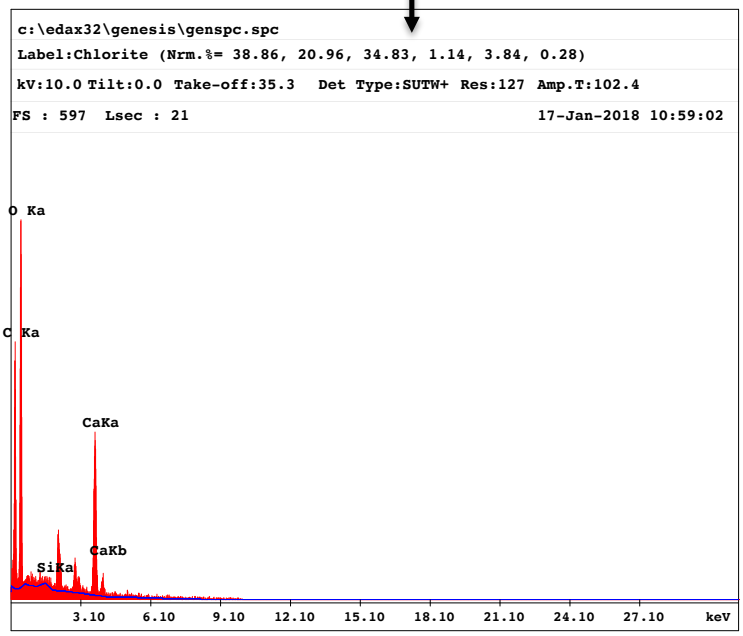
EDAX ZAF Quantification (Standardless)
Element Normalized
SEC Table : Default

Element	Wt %	At %	K-Ratio	Z	A	F
C K	42.88	59.02	0.2187	1.0275	0.4961	1.0004
O K	27.80	28.72	0.0381	1.0103	0.1355	1.0000
SiK	0.95	0.56	0.0074	0.9685	0.7968	1.0055
CaK	28.37	11.70	0.2724	0.9394	1.0218	1.0000
Total	100.00	100.00				

Element	Net Inte.	Bkgd Inte.	Inte. Error	P/B
C K	101.94	1.17	2.12	87.23
O K	46.75	1.75	3.22	26.67
SiK	12.05	7.91	9.29	1.52
CaK	278.32	4.67	1.29	59.54

Figure 45:SEM-EDX analysis of biological mortar after curing for 28 days. Orange colored arrows show embedded bacterial bodies





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

Element	Wt %	At %	K-Ratio	Z	A	F
C K	22.64	35.11	0.1541	1.0502	0.6478	1.0007
O K	41.24	48.03	0.1336	1.0265	0.3157	1.0000
SiK	0.37	0.24	0.0033	0.9670	0.9179	1.0035
CaK	35.75	16.62	0.3324	0.9256	1.0044	1.0000
Total	100.00	100.00				

Element	Net Inte.	Bkgd Inte.	Inte. Error	P/B
C K	68.00	3.96	2.77	17.15
O K	111.00	4.34	2.13	25.59
SiK	2.15	6.76	39.85	0.32
CaK	83.86	2.85	2.44	29.48

Figure 46:SEM-EDX analysis of mortar mixture without bacteria

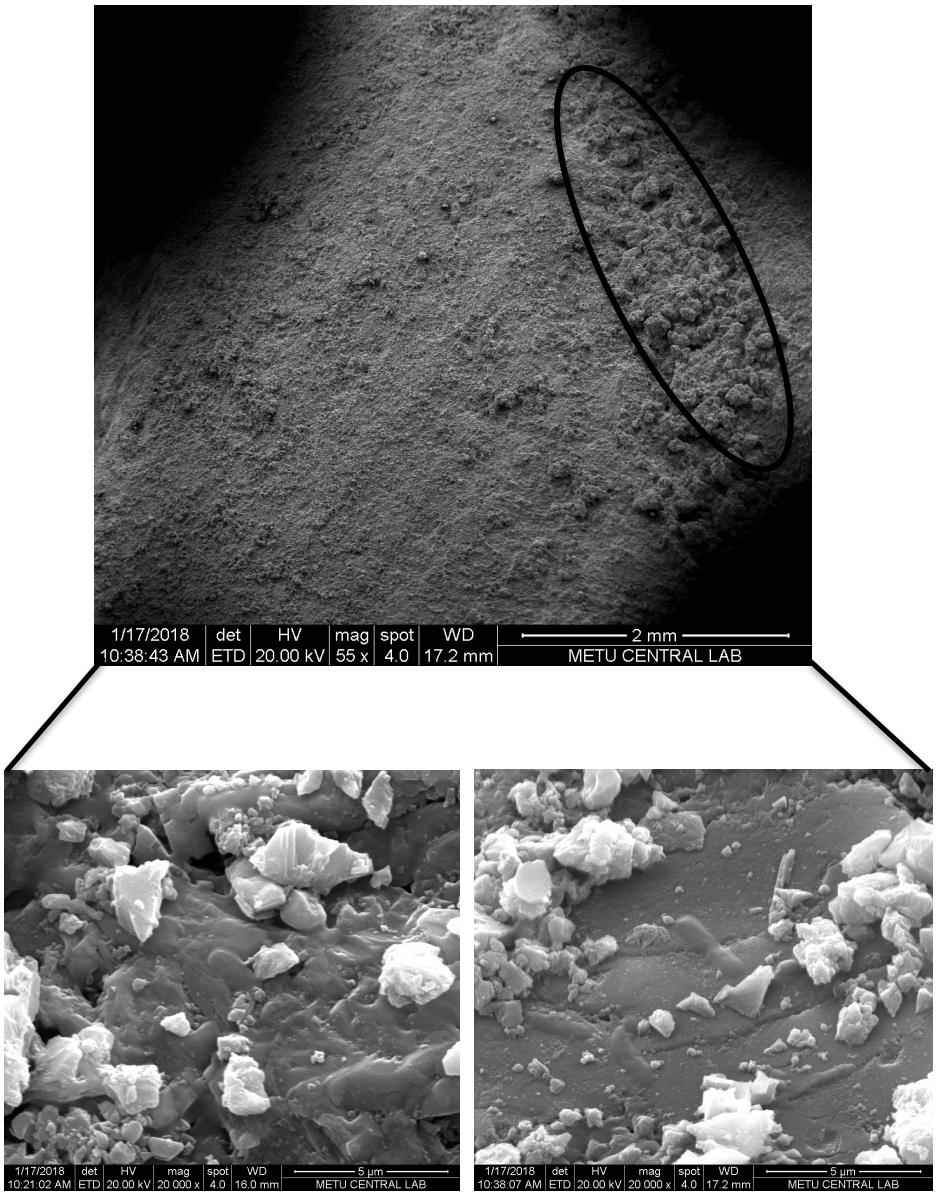


Figure 47: SEM-EDX analysis of biological mortar applied to crack of test stone (T14)

3.3.3. Other Evaluation Criteria

Several evaluation criteria were investigated both for biological mortar and its application to test stones 5 days after curing. When the optimum concentration of mortar components was obtained, resultant table was as follows;

Table 30: Yes/No analyses conducted based on other evaluation criteria

Parameters	Biological mortar cubes		Biological mortar application	
	Yes/No	Additional comment if any	Yes/No	Additional comment if any
Cohesive consistency	Yes		Yes	
Crack formation	No		No	
Smell	No	Only in the beginning of curing period.	No	Only in the beginning of curing period.
Fungal growth	No		No	
Excess of liquid	No		No	
Material integrity	Yes		Yes	
Disintegration	No		No	

3.4. Feasibility Study

To determine the practicability, applicability and usability of the developed biological mortar, a feasibility study was conducted considering technical, economical and timing aspects.

In relation with the assessment of technical feasibility, an instrument list used in this study was generated as below showing the capacity of the institute.

- Scanning electron microscope
- X-ray diffraction instrument
- Biological safety cabinet
- Shaking incubator
- Low temperature incubator
- Medical Refrigerator (+4)
- UV/VIS Spectrophotometer
- Ultrasonic pulse velocity tester
- Centrifuge
- Light microscope
- Stereomicroscope

Regarding economic feasibility, as previously mentioned, cost analysis of biological mortar application was conducted based on the cost of each component and the quantity of the mortar applied (Table 31).

Table 31: Cost analysis of biological mortar and its application[1]

Biological mortar	Approximate Costs	Cost Analysis of a Case Study
Bacterial sediment	Field study Laboratory equipment: 78.00 $ Staff: 156.00 $ Isolation Equipment: 156.00 $ Identification MALDI-TOF technique: 1,250.00 $ Selection Laboratory equipment: 156.00 $	If biological mortar is prepared in specified amounts (See Chapter 2) If you are starting from the very beginning: Field study+ Isolation+ Identification + Selection + Laboratory equipment + Medium ingredients = 1,000.00 $ If you have isolated strain: 3 g bacteria+ 3 g nutritive medium+15 g stone powder = 30.00 $
Nutritive medium	Medium ingredients: 600.00 $	
Stone powder	NA	

Based on a case study, 5 g of biological mortar was applied to micro-cracks having a total volume 2142 mm^3 (Table 32). Considering the cost of 21 g biological mortar is about 30.00 $ (Table 31), unit cost of the mortar was calculated as 0.0033 $ /mm^3.

[1] Calculated by Indicative Exchange Rates Announced at 15:30 on 09/18/2018 by the Central Bank of Turkey

Table 32: Calculation of biological mortar applied volume in test stones

Sample	Biological Mortar Applied Volume
T2	66 mm^3
T5	38 mm^3
T6	78 mm^3
T36	132 mm^3
T37	264 mm^3
T38	164 mm^3
T34	442 mm^3
T35	288 mm^3
T36	218 mm^3
T14	36 mm^3
T20	52 mm^3
T9	202 mm^3
T11	6 mm^3
T33	156 mm^3
Total Volume	2142 mm^3

On the other hand, total cost of the staff planned to work in the study is calculated based on the training cost of the conservation scientist and working days of restorer in the field (Table 33).

Table 33: Cost analysis of staff[2]

Staff	Cost analysis
Conservation Scientist	Training of restorer: 250.00 $ / day Biological mortar preparation: 250.00 $ / day 1 working day = 9 hours 9 hours = 63 g biological mortar
Restorer	Restoration works: 250.00 $ / day 1 working day = 9 hours 9 hours = 6426 mm^3 applied mortar volume
Total	750.00 $ / day

The assessment of timing feasibility indeed directly related with the working days of the restorer and conservation scientist. In addition, for biological mortar applications on-site, the recommended season has a mild climate such as minimum daily temperature as 15 °C, daytime temperature difference about 10-15 °C, low rain and little wind with an average level of humidity.

[2] Calculated by Indicative Exchange Rates Announced at 15:30 on 09/18/2018 by the Central Bank of Turkey

CHAPTER 4

DISCUSSION

In this chapter, the process of biological mortar development, characteristics of test stones, and performance measurement of repair material were discussed based on the experimental results of this study.

4.1. Development of Biological Mortar

Process of generating biological mortar by optimization of the mortar components and bringing these components together at appropriate concentrations were discussed in detail based on the outcomes of the related experiments.

Isolation and Identification of Calcifying Bacteria

Biological mortar has three main components as stone powder, bacterial sediment and feeding solution. These three components were needed to be optimized to achieve a usable and applicable mortar.

In Pamukkale travertines region, samples from various thermal spring water sources were taken and analyzed for their bacterial content. As previous research suggested, *B. cereus* had been selected as the target bacterial species due its high calcite precipitation capacity (Castanier et al., 1999; Le Metayer-Levrel et al., 1999b, 1999b)

In addition to the feature of calcium carbonate precipitation, *B. cereus* was preferred due to number of reasons such as their capability to be used industrially, not being

harmful to human health and environment and being adaptable to harsh conditions (Bibi et al., 2018; Orial et al., 2003b).
Bacteria from collected water samples were isolated using standard filtration technique followed by culturing on LB solid medium and selected colonies were identified by MALDI-TOF technique. At the end we were able to isolate an environmental *B. cereus* strain.

Based on the objective of the study, isolation process was designed for obtaining culturable bacteria that are facultative anaerobes. Therefore, bacterium that needs special growth medium contents might not be cultured during isolation process from different thermal spring water sources. Since MALDI-TOF MS instruments library mostly involve clinical references and due to the lack of reference strains for environmental isolates, all isolates couldn't be identified. Besides, the presence of viable but non-culturable bacteria is another issue that needs to be taken into account (Asangba, 2015). Considering these parameters, the existence of unidentified bacterium during identification studies was an expected result (Table 12). Eventually, making an assumption about the entire microbial community in thermal spring water sources might not be appropriate, as it was not the main purpose this study.

In collected water samples, the mixture of cultured isolates was from two phyla; Proteobacteria and Firmicutes that belong to moderately thermophilic population. It is seen that these cultured isolates were dominated by species belonging to Proteobacteria (Table 14). As mentioned above, although the distribution of bacterial population didn't represent the entire microbial community, divisional determinations were similar with the previous studies conducted on karstic environments. (Banks et al., 2010; Fouke, 2011; Fouke et al., 2003; Okyay and Rodrigues, 2015).

More specifically, in the studies of Okyay and Rodrigues (2015), the samples were collected from ponds in Pamukale travertines located in Denizli and a pond in the cave located in Mexico. Based on 16S rRNA analysis, six different classes (two different phyla) were determined as *Bacilli*, *Sphingobacteria, Flavobacteria* and α-, β-, and γ-*Proteobacteria.* And, contrary to the present study, Firmicutes was observed as the

dominant phyla. This could be the result of the difference in sampling locations where temperature was around 28°C. And, in relation with this, difference in dominancy of phyla might be explained by the temperature preferences of isolates depending on their growth characteristics.

Of the 16 isolates, ~ 44% were able to precipitate calcium carbonate and only two of the 7, named *Bacillus cereus* and *Pseudomonas fluorescens* were used in previous conservation studies (Table 15). Besides, most of them might cause infections in humans which might explain why only these two species were preferred in previous conservation studies.

The survival of microorganisms is largely dependent on physical and chemical conditions of (pH, temperature, availability of nutrient) thermal spring water (Fouke, 2011). Based on the data recorded during field studies, temperature and pH values of different thermal spring water sources were found to be close to each other varying in the range of 33.5-34.6°C and 5.85- 6.01, respectively. Only in the spring water outlet point the temperature decreased to 30.6°C without a change in usual pH value. Major cations were Ca and Mg, while major anions were HCO_3 and SO_4 in thermal spring waters (Akan and Simsek, 1997).

Based on Table 13 that linked environmental isolates with their sampling locations, there was not any distinct pattern regarding the phyla of the isolates changing with type of water source, except the dominancy of Proteobacteria. In the studies of Asangba (2015), Proteobacteria were dominated in active travertine system, while Firmicutes were dominated in the Pleistocene-aged travertine.

However, in the present study, the dominancy of Proteobacteria phyla and rare existence of Firmicutes throughout the sampling sources might be explained by two processes which are not limited with; (1) Changes in physical and chemical conditions in the course of conversion of sediment to sedimentary rock, (2) Necessity to resist in changing environmental conditions by microbial population of Firmicutes via producing endospores (Asangba, 2015).

Selecting and Analyzing the Target Bacteria

As a well-known calcifying strain, *B. cereus* was the target bacteria to be used for the biological mortar development in the present study since this bacterium had been tested for its ability to induce precipitation of $CaCO_3$ in several studies particularly in the fields of biomineralization and biological mortar (Castanier et al., 1999; Jimenez-Lopez et al., 2008; Le Metayer-Levrel et al., 1999b; Orial et al., 2003b; Rodriguez-Navarro et al., 2003b).

B-4 (both liquid and solid) and Urea-$CaCl_2$ (solid) were the selected media in this study for the investigation of the crystal type and crystal production capacity of bacteria. Since these mediums have been designed to stimulate nitrogen cycle pathways, both of them are widely used in isolation of calcifying bacteria and to determine calcite production capacity of bacteria (Chalmin et al., 2007; De Muynck et al., 2010b; Marvasi et al., 2012; Park, 2013; Zamarreno et al., 2009). It is important to point out that other growth media, without specific Ca^{+}, nitrogen and carbon sources, would not allow to observe biogenic calcite formations depending on the characteristic metabolisms of the bacterial isolated from thermal spring waters and cave environments.

More specifically, B-4 medium includes calcium acetate as an energy and Ca^{2+} source to be used by the bacteria to produce calcium carbonate and glucose to speed up the reaction as a carbon source (Cacchio et al., 2012). Moreover, this medium has a buffering capacity due to its high protein content with yeast extract that supports or prevent formation of the crystals based on the pH of the environment (Marvasi et al., 2012).

On the other hand, *B. cereus* is known to produce urease enzyme by the induction of urea in the process of calcite precipitation (Anitha, 2018). In this process ammonia (NH_3) and carbonic acid (H_2CO_3) are produced as a result of urea hydrolysis. Furthermore, carbonate ions (CO_3^2) are formed as a result of dissociation of the carbonic acid. Then, following to carbonate ions reaction with calcium ions, calcium carbonate is precipitated (Bibi et al., 2018). Due to this reason, mechanism of CaCO3

production could be directly related with the hydrolysis of urea. Based on these facts, Urea-$CaCl_2$ medium was also preferred to determine the calcite production ability and capacity of *B. cereus*.

SEM-EDX analysis showed that *B. cereus* produced large calcium carbonate crystals in B-4 liquid medium (Figure 27). Moreover, optical microscope images presented calcite and aragonite forms that are most common polymorphs of calcium carbonate crystal, in a drop of B-4 liquid medium (Figure 26). In relation with this, the dominancy of calcite could be explained by its more stable structure than aragonite. Therefore the crystal tends to be in calcite form in the medium as well as in nature (Dai et al., 2017).

Optical microscope images in B-4 agar medium and naked eye observations in Urea-$CaCl_2$ medium clearly showed calcite precipitation both in the surrounding of (Figure 29) and within parent colony (Figure 28).

Obtained SEM and optical microscope images (Figure 26, Figure 27, Figure 29), although not clear, support three hypotheses regarding to the role of bacteria in precipitation of calcium carbonate; (1) mineralization is the by-product of bacterial metabolism in passive processes which involves either autotrophic and heterotrophic pathways. In the course of these passive processes, metabolic reactions increase the pH of the environment which leads bicarbonate-carbonate equilibrium to proceed in the direction of more carbonate production. Then, this will be followed by the $CaCO_3$ precipitation if Ca^{2+} cation is available in the environment (Castanier et al., 1999), (2) in the presence of Ca^{2+} cation, nucleation occurs in the cell membrane of bacteria either by ion exchanges through membrane or adsorbing cations as Ca^{2+} by negatively charged cell wall structure (Castanier et al., 1999), (3) extracellular polymeric substances (EPS) play critical role in calcium carbonate precipitation either by attracting calcium ions or inducing different polymorphs of $CaCO_3$ by specific proteins in their structure (Kawaguchi and Decho, 2002).

Preparation of Bacterial Sediment (Binder)

Optimum incubation time, bacterial concentration and biomass were the important parameters for efficient bacterial sediment and therefore biological mortar.

Based on growth curve of *B. cereus* the hours between 32nd and 44th, which is just before the stationary phase, were determined as optimum period for bacteria yielding since bacterial deaths were seen to start after 44th hour. Indeed, bacterial sediment prepared with the bacteria in the death phase, neither crystal formations nor bacterial bodies were observed when analyzed by SEM-EDX.
On the other hand, based on values of CFU measurements, the concentration of bacterial cell at the optimum hours was about 10^8 CFU/ml which is the required value for the process of calcium carbonate precipitation (Le Metayer-Levrel et al., 1999b). Indeed, some trials were also conducted with lower concentrations of bacterial cell but the results were not promising based on SEM-EDX analysis and other evaluation criteria.

The produced bacterial sediment in this study was 3 g/L using conventional 2 L flasks on regular benchtop shakers. This was found to be sufficient for mortar applications and further analyses. In others studies like Orial (2003), researchers could obtain 25 g/L bacterial sediment using a bioreactor. Although satisfactory results were obtained in relation with the calcium carbonate production in mortar with the defined amounts of bacteria, yielding much bacteria might increase the calcification activities in mortar which in turn improve the strength of the biological mortar. Therefore, increase in strength could support the self-healing ability of mortar when exposed to heavy rains. In other words, if a biological mortar composed of more concentrated bacterial sediment, quantity of calcium carbonate production increases which keeps the aggregate of mortar together preventing from disintegration.

Preparation of Nutritive Medium (Water Source)

Since the main focus of this study was to investigate whether a biological mortar could be developed from an environmental isolate, we used an already defined nutritive

medium for its well-known properties in stimulating the production of calcium carbonate (Orial et al., 2003b). This medium had been originally generated to activate passive calcium carbonate precipitation by nitrogen cycle pathways. Considering the possible pathways of nitrogen cycle, peptone & yeast extract had been added for oxidative deamination of amino acids in aerobiosis and potassium nitrate for the dissimilatory reduction of nitrate in anaerobiosis or microaerophily to the medium. Furthermore, fungicide in the medium prevents contamination of fungi either from stone or air (Orial et al., 2003b).

Based on the results of microstructural analyses and other evaluation criteria, content and concentration of the nutritive medium was found to be satisfactory for stimulating carbonatogenic pathways of bacteria. However, a specific activator, known to stimulate nitrogen cycle pathways, might increase the calcite formation, and therefore strength of mortar.

Preparation of Stone Powder (Aggregate)

While adjusting the grain size of stone powder, gap size between grains is important for bacteria in terms of providing enough space for filling with calcium carbonate production and reaching of nutritive medium to the core of biological mortar (Orial et al., 2003b).

As previously mentioned, the present study was focused on developing a biological mortar with a calcifying bacterium isolated from environment. Therefore, at this stage, a granulometry between 38-150 μm was preferred since it was close to the optimum granulometry range known to work well in the previous studies (Orial et al., 2003b).

There were two critical points as quarry of stone and granulometry of stone powder in preparation of aggregate and based on the results of examination methods, it was found that preferred granulometry range was appropriate for bacterial survival and calcification. In the studies of Orial et al., (2003), granulometries of 40, 60, 250 and 400 μm were tested in mortar mixture. Based on several parameters such as optimum space for bacteria to produce calcite and bind the aggregates, and penetration of

nutritive medium to the entire mortar mixture, as well, 40 and 160 μm were determined as the best granulometry for biological mortar.

Preparation of Biological Mortar

Optimization of mortar components performed simultaneously with the biological mortar preparation process like gathering together all components and testing for their best. Therefore, weight optimization of the components in the mortar mixture already conducted several times during preparation of each mortar component.

The best proportions of the ingredients were determined as X g bacterial sediment, X g nutritive solution, and 5X g stone powder depending on the results of microstructural analyses and other evaluation criteria. When the stone powder proportion was higher than 5X g, excess of liquid was observed, but when it was lower than 5X g, micro-cracks were observed on the surface of the biological mortar.

Considering the final proportions of biological mortar mixture in the studies of Orial (2003) which is as X g bacterial sediment, X g nutritive solution, and 2X g stone powder, proportional difference in mortar mixture from the present study might be due to type of aggregate which was obtained from stones having rather low porosity which might need less water while generating biological mortar.

4.2. Characteristics of Test Stones and Performance Measurement of Repair Material

Results of examination methods either for test stones or repair material together with its application were discussed in detail to show the characteristics of test stones & repair material and performance of the application, as well.

4.2.1. Physical and Physico-Mechanical Properties

Properties of test stones, biological mortar with its application and changes in these properties before and after artificial salt crystallization tests were discussed based on the results of conducted experiments.

Effective Porosity and Density

Porosity and density are the basic properties that affect the strength of rocks. Pore spaces in the rock specimens can decrease the strength while increase the susceptibility of rock to deformations. In other words, little changes in the pore spaces or voids can significantly affect the mechanical strength and properties of the rocks. Since many rocks display density properties close to each other, values of porosity and density also show a significant relationship with each other.

Besides, pore spaces in the rock also affect the unit weight of the rock. Therefore, although the values obtained from the porosity tests give information about properties of the rocks, the assessment together with the density values would be more accurate for an integrative definition.

Travertine stones from Kocabaş (Denizli) quarry had moderate density and porosity values according to the classification of Anon (1977) (Table 17, Table 18, Figure 31). Effective porosity of the travertine cubes was found to be changing between 6,49 % to 10,65 % with an average value 8,31 ± 0.95 %. On the other hand, density of the travertine cubes was found to be changing between 2,34 g/cm^3 to 2,45 g/cm^3 with an average value 2,42 ± 0.02 g/cm^3.

When the porosity and density values of these samples are evaluated together, there is a consistent relationship with each other that the porosity values up to 10 percent also bring moderate density values.

After 30 cycles of artificial salt crystallization effective porosity and density measurements were performed again. When the average values are considered, the average porosity value before the sodium sulphate experiment was 7,85 ± 0.68 %, whereas it was determined as 7,26 ± 0.69 % after the aging experiment. Similarly, the average density value of 2,42 ± 0.02 g/cm^3 before Sodium Sulfate experiment was determined to be 2,38 ± 0.02 g/cm^3 after the aging experiment (Figure 32, Table 19). Based on these values, slight decrease in average density value represents the degradation, which is a natural process, of the samples exposed to salt crystallization cycles. On the other hand, decrease in average porosity values, which is unexpected result, might be explained by the fact that salt crystals still exist in the pores due to insufficient washing at the end of salt crystallization test.

Ultrasonic Pulse Velocity (UPV)

This experiment provides information about the state of structural degradation and discontinuities in the rocks based on the propagation velocity of sound in solid, liquid and gaseous media ($V_s > V_l > V_g$). By means of the sonic velocity experiment, information about the elasticity, anisotropy state, porosity and decomposition state of the rocks are obtained by means of the sonic velocity experiment. In addition to this information, this test is also used with the purpose of observing the decomposition and changes in the rock during cyclic experiments as aging.

When the dry and saturated sonic velocity values of the travertine cubes are compared, it is seen that the dry sonic velocity values (3774,68 ± 481,63 m/s) are lower than the values in the saturated state (4896,37 ± 136,29 m/s) (Table 20, Table 21, Figure 33). Since propagation velocity of sound acts like $V_{solid} > V_{liquid} > V_{gaseous,}$ this can be interpreted as there was no structural alteration, softening, or degradation within the test cubes due to the interaction of water with the pore spaces or voids.

On the other hand, average value of UPV measurements for dry test stones was decreased 35,98% while 25.29% decrease was observed for saturated test stones after artificial aging cycles by sodium sulfate (Table 22, Table 23, Figure 34, Figure 35).

This decrease in UPV values is an expected situation that resulted from the deterioration of the test cubes. Besides, relatively lower decrease of UPV values in saturated test stones could be explained by the water that fills the voids.

In addition to test cubes, this test was also performed in biological mortar cubes (2.2 cm x 2.2 cm x 2.2 cm) 7^{th}, 14^{th} and 28^{th} days after molding. According to the test results, it was determined that overall UPV values of biological mortar cubes are increasing throughout the weeks except minor differences (Table 24). The reason of increase in UPV value being so slight might be the result of evaporation of water in the biological mortar and filling of voids by the calcification activities of bacteria concurrently.

Uniaxial Compressive Strength (UCS)

UCS tests were conducted on dry and water saturated test stones before and after artificial salt crystallization cycles. Before salt crystallization cycles, the average strength of dry and saturated test stones was determined as 60,41 ± 11,74 MPa and 55,61 ± 10,69 MPa, respectively (Table 25). But after the salt crystallization cycles, UCS values of dry and saturated test stones were determined as 48.57 ± 14,72 MPa and 44.66 ± 14.66 MPa, respectively (Table 27). Considering the strength values before and after sodium sulfate test, the decrease in UCS values was an expected situation that resulted from the deterioration of the test cubes.

According to ISRM (1981), both dry and saturated strength of the rocks tested in this study are in the category of "strong rock" before and after salt crystallization cycles when standard deviation factor is considered (Table 26).

In addition, significant differences in UCS values of dry and water saturated test stones observed either before or after salt crystallization cycles could be explained by heterogeneity (porosity and microfracture differences) of the samples (Table 25, Table 27).

Point Load Strength Index (Is)

This test was conducted in order to obtain information about impact of mortar on rock in terms of strength. Considering the average UCS value of test stones (48.57 MPa), biological mortar strength determined as 1,2 MPa (very weak) which does not make a significant contribution to the rock strength (Table 28).

Colorimetric measurements

Even ΔE* values presented higher differences when target color was adjusted to Ref 2, color measurements conducted based on Ref 1 showed that produced repair material is suitable (ΔE* < 5) to be used in conservation interventions (Uni Normal-43/93). Because, high values of ΔE* might be the result of residues of salt crystals and deteriorated zones on stone surfaces of artificially aged test stones.

4.2.2. Microstructural Properties

Results of XRD, SEM-EDX, thin section and cross section analysis, conducted on biological mortar, biological mortar applied samples and test stones before and after artificial salt crystallization tests, were discussed comprehensively.

X-Ray Diffraction (XRD) Analysis

Powders of sound & aged travertine stone and biological mortar were analyzed by XRD analysis and all these samples showed only characteristic peak of calcite ($CaCO_3$) (d value: 3.02^{o}A and 3.03^{o}A) as an indication of the main mineral (Figure 37, Figure 38, Figure 39). Therefore, based on XRD diffractograms, samples used in this study were found to be homogenous.

Thin Section Analyses by Optical Microscopy

Sound (T7) and artificially aged (T42) test stones were observed under optical microscopy and both of the samples showed same minerology as consisting of calcite minerals homogenously. It was determined that some organic materials also existing in the stone material. (Figure 40). Thus, information obtained regarding crystalline structure and mineralogical character of test stones were found to be consistent when results of XRD analysis were evaluated together with thin section analysis.

Thin sections of biological mortar applied test stones (T15&T38) were also examined under optical microscope and some secondary calcite minerals were observed throughout the micro-cracks. When thin sections of biological mortar applied test stones (T15&T38) are compared with not applied ones (T7&T42) secondary calcite minerals are observed larger in biological mortar applied samples (Figure 40). This could be evidence of calcification activities of *B. cereus* in biological mortar. However, some further studies are needed to support this conclusion.

Cross Section Analyses by Stereomicroscopy

Cross sections (perpendicular to the sample surface) of samples prepared by molding with a fragment of stone ($T27_{M1}$ & $T27_{M2}$) and applying onto a fragment of stone ($T29_{F1}$ & $T29_{F2}$) were examined under stereomicroscope.

Based on microscopic images, it was observed that a strong bond generated between the grains and matrix of biological mortar. This might be the result of calcium carbonate production of *B. cereus* in biological mortar. Besides showing similar texture, interface of biological mortar and rock surface seem to be continuous and coherent in all samples (Figure 43). In addition, no difference was observed between the samples cured in laboratory and open-air conditions.

Scanning Electron Microscopy (SEM) Analyses Coupled with Energy Dispersive X-Ray (EDX) Analyzer

SEM-EDX analysis was conducted on precipitates in B-4 liquid medium, biological mortar (as cubes and in cracks), mortar mixture without bacterial sediment, and non-applied stone surface.

Rhombohedral crystals were determined when precipitates of B-4 liquid medium analyzed by SEM. Besides, elemental analysis of this crystal by EDX pointed out $CaCO_3$ mineral which might the evidence of calcite formation by *B. cereus*, clearly (Figure 44).

When biological mortar as cubes and applied forms in cracks were analyzed by SEM-EDX, calcium carbonate coated *B. cereus* bodies (endospore) were determined in the matrix, easily. This indicates that *B. cereus* fulfills their role by acting as a binder of stone aggregates in biological mortar (Figure 46 and Figure 48).

Control groups of the present study were designed to include mortar mixture without bacteria and non-applied stone surface and these samples were analyzed by SEM-EDX. Based on the images of SEM and EDX analysis, secondary calcite formation due to bacterial activities didn't observed. Besides, mortar mixture showed a tendency to break apart during sample preparation process for SEM analysis. This might be the the indication of the decrease in strength resulted with the loose bonding in matrix of biological mortar (Figure 45 and Figure 47).

4.2.3. Other Evaluation Criteria

In addition to physical, physico-mechanical and microstructural examination methods, designing a yes/no table was found to be practical and efficient for evaluation of biological mortar and its application. Indeed, this table was also useful for archiving results of biological mortar optimization and biological set-up processes.

The table consisted of several parameters such as crack formation, smell, fungal growth, excess of liquid, material integrity, cohesive consistency and resistance to

disintegration. However, content could be extended based on the needs of performance measurement scope.

When biological mortar was optimized in terms of component and concentration either individually or as a whole, there wasn't any crack formation, fungal growth, excess of liquid and disintegration in generated and applied biological mortar. Moreover, formed material displayed cohesive consistency and strong material integrity.

4.3. Feasibility Study

A feasibility study was conducted to evaluate the potential success of the study considering technical, economical and timing aspects. In relation with technical feasibility it is found that the institute planning to conduct this study must have the listed instruments as a production substructure.

Considering economic feasibility, which have two parameters as product and staff, it is obvious that the study is cost-effective and suitable to be applied in today's conditions.

On the other hand, timing feasibility of the biological mortar was evaluated based on time and period to complete the conservation intervention. Considering the amount of the product produced per day and the volume of the mortar to be applied, it can be definitely stated that the application of biological mortar is a realistic approach that suits one's standard daily work tempo. In addition, determination of the most suitable period for the application on-site is of great importance in terms of functionality of the mortar. The best period is defined as minimum daily temperature about 15 °C, daytime temperature difference about 10-15 °C, low rain and little wind with an average level of humidity.

CHAPTER 5

CONCLUSION & RECOMMENDATIONS

This thesis conducted for the development of biological mortar including an environmental calcifying isolate as a binder.

Based on the results of the tests carried out in the context of this study, following conclusions are drawn in order of sections in methodology.

In collected thermal spring water resources, dominance of Proteobacteria and Firmicutes in the mixture of cultured isolates, are not surprising, given that these phyla are known as moderately thermophiles (facultative thermophiles).

During conducting a pre-study with *B. cereus* to understand its growth pattern characteristics, the concentration of bacterial cell at the optimum hours (between 32nd and 44th) was determined as 10^8 CFU/ml. In addition, 1 L of LB liquid medium with this concentration gave 3 g of bacterial precipitate. Although this amount was satisfactory for mortar applications, further analyses with the use of a bioreactors would definitely increase the end yield. For instance, increase in strength could support the self-healing ability of mortar when exposed to heavy rains. This is significant because during curing period of a trial set in open-air conditions, mortar applied test stones were exposed to very heavy rain and strong wind. This harsh climatic condition resulted with dissolution of mortars in cracks. If calcifying strains were existing high enough to keep the aggregate of mortar together, the damage would be less.

In a parallel study, content and concentration of the nutritive medium and granulometry of stone powder was defined in the manner of stimulating bacterial calcification and ensuring bacterial survival.

The best proportions of the biological mortar components were defined as X g bacterial sediment, X g nutritive solution, and 5X g stone powder based on the results of microstructural analyses and other evaluation criteria. However, as previously mentioned, a more concentrated bacterial sediment might directly affect the calcite production quantity which affects the strength properties of the biological mortar. To have an effective result from this repair material, BM set up and its application procedure given in the chapter of materials and methods should be fully respected.

UPV measurements conducted on biological mortar cubes 7^{th}, 14^{th} and 28^{th} days after molding and overall UPV values displayed a slight increase through this curing period. The reason of increase in UPV value being so slight might be the result of evaporation of water in the biological mortar and filling of voids by the calcification activities of bacteria concurrently.

Point load test was performed in mortar applied test stones in order to determine the contribution of mortar on rocks in terms of strength. Since biological mortar strength determined as 1,2 MPa (very weak), it can be concluded as mortar does not make a significant contribution to the rock strength.

Thin section analysis of sound & artificially aged test stones and biological mortar applied test stones were performed using optical microscopy. Sound & artificially aged test stones showed same minerology with consisting of calcite minerals homogenously. On other hand, thin sections analysis of biological mortar applied test stones displayed secondary calcite mineral formations through micro-cracks which might be the evidence of calcite formations due to the activities of *B. cereus*. However, some further studies as are needed to support this conclusion.

Samples molded with a fragment of stone and applied onto a fragment of stone were examined by stereomicroscope. Microscopic images revealed strong bond generated between the grains and matrix of biological mortar in all type of samples. In addition to rock-mortar texture similarity, interface of biological mortar and rock surface showed continuous and coherent structure in the samples cured in laboratory and open-air conditions.

Precipitates of *B. cereus* inoculated B-4 liquid medium, biological mortar (as cubes and in cracks), mortar mixture without bacterial sediment, and non-applied stone surface were analyzed by SEM-EDX. Precipitates obtained from liquid medium observed as rhombohedral crystals with $CaCO_3$ composition. On the other hand, calcium carbonate coated *B. cereus* bodies (endospore) were determined in biological mortar cubes and from mortar applied cracks of test stones. These findings support the idea of calcite production ability and promoting binding of stone aggregates in biological mortar by *B. cereus*. In SEM-EDX analysis of control groups (mortar mixture without bacterial sediment, and non-applied stone surface), neither embedded bacterial bodies nor secondary calcite formations were observed.

Considering other evaluation criteria, no crack formation, fungal growth, excess of liquid and disintegration were observed in generated and applied biological mortar. Although, formed material displayed strong material integrity and cohesive consistency.

Regarding application of the biological mortar on site, the recommended season has a mild climate such as minimum daily temperature as 15 °C, daytime temperature difference about 10-15 °C, low rain and little wind with an average level of humidity.

Considering biological mortar application in an outdoor environment, all components of mortar should be prepared in laboratory conditions and transfer to the field where conservation intervention planning to be carried out. It is appropriate to mix the components just before the application since biological mortar hardens quickly due to

calcification activities inside the repair material and evaporation of water inside. In addition, if the weather is rainy and/or windy, it is appropriate to protect the biological mortar application by protective cover for 4 weeks until the bad weather conditions passes since estimated carbonatogenesis lasts for 4 weeks.

On the other hand, when biological mortar application is planning to be performed in an indoor environment, area of application should be closed for a while to prevent contamination of biological mortar from humans. The exact period of this closing process is not known but could be determined with further studies.

Future studies

Development of biological mortar, its application to test stones and eventually measurement of the BMs performance could be stated as a pioneering study for medium-highly porous stone repair problems. Therefore, a number of recommendations could be given briefly in order to enhance the efficiency of this repair material and create steps for future work.

To increase the rate and concentration of bacterial calcite precipitation in mortar, some parameters in the experimental procedure could be improved by several approaches. The first of these could be adding specific enzymes or macromolecules having potential to induce nitrogen cycle pathways to the nutritive medium after a detailed research. However, as a prior study, it would be a straight approach to develop a methodology for the measurement of the bacteria reaction rate which is known to be controlled by the content of the media, pH and temperature. The other might be using large fermenters that could yield high quantities of bacteria. Furthermore, using multiple bacterial assembles might also increase the amount of calcite production. Last but not least, the genes related with the mineral production process could be identified and with the adjustment of these genes to bring out large quantities of macromolecules might induce calcite precipitation.

When above mentioned improvement alternatives do not give satisfactory results, a combination of a traditional method and with a biological mortar might be used to reach a biological mortar having desired quality.

If the resistance of biological mortar to heavy rain is increased with the improvement studies of mortar, scientific examination methods (UCS, density & porosity, modulus of elasticity, etc.) to evaluate performance of this repair material could also be broadened which in turn enable the comparison of the biological mortar with the traditional ones, comprehensively. In addition, conducting UPV measurements after BM application to cracks of test stones could give information about the characteristic of this repair material regarding filling of the cracks, gaps/voids.

On the other hand, methods for biogenic calcite determinations and calcite production role and rate of bacteria in travertines could be studied as a sub topic since outputs of these studies would make significant contribution to the current study.

Furthermore, penetration depth of the biological mortar could be studied further to determine the success of mortar in changing depths of conservation interventions.

Since produced mineral by bacteria is $CaCO_3$, biological mortar development and its application could be tried also in other carbonate stones, composed primarily of carbonate minerals, as limestones.

Considering the biological mortar application to the layered surfaces (stones having painting layers), the suitability of the application should be investigated in detail not only for interaction but also for compatibility of mortar with the layered surfaces.

Although *Bacillus cereus* strain used in this study is an environmental isolate which is not harmful to human health, characterization of this bacteria for its pathogenic properties could be investigated comprehensively in future studies.

In conclusion, although the constraints are greater than traditional mortars, the outputs of the study are notable given that biological mortar in conservation field is the first in Turkey and still in development. Considering the context of the conservation interventions, based on the feasibility study of the biological mortar, this repair material is appropriate for remediation of micro-cracks (width < 1mm, depth ≅ 2mm) in qualified historical travertine structures such as sculptures, ornaments, figures, capitals, and elements. Besides, perfectly integrating with the test stones, its application fits the minimum-intervention principle which is commonly accepted approach in conservation.

This thesis presents the outcomes of a multidisciplinary team composed of conservation architects, geological engineers, physicists, biologists majored in microbiology, molecular biology and genetics, therefore it should continue with the contributions of the researchers from these disciplines.

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