USE OF BIOMINERALIZATION IN SELF-HEALING

CEMENT-BASED MATERIALS

A Thesis

by

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ABSTRACT

Factors affecting the durability of concrete structures are generally associated with each other. Due to its brittle nature, concrete can crack when stress is applied. Recent research in the field proposes that it might be possible to develop a smart, cement-based material that can self-heal itself. Self-healing is the ability of concrete to heal the cracks without any external intervention. Self-healing property of concrete can be obtained via different approaches. Use of biomineralization is a novel technique to provide self-healing in cement-based materials. Biomineralization is a biochemical process in which microorganisms stimulate the formation of minerals. In this system, calcium carbonate ($CaCO_3$) is induced by leveraging the metabolic activity of microorganism and self-healing is obtained by sealing of the cracks with $CaCO_3$. A limited number of studies have been conducted to evaluate self-healing by biomineralization as means of recovery in compressive strength. However, there is not enough information regarding the influence of this system on flexural strength. In addition, none of the previous work to date studied the interactions amongst concrete admixtures, biomineralization, and the resultant self-healing ability. At last, the durability of the $CaCO_3$ precipitate under weathering conditions is controversial. The goal of this study was to investigate these abovementioned issues in depth and fill the knowledge gap in the literature. The ultimate goal was to take novel method one-step further into practical use. In this study, the bacteria was introduced to cement paste with its growth media without any additional manipulation such as encapsulation. With this approach, the flexural cracks on mortar surface were sealed with the $CaCO_3$, the permeability of the healed specimen was decreased compared to the cracked samples. However, there was no significant flexural strength regain after crack healing. This was attributed to the soft and brittle nature of the

CaCO$_3$ as well as the weak bonding between the precipitate and the mortar. The precipitate, CaCO$_3$, was found to be durable against rain and freeze-thaw cycles. However, the resistance of the CaCO$_3$ sealant against sunlight was lower than its resistance to other weathering conditions.

ÖZETÇE

Beton yapıların servis ömrünü etkileyen faktörler birbirleriyle genellikle bağlantılıdır. Beton gevrek ve kırılgan doğası yüzünden, gerilmeler altında çatlayabilir. Son yıllarda yapılan araştırmalar kendiliğinden iyileşen çimento esaslı malzemelerin üretiminin mümkün olduğunu göstermiştir. Kendiliğinden iyileşme özelliği, betonun oluşan çatlakları kendiliğinden kapatabilmesidir. Bu amaçla kullanılabilecek yenilikçi yöntemlerden biri biyomineralizasyondur. Biyomineralizasyon için kullanılan metabolik aktiviteleri sonucu ürün olarak kalsiyum karbonat ($CaCO_3$) oluşur ve oluşan ürün/$CaCO_3$ çökeltisinin çatlakları doldurması ile kendiliğinden iyileşme elde edilir. Biyomineralizasyon ile kendiliğinden iyileşmenin basınç dayanımına etkisi ile ilgili yapılmış araştırmalar vardır, ancak eğilme dayanımı üzerindeki etkileri yeterince açıklanmamıştır. Ayrıca, beton üretiminde sıkça kullanılan kimyasal katkı malzemelerinin biyomineralizasyona, dolayısı ile kendiliğinden iyileşme yeteneğine nasıl bir etkisi olduğu bilinmemektedir. Son olarak, kendiliğinden iyileşmeyi sağlayan çökeltini dayanıklılığı da yanıtlanmamış konulardan biridir. Bu projenin amacı, çimento esaslı malzemelerde çatlakların biyomineralizasyon ile sağlanan kendiliğinden iyileşme mekanizmasının daha detaylı bir şekilde incelemektir. Bu projede çimento harcı içine katılacak bakteriler besi yerinde büyütüldükten sonra hiç bir işlem uygulanmadan, çimento ile karıştırılmıştır. Ardından çimento harcı içine karıştırılan bakteriler eğilme altında oluşturulan çatlakları kapatabilmiş, ürün/$CaCO_3$ çatlakları doldurmuş, geçirgenliği azaltmış ancak eğilme dayanımında belirgin bir artış sağlanamamıştır. Bu oluşan ürün/$CaCO_3$'ın çimento harcı ile yaptığı bağın (aderans) zayıflığına ve yumuşak yapısı ile ilişkilendirilmiştir. Çatlakları kapatan ürün/$CaCO_3$'ın yağmur suyu ve donma-çözünme gibi doğal olaylardan etkilenmediği gözlemlenirken, ürün/$CaCO_3$'ın ışığa karşı direncinin diğer doğal olaylara göre daha az olduğu saptanmıştır.

DEDICATION

This thesis is dedicated to my parents.

ACKNOWLEDGEMENT

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Chapter I

Introduction

Concrete is the cheapest and relatively the strongest building material used in construction. It is one of the most used man made material in the world [1]. Although cement-based materials are commonly used, their negative effects on the environment during their production process create problems in terms of sustainable development. The massive production on big scales and transportation result in with a large amount of energy consumption and greenhouse gas emission. It is estimated that 7% of CO_2 emission to the environment is due to cement production [1]. To reduce this emission, one of the actions that could be taken is to extend the service life of the cement-based materials, which could lead to a decrease in cement production.

A concrete structure, which is designed properly, could be inherently strong and durable. However, cracking is inevitable in the cement-based matrix in most cases due to internal and external stresses induced such as shrinkage, excessive loading. The cracks in concrete structures not only decreases the strength of the material but also provide internal access to the aggressive material (e.g. chloride ions), which will decrease the service life of the structure. Generally, there are several commonly used methods for surface cracks such as epoxy injection, coating, sealants, etc. to remediate the cracks[2]. However, above mentioned methods for repair not only require time, but also, they might be inadequate for very narrow microcracks. Still, concrete has a natural mechanism that can remediate the cracks by triggering latent reaction to a certain extent called autogenous healing. So called autogenous self-healing can be triggered by 4 main factors; (1) further hydration of unhydrated cementitious material (2) transported impurities or sedimentation

of fractured pieces clogging the cracks (3) transported impurities or sedimentation of fractured pieces making crack walls to swell (4) $CaCO_3$ formation through the carbonation of calcium hydroxide (CH) [3]. Figure 1.1 illustrates these 4 factors triggering the autogenous healing of concrete cracks.

Physical factors	Chemical factors		Mechanical factors
Swelling of the crack walls	Continued hydration	Calcium carbonate formation	Accumulation of fine particles: Broken off from fracture surface / Orginially in the water
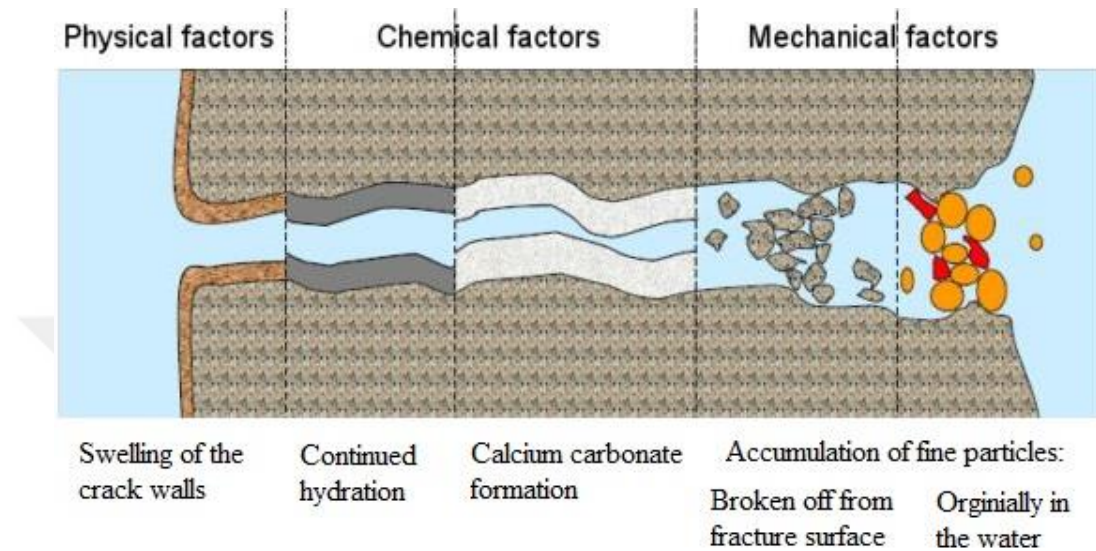
Figure 1.1: Factors triggering the autogenous healing of cracks in concrete [4].

One of the limitations of possible autogenous healing is the crack width. Up-to-date, researchers showed that the autogenous healing ability of cement-based materials could occur in cracks that had a width of 10 μm and 300 μm [5]. This performance of autogenous healing in concrete is variable and hardly predictable. A more systematic analysis of autogenous healing dates back to 1926 and was executed by Glanville, already at that time a distinction was made between self-healing and self-sealing[6]. In the first case, the original strength of the concrete is completely recovered, whereas in the second case cracks was closed but there was not any recovery in strength.

Advanced methods were developed to achieve stable, controllable and predictable self-healing performances. Stimulation and improvement of autogenous healing were one of the strategies for the development of self-healing concrete. Researchers used superabsorbent polymers, shape memory alloys, micro fibers and natural fibers to control

the autogenous healing. Recent studies revealed that microorganisms could be used to trigger self-healing in cement-based materials via biomineralization. Biomineralization is a series of complex bio-chemical reactions in which microorganisms induce mineral precipitation [7]. In this particular case, the microorganisms stimulate the formation of $CaCO_3$, which is also known as Microbial Induced Calcium Carbonate Precipitation (MICP) [7]. There are many bacterial species that can induce biomineralization in nature. However, the main challenge is to find a species that can survive in cement-based matrix and can actually induce MICP [8]. One of the main problems regarding cement-based materials is the high pH environment. The pore solution of cement paste is known to be pH 13, which is an extremely alkali environment for most of the bacterial species [9]. Moreover, there can be physio-chemical stresses induced on the bacterial cells due to hydration and hardening process which can affect bacterial metabolic activity and growth [10]. At last but not the least, there will be nutrient depletion for bacteria after hardening [11].

Considering these factors affecting bacterial growth, viability and $CaCO_3$ precipitation, choosing the right type of bacteria for biomineralization in cement-based material can be a challenge. The most commonly used species in cement-based materials is *Sporosarcina pasteurii* (*S. pasteurii,* formally known as *Baciullus pasteurii)*, which is a non-pathogonic, alkaliphilic and endospore forming soil microorganism[12]. Moreover, there are also various type of microorganisms that could induce biomineralization in cement-based materials such as; *Bacillus cohnii* [13]*, Bacillus megaterium* [14]*, Bacillus subtilis* [15]*, Shewanella* [16]*, Bacillus alkalinitriculus* [18].

Another challenge of the application is the incorporation of the cells in cement-based matrix. An early approach was simply incorporating endospores rather than vegetative cells (metabolically active) [17]. These endospores were found to be viable up

to 4 months without any protection [7]. Then, concerns regarding the viability of the endospores within the restrictive and high pH environment of cement-based materials have led researchers to propose encapsulation for the endospores. The encapsulation methods consist of embedding the endospores in a protective covering, e.g. inorganic lightweight porous aggregates [18] (LWAs), polymeric membrane [19], microcapsules [20] and hydrogels [21]. Amongst all these approaches, LWAs [18] and hydrogels have shown the most promising developments regarding the viability. However, both of these approaches decreased the compressive strength of mortar. Interestingly, previous studies have shown that with the proper selection of species and incorporation of nutrients the vegetative cells could survive in cement paste without any encapsulation[22].

Even though the cells could stay viable without any encapsulation, use of nutrients in the cement paste mix have some drawbacks in the application [22]. Generally, the yeast extract is being used in the bacterial cultures [23]. Besides the fact that yeast extract induced a negative impact on setting and strength, it is one of the most expensive ingredients of nutrient medium. Almost 60% of total operating cost of the medium is due to use of yeast extract [1]. Thus, it is crucial to replace yeast extract with a cheaper alternative, which will also provide the same efficiency in bacterial growth and bio-mineralization reactions.

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1.2   Goals and Objectives

Limited research on biomineralization in cement-based systems has shown promising results and suggested that $CaCO_3$ could seal the surface cracks and leading to a decrease in permeability[24]. While this is encouraging, there is not enough information, whether there could be alternatives for yeast extract and if the flexural strength of the self-healing mortar can be improved via self-healing. Moreover, it is still not known how long this biogenic sealant can resist *within* the cracks after it is exposed to weathering conditions such as sunlight, rain and freeze and thaw. At last but not the least the influence of chemical admixtures on biomineralization and self-healing are not completely known.

The ultimate goal of this study was to develop a sustainable and cost-efficient bio-based self-healing agent. To achieve this goal following objectives were targeted:

1- The possibe use of corn steep liquor (CSL) as an alternative carbon source in stead of yeast extract

2- The influence of bio-based admixture on performance of cement-based materials was evaluated in terms of setting, chemical composition and strength

3- The self-healing ability of the biomineralization product ($CaCO_3$) was investigated by comparing flexural strength regain and changing water absorption coefficient

4- The effects of commonly used additives such as superplasticizer and air entraining additives (AE) on the biomineralization and self-healing ability was evaluated

5- The durability under product ($CaCO_3$) under natural conditions such as light, rainwater and freeze-thaw cycles.

1.3   Outline of the Thesis

This thesis includes 5 chapters, Chapter 1 is mainly the *introduction*. Chapter 2 is the *summary of the literature* on use of biomineralization in cement-based materials, microorganism and nutrient selection, definition of self-healing and applications of self-healing in cement-based materials using microbial induced calcium carbonate(MICP) and effect of admixtures on self-healing applications. Chapter 3 explains *materials and methodology* including microorganism and nutrient medium selection for this study, bacterial growth procedure, *in-vitro* $CaCO_3$ precipitation and crystal characterization, addition of bacterial culture into cement paste and mortar, test methods on fresh and hardened cement. Chapter 4 investigates the development of self-healing in concrete and the relevant results. This chapter focuses on the results of bacterial growth in substituted corn steep liquor nutrient medium(UCSL), effect of bacterial culture on the mechanical properties of mortar and hydration kinetics, self-healing development and the properties of precipitated $CaCO_3$ and the durability of precipitated material in different environments. At last, Chapter 5 includes conclusion and future work.

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Chapter II

Literature Review

Even though concrete is one of the most used materials in the construction industry; microcracks could be induced due to internal and external stresses. These microcracks not only provide pathways for chemicals and water to penetrate, they are hard to repair. While common repair methods are sufficient to remediate macro cracks, the healing agents might not be able to fully penetrate through microcracks. In such a case, autogenous healing could be a method to remediate cracks. Autogenous healing could be obtained via (1) further hydration of unhydrated cementitious material (2) transported impurities or sedimentation of fractured pieces clogging the cracks (3) transported impurities or sedimentation of fractured pieces making crack walls to swell (4) $CaCO_3$ formation through the carbonation of calcium hydroxide (CH) [3]. As mentioned before, one of the key factors restricting the autogenous healing in concrete was the crack width and there was a limit for autogenous healing to occur.

Recent researches in the field showed that it might be possible to develop a smart self-healing cement-based material that could trigger autogenous self-healing via biomineralization [13,25–27]. Biomineralization is a series of complex bio-chemical reactions in which microorganisms induce mineral precipitation [7]. In this particular case the microorganisms stimulate the formation of $CaCO_3$, which is also known as MICP [7].

2.1. Mechanism of MICP in nature:

Biomineralization could be observed due to their unique surface structure and functional units on the bacterial cell wall in nature. It also, could be obtained through metabolic activities of microorganisms. There are many pathways to induce biomineralization if the suitable conditions are provided to the bacteria. Heterotrophic bacteria can induce mineral precipitation via nitrogen or sulphur cycles. Nitrogen cycles can be simply explained by ammonification of amino-acids, degradation of urea and reduction of nitrates[28].

Among the abovementioned pathways relevant to MICP, degradation of urea has been commonly used in cement-based materials. In this case, microorganisms possessing urease enzyme can degrade urea into ammonia and carbon dioxide (1). This is followed by an increase in pH and if dissolved calcium $[Ca^{+2}]$ is present in the environment, then this pH increase triggers MICP (2) [29].

$$CO(NH_2)_2 + H_2O \xrightarrow{urease} 2NH_3 + CO_2 \qquad (1)$$

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad (2)$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \qquad (3)$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \qquad (4)$$
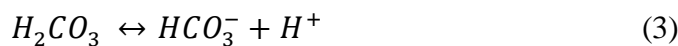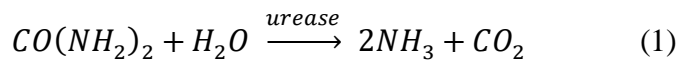
These reactions lead to calcium carbonate precipitation (Equation 5)

$$CaCO_3 \leftrightarrow Ca^{2+} + CO_3^{2-} \qquad (5)$$
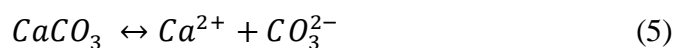
Here, ammonia is the main nitrogen source and it is consumed directly by the bacteria. While urease–active microorganisms can decompose urease ammonia and dissolved inorganic carbon (DIC), their surface charge can attract positively charged calcium ions acting as a heterogeneous nucleation site for MICP. There are several factors influencing these physio-chemical reactions such as microorganism type, $[Ca^{+2}]$ and DIC concentration, pH of the environment and availability of nucleation sites [30,31]. These factors not only influencing the rate of precipitation, they play a key role in change in morphology of $CaCO_3$ precipitate.

There are 6 known polymorphs of $CaCO_3$: Calcite, vaterite, ikate, aragonite monohydrocalcite and amorphous calcium carbonate (ACC). Among these known 6 polymorphs, calcite and aragonite are the most stable polymorphs, while ikaite and ACC are rarely observed. In case of MICP, calcite and vaterite are the most commonly observed polymorphs [10,32].

Mortensen et al. [33] evaluated the effects of environmental factors on MICP by suspending *S.pasteurii* cells in fresh water and sea water separately. To understand the influence of different parameters on MICP, the authors tested different ammonium and oxygen concentrations, as well as the viability of cells. It was concluded that uniform MICP was achievable in many different environments and ureolytic microorganisms were able to remain viable in different salinity conditions. Ureolytic bacteria could be efficient even in high ammonium concentration and limited oxygen environments[33].

According to L. Addadi et al.[32], for most of biological amorphous $CaCO_3$, the infrared spectra were the same. He also, investigated samples collected from sea organism such as *Pyura pachydermatina* and *Orchestia cavimana*. However, it was found that the major distinction between different amorphous $CaCO_3$ was whether they could transform to more stable phase of crystalline structure during a period of time[32]. This concept

could explain the unusual nature of infrared spectroscopy in biogenic $CaCO_3$ production. Stable amorphous $CaCO_3$ has absorption peaks at 866 cm^{-1} and 710 cm^{-1}, whereas, well-formed calcite crystal has a sharp peak at 713 cm^{-1} and the absence of 713 cm^{-1} peak in an infrared spectrum shows well-ordered calcite is not present in the material [32].

2.2. Microorganism selection

The list of microorganisms that could facilitate biomineralization is extensive. However, considering the factors affecting bacterial growth, viability and $CaCO_3$ precipitation, choosing the right type of bacteria for biomineralization is particularly important for the application.

Since the environmental conditions in cement-based materials is much more complex compared to the existence of the cells in nature, the main challenge is to find a microorganism that can tolerate these highly alkaline conditions and survive the mixing process, and can remain viable with a limited access to nutrients. In particular, alkaliphilic and endospore forming microorganisms can tolerate the stresses induced within the cement-based materials.

Table 2.1 summarizes the different bacterial strains used for applications in cement-based materials.

Table 2.1: Type of microorganisms used in biomineralization applications in cement-based materials

Bacteria type	Approach	Use	Viability in cement based mortar	Reference
Shewanella sp.	Cells were suspended in tap water	Crack healing	Survived 6-7 days	[16]
Bacillus megaterium	Cells were mixed in mortar by vegetative inoculation method	Compressive strength	Approximately 0.1% of cell retained in mortar	[14]
Bacillus cohnii	Encapsulated cells mixed in mortar	Crack healing	2% survived after 10days of mixing	[13]
Bacillus pseudofirmus	Encapsulated cells mixed in mortar	Crack healing	2% survived after 10days of mixing	[13]
Sporosarcina pasteurii	Cells were suspended in mixing water	Crack healing	Not reported	[16]
Bacillus subtilis	Cells were suspended in mixing water	Compressive strength	Not reported	[15]
Escherichia coli	Cells were suspended in mixing water	Compressive strength	Not reported	[34]
Lysinibacillus sphaericus	Cells were immobilized in diatomaceous earth (DE) in mortar	Crack healing	Not reported	[9]
Bacillus alkalinitriculus	Cells were immobilized in LWAs	Crack healing	Up to 100 days	[18]

2.3.   Nutrient medium selection:

Since MICP is directly affected by microorganism type, nucleation sites, viable cell concentration and nutrient source, selection of an efficient nutrient source is inevitable factor to keep microorganisms in vegetative state and metabolically active.

To trigger MICP through urea hydrolysis, bacterial cells require a nitrogen and carbon source. As followed by the bio-chemical reaction (*see* Section 2.1), urea is the main nitrogen source for the cells. Urea is decomposed by bacterial urease enzyme, which results in the production of ammonium (NH_4^+) and then used by the cells. In most of the cases yeast extract has been used as a carbon source for the bacterial source. Yeast extract can provide many amino acids and vitamins essential for bacteria survival [14]. However, substantial extension of the induction period in hydration was observed when yeast extract was used in the mix[20,27,35]. This was attributed to the sugar content and other carbohydrates that were known to be effective retarders[36,37].

Besides the fact that yeast extract induced a negative impact on setting and strength, it is one of the most expensive ingredients of UYE medium. Almost 60% of total operating cost of UYE medium is due to use of yeast extract [1]. Thus, it is crucial to replace yeast extract with a cheaper alternative, which will also provide the same efficiency in bacterial growth and bio-mineralization reactions. An alternative carbon source instead of yeast extract could be lactose mother liquor (LML), which is a by-product of dairy industry. According to Achal et al.[38], use of LML in the nutrient medium resulted with a better performance in bacterial growth and MICP compared to nutrient media containing nutrient broth or yeast extract. CSL, as being a waste product of corn industry, can be a cheap and a sustainable alternative for yeast extract. Achal et al. [39] showed a significant reduction in total cost by using CSL as a carbon source for *S. pasteurii.* Moreover, it was found that use of CSL in the nutrient medium improved the

urease activity and calcite production of *S. pasteurii* cells [39]. However, there is a very limited information regarding use of CSL for biomineralization applications.

2.4. Influence of [Ca^{+2}] source on MICP:

One of the factors affecting MICP and the morphology of the precipitate, is the calcium ion concentration in the environment. It was reported, precipitation of vaterite was favored over calcite when the calcium source was provided as calcium acetate instead of calcium chloride [40]. Cement-based materials naturally have sources of calcium, although, additional calcium sources are often added for MICP applications [16,41]. These reported calcium sources could be listed (a) calcium chloride($CaCL_2$), calcium nitrate($Ca(NO_3)_2$), and calcium lactate ($CaC_6H_{10}O_6$) [18,41]. According to Wiktor and Jonkers [18] use of calcium lactate did not have any negative effect on the compressive strength of cement-based mortar. Even though, $CaCl_2$ worked more efficiently in MICP application, also, $CaCl_2$ [41] could cause corrosion for reinforcement steel inside the concrete.

2.5. MICP applications in cement-based materials:

Earlier studies in the literature have shown that MICP could be used to bind non-cohesive sand particles and improve their properties under shear [24,42]. Following the applications in soil mechanics, MICP has been used in cement-based materials to remediate surface microcracks [20,43,44].

There are different approaches to remediate cracks in cementitious material throughout in the literature. Ramachandran et al. [43] proposed a procedure for crack healing in cement based cubes and beams by filling cracks with sand and *S. pasteurii* cells with urea-$CaCl_2$ solution at different concentrations (10^7-10^8cells/cm^3). Results suggested

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that biomineralization could be applicable for surface cracks meanwhile the efficiency can be limited by crack width. In addition, biomineralization was more effective in crack healing than compressive strength.

Another approach was conducted by Bang et al.[19] and remediation of cracks was obtained by using *S. pasteurii* microorganism in glass beads. Microorganisms were encapsulated in glass beads and injected into cracks with nutrient media and calcium chloride. Initiated cracks on beams had 3.17 mm and 12.7 mm of width and depth. Also, cracks with width and depth of 3.17 mm and 25.4 mm were initiated on cubic mortar samples. This research resulted with an increase in compressive strength and stiffness for bacterial samples comparing to control samples.

Then studies were much more focused on developing a self-healing material by actually incorporating the cells in mortar or concrete. An early approach was incorporation of *Bacillus pseudofirmus* and *Bacillus cohnii* endospores by suspending them in mixing water of mortar [13]. With this approach, crack sealing was observed due to robust mineral precipitation but the permeability of the mortar after self-healing was not investigated. These endospores were found to be viable up to 4 months without any protection; however these endospores with their nutrient sources reduced the compressive strength of mortar [13]. One of the possible reasons to observe this strength decrease due to the addition of endospores could be explained by the degradation of proteins by pH and induce formation of air bubbles [45]. Then, concerns regarding the use of the endospores within the restrictive and high pH environment of cement-based materials have led researchers to propose encapsulation for the endospores. The encapsulation methods consist of immobilizing the bacterial endospores in a protective covering, such as inorganic lightweight porous aggregates (LWAs), polymeric membranes [46,47], hydrogels [21] and microcapsules [20].

Wang et al. [21] proposed a biocompatible hydrogel encapsulation for *Bacillus sphaericus* endospores to induce self-healing in cement-based mortars. It has been shown that these hydrogels were able to keep the endospores viable *within* the cement paste matrix and the microorganisms were able to self-heal cracks as large as 0.5 mm within 7 days and decrease water permeability. However, the strength recovery of the cracked mortar samples was not investigated in this work. On a related note, Wang et al. [47] conducted a series of tests to determine the self-healing ability of *B. sphaericus* endospores encapsulated micro silica gel and polyurethane membranes when they were introduced through glass tubes embedded in mortar. The results showed that polyurethane membranes showed a higher self-healing efficiency compared to silica gels in terms of strength recovery and reduction in permeability [47].

Wiktor and Jonkers [18] used LWAs to encapsulate *Bacillus alkalinitriculus* endospores and their nutrient source, calcium lactate. With this approach, the researchers could extend the viability of the bacteria; however, incorporation of LWAs decreased the compressive strength of the material. Another objective of their study was to evaluate if the immobilization of bacteria inside expanded clay could help the time dependency microorganism viability. As a result, it was observed that the LWAs are capable of increasing functionality of self-healing from 7 days to 100 days[18].

Up-to-date, most of the self-healing applications in cement-based materials targeted to remediate cracks induced after 28-days of casting, which still can be considered as an early age application for cement-based materials. In such a case, it might not be necessary to encapsulate the microorganisms. Studies shown that with a proper microbial selection and nutrient medium, 2% of the initial bacterial inoculum remained viable for 11 months after mortar mixing [22,48]. The inoculated *S. pasteurii* cells were able to precipitate $CaCO_3$ within the cement paste and remediate internal microcracks

[23]. However, incorporation of urea-yeast extract (UYE) medium significantly delayed the initial set of the cement-paste and decreased the compressive strength of the material. Even though the addition of *S. pasteurii* cells improved the compressive strength, they made delay in initial set was more pronounced [14,23].

2.6. Influence of chemical admixtures on self-healing

Studies on developing self-healing mortar has shown promising outcomes in terms of crack repair and reducing the permeability, yet there are still more questions to carry on this application to the field. One of the major key questions is the compatibility of this bacterial culture with the chemical admixtues used in production of concrete such as superplasticizers, AE, accelerators etc.

Previously, Ersan et al.[45] have tested the performance of AE (BASF MasterAir 100) as a protection method for microorganisms embedded in cement paste matrix. The study has only focused on the effects of the AE (1% w/w of cement) in terms of its influence on compressive strength and setting of mortar samples. Use of AE as a protection method decreased the compressive strength of mortar and further analysis on microorganism viability was not conducted. This study was followed by a deeper investigation of influence of AE on survival rate of *S. pasteurii* [49]. *In-vitro* experiments revealed that morphology of $CaCO_3$ crystals was significantly affected by the addition of AE, while it does not affect the zeta potential of *S. pasteurii* cells. The cell viability was highly influenced by incorporation of AE such that there was a significant decrease in viable cell concentration with the addition of 0.2% AE (w/w cement) [49].

Moreover, the effect of admixtures was conducted by Stuckrath et al. [50] and the research was designed to use two component self-healing agents based on $CaCO_3$ precipitation in LWA. Two common chemical admixtures superplasticizer and AE with two different self-healing agents (chemical and biological) were used to evaluate the

effects of chemical admixtures on self-healing. *B. pseudofirmus* LMG 17944 (Belgian coordinated collections of microorganisms, Ghent) with calcium lactate embedded in LWAs (expanded clay sieved between ASTM standards n°4 and 16) were used as a self-healing agent. A calcium lignosulfonate based superplasticizer and sodium lauryl ether sulfate based AE were used in this research. Results of this research showed that rhombohedral precipitations of $CaCO_3$ in samples without any chemical admixtures. However, incorporation of AE agent resulted with needle a crystals of $CaCO_3$, which could be aragonite and using superplasticizer resulted with irregular agglomeration of rhombohedral precipitates[50]. Thus, it was found that use of chemical admixtures could affect the morphology of $CaCO_3$.

Chapter III

Materials and Methods

3.1 Microorganism and nutrient medium selection:

Leibniz Institute- German Collection of Microorganisms and Cell Cultures: *S. pasteurii* (DSMZ 33) was used in this study. This particular species can be easily obtained from the soil and non-pathogenic. *S. pasteurii* was frequently used in biomineralization studies due to its high urease enzyme activity and its negative surface charge [51]. *S. pasteurii* can tolerate highly alkaline conditions, can survive the mixing process, and can remain viable with limited access to nutrients [49]. In particular, alkaliphilic and endospore forming microorganisms can tolerate the stresses induced within the cement-based materials.

The bacteria used in this study were grown aseptically under specified batch cultivation conditions (*see* Section 2.2). Then, these cultures were kept as a -80°C stock culture in glycerol solutions (filled in 1.5 mL sterile vials).

3.2 Cultivation of vegetative *S. pasteurii* cells:

In this study, 2 different nutrient media were used: Urea-Yeast Extract (UYE) medium and Urea-Corn Steep Liquor (UCSL) medium. UYE and UCSL media were composed of Tris base, urea and yeast extract/CSL with distilled (DI) water.

UYE medium was composed of Tris base (15g), urea (10g) and yeast extract (20g) per liter of distilled (DI) water. UCSL medium was obtained by adding Tris base (15g), urea (10g) and CSL (15g) to a liter of DI water. The medium was adjusted to pH 9 by adding 0.1M HCl after the Tris base was added to 1L of DI water. CSL was provided in liquid form as a commercially available product from Sigma Aldrich and the chemical composition was not specified by the manufacturer [52]. These media were sterilized by

an autoclave (HIRAYAMA HV 25-L, Japan) at 121 °C and 100 kPa. For preparation of the agar plates, the solution containing the Tris base was divided into 3 equal volumes and added urea, YE/CSL and agar. DSMZ No: 33 *S. pasteurii* strain was added to the sterile nutrient media and multiplied. *S. pasteurii* cells were inoculated in 600 mL of UYE media or UCSL media separately and incubated aerobically with shaking conditions (180 rpm) at 30°C using an IKA KS 4000 model incubator. Sample aliquots were taken from these media periodically and plated on agar plates. Samples for viable plate counts were serially diluted (10^0-10^{-7}); and the cell concentration was obtained by viable plate counts and represented as colony forming units (CFU/mL). Bacterial growth curves were developed in terms of CFU/mL vs. time. Growth experiments for both media were conducted as triplicates.

To determine the influence of nutrients and chemical admixtures on surface charge of bacterial cells, *S. pasteurii* cells were grown in UYE and UCSL media until a concentration of 2 x 10^8 CFU/mL was reached. Then, cells were harvested by centrifugation at 6300g for 15 minutes, washed by sterile DI water and resuspended in 6 different media: *UYE, UYE+SP, UYE+AE, UCSL, UCSL-SP* and *UCSL+AE*. A Malvern Zetasizer Nano ZS (Malvern, Worcestershire, United Kingdom) was used to determine the influence of chemical admixtures and nutrient media on zeta potential of the cells.

## 3.3   *In-vitro* CaCO$_3$ precipitation and crystal characterization

To examine the formation of biomineralization, the *S. pasteurii* cells were grown in a 200 mL nutrient media (UYE and UCSL) to an exponential growth (log phase) stage and then calcium ($[Ca^{+2}]$) source was added to this nutrient medium. For this step 3 different type of calcium sources were evaluated: Calcium chloride- CaCl$_2$ (20 g/l), Calcium lactate- C$_6$H$_{10}$CaO$_6$ (37.5 g/l), Calcium nitrate- Ca(NO$_3$)$_2$.4H$_2$O (40 g/l). the

amount of added $CaCl_2$, $Ca(NO_3)_2.4H_2O$ and $C_6H_{10}CaO_6$ into UYE and UCSL were calculated to have 0.167 M calcium $[Ca^{+2}]$. One molar (M) of urea results in 1 M of carbonate $[CO_3^{-2}]$ and for biological $CaCO_3$ precipitation, calcium sources were calculated to result with 1 M calcium $[Ca^{+2}]$. After 24 hours, precipitations were collected by 6300 g (15 min) speed centrifuge. Collected samples were investigated by scanning electron microscope (SEM, JEOL JIB-4501 Multi-Beam Focused Ion Beam Scanning Electon Microscope- Freising, Germany) and X-ray diffraction analysis (X-ray diffraction analysis - XRD BRUKER D8 Advance X-ray Diffractometer (Karlsruhe, Germany)). The accelerating voltage was kept at 5 kV while the working distance was held at 9-12 mm at various magnifications. For XRD analysis, collected precipitates were kept in a drying chamber at 40°C for 24 hours prior to testing. Then, the samples were placed and compacted into a sample holder and analysis was conducted at angles from 10-90° 2θ at a step size of 0.02° 2θ.

3.4 Addition of bacterial culture into cement paste

S. pasteurii cells, were grown until they reach the station phase. As it was mentioned in section 3.2, *Bacterial Paste* was prepared by replacing the water of the cement paste with the bacterial culture and *Nutrient Paste* was prepared by replacing the water of the cement paste either with UYE or UCSL media. The solution to cement ratio (s/c) for all pastes was kept constant at 0.45 (w/w). The solution portion of the s/c ratio refers to the aqueous component of the all paste mixes. Thus, the solution portion for the *Neat Paste, Nutrient Paste*, and *Bacterial Paste* are tap water, nutrient media, and the bacterial culture, respectively. An important fact here is that the 96% of this bacterial culture was water and the negligible mass of bacteria cells would not affect the water to cement ratio.

BASF MasterGlenium superplasticizer (0.9 kg/kg cement) and BASF MasterAir air entraining agent (0.1 kg/kg cement) were added to required samples when needed. Mortar samples with Ordinary Portland Cement (CEM I 42.5R) and crushed sand. ASTM C128-15 Standard Test Method for Density, Relative Density (Specific Gravity) and Absorption of Fine Aggregate was used to determine the absorption coefficient of the sand [53] and ASTM C136-14 Standard Test Method for Sieve Analysis of Fine and Coarse Aggregates was used to determine the particle size distribution (PSD) of the sand [54]. Absorption capacity for the sand was found 0.67% and specific gravity was found as 2.6. Figure 3.1 shows the PSD curve for the sand and fineness modulus of the sand was calculated as 2.8.
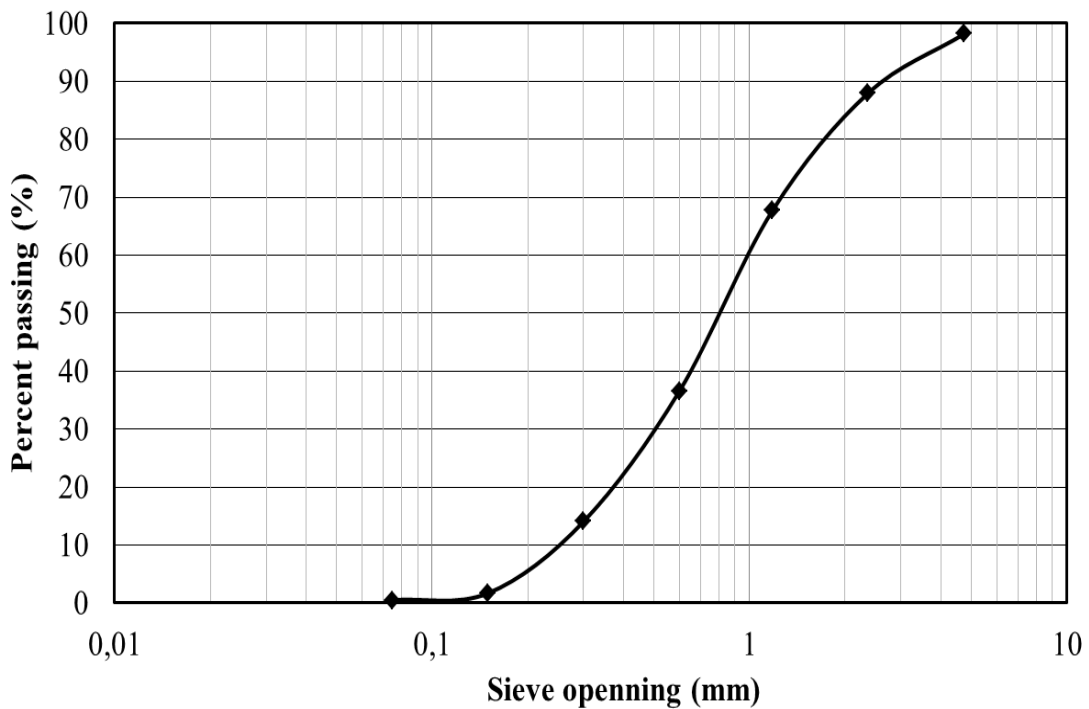
Figure 3.1: PSD for fine aggregate used in the mortar mix.

Table 3.1 describes the different cement paste samples prepared for this project, showing materials used during the mix such as nutrient media, admixtures and calcium source.

Table 3.1 Composition of each cement paste series for testing. SP: Superplasticizer; AE: Air-entraining agent; UYE: Urea+ Yeast extract; UCSL: Urea+ Corn Steep Liquor

Series		Ingredients					Admixtures		$[Ca^{+2}]$ source
		Water	UYE	UCSL	Bacterial Culture-UYE	Bacterial Culture-UCSL	SP	AE	$Ca(NO_3)_2$
Neat Paste	Neat	x							
	Neat-SP	x					x		
	Neat-AE	x						x	
	Ca-Neat	x							x
	Ca-Neat-SP	x					x		x
	Ca-Neat-AE	x						x	x
Nutrient Paste	UYE		x						
	UYE-SP		x				x		
	UYE-AE		x					x	
	UCSL			x					
	UCSL-SP			x			x		
	UCSL-AE			x				x	
	Ca-UYE		x						x
	Ca-UYE-SP		x				x		x
	Ca-UYE-AE		x					x	x
	Ca-UCSL			x					x
	Ca-UCSL-SP			x			x		x
	Ca-UCSL-AE			x				x	x
Bacterial Paste	Bac-UYE				x				
	Bac-UYE-SP				x		x		
	Bac-UYE-AE				x			x	
	Bac-UCSL					x			
	Bac-UCSL-SP					x	x		
	Bac-UCSL-AE					x		x	
	Ca-Bac-UYE				x				x
	Ca-Bac-UYE-SP				x		x		x
	Ca-Bac-UYE-AE				x			x	x
	Ca-Bac-UCSL					x			x
	Ca-Bac-UCSL-SP					x	x		x
	Ca-Bac-UCSL-AE					x		x	x

3.5 Test on fresh cement paste

3.5.1   Vicat needle test

A modified ASTM C191-13 Standard Test Methods for Time of Setting of Hydraulic Cement by Vicat Needle test was conducted to determine the setting time of the cement paste samples described in Table 3.1 (*see* section 3.4) [55]. Instead of determining the s/c ratio that will yield a "normal consistency" paste as suggested in the standard, the s/c ratios were kept constant at 0.45 to be consistent with the ratios used throughout the other analysis conducted in the study. Six hundred fifty grams of cement were mixed with 292.5 g of water for *Neat Paste* control mixes. Similarly, 650 g of cement were mixed with 292.5 g of UYE or UCSL media and bacterial culture grown in UYE or UCSL media (10^8 CFU/mL) for the *Nutrient* and *Bacterial Paste* samples respectively. The initial setting of the cement paste samples was determined according to penetration depth of the Vicat needle. Analyses were conducted based on triplicates of samples.

3.6 Tests on hardened cement paste and mortar:

3.6.1   Thermogravimetric analysis (TGA) of cement pastes:

Cement paste samples were prepared as previously described in Section 3.4. Following the mixing, samples were cast into prismatic molds (2 x 3 x 4 cm). The specimens were initially cured at 100% relative humidity at 21°C for 24 h. Then the molds were removed, and the samples were further cured by submerging them in UYE or UCSL media (pH 10) depending on the medium used in the mixing process. The samples were kept in the nutrient medium curing until the tests were conducted. The analysis was conducted on 3, 7 and 28 days old samples.

At the time of testing, the specimens were removed from the curing solution and a representative sample was taken out from the core of the specimens. Then, the samples

were pulverized with a pestle and mortar to provide a homogenous form. The powdered sample was ground in the presence of ethanol to stop hydration and preserve the chemical composition of the samples [24]. The samples were kept in a vacuum desiccator for 24 h and then tested in Netzsch STA 449 Jupiter TGA-DTA Analyzer (Germany). The analysis was conducted by heating the pulverized cement paste samples from 40°C to 1100 °C and mass loss was recorded as a function of time.

Figure 3.2 shows a representative mass loss (TGA) and difference thermogravimetric (DTG) plots for a 3-day old *Bac-UCSL* sample. The samples were heated from 40°C to 1100 °C at a rate of 10°C/min. DTG curve was used to determine the exact temperatures for decomposition for both CH and $CaCO_3$. The mass percentages of CH and $CaCO_3$ were calculated from the TGA curve by using these temperature points pinpointed from DTG curve. Ranges between 400-450°C and 650-800°C respectively were used to determine CH and $CaCO_3$ mass percentages.
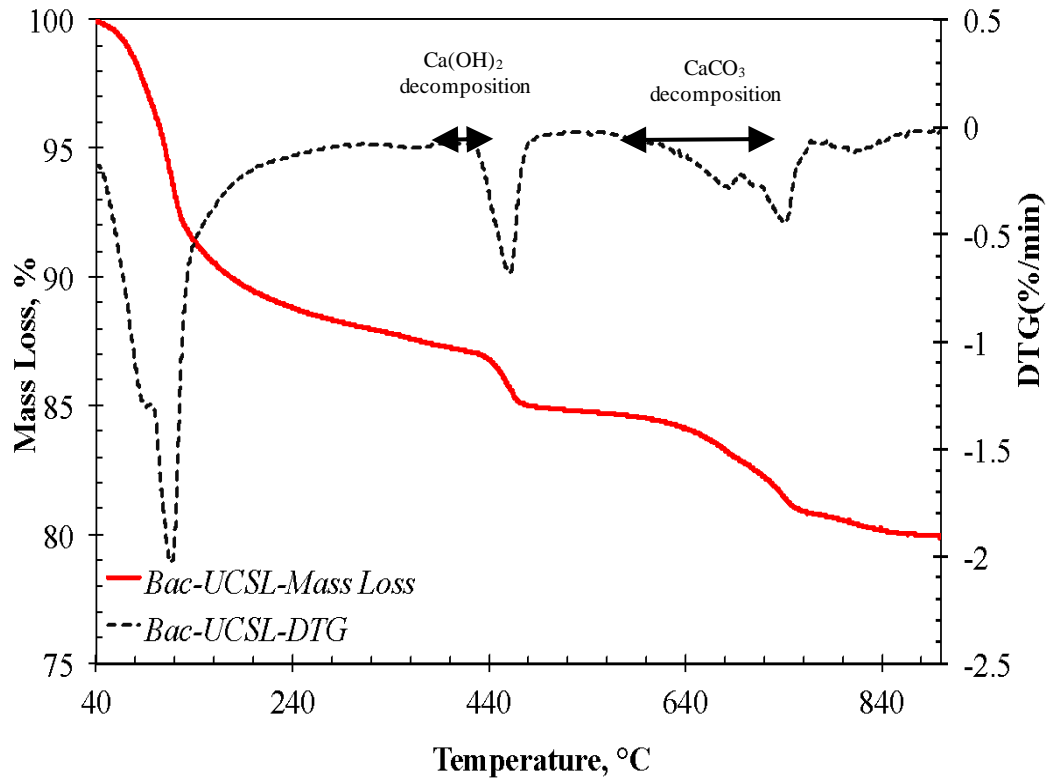
Figure 3.2: Representative mass loss (TGA) and DTG curve for 3-day old *Bac-UCSL* sample to illustrate $Ca(OH)_2$ and $CaCO_3$ content calculation for cement paste samples.

3.7 Compressive and flexural strength tests

For compressive strength testing, mortar samples were prepared by adding sand to the cement paste samples listed in Table 3.1. Mortar samples were prepared by ASTM C305-14 Standard Practice for Mechanical Mixing of Hydraulic Cement Pastes and Mortars were modified by replacing the water content with UYE medium, UCSL medium or bacterial culture grown in these media[56]. The mortar samples were cast in 5 x 5 x 5 cm cubes and kept in humid environment at 21°C for 24 hours. Then the molds were removed and the samples were further cured in UYE or UCSL medium depending on the medium used in the mix until testing (22°C). Compressive strength testing was conducted according to ASTM C109-13e1 Standard Test Method for Compressive Strength of

Hydraulic Cement Mortars (Using 2-in. or [50-mm] Cube Specimens) [57] at 3,7,28 and 90 days on triplicates of samples.

For flexural strength testing, series of 40 x 40 x160 mm mortar specimens were prepared by adding sand Table 3.1 to cement pastes. Mortar samples were prepared by ASTM C305-14 and the standard was modified by replacing the water content with UYE medium, UCSL medium or bacterial culture as it is mentioned above. The samples were kept in humid environment at 22ºC for 24 hours. Then the molds were removed and the samples were further cured in UYE or UCSL medium depending on the medium used in the mix until testing (22ºC). Flexural strength testing was conducted according to ASTM C348-14 at 3,7,28 and 90 days on triplicates of samples.

3.8 Scanning electron microscope (SEM analysis)

SEM analysis was performed on samples collected from fractured surfaces after the bending strength test was conducted. For this purpose, the samples were kept in a desiccator for 24 hours at the first stage. Then the samples were coated with gold; During the analysis, the working distance was set at 9-12 mm and the voltage was set at 5 kV.

3.9 Self-healing investigation

3.9.1 Crack formation and curing conditions

To examine the possible use of microorganism to develop a self-healing mortar, 40 x 40 x160 mm beams were prepared as it was stated in Section 3.7 and kept in humid room for 24 hours. To provide flexural resistance Kord-sa KraTos 6-mm microsynthetic fibers were added to mortar (4 g/ m^3 of mortar).

After demolding, samples were further cured in UYE or UCSL media depending on the medium used in the mix until testing (22ºC) for 7 days. At the end of the 7th day, the samples were cracked under a bending load using a servo hydraulic displacement controlled device (0.05 mm/sec). The crack widths were kept less than 0.3 mm.

Once the cracks were formed, the first set of beams were cured for 50 days in the aqueous media with either UYE or UCSL media depending on the medium used in the mix until testing. In the literature, it is frequently observed that a calcium source is added to the curing environment as well as calcium source added during the mixing (Ramachandran, 2001; Wang, 2014), for the same reason above mentioned second set of specimens were prepared again and it was cured for 50 days in nutrient media containing $Ca(NO_3)_2$ and UYE or UCSL (depending on the media used in the mix).

3.9.2 Visual observation of self-healing- Optical imaging:

To investigate the self-healing in cement-based mortar, prepared beams were periodically observed under optical microscope after crack initiation. Bacterial specimens were taken out from curing solution every 2-week intervals and analyzed for $CaCO_3$ crystal formation within cracks. HIROX-USA, Inc KH7700 DIGITAL MICROSCOPE was used for this purpose.

3.9.3 Quantification of self-healing ability- Flexural strength, characterization of CaCO₃ *and* water absorption

Once the cracks were completely filled with the biogenic $CaCO^3$, the self-healing ability was quantified by determining the change in flexural strength and water absorption. Moreover, the density of the product ($CaCO_3$) formed in the cracks was determined by FT-IR scattering analysis.

The first step of quantification was done by determining the flexural strength regain. To measure the regain in flexural strength, the healed samples were tested under bending after 50 days of nutrient medium curing was completed. The samples were cracked under a bending load using a servo hydraulic displacement controlled device (0.05 mm/sec). The change in flexural strength in repaired sample was then compared with the 7-day uncracked counterpart series.

Water absorption capacity of the healed mortar samples was calculated by RILEM 25 PEM II-6 test. The samples were kept at nutrient medium for 50 days until the cracks are complete sealed. The crack closure was determined via visual inspection under an optical microscope (see Section 3.9.3). Then, the samples were removed from the curing solution and were dried at $40\,^{\circ}C$ until the mass change was reduced to $\pm\,0.1\%$ of periodic mass measurement. All adjacent sides of the cracked region were coated with silicone and then the mass of dry sample was measured (W_d). The cracked uncoated surface was then held in a water bath of 10 ± 1 mm height. The samples were taken out from the water bath and weighed periodically (W_W) after removing the surface water with a wet towel periodically (1,2,4,6,12,24,72,120 and 240 hours). After each measurement, the samples were immediately put back into the water bath. The water absorption coefficient of the samples was calculated by the following formula:

$$k(t^{1/2}) = Q/A$$

k: water absorption coefficient (g.cm^{-2}. s$^{-1/2}$)

Q: periodic mass of water in specimens (W$_W$-W$_d$) (g)

A: submerged surface area (cm^2)

FT-IR spectroscopy was used to evaluate the crystal structure of precipitation and the evidence of CaCO$_3$ presence in precipitation at the crack interface. Samples were taken from crack surfaces and put in samples holder as powder material. FTIR spectroscopy Nicolet™ iS™ 10 FT-IR was used for this purpose.

3.10 Durability of CaCO$_3$ precipitate under changing environmental conditions

To determine the long-term applicability of self-healing, It is important to determine the durability of the biogenic CaCO$_3$ sealant under possible environmental conditions such as sun light, rain and freeze-thaw.

The *Bacterial Mortar* samples were casted by replacing the water content with a bacterial culture grown in UCSL medium (*Bac-UCSL, Bac-UCSL-SP, Bac-UCSL-AE*). Samples were prepared as 40 x 40 x 160 mm beams as it is stated in Section 3.9.1 and kept in 24 h humidity conditions. To provide flexural resistance Kord-sa KraTos 6-mm microsynthetic fibers were added to mortar (4 g/ m^3 of mortar).

After demolding, samples were further cured in UCSL medium until testing (22ºC) for 7 days. At the end of the 7th day, the samples were cracked under a bending load using a servo hydraulic displacement controlled device (0.05 mm/sec). The crack widths were kept less than 0.5 mm. After the cracks were formed, the beams were cured for 50 days in the UCSL nutrient medium. Self-healing of cracks was visually inspected via optical microscopy (*see* Section 4.3).

Once the cracks were completely sealed, then the specimens were separated into 3 groups. To understand the effects of rainwater on the precipitated material, rain water was sprayed periodically every day for 30 days. The second group was kept under light during the day for 30 days. At last but not the least the 3rd group was subjected to freeze-thaw cycles. The freeze and thaw cycles were obtained by keeping the samples at -18oC for 24 hours and 4oC for 24 hours. After 30 days of treatment, the durability of the precipitate was quantified by measuring the change in water absorption. The was absorption capacity was measured based on RILEM 25 PEM II-6 test (*see* Section 3.9.3).

Chapter IV

Result and Discussion

4.1 Microorganism growth and in-vitro CaCO$_3$ precipitation

The possible use of CSL was evaluated by replacing 2% yeast extract (w/w ratio of DI water) content in *S. pasteurii* nutrient medium with 1.5% (w/w ratio of DI water) CSL. Then, *S. pasteurii* was grown in 600 mL of both medium at 30°C (*see* Section 2.1). The growth profile for *S. pasteurii* cells in each media, averaged from triplicates of samples, is presented in **Figure 4.1.**
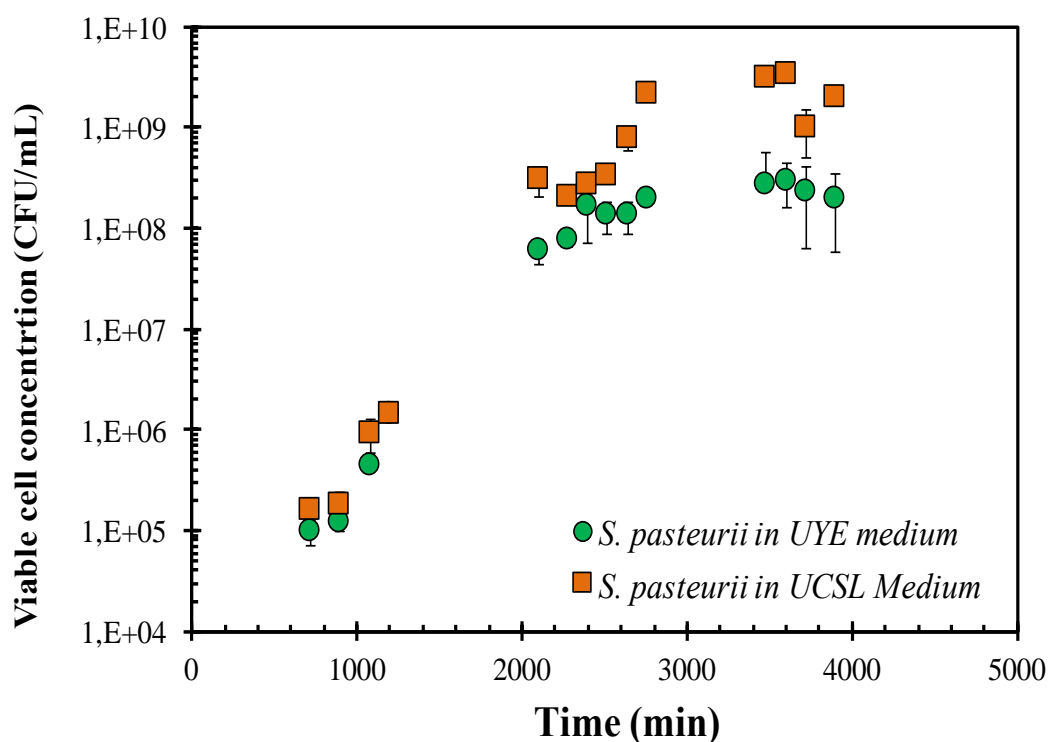
Figure 4.1 Representative growth profile for *S. pasteurii* (DSMZ 33) averaged from triplicates of viable plate counts (cell concentration vs. time) in UYE and UCSL media (pH 9) at 30°C; U: urea, YE: yeast extract, CSL: corn steep liquor; Error bars represent the standard deviation based on triplicates of viable plate counts.

As compared to UYE medium, there was a substantial bacterial growth in UCSL media such that the growth profile was similar. This might indicate that CSL was a good nutrient source which can provide the required carbon, nitrogen, amino acids and vitamins that can stimulate the bacterial growth. Achal et al. [39] also showed that *S. pasteurii* cells had a higher urease activity in a nutrient media with CSL compared to a media with yeast extract. CSL is an inexpensive nutrient media compared to yeast extract and peptone. However, Its variable composition could be a disadvantage in terms of nutrient dosages, thus it is crucial to determine the C:N ratio of the waste material prior to its usage [39].

To determine the possible influence of chemical admixtures and nutrients on surface charge of bacterial cells, zeta potential measurements were taken in bacterial cultures grown in UYE and UCSL media (pH 9) separately and then incubated either in UYE or fresh UCSL medium with or without incorporating admixtures. Results of zeta potential measurements are presented in **Table 4.3**.

Table 4.3 Zeta potential *for S. pasteurii* incubated in *UCSL* or *UYE* media; error represents the standard deviation for triplicate sample. SP: Superplasticizers; AE: Air Entraining Agents; UCSL: Urea-CSL medium; UYE: Urea-Yeast Extract.

Sample	*pH*	*Zeta potential (mV)*
S. pasteurii cells in UCSL	9.0	-29.2±4.9
S. pasteurii cells in UCSL+SP	8.93	-31.4±5.1
S. pasteurii cells in UCSL+AE	8.97	-29.4±4.2
S. pasteurii cells in UYE	9.0	-46.1±6.7
S. pasteurii cells in UYE+SP	8.91	-40.2±5.3
S. pasteurii cells in UYE+AE	8.95	-46.3±6.8

Even though, use of CSL did not have a negative effect on bacterial growth, it significantly reduced the zeta potential, in other words, the surface charge of the *S. pasteurii* cells. As it is mentioned before, surface charge of the cells would impact MICP, thus the morphology of the $CaCO_3$ precipitate. Use of chemical admixtures did not affect the surface of the cells regardless of the medium used in the culture.

Besides the bacterial surface charge and growth, another parameter that could affect MICP in nature is the source of $[Ca^{+2}]$ ions. To investigate the influence of calcium sources on biomineralization, 3 different $[Ca^{+2}]$ sources, $C_6H_{10}CaO_6$ (calcium lactate) $CaCl_2$ and $Ca(NO_3)_2.4H_2O$ were added to the bacteria cultures grown in 2 different media (UCSL and UYE). Figures 4.2 and 4.3 illustrate SEM analysis of collected $CaCO_3$ obtained with using different calcium sources from 2 nutrient media (UYE and UCSL media). Calcite was observed in the cultures in which $CaCl_2$ and $Ca(NO_3)_2.4H_2O$ were added, but vaterite was also rarely observed. However, the use of calcium lactate as a calcium source only resulted with vaterite precipitation.
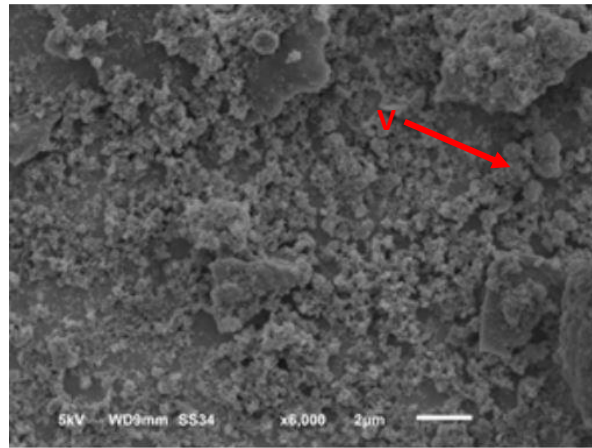
(a)
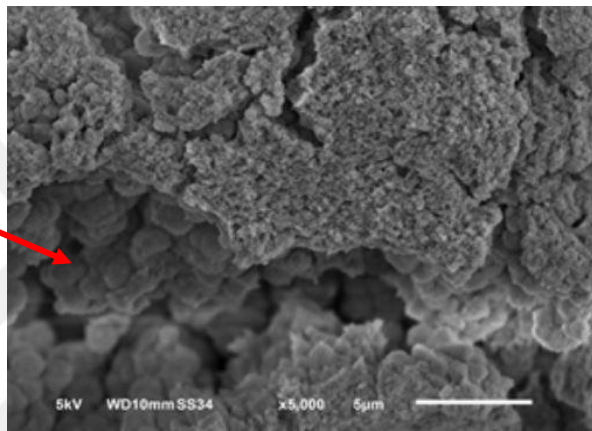
(b)
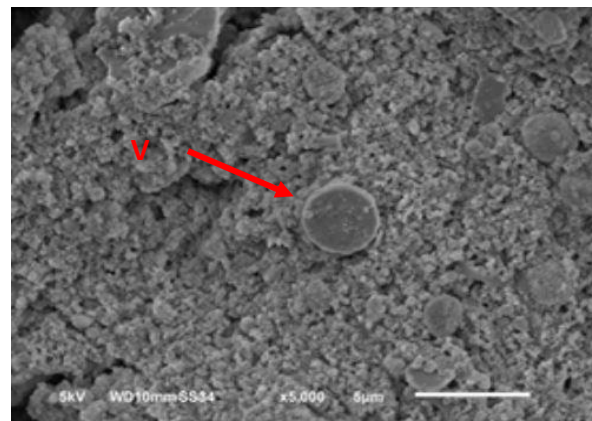
(c)

Figure 4.2 The $CaCO_3$ obtained from S. pasteurii cells and different sources of calcium in the UYE medium (a)$CaCl_2$ (b) $Ca(NO_3)_2.4H_2O$ (c) $C_6H_{10}CaO_6$

Both calcite and vaterite crystals were determined in the prepared UCSL media with the same calcium sources (Figure 4-3).
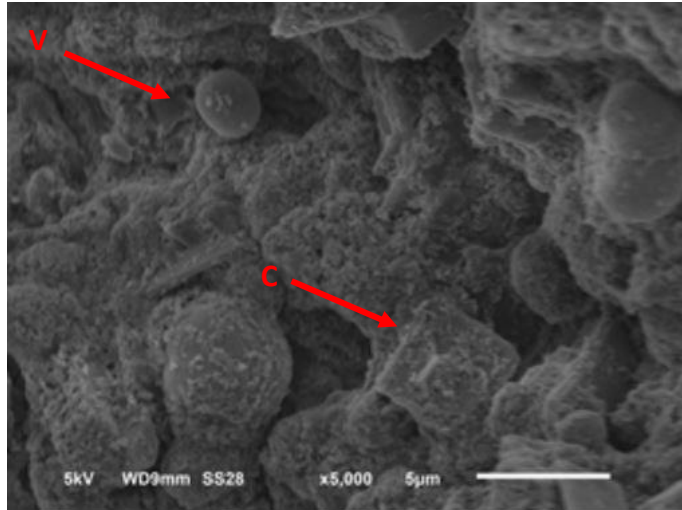
(a)
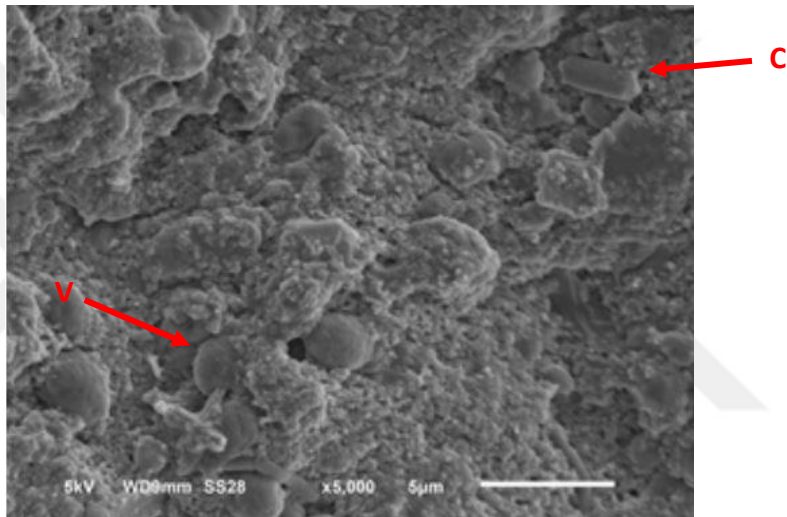
(b)
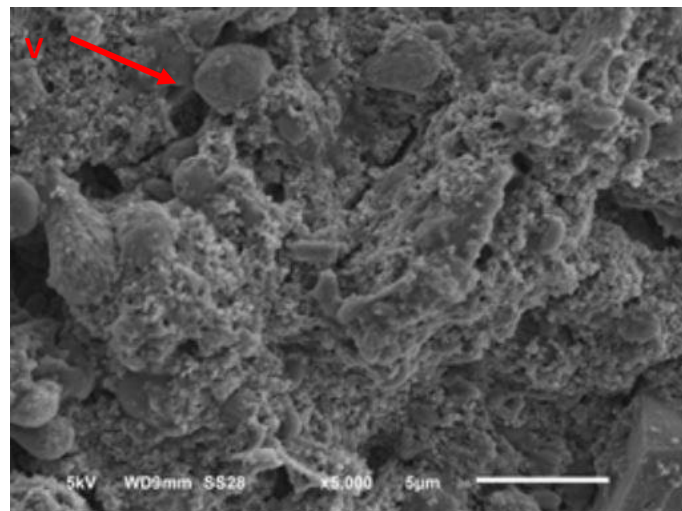
(c)

Figure 4.3 The CaCO$_3$ obtained from *S. pasteurii* cells and different sources of calcium in the UCSL medium *(a)CaCl$_2$ (b) Ca(NO$_3$)$_2$.4H$_2$O (c) C$_6$H$_{10}$CaO$_6$*

XRD analysis was also performed on these samples to support SEM analysis. Figure 4-4 and Figure 4-5 summarize the XRD analyzes of biogenic precipitates obtained by adding $[Ca^{+2}]$ sources to the bacterial cultures grown in UYE and UCSL media. In the solutions prepared with the UYE, it was determined that most of the precipitation products are vaterite crystals (Figure 4-4). $Ca(NO_3)_2$ added to the bacterial culture grown in UCSL media causes only vaterite precipitation, whereas, presence of $CaCl_2$ and calcium lactate resulted with a calcite-vaterite mixture.
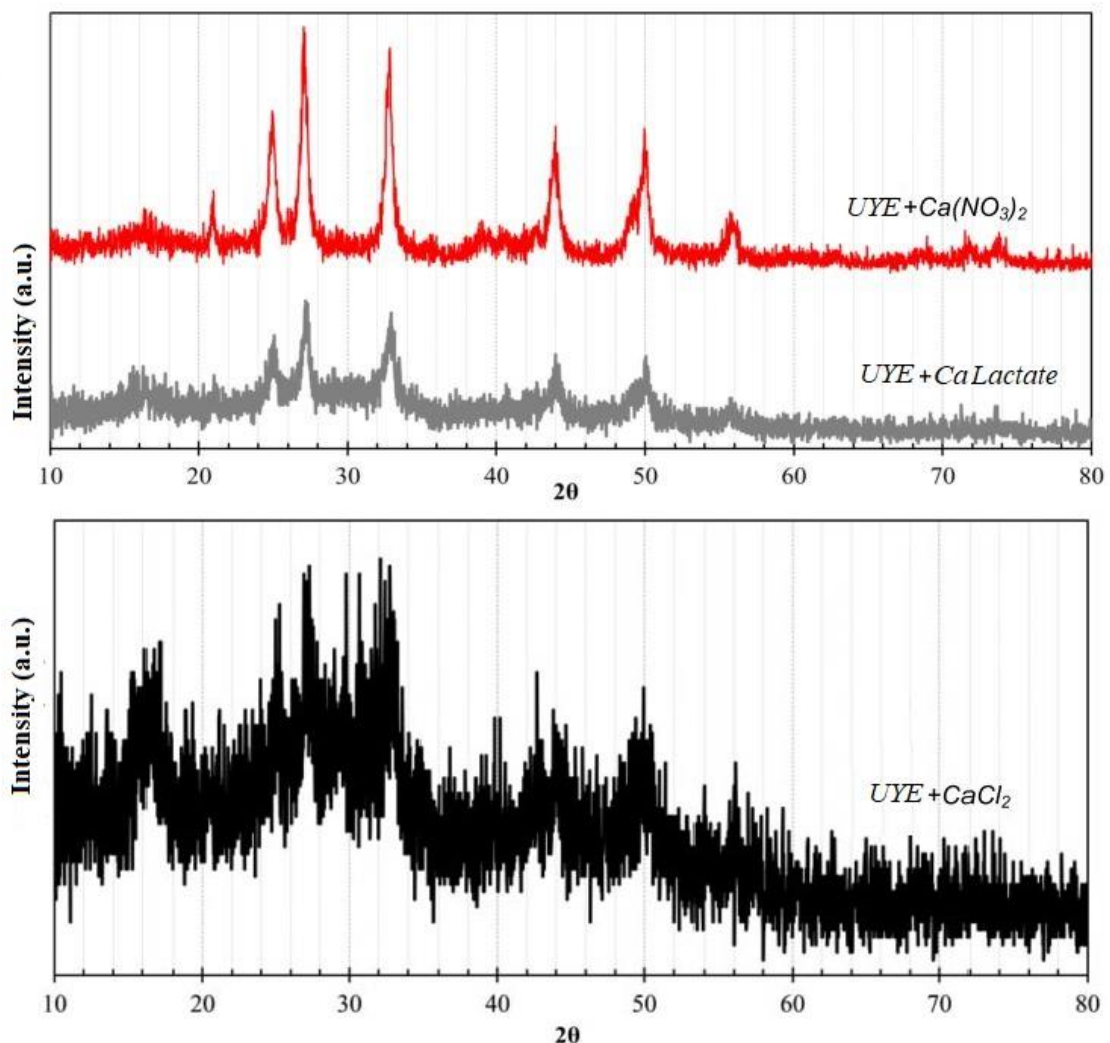
Figure 4.4 XRD analyzes of the precipitation product ($CaCO_3$) obtained from the *S. pasteurii* cells in the UYE medium, YE: Yeast extract
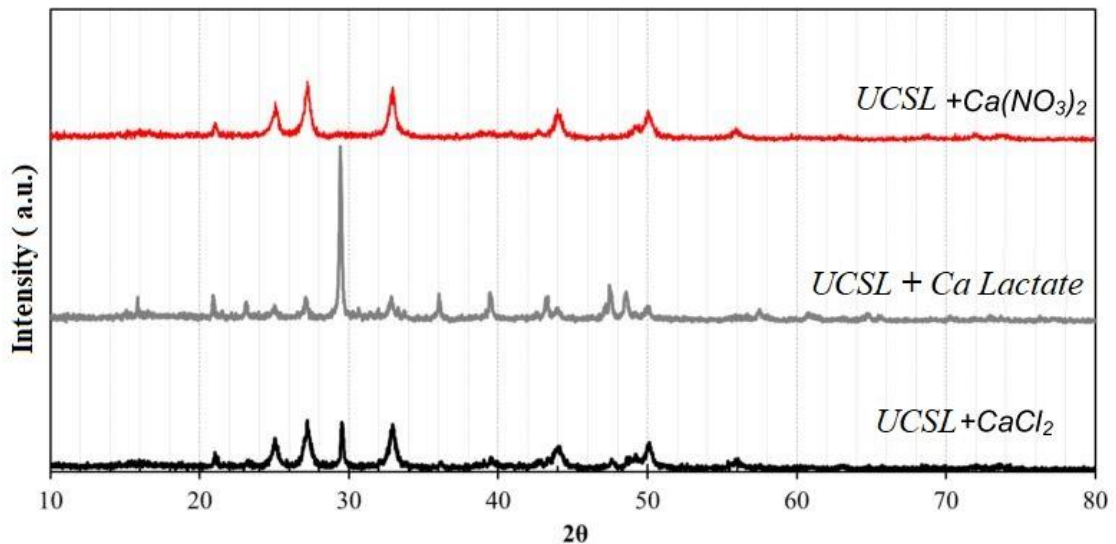
Figure 4.5 XRD analyzes of the precipitation product ($CaCO_3$) obtained from *the S. pasteurii* cells in the UCSL medium. CSL: corn steep liquor.

According to the XRD analyzes, $CaCl_2$ and $Ca(NO_3)_2.4H_2O$ caused calcite and vaterite precipitation in both nutrient media, whereas calcite precipitation was observed in both environment with the addition of $C_6H_{10}CaO_6$. However, $C_6H_{10}CaO_6$ is the most expensive $[Ca^{+2}]$ source in terms of unit price, and the effect of formed lactic acid during the decomposition on the concrete service life is unknown. The addition of $CaCl_2$ in a similar manner poses a risk to the used steel reinforcement. In this regard, the literature continued with the use of $Ca(NO_3)_2.4H_2O$, which is very often used for biomineralization.

Given the unique morphologies obtained from in vitro biogenic $CaCO_3$ precipitation, the type of $[Ca^{+2}]$ also affects the crystallization of $CaCO_3$. Even though, the mass of $[Ca^{+2}]$ sources added to nutrient media were determined in a certain range such that the 0.15M $[Ca^{+2}]$ ions could be provided in the solution. The difference in the $CaCO_3$ crystal morphology could be attributed to different solubility coefficients of these two compounds, as $Ca(NO_3)_2$ having a higher solubility compared to $CaCl_2$ at room temperature. The lower soluble compound $CaCl_2$ resulted with more prominent calcite

peaks while $Ca(NO_3)_2$ induced more prominent vaterite peaks. This might be attributed that different solubilities of these compounds might interfere with the morphology of $CaCO_3$ by affecting $[Ca^{+2}]$. Ogino et al. [58] suggested that an increase in $[Ca^{+2}]$ favored vaterite precipitation and inhibited the transformation of vaterite to calcite. Compounds with a higher solubility might lead to a higher $[Ca^{+2}]$ dissolved in the solution correspondingly increase the ionic strength, contained more vaterite and less calcite than the cultures supplemented with the compounds having lower solubility.

Another point that should be mentioned is the influence of the zeta potential of cells. Based on the zeta potential measurements, the surface charge of cells cultivated in UCSL medium was less than surface charge of cells grown in UYE medium. Only vaterite was observed in UYE medium at pH 9, regardless of the type of calcium source. Even though use of CSL did not affect the pH of the solution the surface charge of the cells decreased significantly. Through the MICP process bacterial cells not only hydrolyze urea but they also act as nucleation sites, any change in surface charge of the cells will interfere with the precipitation process. Presence of calcite precipitates in UCSL medium could be attributed to the difference in cell surface charge.

To investigate the effects of admixtures on biomineralization additives, super plasticizer (22.2 g / L) and AE (2.22 g / Liter) were added to the *S. pasteurii* cells bacterial culture after growing for 7 hours in 200 mL aqueous media (UYE and UCSL media) with presence of $Ca(NO_3)_2.4H_2O$ (40 g / L) (*see* Section 3.2). Figure 4.6 to Figure 4.9 summarize SEM and XRD analyzes of precipitates obtained from bacterial cultures grown in UYE and UCSL media containing chemical admixtures.

(a)

(b)
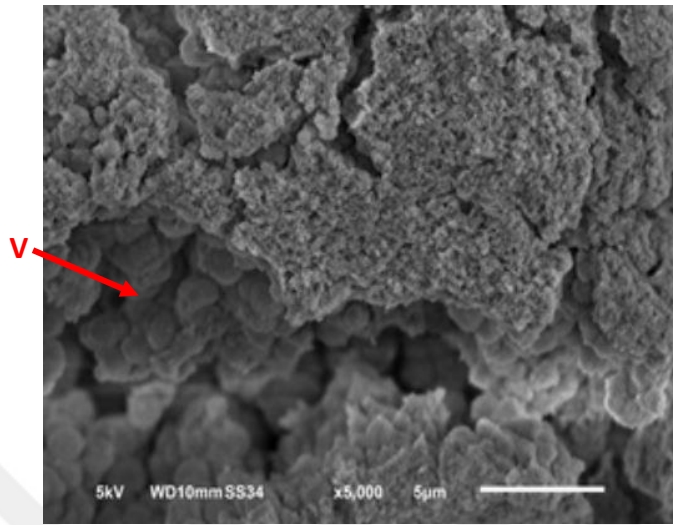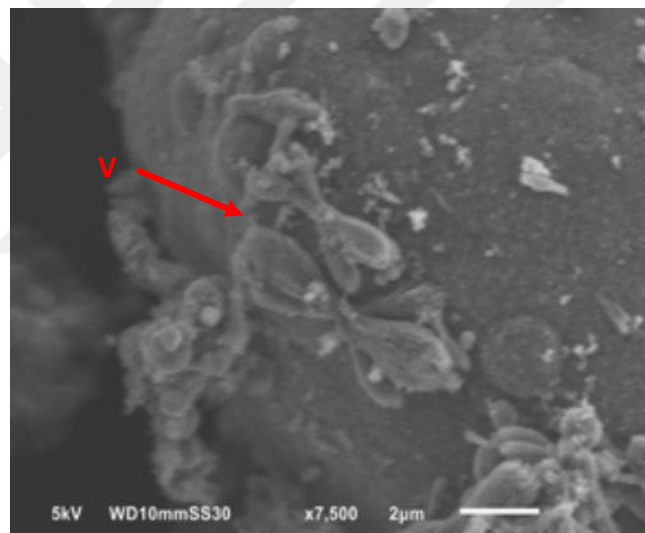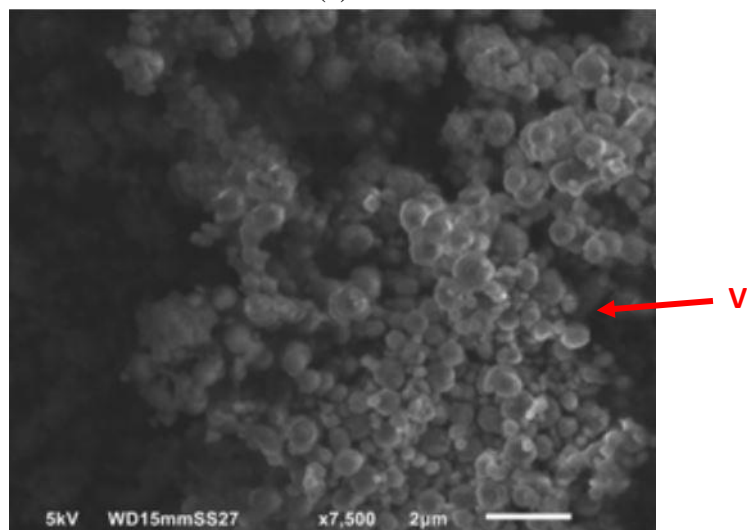
Figure 4.6 SEM images of the precipitation product ($CaCO_3$) obtained from *S. pasteurii* cells and $Ca(NO_3)_2.4H_2O$ at the UYE nutrient media (a) with no additives (b) with air entraining additive (c) with superplasticizer

As shown in Figure 4.6 (b) and 4.8 (b), incorporation of AE in both media resulted with spheroid and layered-flower like shaped- vaterite crystals. Vaterite was observed in samples prepared with AE, which could be related to working mechanism of the admixture. AE can create air bubbles in aqueous environment, which could influence the nucleation of the precipitates. Figure 4.6 (c) and 4.8 (c) shows the result of SP incorporation with both media and the presence of spheroid vaterite in SEM images.

There was no change in the form of precipitation by the addition of $Ca(NO_3)_2$ and AEA in the UYE medium, and mostly, vaterite precipitation was observed (Figure 4.6 and Figure 4.7). However, incorporation of superplasticizers again triggered calcite precipitation. Not only superplasticizers are surfactant materials and could affect the crystal structure of the precipitated product, there is a decrease in the surface charge of the cells compared to cells grown in UYE medium without any admixture. Thus, there was a relationship between the surface charge of the cell and morphology of the precipitate, such that more negative surface charge triggered vaterite precipitation.
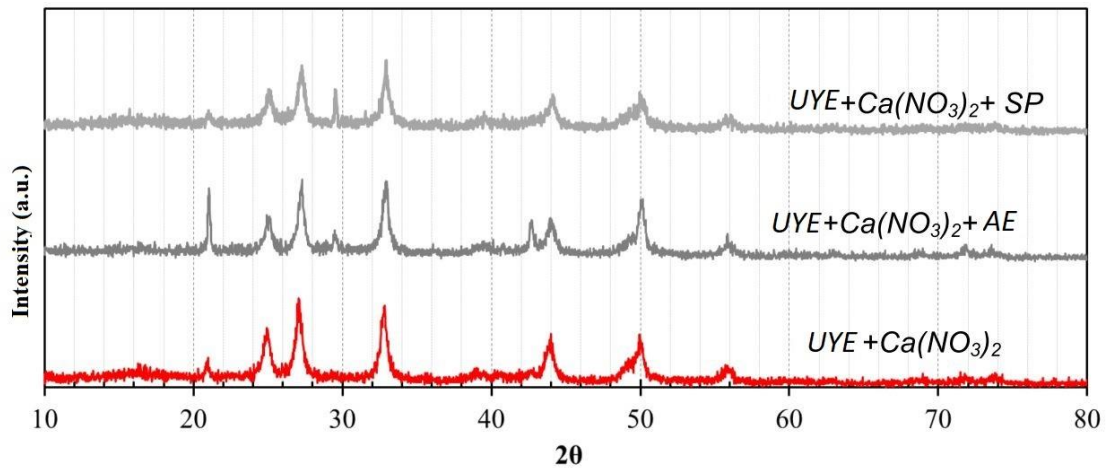
Figure 4.7 XRD analyzes of the precipitation products ($CaCO_3$) obtained from the *S. pasteurii* cells and $Ca(NO_3)_2 \cdot 4H_2O$ in the UYE medium carried out with (a) no additive material, (b) air entraining additive (AE), (c) superplasticizer(SP)
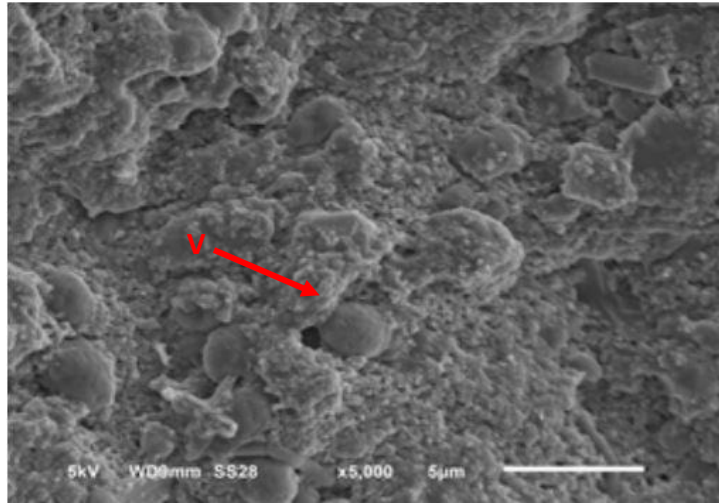
(a)
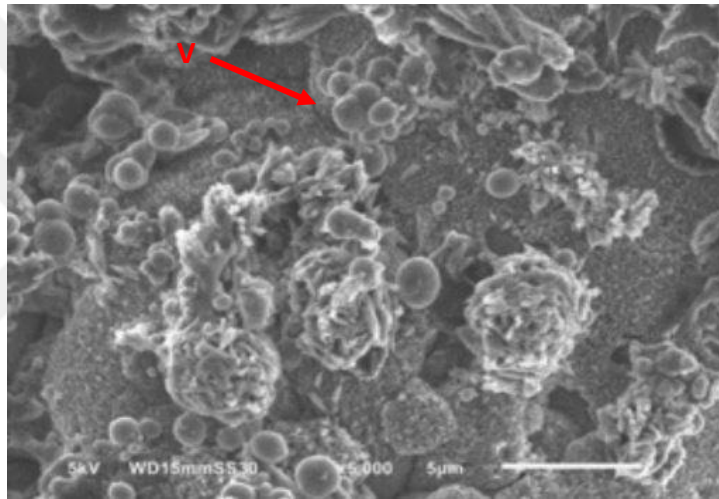
(b)
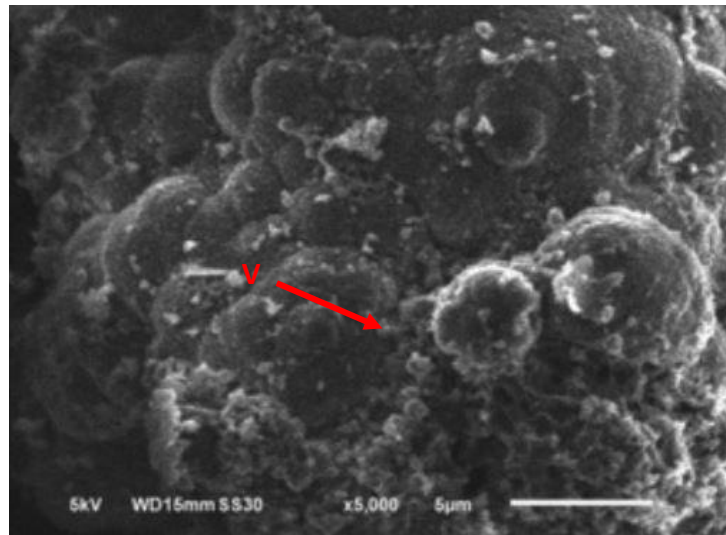
(c)

Figure 4.8 SEM images of the precipitation products ($CaCO_3$) obtained from the *S. pasteurii cells* and $Ca(NO_3)_2.4H_2O$ in the UCSL medium carried out with (a) no additive material, (b) air entraining additive (AE), (c) superplasticizer(SP)
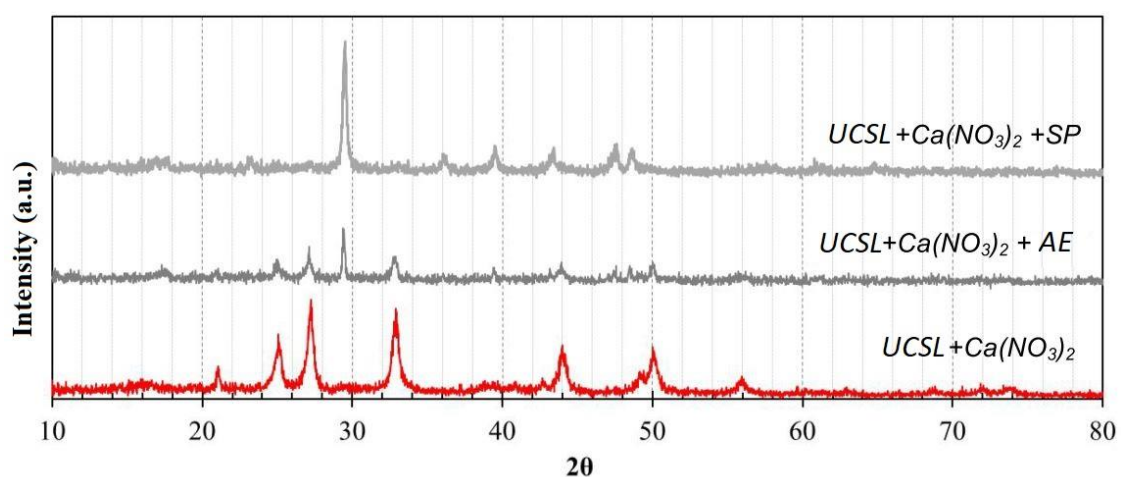
Figure 4.9 XRD analyzes of the precipitation products ($CaCO_3$) obtained from the *S. pasteurii* cells and $Ca(NO_3)_2.4H_2O$ in the UCSL medium carried out with (a) no additive material, (b) air entraining additive (AE), (c) superplasticizer(SP)

Vaterite was obtained when AE was incorporated to the UCSL medium and it was observed that obtained crystal had a more layered structure, rather than a spherical structure. The addition of $Ca(NO_3)_2$ and superplasticizer in UYE medium caused vaterite precipitation with the spherical structure, but there was a significant reduction in the size of the crystals. Variations were observed in the form and size of the crystals produced by $Ca(NO_3)_2$ and the addition of admixtures in UCSL medium (Figure 4.8 and Figure 4.9). Addition of $Ca(NO_3)_2$ to the UCSL medium resulted in only vaterite crystals, whereas AE addition resulted in the formation of vaterite-calcite (Figure 4.8). In addition, according to XRD results, it was determined that the addition of $Ca(NO_3)_2$ and superplasticizer into UCSL medium resulted in only calcite formation and that the calcined calcite structure was not rhombohedral.

4.2 Effect of bacterial culture on the initial setting of cement paste

One of the most important problems in the development of the self-healing mortar is the influence of nutrient medium ingredient on cement-paste hydration. Figure 4.10 shows the effect of bacteria cultures grown in the UYE and UCSL media on the setting time of cement paste samples. The use of CSL instead of the YE in the nutrient medium reduced this effect. The use of UCSL medium (*UCSL sample*) instead of the UYE medium in the cement paste mixture reduced the setting time to 6.5 hours instead of 13 hours. According to Bundur et. al[23]., incorporation of the bacterial culture grown in UYE medium prolonged the setting period significantly. . Similar behavior was observed by Wang et al. [20] such that an increase in YE concentration from 0.35% to 0.85% exacerbated the induction period and decreased the degree of hydration in cement paste with an w/c of 0.50. This delay hardening was attributed to the presence of YE, which is mainly composed of sugar-based carbohydrates [36,37]. Even though incorporation UCSL also retarded the initial set for 7 hours, this retardation in cement paste samples was significantly less than what was found with the addition of UYE medium in both nutrient and bacterial mortars. This can be related to fewer amounts of carbohydrate and sugar in CSL comparing with YE since carbohydrates can act as a retardant agent [59]. Thus, it is crucial to find an alternative carbon source that can be used instead of YE.

Addition of *S. pasteurii* cells with both UYE and UCSL media (*Bac-UYE* and *Bac-UCSL* samples) exacerbated the delay initial setting. Since the microorganisms were grown in these media, there might be changes in both media due to the metabolic activity of cells. The changes in the nutrient medium induced by the metabolic activity of cells might influence the ionic concentration of the medium, thus affecting the initial set time.
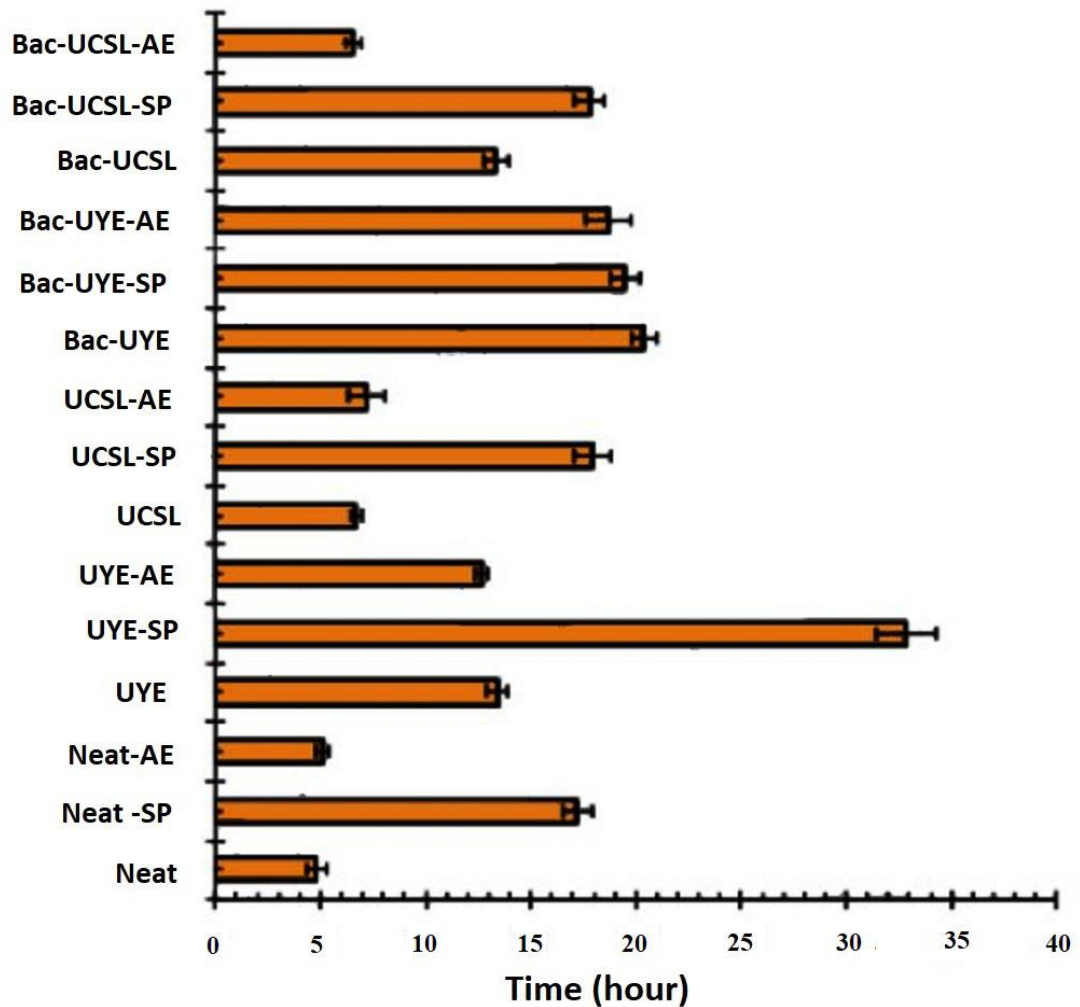
Figure 4.10 The effect of bacterial cultures grown in the UYE and UCSL media on the setting time of cement paste. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture. The bars represent the average obtained from three replicates, and the error bars represent the standard deviation.

In terms of hardening and initial setting, CSL was found to be a suitable replacement for YE. While there is not enough information regarding UCSL medium in MICP applications, these results were promising in developing a self-healing cement-based material.

In addition, this study focused on interaction between the bacterial culture and chemical admixtures. As expected, incorporation of superplasticizer (SP) to *UCSL-SP*

(1% by weight of cement) extended the setting time period compared to UCSL sample. This could be attributed to the dispersion of cement particles; however, this delay was more than what was generally observed with incorporation of superplasticizers. While the main reason is not known, this phenomenon was more severe in samples including UYE medium. Further studies have to be done in order to understand the interaction with nutrient media, bacterial culture and superplasticizers.

Another chemical admixture tested was AE (0.1% of the cement weight) and incorporation of AE did not affect the initial setting time in contrast to superplasticizers.

Finally, use of a calcium source was investigated (*see* Figure 4.11). Incorporation of $Ca(NO_3)_2$ as a calcium source (up to 1% of the cement weight) shortens the setting time for 1-2 hours for each sample.
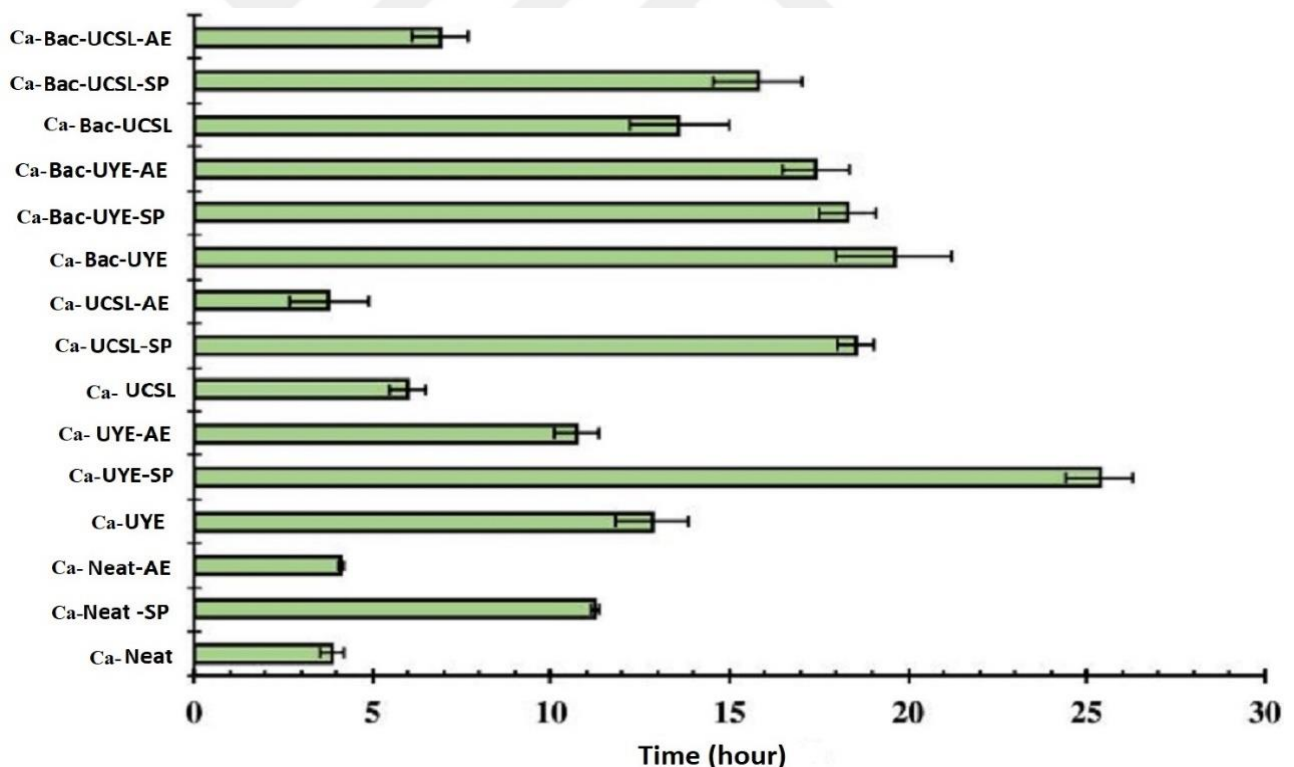
Figure 4.11 The effect of bacterial cultures grown in the UYE and UCSL media incorporated with $Ca(NO_3)_2$ on the setting time of cement paste. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture. The bars represent the average obtained from three replicates, and the error bars represent the standard deviation.

4.3 Microstructural changes in cement paste: $CaCO_3$ formation

Since vegetative cells were incorporated to cement paste with their nutrients, possible MICP within the cement paste was also investigated. TGA and DTG curves were obtained as it is stated in Section 3. 6.1. DTG curve was used to determine the exact temperatures for decomposition for both CH and $CaCO_3$. The mass percentages of CH and $CaCO_3$ were calculated from the TGA curve by using these temperature points pinpointed from DTG curve (*see* Figure 3. 2 in Section 3. 6). Results of the analyses are summarized as percentages of CH and $CaCO_3$ in cement paste samples in Figures 4.12 and 4.13.

(a)

(b)

(c)
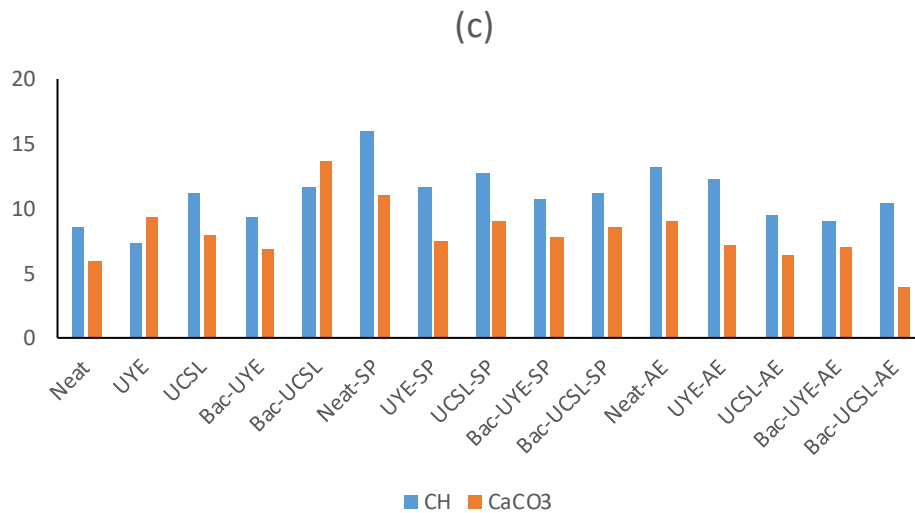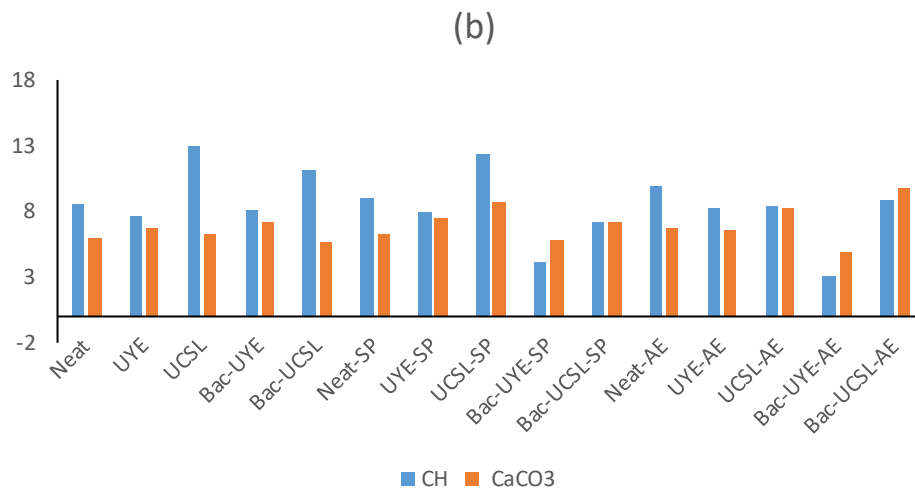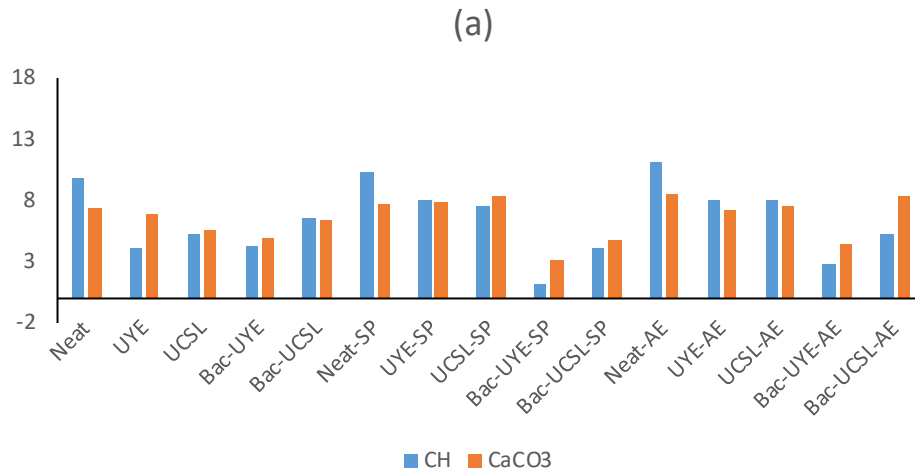
Figure 4.12 *CH, and CaCO₃* amounts for (a)3-days, (b)7-days and (c)28-days days for all cement paste samples without [Ca^{+2}] source addition into mix. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.
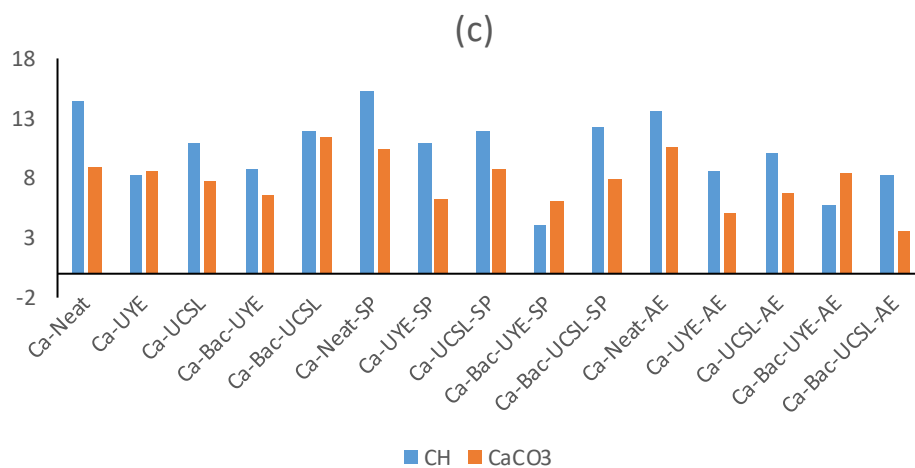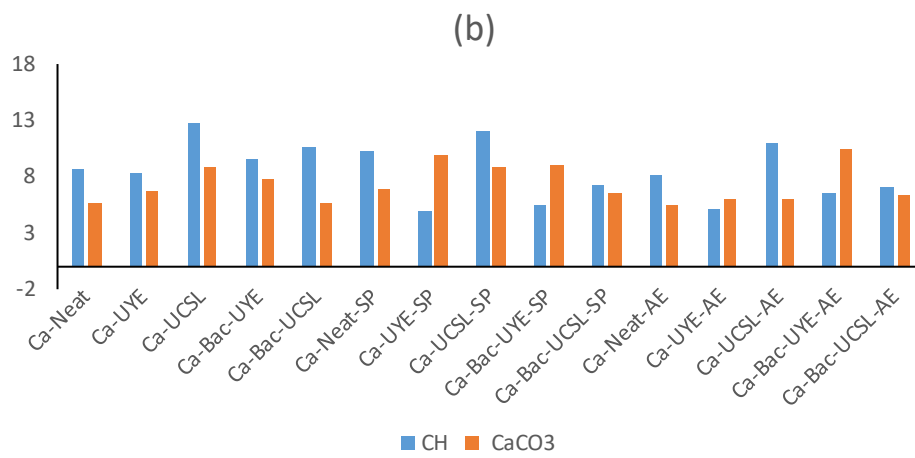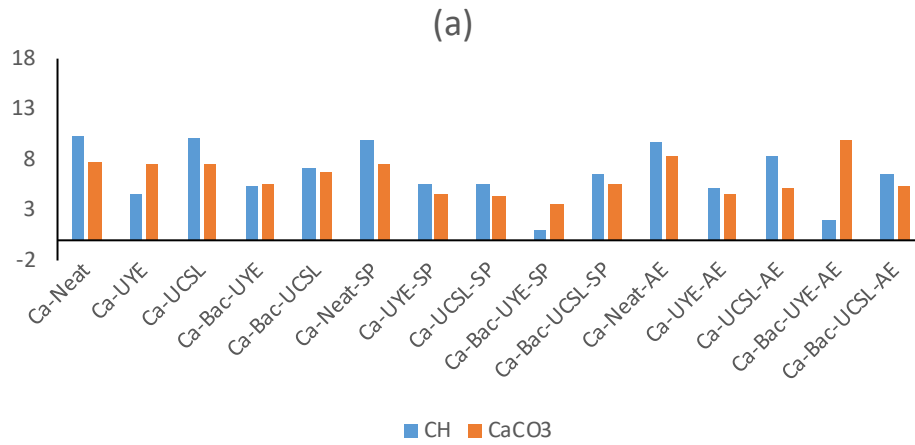
Figure 4.13 CH, and CaCO$_3$ amounts for (a)3-days, (b)7-days and (c)28-days days for all cement paste samples with [Ca^{+2}] source addition into mix. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.

At 3-days, there was a slight decrease in CH content in *UYE and Ca-UYE* sample compared to both *Neat paste* and *Nut-UCSL* samples. This decrease might be attributed to the delay in the initial set, which is mainly related to the formation of hydration products [20]. In contrast, the CH content in *UCSL* samples were on par with *Neat paste* samples, which again in line with the initial setting times for both samples. The decrease in CH content at 3-days was more pronounced in *Bac-UCSL* and *Bac-UYE* samples, which could again be attributed to a longer initial setting time [23].Thus, it could be concluded that there is a relation between the initial setting times of the samples and the CH content, particularly at 3-days.

Interestingly, there was not increase in $CaCO_3$ content in any 3- old day sample compared to control *Neat paste* at 3-days. Incorporation of cells in *Bac-UCSL* sample slightly increased the $CaCO_3$ content comparted its control *Nut-UCSL* but an opposite trend was observed in samples prepared with UYE. Even though, the reason causing these opposite trends was not known, it might be related to viability of cells.

At 7-days, considering CH formation values for samples showed a noticeable jump for CH amount in all samples except for *Neat paste*. Again, CH content in *UCSL* sample comparing to other samples was significantly increased. Bacterial paste samples (*Bac-UYE* and *Bac-UCSL*) in comparison to *UCSL* and *UYE* had less amount of CH content. Use of UYE medium induced a higher $CaCO_3$ content within the cement paste sample compared to use of UCSL medium.
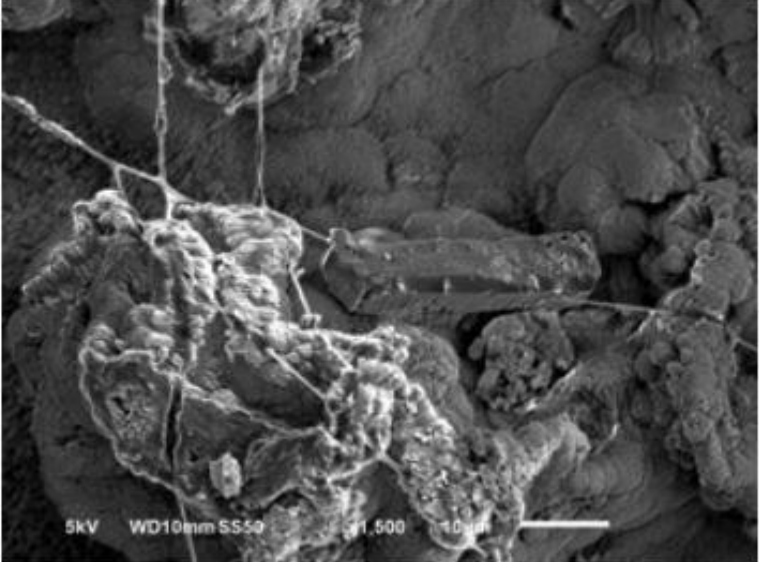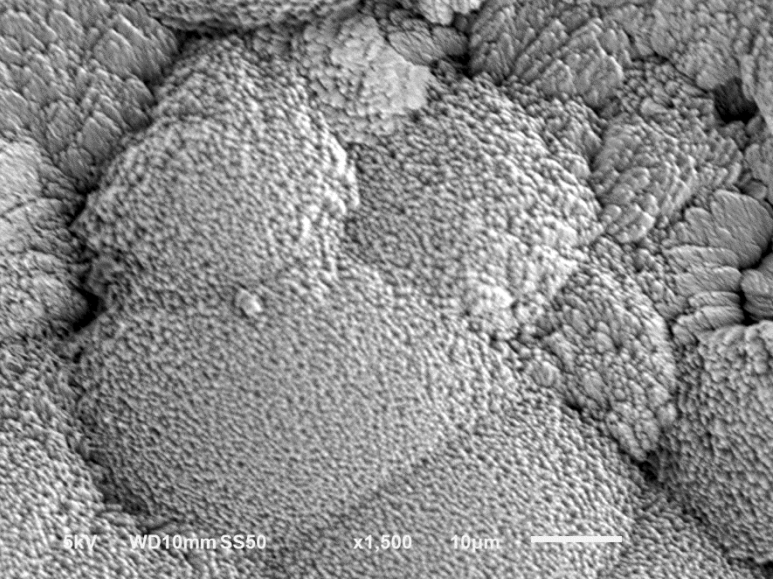
At 28-days, the CH contents in *UYE* and *CSL* samples were decreased comparing to other samples at the same age. This might be caused by curing process with full submersion of samples in curing solution due to CH being leached out. However, there was an increase in CH content in bacterial paste samples (*Bac-UYE* and *Bac-UCSL*). By 28-days, all cement paste samples had similar levels of CH, which indicates same

hydration level for samples at this age. At the end of 28 days, *Bac-UCSL* sample had the highest $CaCO_3$ content among the samples indicating a higher carbonation rate, which should be evaluated in the future considering possible durability problems.
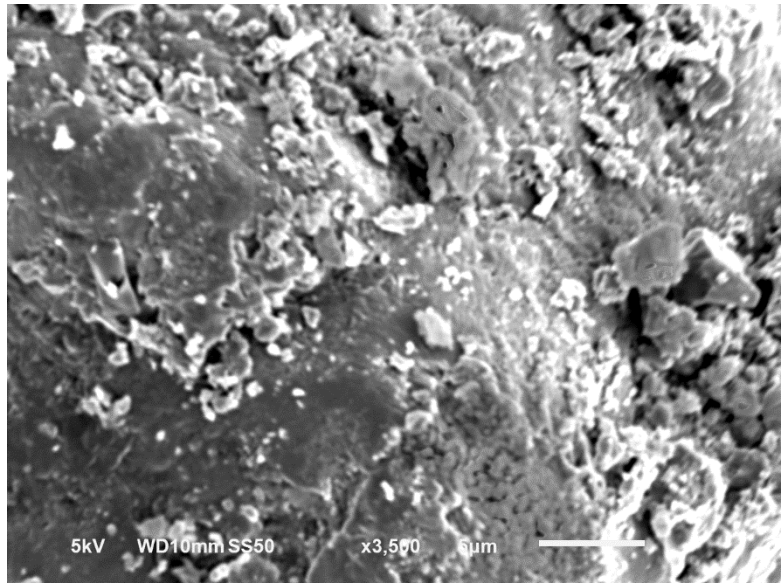
In general, the results obtained from TGA analysis revealed that maximizing biogenic $CaCO_3$ precipitation could not actually be increased within the cement paste when vegetative bacterial cells were inoculated with their nutrients. Even addition of Ca $(NO_3)_2$ into the mixture did not impact neither CH nor $CaCO_3$ content (*see* Fig. 4.12). However, there was a decreasing trend in biogenic $CaCO_3$ through aged samples, which might be attributed to cell death or endospore formation due to limited access to nutrients. Previously, Bundur et al. [49] have observed that a fraction of the inoculated cells can persist in cement for almost one-year, however, there was a decreasing trend in the concentration of viable cells within the cement paste. This might suggest that to induce biogenic $CaCO_3$ precipitation within the cement-paste there is a threshold value for cell concentration, which might indicate that self-healing of cracks can be obtained potentially at early ages via MICP.

SEM studies were carried out to investigate the microstructure effects of the media and bacterial cultures added to the cement paste. Table 4-4 and Table 4-5 summarize SEM images of 28 day for bacterial samples.

Table 4.4 SEM images for 28 day samples taken from fracture surfaces of bacterial specimens. Bac: Bacterial mortar; YE: yeast extract, CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture. Rest of the images are presented in Appendix A.

Bac-UYE	
Bac-UYE-SP	

Bac-UYE-AE	
Bac-UCSL	
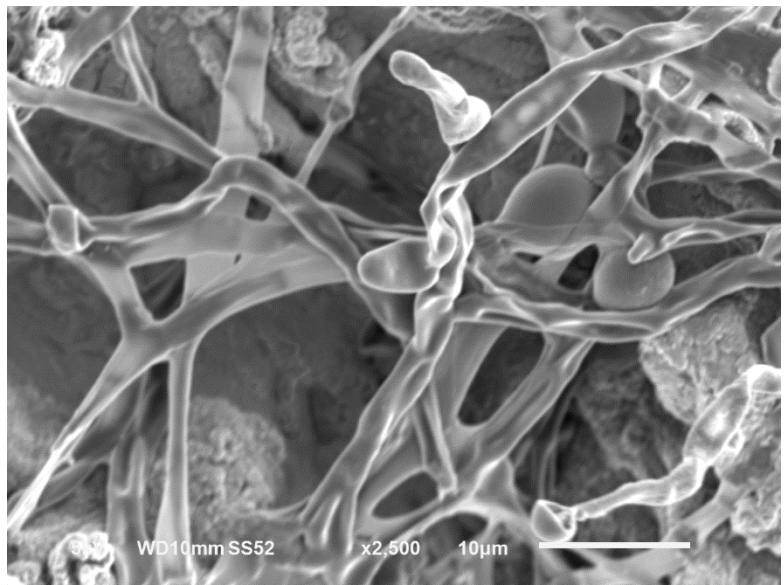
|	
Bac-UCSL-SP	
Bac-UCSL-AE	
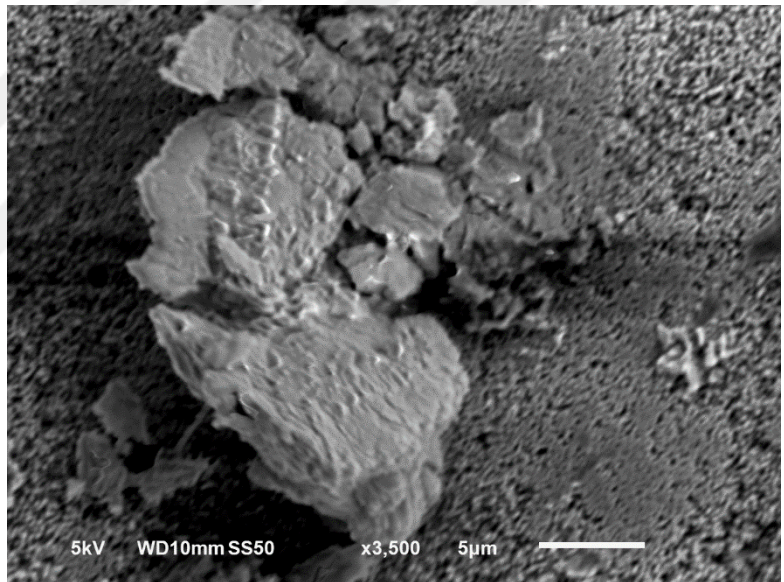
Table 4.5 SEM images for 28 day bacterial samples taken from fracture surfaces bacterial specimens containing calcium source. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.

Sample	SEM image	Sample	SEM image
Bac-UCSL		Ca-Bac-UCSL	
Bac-UCSL-AE		Ca-Bac-UCSL-AE	

SEM images were provided by the samples taken from the crack surfaces initiated on the specimens under bending machine. Even without an increase in the amount of $CaCO_3$ determined by TGA analyzes, $CaCO_3$ crystals were observed in samples containing bacteria (*Bac-UCSL, Ca-Bac-UCSL, Ca-Bac-UCSL-AE*). This, indicates that biomineralization takes place in the cement paste.

4.4 Effect of bacterial culture on the compressive and flexural strength

The influence of *S. pasteurii* and nutrient media on compressive and flexural strength of mortar samples is given through Figure 4.14 to 4.17.
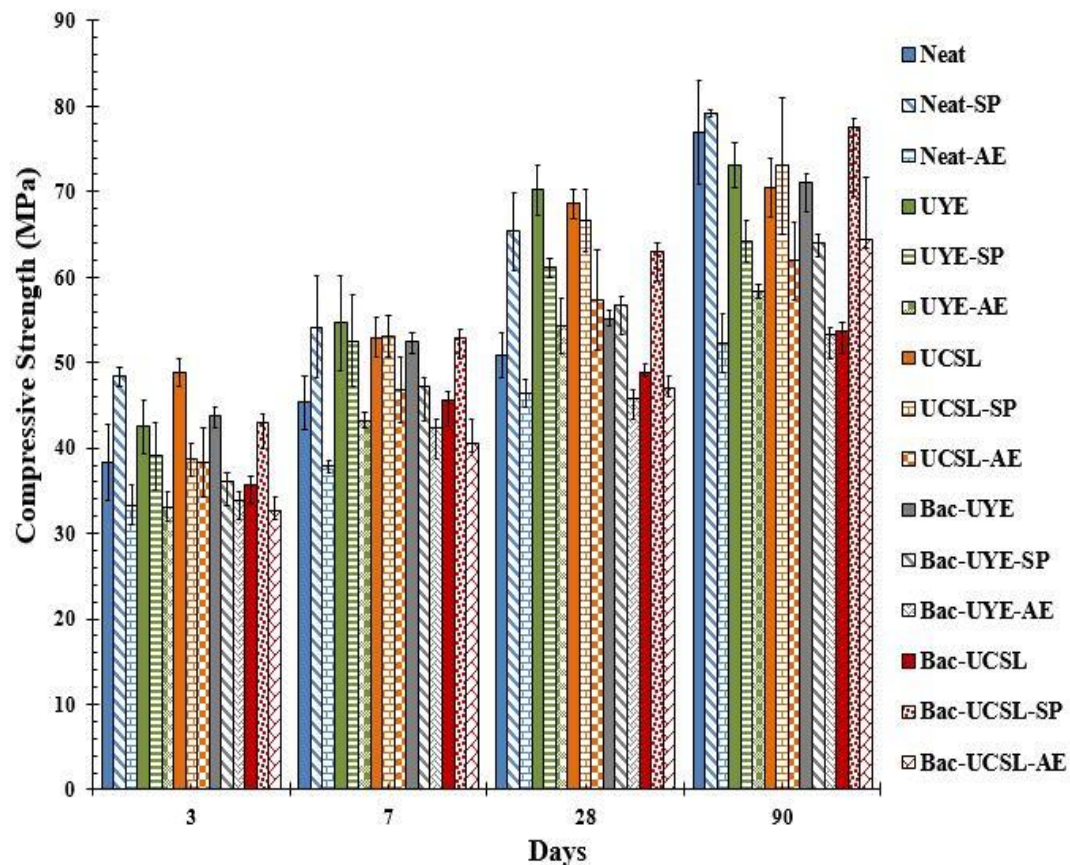
Figure 4.14 Effect of bacterial culture on the cement mortar without Ca^{+2} addition for 3, 7, 28 and 90 days. The cement ratio was 0.45. The bars represent the average obtained from three replicates, and the error bars represent the standard deviation. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.

Based on the compressive strength test result, use of UCSL medium (*UCSL* sample) instead of UYE medium (*UYE* sample) did not have negative effect on the compressive strength. Moreover, there was a substantial increase in compressive strength of *UCSL* sample compared to *UYE* and *Neat* samples at all ages. A similar behavior was also observed in the specimens containing superplasticizer or AE. However, the

compressive strength of both Nutrient mortar samples, *UYE* and *UCSL*, were slightly lower than *Neat mortar* at 90 days, which suggested that the rate of strength development in *Nutrient mortar* samples were higher at early ages, then it slows down. This might be due to decomposition of nutrient medium and side products of them.

Compressive strength, by addition of *S.pasteurii* cells to mortar with the UCSL medium (*Bac-UCSL* sample) was lower than *Neat mortar* sample, also showed a lower compressive strength than the *UYE*, *UCSL* and *Bac-UYE* mortar samples at all ages. Meanwhile, this behavior has not been observed in *Bac-UCSL-SP* and *Bac-UCSL-AE* samples containing superplasticizer and AE.

Based on the TGA analysis, biomineralization could only occur in cement paste under certain conditions. Previously, Bundur et al. [27] showed that incorporation of S. pasteurii cells actually increased the compressive strength and this was attributed to the increase in the amount of $CaCO_3$ in the cement paste. Even though, a similar type of approach was used in this study, there was not any significant change in neither $CaCO_3$ content nor the compressive strength. This difference might be due to use of CEM I 42.5R cement instead of ASTM Low Alkali Type I/II cement. Not only CEM I 42.R have a higher C_3A content compared to Type I/II cement, it also has a higher alkali content. These parameters could affect the cell viability, thus the possible MICP process. In fact, Bundur et al. [49]showed that at 28-day the cell viability with CEM I 42.5 was found to be lower compared to the cell viability recorded in 28-day mortar sample prepared with Type I/II cement[48]. Moreover, incorporation of cells (*Bac-UYE* and *Bac-UCSL* samples led to a decrease in compressive strength compared to their *Nutrient mortar* control samples. This shows the ionic changes in the bacterial culture due to bacterial metabolic activity affected both hardening and strength development of cement paste.

Addition of Ca(NO₃)₂ did not increase the amount of $CaCO_3$ in the cement paste, for the same reason addition of bacterial cells to the specimens containing calcium source did not cause an increase in the compressive strength. A similar trend was observed with the samples when Ca(NO₃)₂ was added. This again was in line with the hypothesis that the increase in compressive strength was directly related to the increase in $CaCO_3$ content.
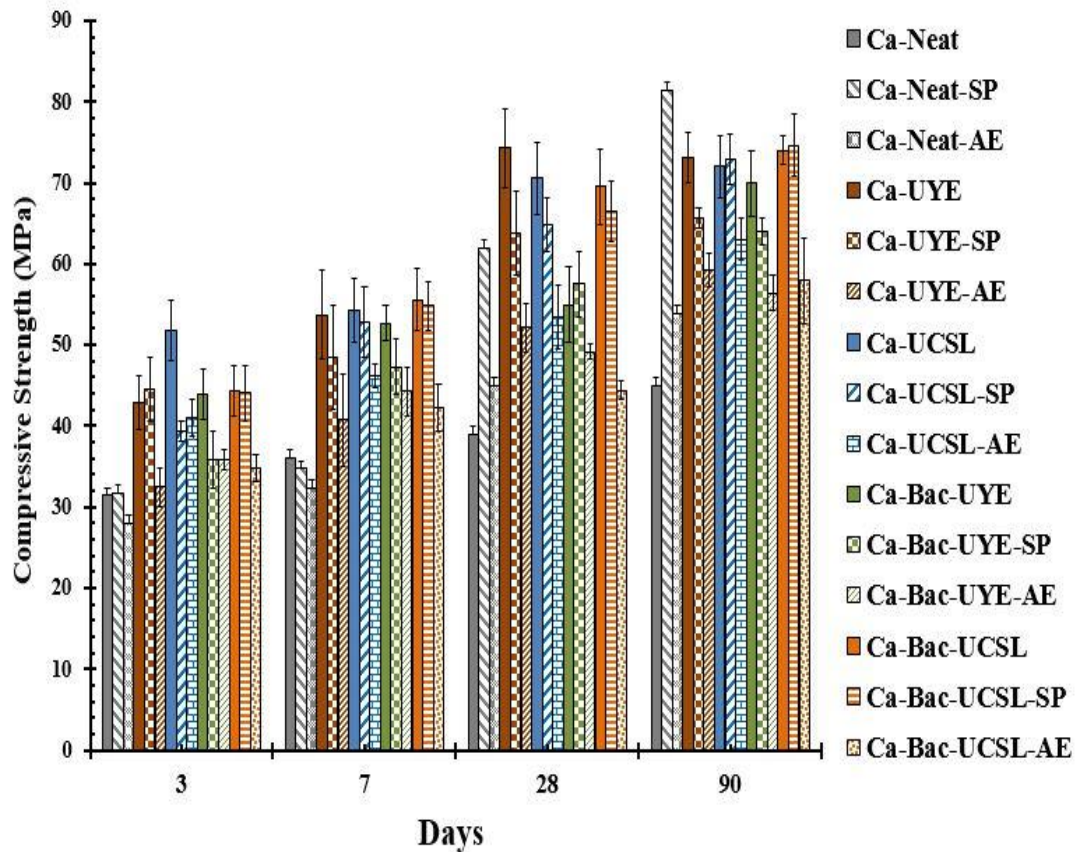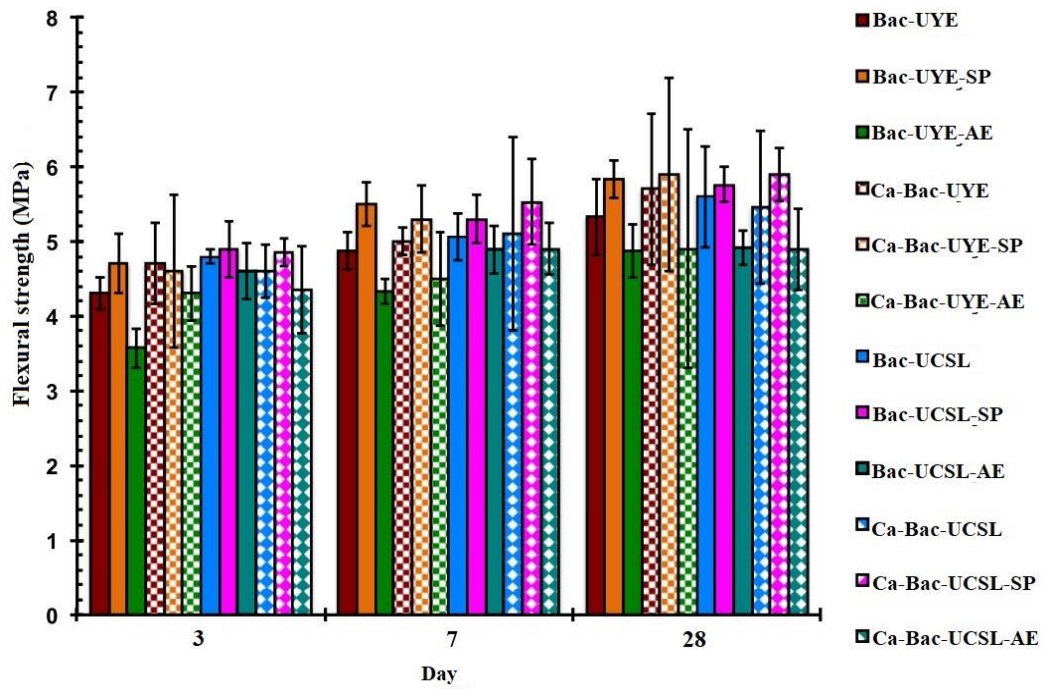

Figure 4.15 Effect of bacterial culture on the cement mortar with [Ca^{+2}] source addition for 3, 7, 28 and 90 days. The cement ratio was 0.45. The bars represent the average obtained from three replicates, and the error bars represent the standard deviation. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.

Figure 4.16 and figure 4.17 show the influence of nutrient media and bacterial culture on the flexural strength of mortar. It was observed that the flexural strength decreased when both nutrient media and bacterial culture were added to mortar. Even though the actual reason for this behavior is unknown, it might be due to different ionic concentrations in the pore solution influencing the bonding between the phases. Based

on the performance of *Bacterial mortar*, it could be concluded that application of self-healing bacterial mortar could be more suitable for non-structural members, such as grouting, instead of structural members Further studies should be conducted to improve the performance of bacterial mortar for structural applications.
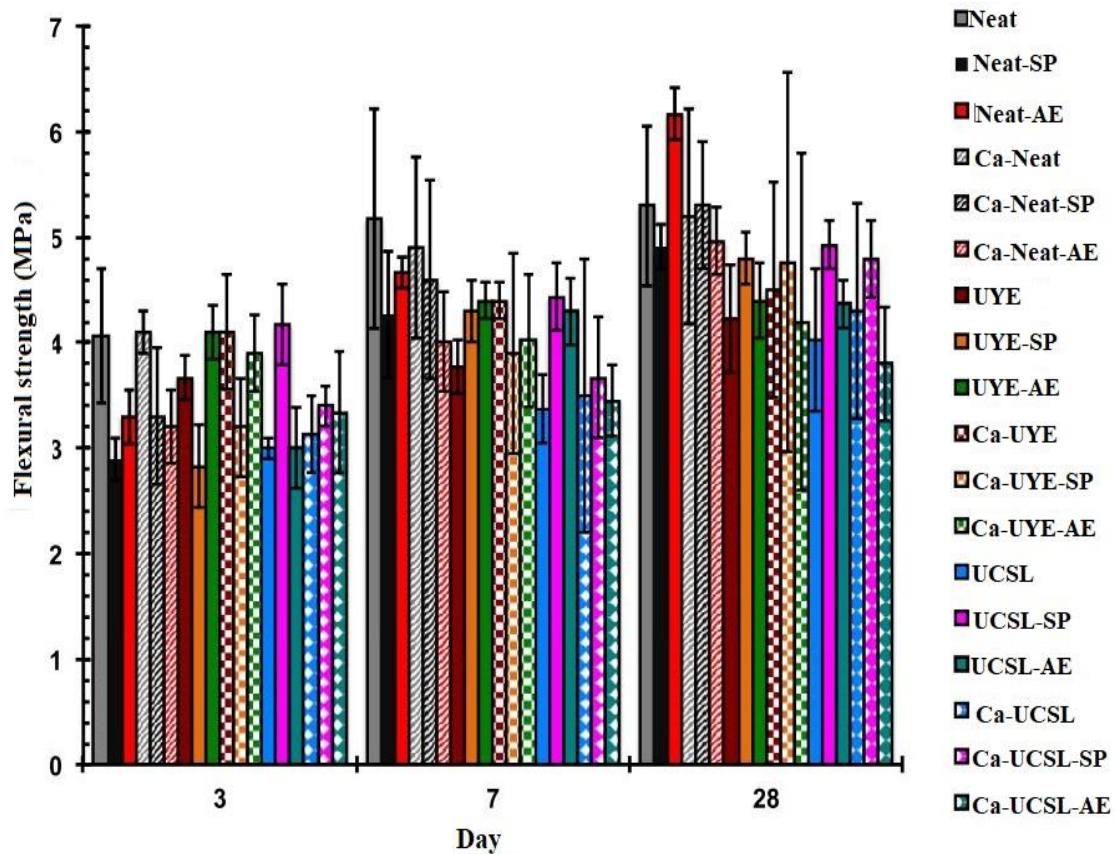
Figure 4.16 Flexural strength data for samples containing water/UYE/UCSL, $[Ca^{+2}]$ source and admixtures. The cement ratio was 0.45. The bars represent the average obtained from three replicates, and the error bars represent the standard deviation. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.

Figure 4.17 Flexural strength data for samples containing $[Ca^{+2}]$ source, admixtures and bacterial culture grown in UYE/UCSL. The cement ratio was 0.45. The bars represent the average obtained from three replicates, and the error bars represent the standard deviation. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.

## 4.5 Development of self-healing mortar

The ultimate goal of this project was to develop a self-healing mortar by using biomineralization. To achieve this goal, the main objective was to observe crack remediation in *Bacterial mortar* samples. The investigations were taken as 3 steps: (1) Visual inspection of crack sealing (2) Flexural strength recovery (3) Characterization of the precipitate collected from the crack surface.

Table 4.6 and Table 4.7 show the optical microscope images taken before and after curing of cracks formed by flexural loading.
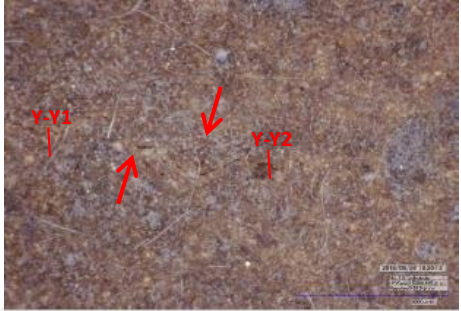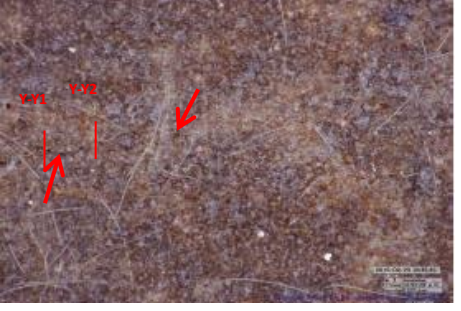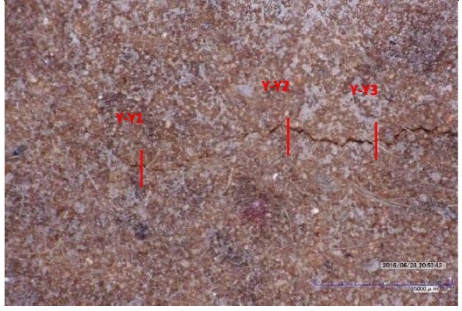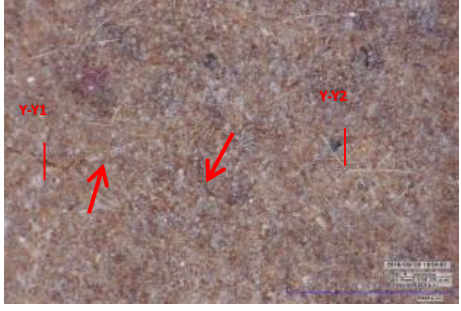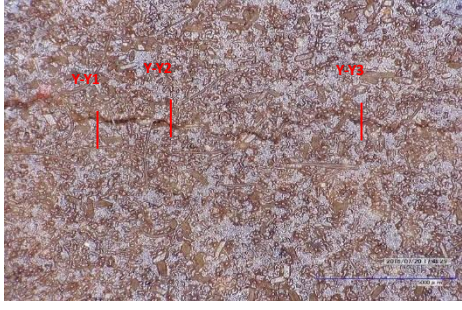
Table 4.6 Cracks occurring on day-7 for control series and cracks after 50 days curing. Triplicate Optical images were taken from samples. The crack size is between 0.3-0.5 mm; Scale: 1 / 5000µm. The samples were kept under curing in the nutrient medium. CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.
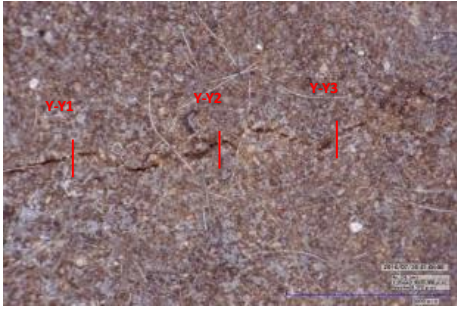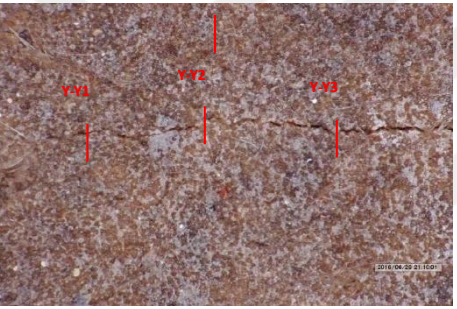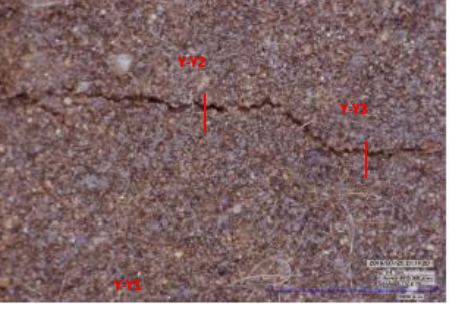
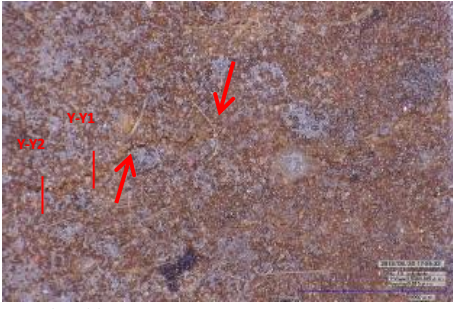| Name	7-day crack	After 50-day curing
Neat		
Neat-SP		
Neat-AE		

UYE		
UYE-SP		
UYE-AE		
UCSL		
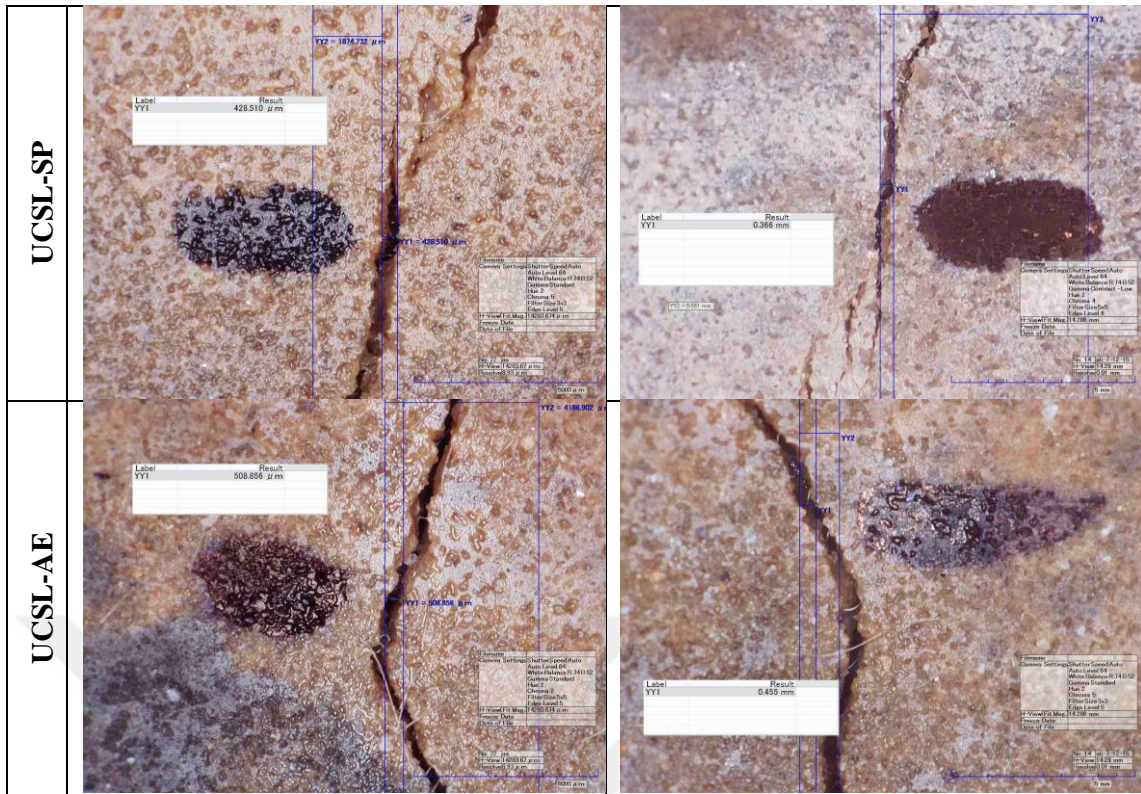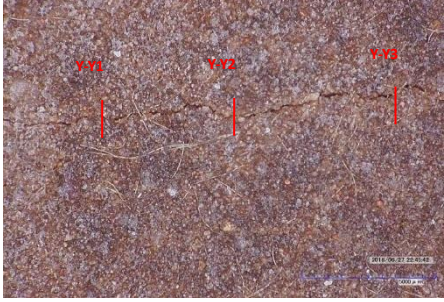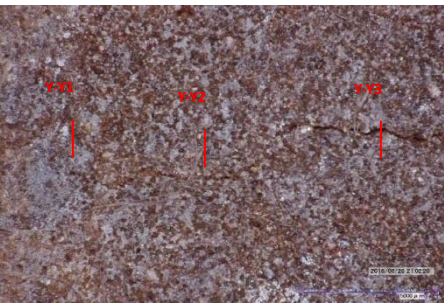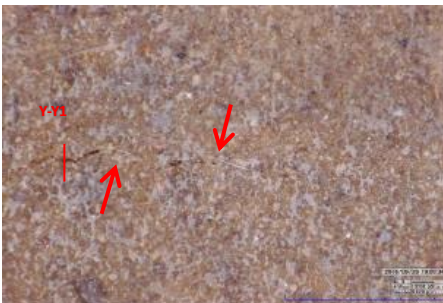
Table 4.7 Cracks occurring at day 7 for bacterial samples and cracks after 50 days of curing. Optical images were selected from triplicate images. The crack size is between 0.2-0.3 mm; Scale: 1 / 5000μm. The samples were kept under curing at the juicy feeder. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.
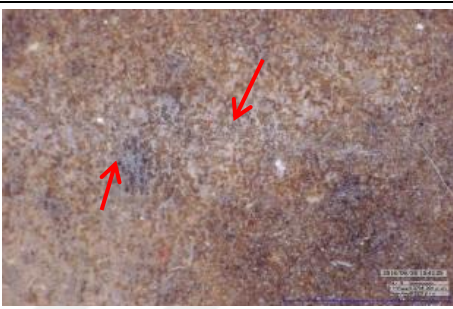
Name	*7 day crack*	*After 50 day curing*
Bac-UYE	Crack width- 0,41 mm	Crack width- 0,23 mm
Bac-UCSL	Crack width- 0,43 mm	Crack width- 0,24 mm
Ca-Bac-UYE	Crack width- 0,41 mm	Crack width- 0,29 mm
Ca-Bac-UCSL	Crack width- 0,39 mm	Crack width- 0,32 mm

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Bac-UYE-SP	Crack width- 0,42 mm	Crack width- 0,32 mm
Bac-UCSL-SP	Crack width- 0,36 mm	Crack sealed
Ca-Bac-UYE-SP	Crack width- 0,40 mm	Crack sealed
Ca-Bac-UCSL-SP	Crack width- 0,43 mm	Crack sealed
Bac-UYE-AE	Crack width- 0,44 mm	Crack width- 0,27 mm

Bac-UCSL-AE	Crack width-0,41 mm	Crack width- 0,25 mm
Ca-Bac-UYE-AE	Crack width- 0,39 mm	Crack width- 0,27 mm
Ca-Bac-UCSL-AE	Crack width- 0,40 mm	Crack sealed

As expected, there was not any crack remediation observed in control samples without any microorganism (see Table 4.6). Basically, the crystals deposited in the crack could be simply CH, which was formed due to submersion of the samples in water or nutrient media. In contrast, cracks were almost sealed when microorganisms were incorporated with their growth nutrient media (UYE or UCSL).

As it was stated before one of the parameters affect MICP process was the concentration of $[Ca^{+2}]$ ions (see Section 2.4). To increase the amount of the biogenic precipitate, $Ca(NO_3)_2$ was incorporated to the mix design. Based on the optical images, incorporation of additional calcium source increased the amount of precipitate and improved the crack remediation (compare *Bac-UYE* sample with *Ca-Bac-UYE* sample).

$Ca(NO_3)_2$ is a commonly used calcium source in biomineralization applications due to its negligible impacts on corrosion compared to $CaCl_2$. In addition, use of $Ca(NO_3)_2$ might improve the freeze and thaw resistance of the material.

In general, self-healing was obtained in *Bacterial mortar* samples when required conditions were provided. CSL was found to be a suitable alternative for YE in terms of self-healing applications in cement-based materials. The cracks were sealed in samples regardless of the nutrient media used in cultures. Almost most of the cracks completely sealed and crack width was significantly reduced in samples even if complete recovery was not observed.

It should be noted that, crack healing was observed in 7-day old mortar samples. However, a similar recovery was not observed in samples older than 7-days (i.e. 14 and 28 days) when microorganisms were incorporated without any encapsulation. This is not only related to cell viability but also related to the concentration of remaining cells. According to Bundur et. al [48], microorganism can survive up to 11 months inside cement mortar without any encapsulation. However, the viable cell count was determined to be 2% (2×10^3 CFU / mL) of the bacteria present in the first mixture. According to Ersan et. al [60] the biomineralization occurs at a time when the number of cells per gram mortar is at least 10^6 cells/ kg cement. Similarly, Bundur et al.[49] showed that the cell concentration should be at least on the order of 10^4 cells per gram of mortar.

The number of cells inoculated without a protection barrier would expected to be decreasing due to stresses such as high pH, hydration, nutrient depletion, lack of oxygen and space etc.[22]. Thus, even though viable cells were detected in samples as old as 11 months, it might not be enough to trigger MICP. Viable cell counts were not done directly after casting however since the methodology and the cement type was similar, it was assumed that the remaining cell concentration in hardened mortar would be the same with

the complementary study conducted with Ghent University. The low cell concentration limits the service life expectancy of this method, such that the cell concentration drops significantly after 7 days. *Thus, it was concluded that self-healing could only be obtained with unprotected cells at early ages.* If crack repair is planned for older ages, the use of endospores or protection barriers should be considered.

Interestingly, the use of superplasticizers visually improved self-healing ability of cells regardless of the nutrient media used in the mixing. Such that, the cracks were almost completely sealed with the addition of superplasticizers (see Table 4.7). This might be again due to the surfactant characteristics of the admixture, improving cell agglomeration and possible nucleation sites. However, a deeper investigation on the effect of superplasticizers on bacterial viability and biomineralization mechanism is needed to better explain this interaction.

AE was another chemical admixture used in this project. Compared to superplasticizers, AE created a negative effect on self-healing ability cells especially when it was used with UYE medium. Partial crack sealing and $CaCO_3$ precipitation was observed in samples containing AE (comparing *Bac-UYE*, and *Bac-UYE-AE* samples in Table 4. 7). However, such a negative effect was not observed when AE was used in samples prepared with UCSL medium (Comparing *Bac-UCSL-AE* and *Ca-Bac-UCSL-AE* samples with *Bac-UYE* and *Ca-Bac-UYE-AE* samples in Table 4.7). This decrease in self-healing ability was attributed to the decrease in cell viability when AE was added to Bacterial mortar prepared with UYE medium. Bundur et al. [49] hypothesized that use of AE could actually increase the cell viability by providing extra space for cells. However, their results showed an opposite trend such that use of AE actually reduced the viability of bacteria when the cells were inoculated in mortar with UYE medium. The researchers have linked this to the working mechanism of AE, such that the entrained air voids were

created with hydrophobic nature of the surfactant. Surfactant is an additive which can form a layer within the area of hydrophobic shell and hydrophilic water core decreasing the repelling force between these 2 layers[61]. Microorganisms could easily fit into the entrained air voids however the nutrient medium could not due to the hydrophobic tails of the AE. Thus, it would be more convenient to use endospores than vegetative cells since endospores do not require nutrients to stay viable. AE generally added to water phase of the mixture and it can be activated in an aqueous solution. However, CSL has a higher viscosity compared to water and UYE medium, thus it might interfere with the working mechanisms of AE. This might be the reason for not seeing such a negative effect with the use of UCSL. Further investigation should be done to analyze the interaction with UCSL medium and AE.

At the end of the visual analysis, it was determined that the UCSL medium did not have any negative effect self-healing ability of cells and crack closure. Therefore, the rest of the analyzes were only conducted on the samples prepared by bacterial culture grown in UCSL medium.

Once the crack closure was identified, characterization of the precipitate filling the cracks were done by FT-IR analysis. Figure 4.18 shows FT-IR analyses of the precipitate collected from the crack surfaces after the cracks were sealed.
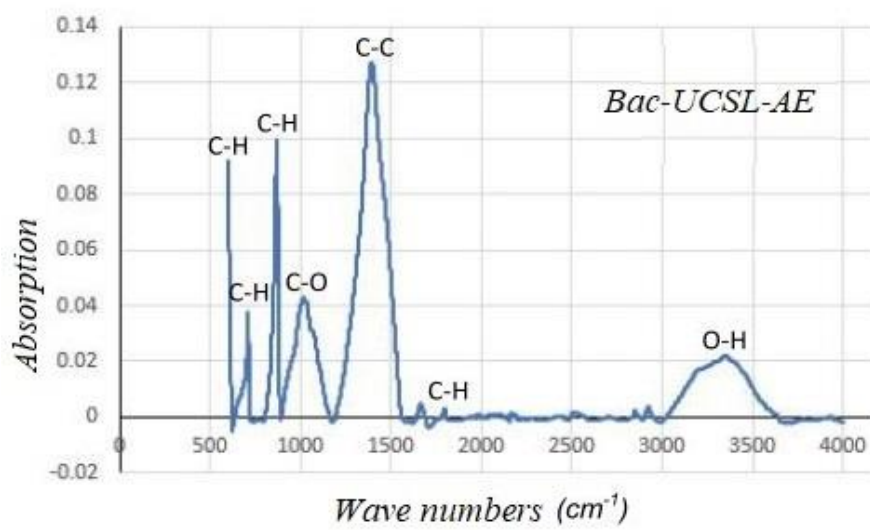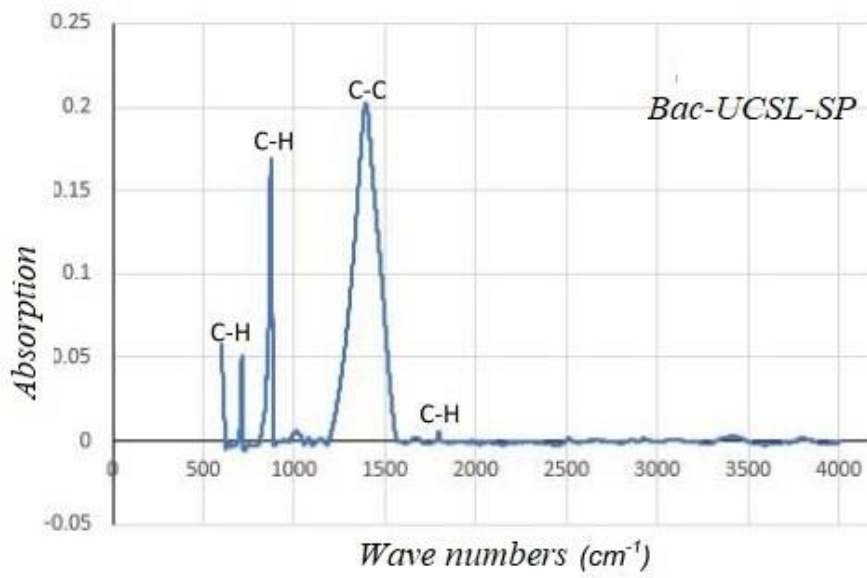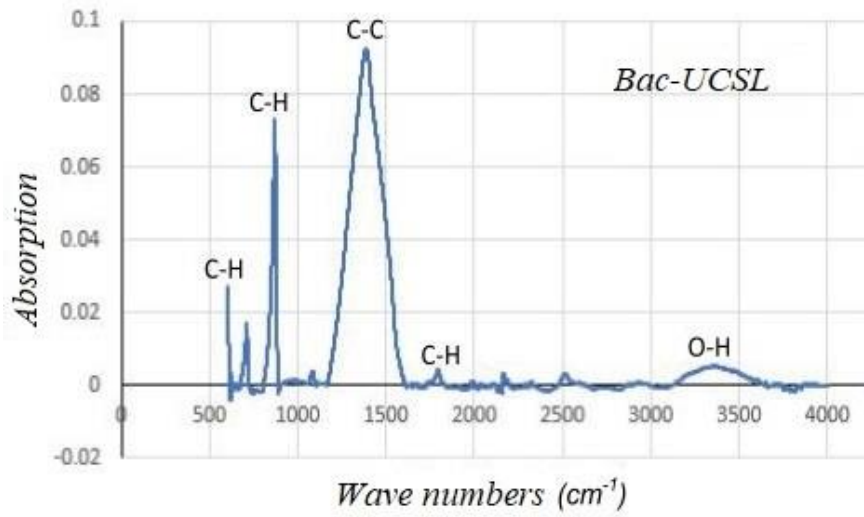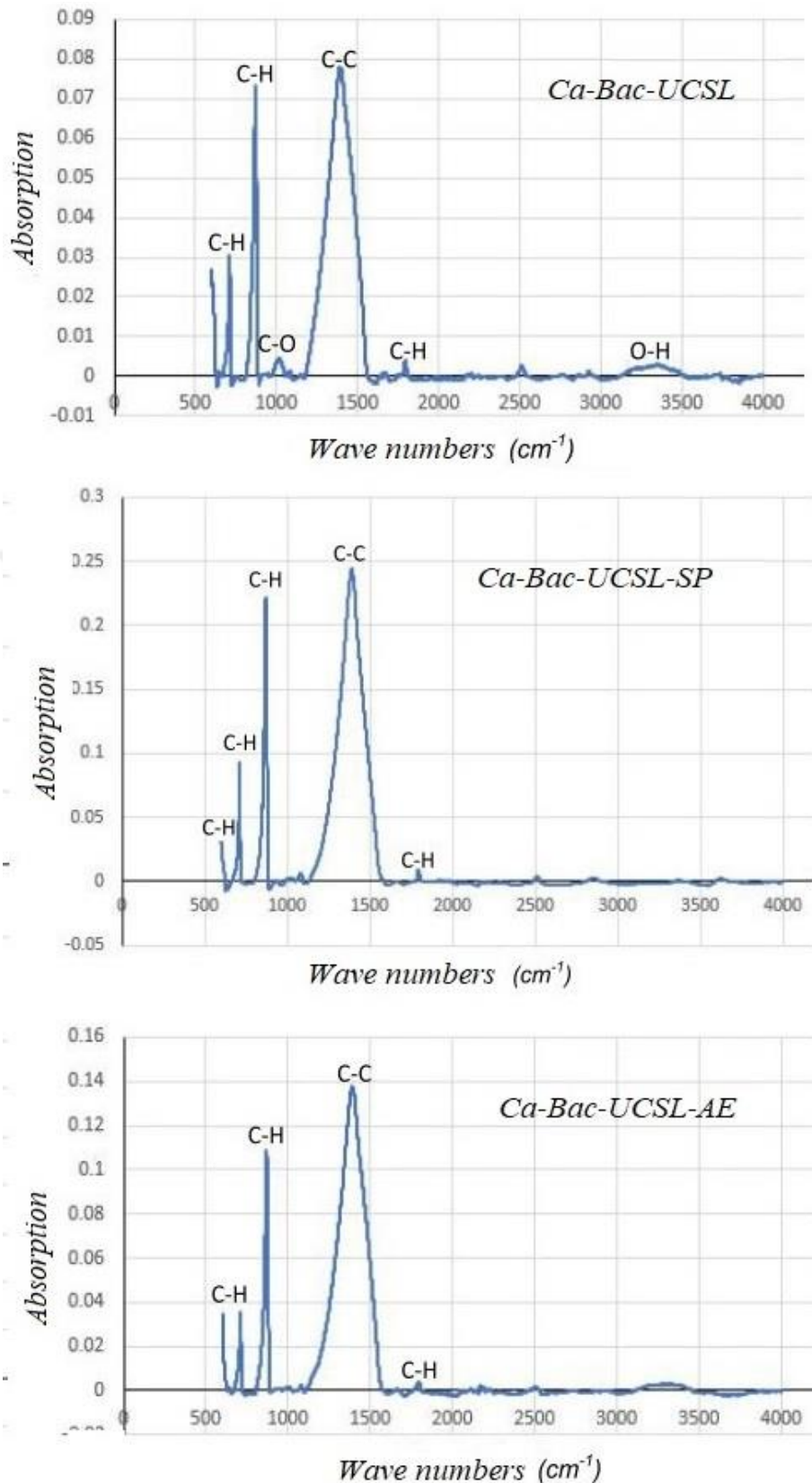
Figure 4.18 FT-IR scattering analysis of the product ($CaCO_3$) precipitated in the crack of S. pasteurii cells grown in the UCSL medium. The solution / cement ratio was used at 0.45. SP: Superplasticizer AE: Air Entraining Agent. C-H: Calcium hydroxide; C-C: Calcium carbonate.

CaCO$_3$ and CH were the main products identified *within* the crack surface. Calcite was the observed polymorph of CaCO$_3$, which showed that the precipitate was chemically stable.

As in line with the visual inspection a denser CaCO$_3$ precipitation was determined in *Bac-CSL-SP* and *Ca-Bac-CSL-SP* samples compared to rest of the samples. Similarly, the amount of CaCO$_3$ in the cracks of specimens containing air-entraining agent were higher than precipitations on untreated samples. However, optical images showed that AE use has no effect on crack repair incorporation with UCSL. With the use of superplasticizer with or without [Ca^{+2}], the amount of CaCO$_3$ formed in the crack increased by 200% while a 75% increase was observed with the use of AE compared to specimens without any chemical admixtures. Addition Ca(NO$_3$)$_2$ as an additional calcium source did not significantly change the density CaCO$_3$ in the crack, which agreed with the visual inspection. By the end abovementioned analysis, it could be concluded that cracks occurred due to early bending stresses of 0.3 mm in width (25-30 mm depth) could be sealed by using biomineralization. *As so, a sustainable and cost-efficient bio-based additive was developed to design self-healing mortar.* However, another challenge was to clarify if this so-called self-healing could actually remediate flexural strength rather than visually sealing the cracks. To determine the change in flexural strength, samples were tested under 3-point bending after crack closure was observed by visual inspection (*see* Section 3.9.3) Table 4.8 shows the flexural strength obtained for all series cement mortars without cracking at 7-day and after the cracks are visually closed.

Even though, cracks were sealed with using MICP, self-healing could not be achieved in terms of recovering the flexural strength. This indicated that the bonding strength between the mortar and CaCO$_3$ precipitate was not enough to overcome flexural stresses. In general, flexural strength regain could be obtained by using more ductile

healing materials such as methyl methacrylate [62] however biogenic CaCO$_3$ is hard and brittle structure compared to most crack repair materials. Due to its brittle nature CaCO$_3$ could only fill the cracks rather than bridging the crack surfaces; establish a bonding with the substrate and improve the flexural strength of the material. *Thus, it could be concluded that with this approach, only "self-sealing" of cracks could be obtained in cement-based materials.* Further studies were conducted if this sealing ability could actually decrease the water absorption of the material after the cracks were filled with the precipitate (*see* Section 4.6)

Table 4.8 Pre- and post-repair bending strength for all series of mortar samples. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.

Samples name	7day un-cracked Flexural strength (MPa)	50 day cracked Flexural strength (MPa)	After curing Flexural strength (MPa)
Neat **Neat-SP** **Neat-AE**	5.17±1.04 4.27±0.60 4.67±0.15	No crack healing	No crack healing
Ca-Neat **Ca-Neat-SP** **Ca-Neat-AE**	4.90±0.86 4.6±0.94 4.01±0.47	No crack healing	No crack healing
UCSL **UCSL-SP** **UCSL-AE**	3.37±0.32 4.43±0.32 4.30±0.50	No crack healing	No crack healing
Ca-UCSL **Ca-UCSL-SP** **Ca-UCSL-AE**	3.50±1.3 3.67±0.57 3.45±0.34	No crack healing	No crack healing
Bac-UCSL **Bac-UCSL-SP** **Bac-UCSL-AE**	5.06±1.23 5.30±0.54 4.89±0.39	0.32 0.44 0.59	0.50±1.60 0.53±0.65 0.46±1.20
Ca-Bac-UCSL **Ca-Bac-UCSL-SP** **Ca-Bac-UCSL-AE**	5.10±1.05 5.53±0.66 4.90±0.49	0.41 0.57 0.38	0.53±1.53 0.55±1.16 0.50±099

4.6   Durability of biogenic CaCO$_3$ precipitate

In order to investigate the durability of precipitated product ($CaCO_3$) inside cracks, specimens with UCSL bacterial culture incorporated with $Ca(NO_3)_2$ were prepared. After 7 days from mixing, the prepared beams were cracked under flexural stress and cured by submerging for 50 days in UCSL medium. It was then visually identified under an optical microscope that the cracks were completely closed. After the cracks were completely closed, the product ($CaCO_3$) has been tested for durability in natural conditions such as light, rainwater and freezing & thawing. Durability is related to the permeability factor. The permeability was investigated based on RILEM 25 PEM II-6. The water absorption coefficient of the recovered samples, is calculated by the following formula:

$k(t^{1/2}) = Q/A$

k: water absorption coefficient ($g.cm^{-2}.\,s^{-1/2}$)

Q: periodic mass of water in specimens $(W_W - W_d)$ (g)

A: submerged surface area (cm^2)

The weight was measured periodically and finally the Q/A and $t^{1/2}$ graphs were plotted for each sample. The slope of this graph shows the water absorption coefficient (k) value. Figure 4-19 shows the graph plotted for the *Ca-Bac-UCSL* sample, periodically repaired with rain water for 30 days. An average absorption capacity was determined by the slopes obtained from the plotted graphs for three different samples. The water absorption capacity for rain water Ca-Bac-UCSL was determined 0.0022 ± 0.006 ($g/mm^2/h$). (*excluding the data point for 0.0015 for its high variance*)
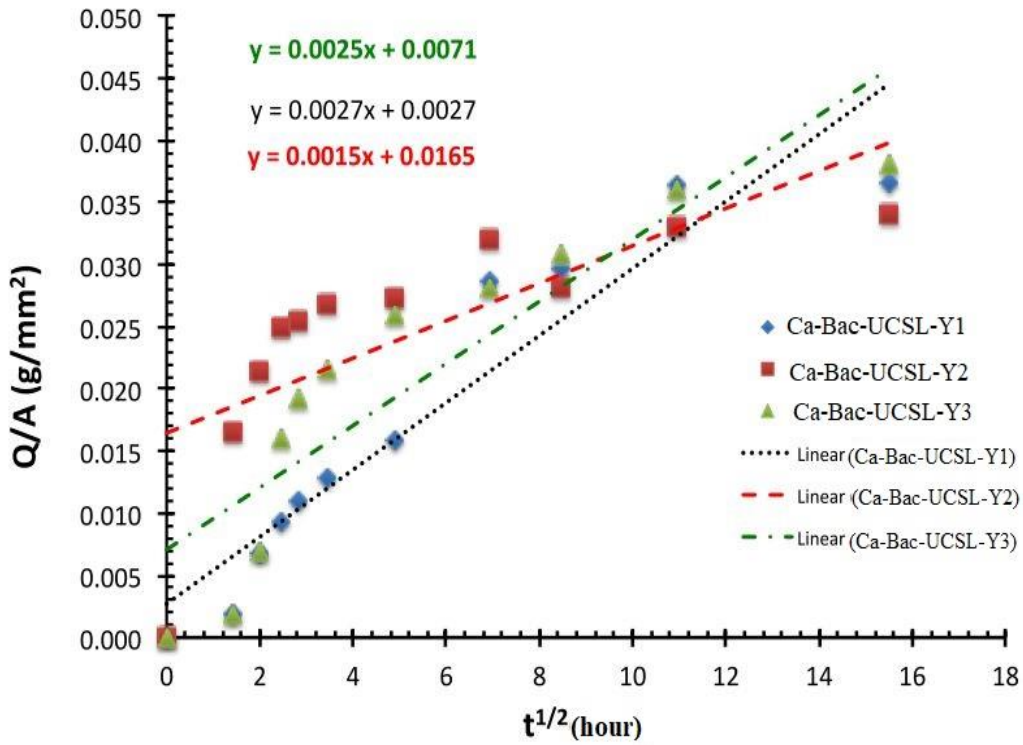
Figure 4.19 Q / A-$t^{1/2}$ curves for Ca-Bac-UCSL sample, which is periodically kept in rain water. The samples were kept in the rain water for 30 days. Y: The rain water was periodically sprayed for 30 days. The tests were repeated with 3 samples. A complete set of graphs are included in Appendix B.

Figure 4-20 summarizes the water absorption coefficients (k) calculated in the above manner for the *Ca-Bac-UCSL, Ca-Bac-UCSL-SP and Ca-Bac-UCSL-AE* specimens kept under natural conditions.
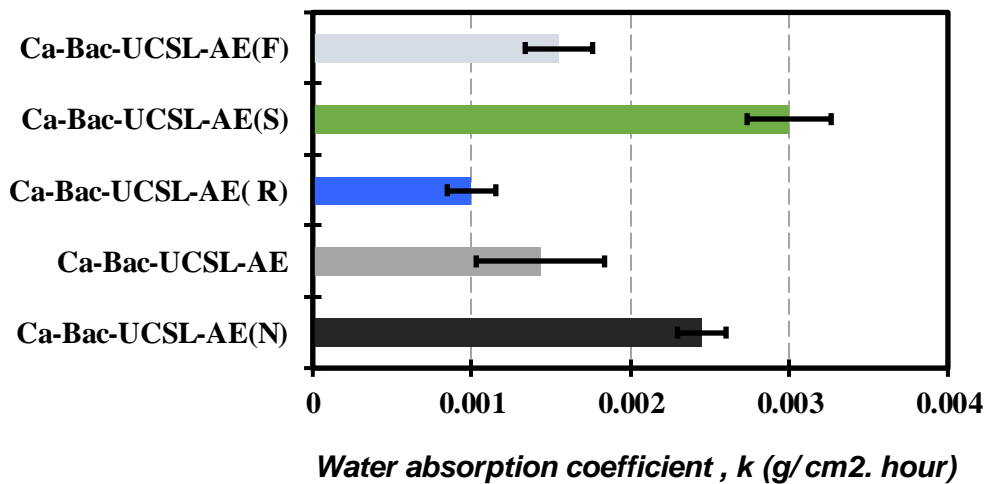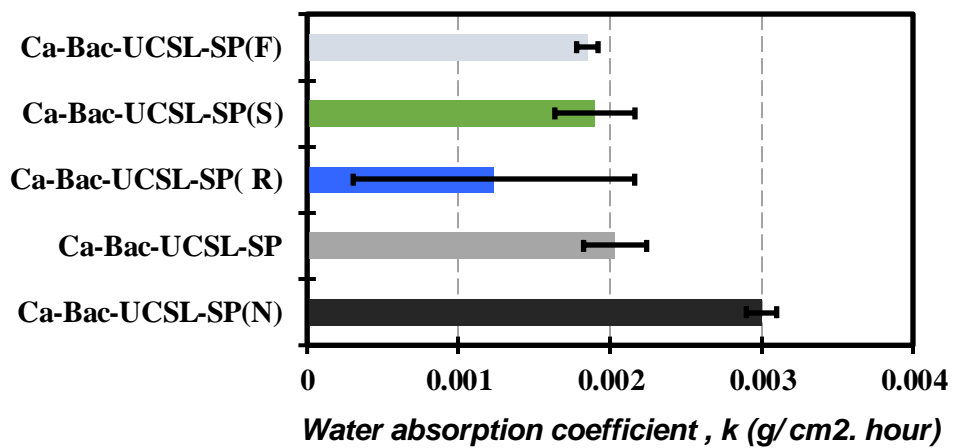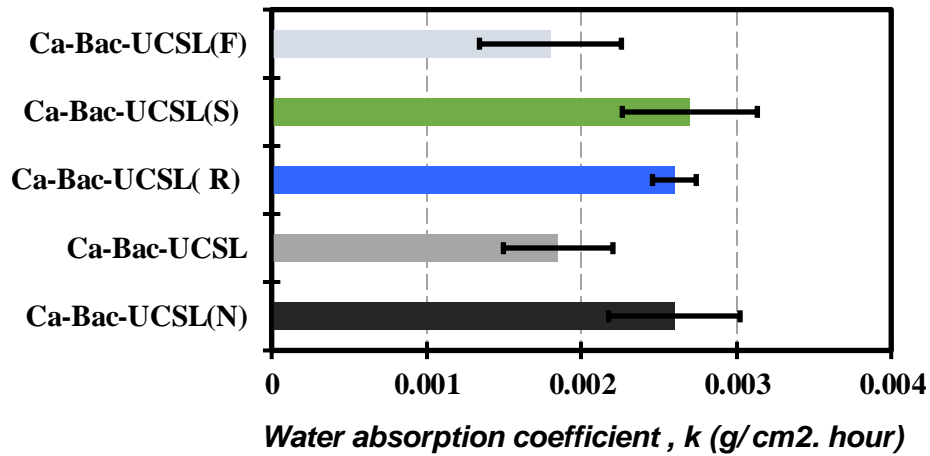
Figure 4.20 Absorption coefficients (k) obtained after repair and natural conditions for all series of cement mortars. F: freeze- thaw condition, S: Sunlight exposure, R: rain water exposure, N: untreated specimens. Bac: Bacterial mortar; CSL: Corn Steel Liquor; SP: Superplasticizer; AE: Air Entraining Admixture.

. Although, flexural strength recovery was not observed via self-healing obtained by using biomineralization, the water absorption capacity of the samples was decreased after the crack sealing. Such that the water absorption capacity of *Ca-Bac-UCSL* sample decreased 19% after crack was sealed. *This suggested that use of biomineralization in developing self-healing mortar is more suitable in terms of reducing the permeability rather than improving mechanical properties*. Incorporation of superplasticizers or AE did not have negative effect on the self-healing ability in terms of water absorption, as it was expected. Based on FT-IR analysis density biogenic $CaCO_3$ precipitation was higher when superplasticizers were incorporated. With the use of superplasticizer, the amount of $CaCO_3$ formed in the crack increased by 200% while an increase of 75% was observed with the presence of AE. Along with this result, water absorption capacity was reduced 33% in *Ca-Bac-CSL-SP* sample compared to cracked *Ca-Bac-CSL-SP* sample. A similar change was observed with samples containing AE (*Ca-Bac-CSL-AE*) such that, there was a 40% decrease in water absorption after the cracks were sealed with biogenic $CaCO_3$. This suggested that the decrease in water absorption capacity was directly related to the density of the precipitate *within* the crack and independent of bonding of the precipitate with the mortar substrate.

After subjecting the self-healed mortar samples to rainwater after 30 days, the water absorption coefficient was increased by almost 40% in all specimens regardless of the type of admixture. Interestingly, the use of superplasticizer and AE improved the durability of the precipitate, $CaCO_3$, against rainwater. This endurance could be again explained by the increase in $CaCO_3$ concentration (*see* Figure 4.16). Such that, the durability of the precipitate directly related to its resistance to abrasion. A higher density of $CaCO_3$ might improve the resistance against abrasion because it might take longer to damage all the precipitate. Also, since the samples were already submerged in an aqueous

environment for curing, they might be already resistant to rainwater. In fact, presence of additional moisture might even trigger further healing, thus increasing the water absorption coefficient.

However, such an effect was not observed in the samples that were exposed to light. Keeping the *Ca-Bac-UCSL* specimen under 30 days of light after the recovery was completed, increased the absorption coefficient by 46% compared to an untreated sample. Likewise, the absorption coefficient was doubled when recovered *Ca-Bac-UCSL-A* specimen was kept under light for 30. Although the actual reason is not known, it is thought that the precipitated product ($CaCO_3$) filling the cracks was dried due to increasing temperature. Calcite is one of the most durable material against sun light exposure, the increase in water absorption after light exposure could be an indication of drying of aqueous medium rather than abrasion of the mineral. Interestingly, incorporation of superplasticizers improved the resistance against light. Even though, this positive effect might be due to higher density of the precipitate, the actual reason is not known. Further studies have to be done to evaluate the interaction between the superplasticizers and biomineralization.

At last but not the least, the $CaCO_3$ precipitate was resistant to freeze and thaw cycles such that the water absorption capacity did not change after subjecting the samples to freeze and thaw cycles for 30 days. These findings were promising in terms of future applications of self-healing mortar. One of the concerns was whether this sealant could resist the natural conditions, once the cracks were sealed. Considering, all the abrasive environments, the most efficient mix design was obtained with *Ca-Bac-UCSL-SP* samples, which includes $Ca(NO_3)_2$ for additional calcium source, bacterial culture grown in UCSL medium and superplasticizers.

Chapter V

Conclusion and Suggested Work

5.1 Conclusion

In this study, we investigated influence of *S. pasteurii* grown in nutrient medium and added to mixture without any protection on the self-healing of cement-based materials. With the data obtained at the end of this project, the mechanism of crack repair and biomineralization was better understood and a more specific method for practical application was obtained. It has been shown that the biomineralization developed by this project can be used for crack repair by a simple method at early ages. The key findings of this project can be listed as follows:

1- One of the most important differences of this process is the use of alternative waste material such as CSL in self-healing applications, while it has been shown in the literature that the YE was usually used to grow *S. pasteurii* for biomineralization. The use of CSL did not have a negative effect on growth of *S. pasteurii* cells and biomineralization. The use of CSL instead of YE not only did not have the negative impact on performance of the material and also reduced the unit price of the developed biological additive material without sacrificing the performance. Also, there was a relationship between the surface charge of the cell and morphology of the precipitate, such that more negative surface charge triggered vaterite precipitation.

2- Chemical additives such as superplasticizer and air entraining additive, which are frequently used in the field, did not have a negative impact on biomineralization when they were used with UCSL medium. However, air-entraining additive decreased the crack sealing ability of the microorganisms when it was used with UYE medium.

3-   Flexural cracks as large as 0.3 mm were recovered in 7-day old mortar by self-healing triggered. The formation of $CaCO_3$, particularly calcite, within the cracks was determined by FT-IR analysis after the cracks were visually sealed. However, self-healing was achieved only in terms of reducing water absorption without improving the mechanical properties of the material. This was attributed to both the brittle nature of the $CaCO_3$ and the weak bonding with the mortar and $CaCO_3$.

4-   In the mixture, both the UCSL bacterial culture and the UYE bacterial culture were able to precipitate more $CaCO_3$ in the crack by addition of superplasticizer into mixture. The use of superplasticizer has made crack repair more effective. While the use of AE did not affect the self-healing properties of the UCSL bacterial culture, the precipitated product in UYE bacterial culture reduced due to use of AE. In these samples, the cracks could be partially repaired. This effect has been associated with the working mechanism of air-entraining agent and the reduction of cells viability. A similar negative effect was not observed when AE was used with CSL, which was attributed to incompatibility between the high viscosity of the medium and AE.

5-   Finally, the effects of different abrasive conditions on the $CaCO_3$ durability was examined. The durability of the product formed in the crack by biomineralization did not decrease when the rain water was sprayed daily for 30 days. The water absorption capacity of the samples exposed to 30 days of rain water spraying was similar to a sample of with no exposure to these conditions. In addition, the use of superplasticizer and AE increased the resistance of the $CaCO_3$ to rainwater. Similarly, the freeze-thaw cycle did not affect the stability of the $CaCO_3$. However, it has been determined that the precipitated $CaCO_3$ was unstable after exposure to sun light except for the specimen prepared with superplasticizer additive. This was attributed to the drying of aqueous phase in the precipitate. Incorporation of superplasticizers improved the

resistance against light. Even though, this positive effect might be due to higher density of the precipitate, the actual reason is not known. The water absorption capacities of the samples kept under light for thirty days were the same as the untreated samples.

By the end of this project, a biological additive material was developed which will enable the self-healing of early cracks on the surfaces of mortars. The optimum composition of the bio-based additive includes $Ca(NO_3)_2$ for additional calcium source, bacterial culture grown in UCSL medium and superplasticizers.

5.2 Future work

The research presented in this thesis provides useful information about development of self-healing bacterial mortar. However, several areas for further research are recommended:
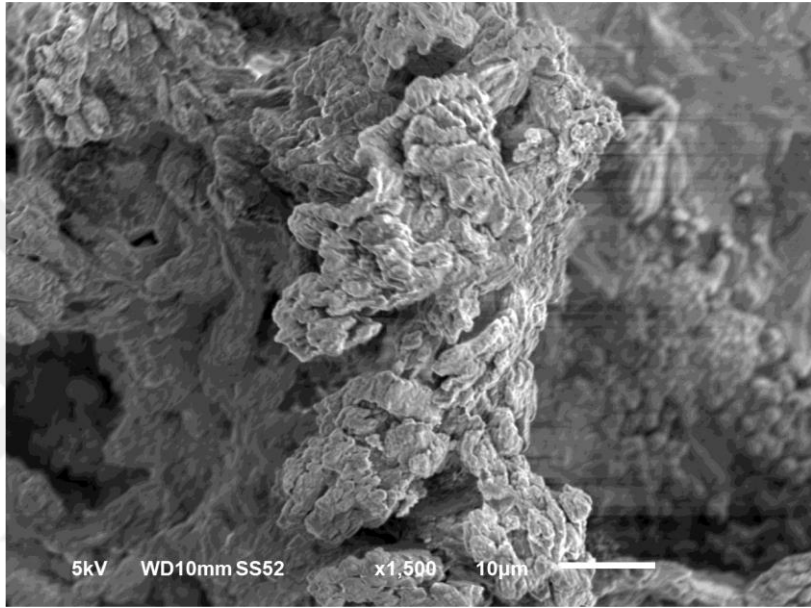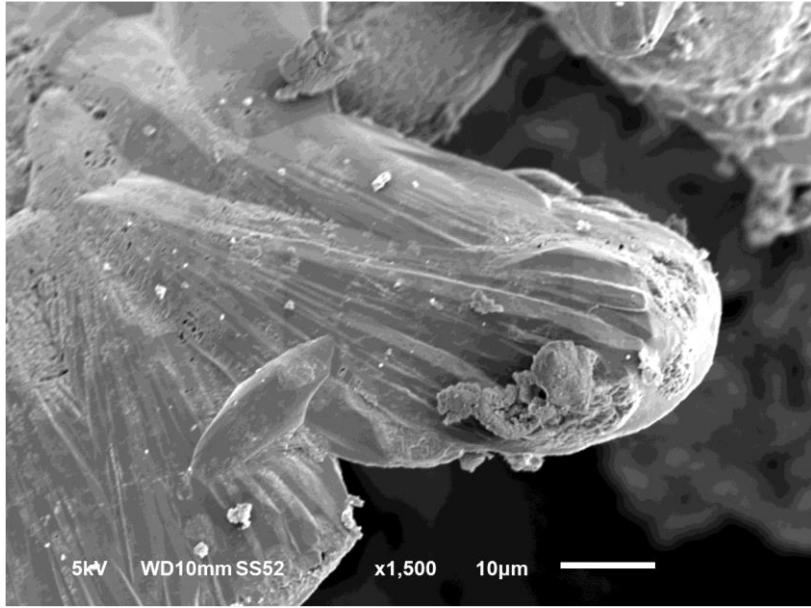
- It has been observed that superplasticizers have a pronounced effect on biomineralization and crack repair. To develop a self-healing concrete, the effects of superplasticizers should be examined further. Similarly, the adverse effects of using air-entraining agents should be investigated.

- With this method, crack repair was provided only at early ages in mortars. This can be an effective area of application for shrinkage cracks in particular. Reduction or repair of shrinkage cracks should be investigated by using microorganism as self-healing agents.

- Natural and cheap protection methods should be developed for the cells to ensure that the application is applicable in mortars at later ages. To extend the service life of the application, the possible use of porous

minerals , like pumice or zeolite, as protection barriers should be examined.
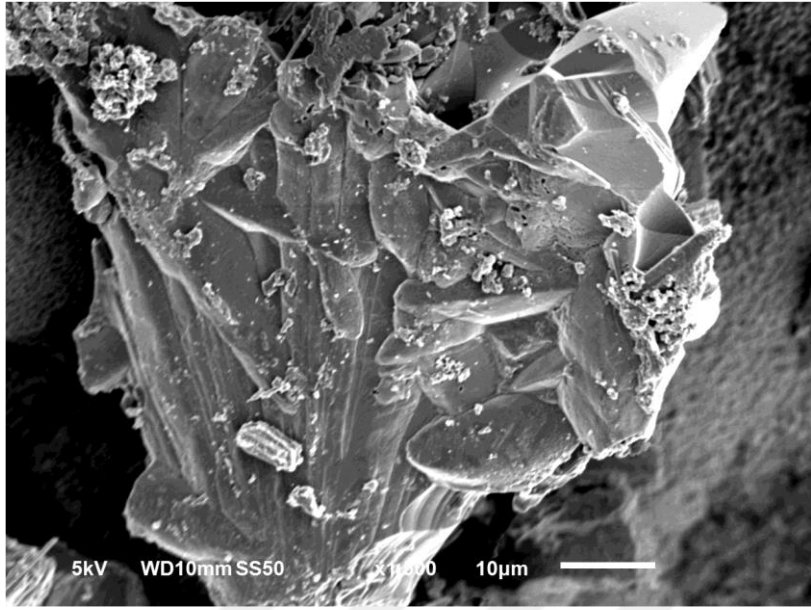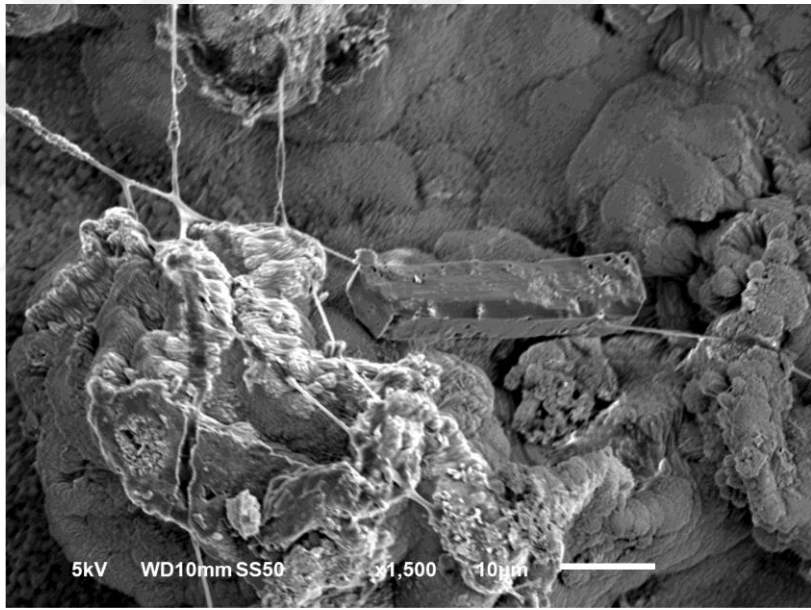
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Appendix A

Table A.1 SEM images obtained at the end of 28 days for the control series. YE: Yeast extract, CSL: Corn Steel Liquor; Ca: Calcium; SP: Superplasticizer; AE: Air Entraining Admixture.
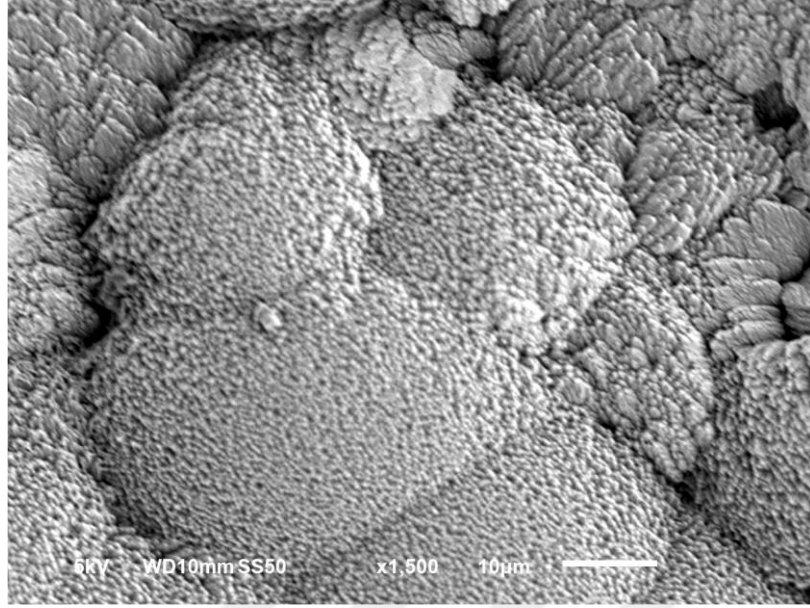
Name	SEM image
Neat	
Neat-SP	

Neat-AE

5kV   WD10mm SS50   X1,500   10μm

UYE

5kV   WD10mm SS50   x1,500   10μm

UYE-SP

UYE-AE

UCSL	
UCSL-SP	

UCSL-AE	
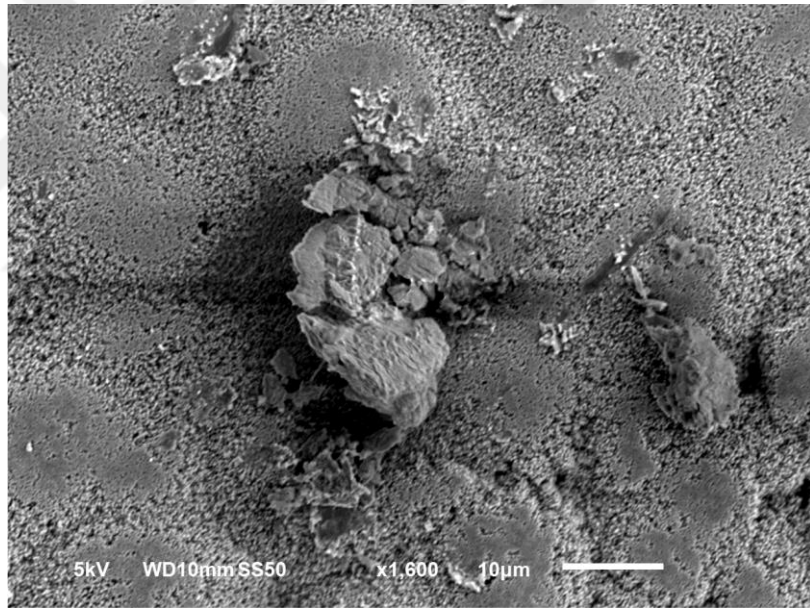
Table A.2 SEM images obtained at the end of 28 days for the bacterial series. YE: Yeast extract, CSL: Corn Steel Liquor; Ca: Calcium; SP: Superplasticizer; AE: Air Entraining Admixture.
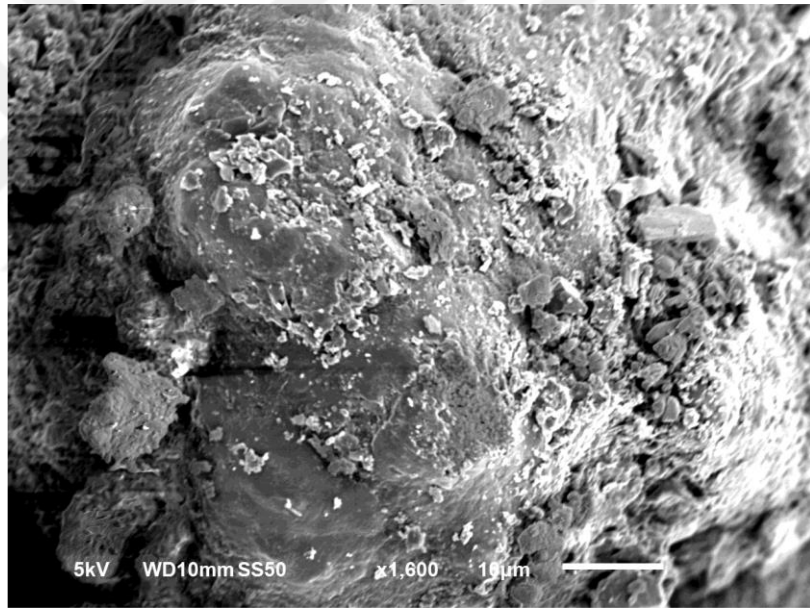
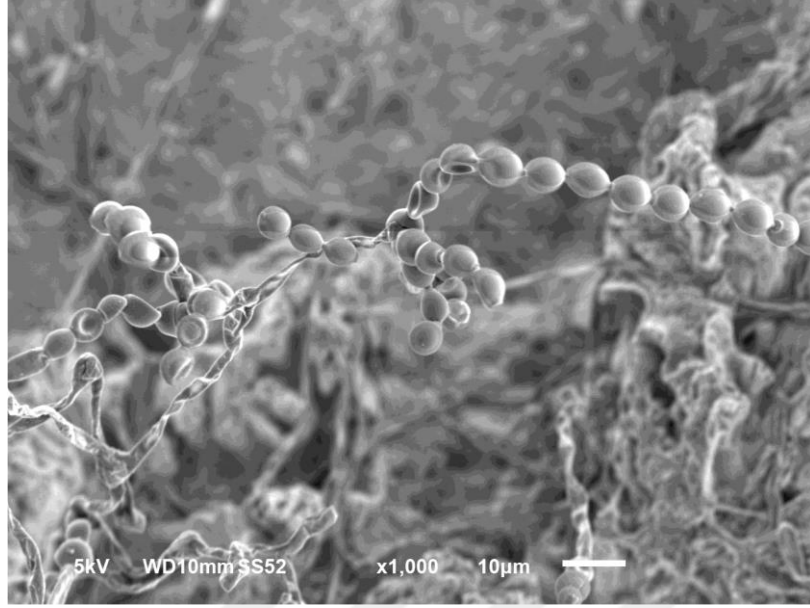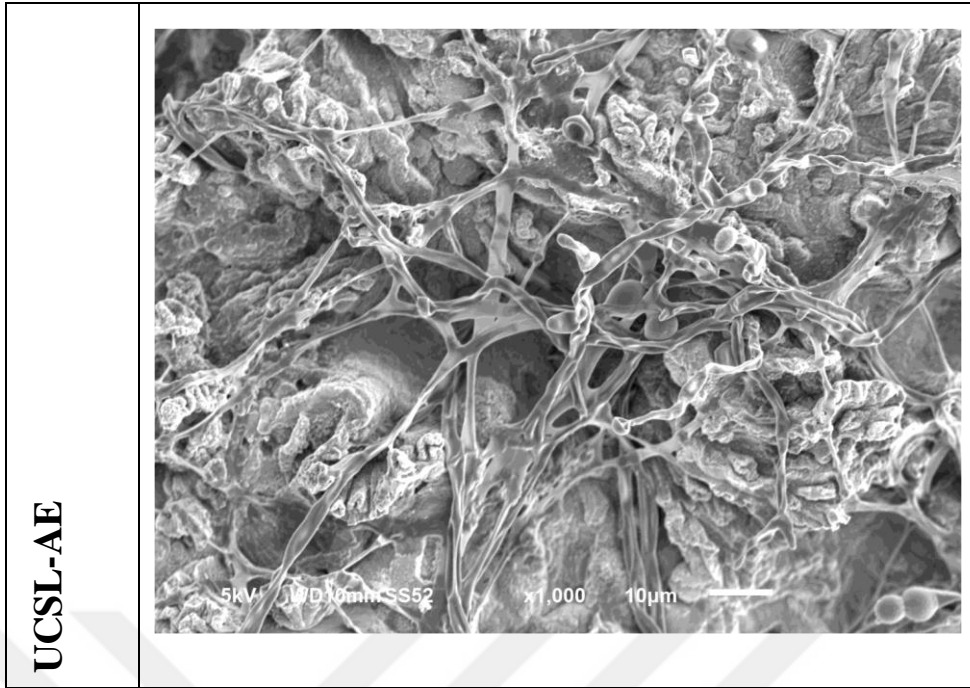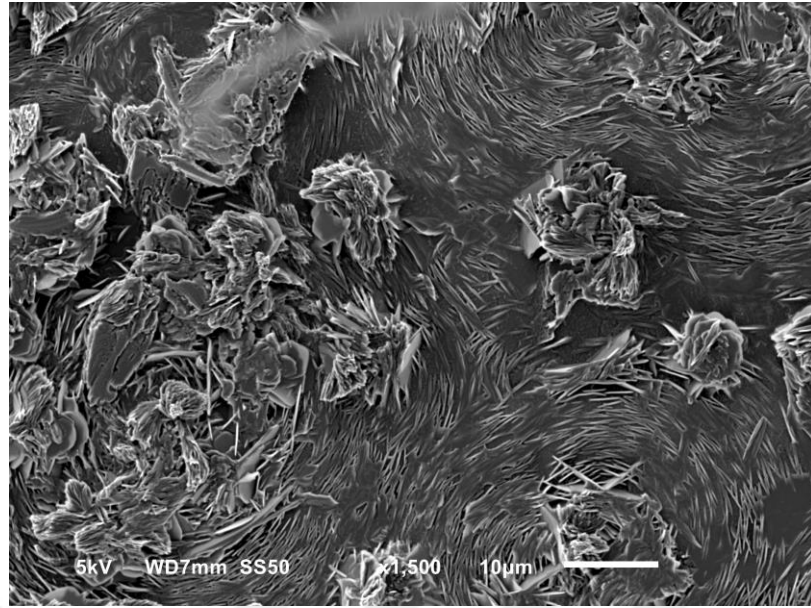| Name	SEM image
Ca-Bac-UYE	

Ca-Bac-UYE-SP

Ca-Bac-UYE-AE

Ca-Bac-UCSL	
Ca-Bac-UCSL-SP	

Ca-Bac-UCSL-AE

5kV   WD7mm SS50   x4,500   5µm
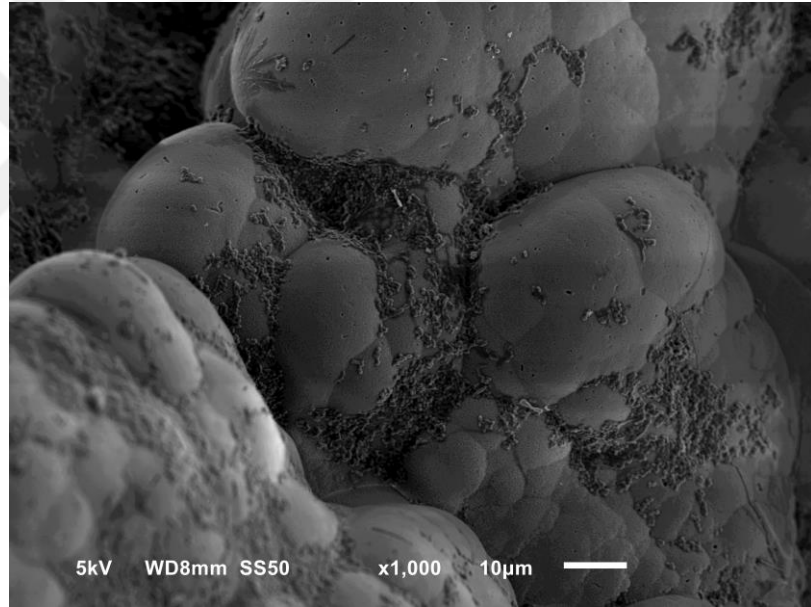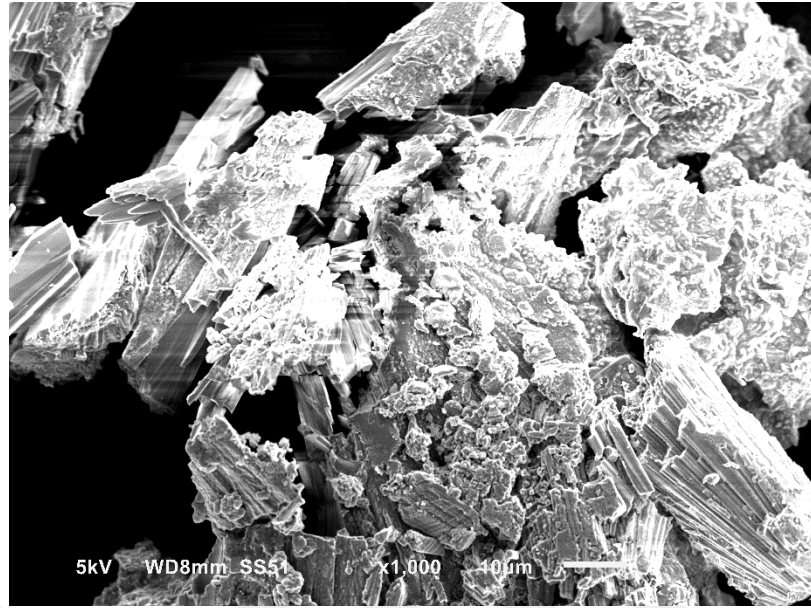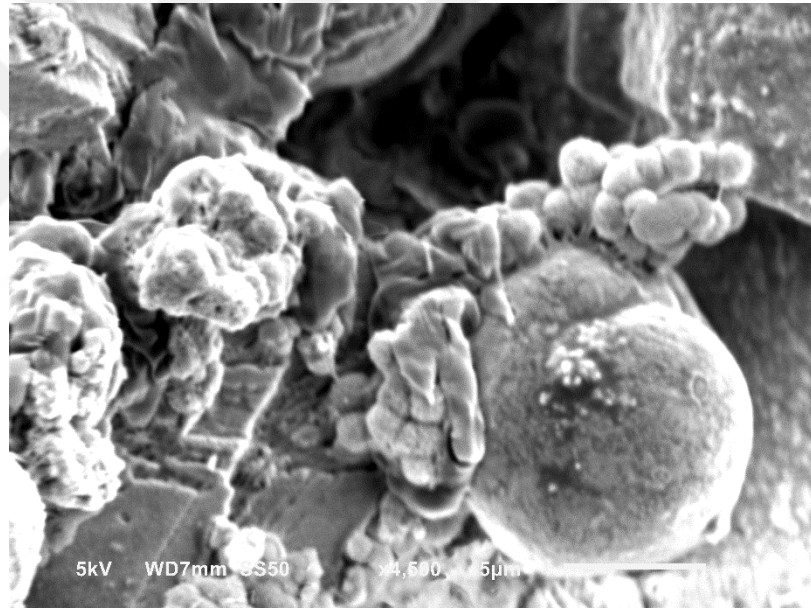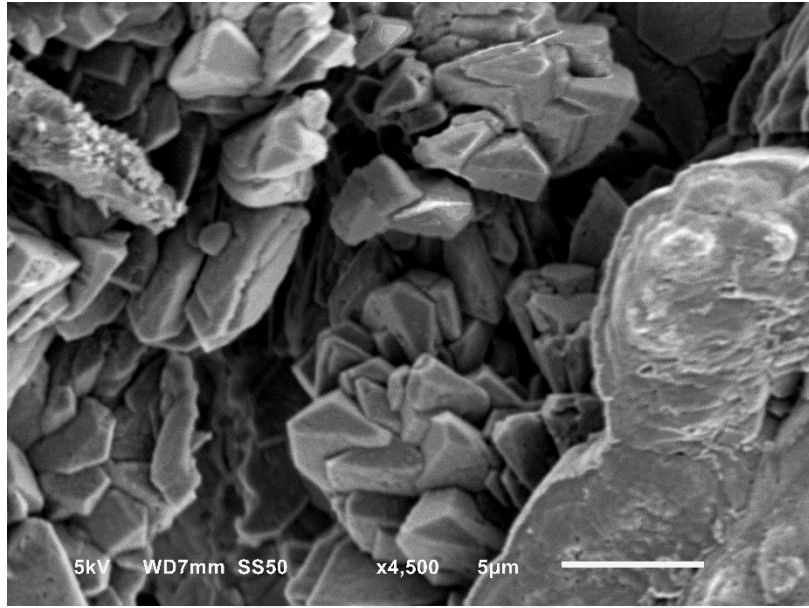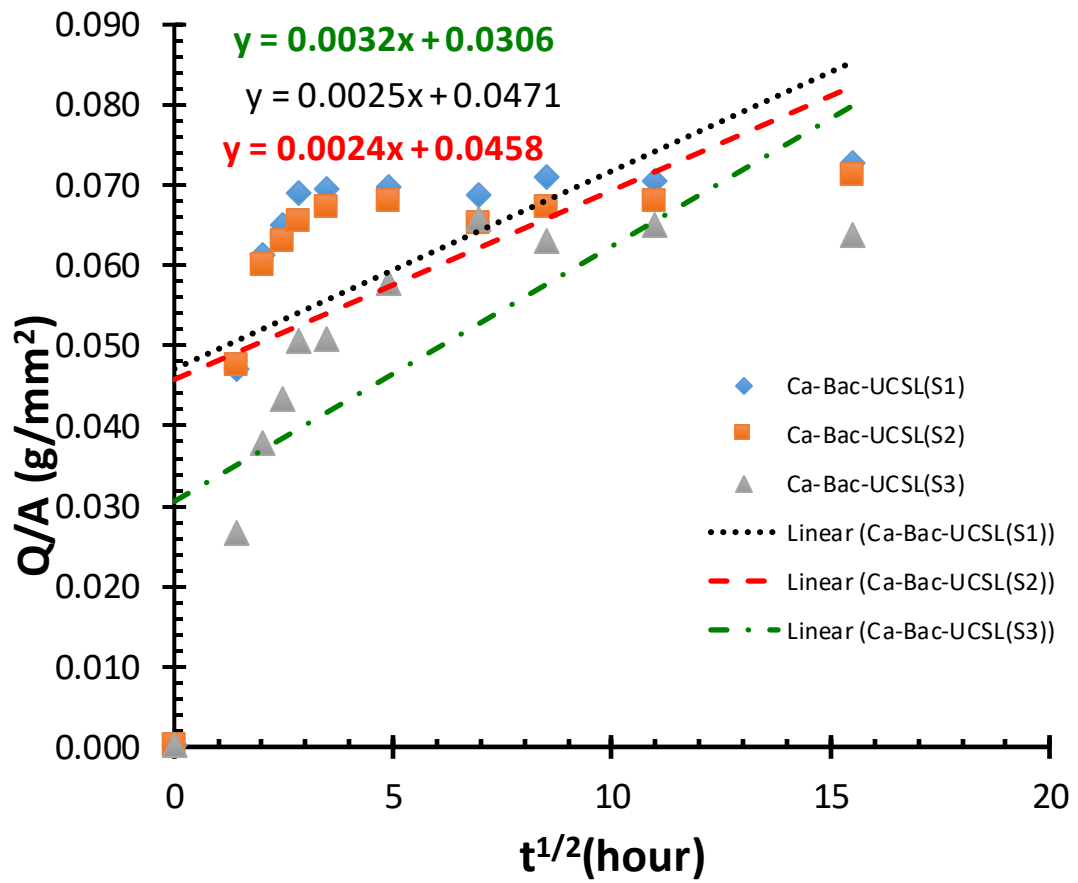
Figure B.1 Q / A-t$^{1/2}$ curves for *Ca-Bac-UCSL* sample, which is periodically kept in light exposure for 30 days
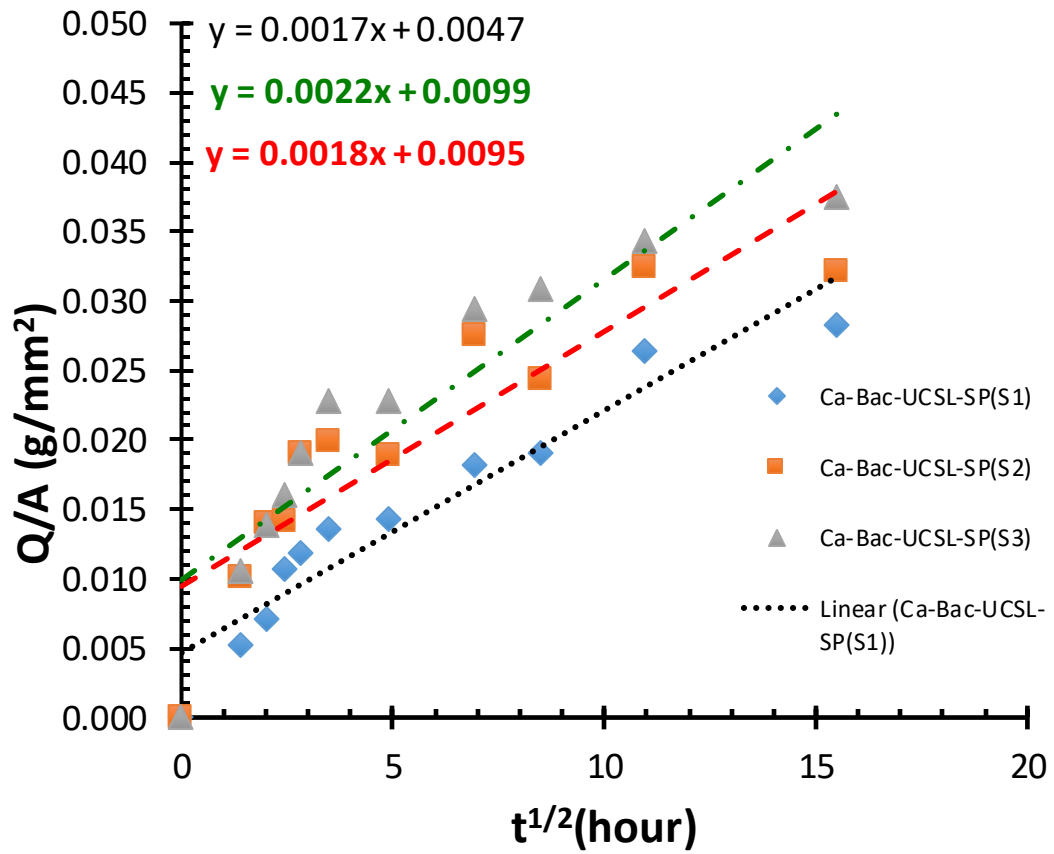
Figure B.2 Q / A-t$^{1/2}$ curves for *Ca-Bac-UCSL-SP* sample, which is periodically kept in light exposure for 30 days
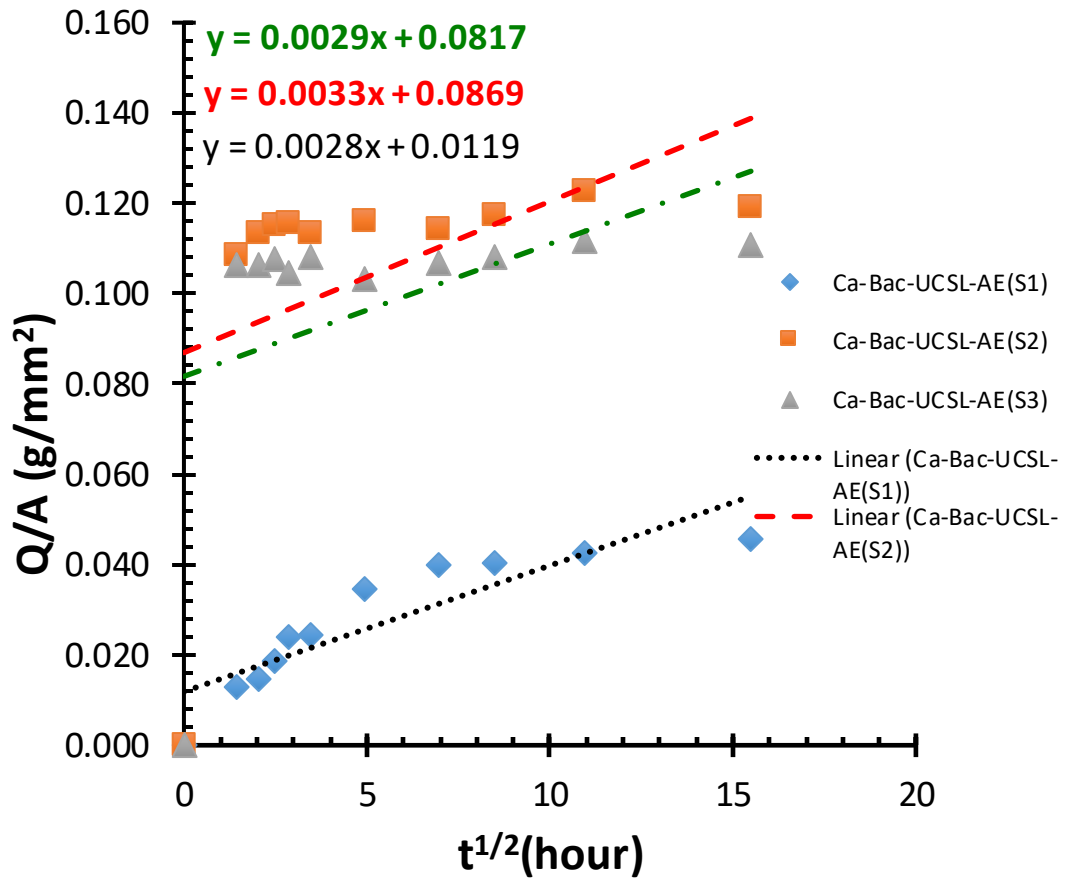
Figure B.3 Q / A-t$^{1/2}$ curves for *Ca-Bac-UCSL-AE* sample, which is periodically kept in light exposure for 30 days

Figure B.4 Q / A-$t^{1/2}$ curves for *Ca-Bac-UCSL* sample, which is periodically kept in rain water for 30 days

Figure B.5 Q / A-t$^{1/2}$ curves for *Ca-Bac-UCSL-SP* sample, which is periodically kept in rain water exposure for 30 days

Figure B.6 Q / A-t$^{1/2}$ curves for *Ca-Bac-UCSL-AE* sample, which is periodically kept in rain water exposure for 30 days
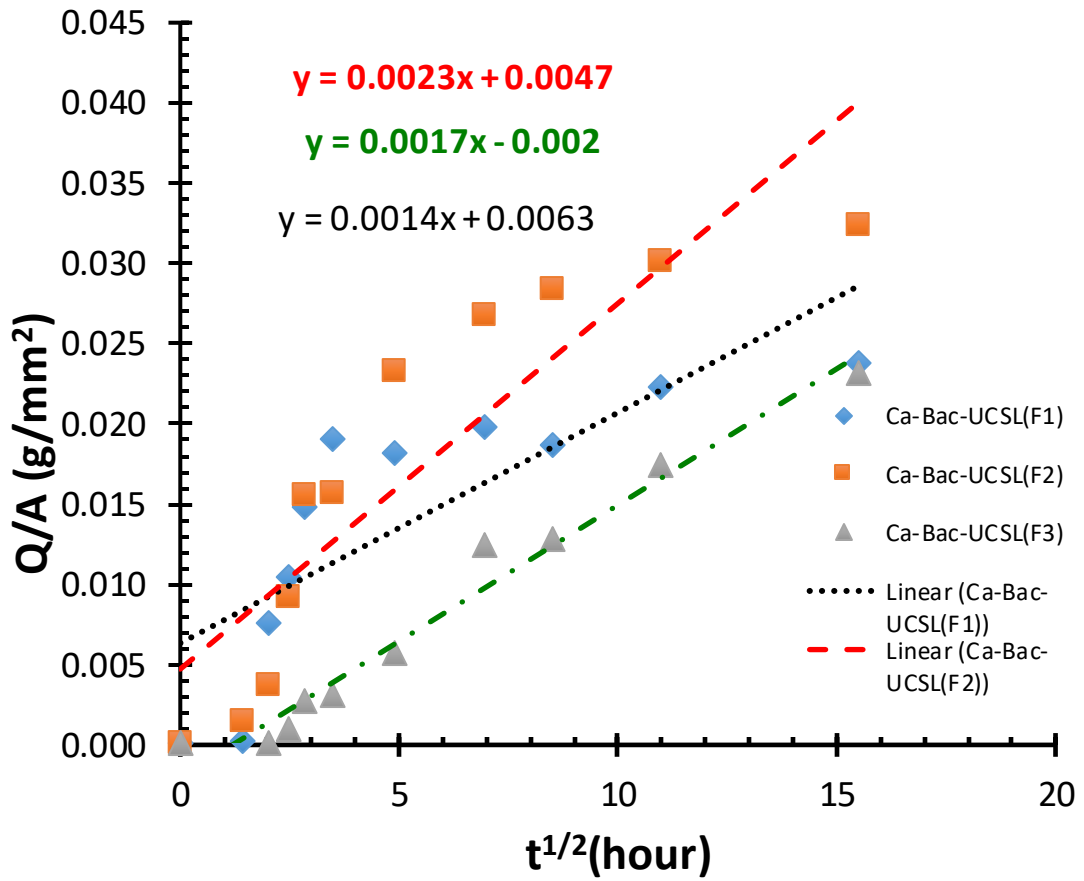
Figure B.7 Q / A-$t^{1/2}$ curves for *Ca-Bac-UCSL* sample, which is periodically exposed to freeze-thaw for 30 days
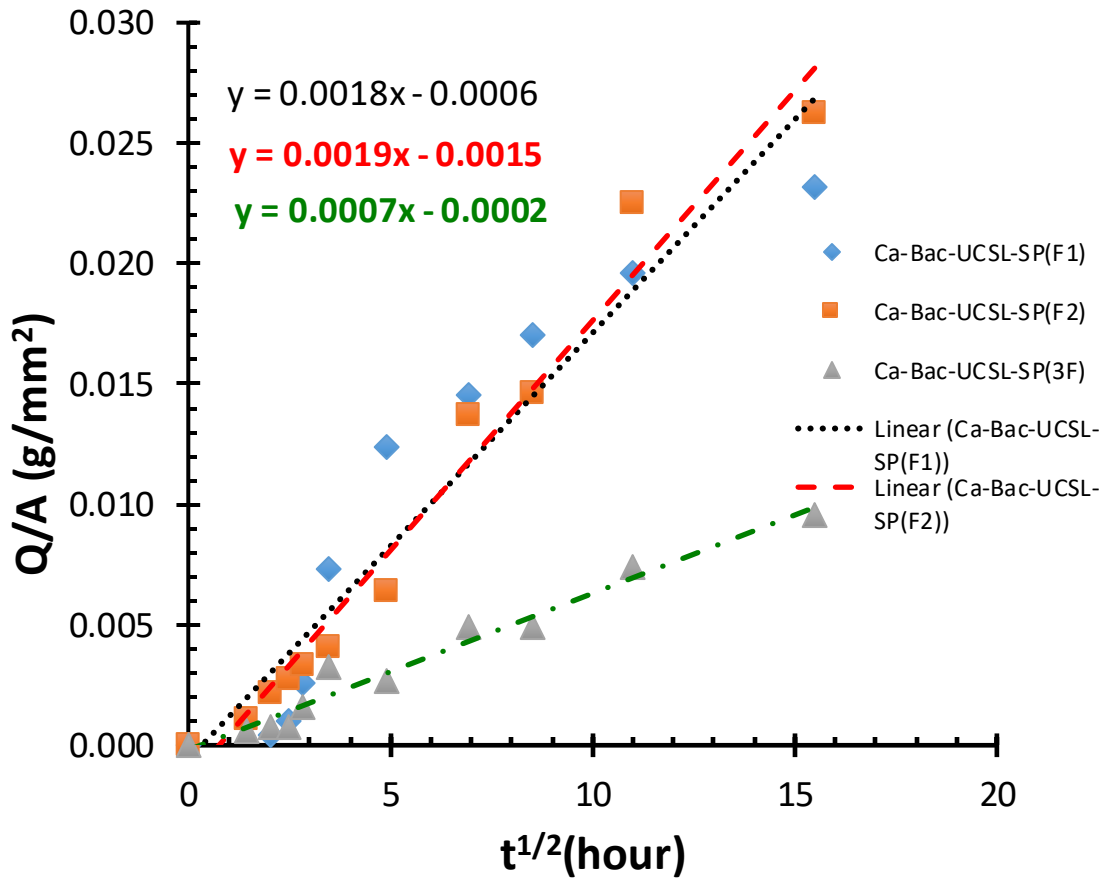
Figure B.8 Q / A-t$^{1/2}$ curves for *Ca-Bac-UCSL-SP* sample, which is periodically

exposed to freeze-thaw for 30 days

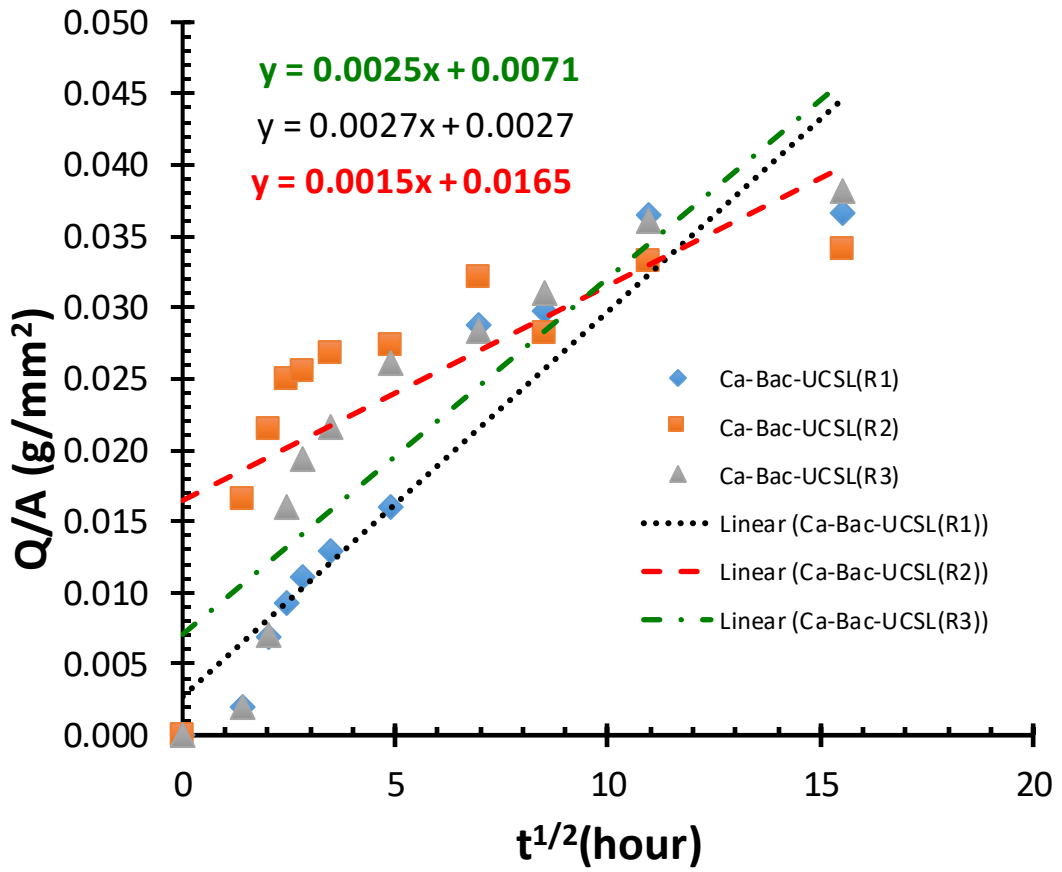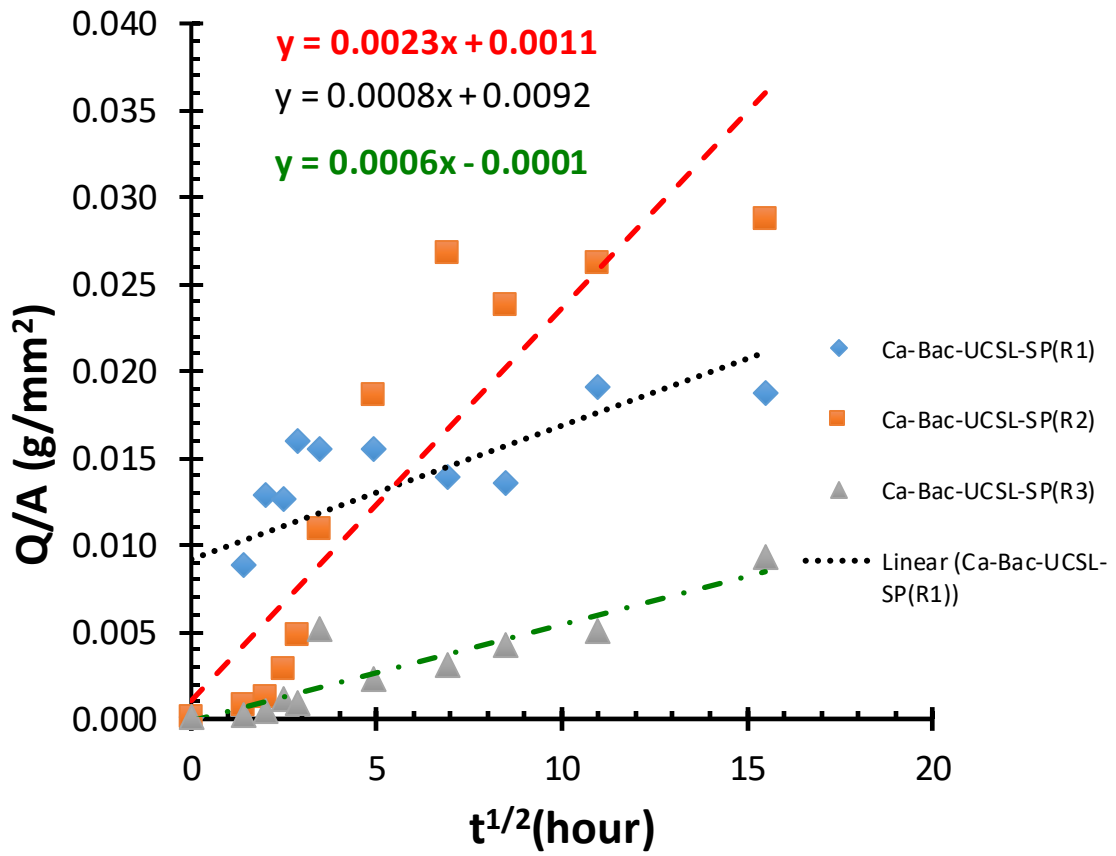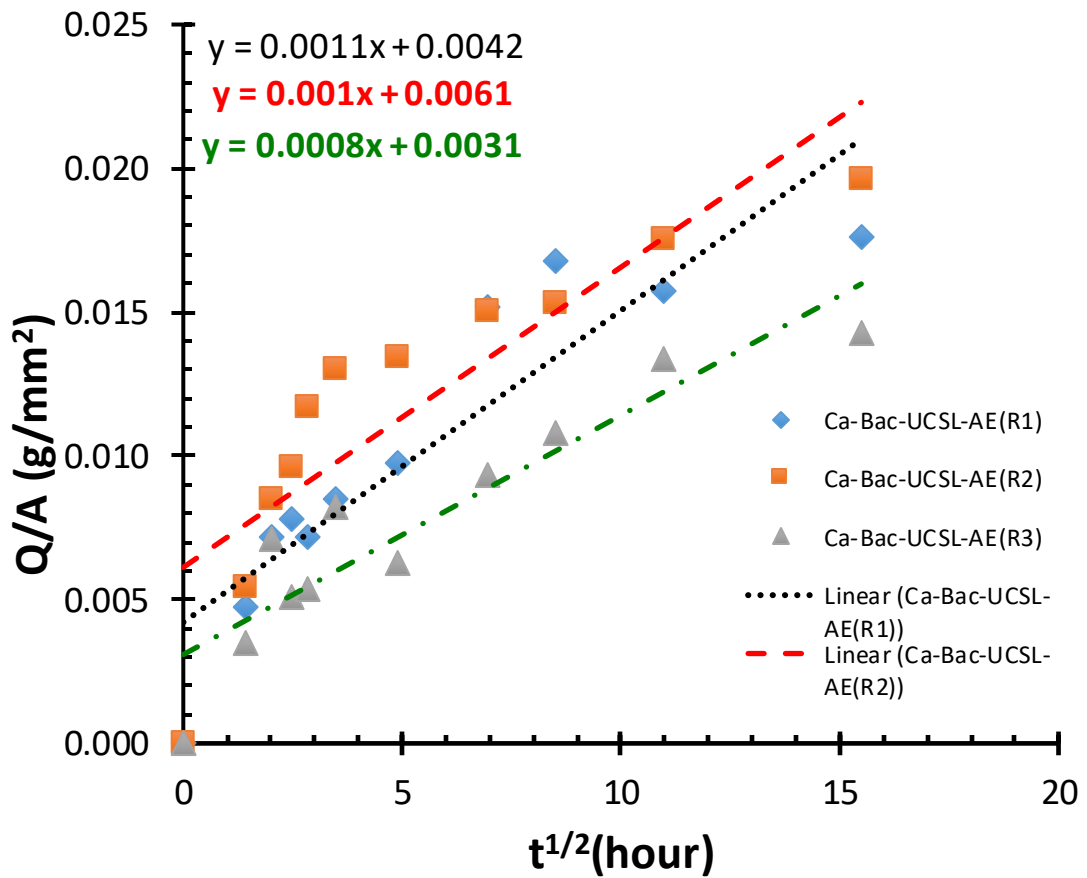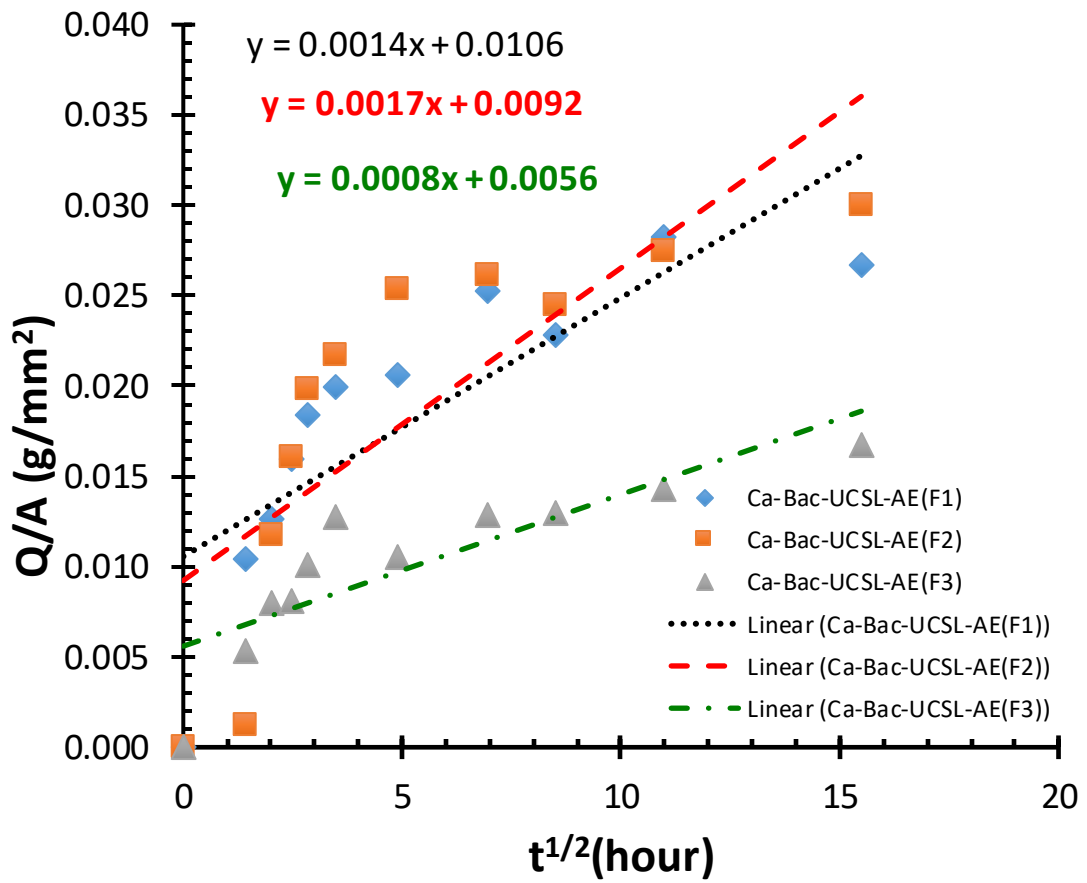**Figure B.9** Q / A-t$^{1/2}$ curves for *Ca-Bac-UCSL-AE* sample, which is periodically exposed to freeze-thaw for 30 days

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