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BIOMINERALIZATION IN CEMENT BASED MATERIALS: INOCULATION OF VEGETATIVE CELLS

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BIOMINERALIZATION IN CEMENT BASED MATERIALS: INOCULATION OF VEGETATIVE CELLS

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Dedication

This dissertation is dedicated to my father H.Memet Basaran.

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BIOMINERALIZATION IN CEMENT BASED MATERIALS: INOCULATION OF VEGETATIVE CELLS

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Recently, self-healing applications of cement-based materials have received a lot of interest. One major area of interest with respect to self-healing applications in cement-based systems focuses on using biomineralization processes. Biomineralization is biochemical process in which microorganisms stimulate the formation of minerals. The existing research on biomineralization in cement-based systems has showed promising results and the studies suggest that biomineralization could be a useful approach for remediation of cracks on the surface of concrete.

This dissertation presents the results of an intensive study undertaken to understand the influence of vegetative bacteria, specifically *Sporosarcina pasteurii* (S. *pasteurii*), when it is incorporated within cement paste. Vegetative *S. pasteurii* cells were suspended in a urea-yeast extract medium and this medium was mixed with cement. The influence of the vegetative *S. pasteurii* cells on Portland cement paste properties, such as

compressive strength, hydration kinetics, and setting time was evaluated. It was determined that the hydration kinetics was highly influenced when the bacterial medium was used to prepare cement paste, and severe retardation was observed. It was also observed that an increase in calcium carbonate precipitation, particularly calcite, occurred within cement paste when the bacterial medium was used. Furthermore, use of the bacterial medium resulted in reducing the porosity and increasing the compressive strength of the hardened paste. Ex-situ culture experiments were conducted to determine the impact of pH and calcium concentration on the morphology of calcium carbonate precipitate; the results indicated that the morphology of the precipitate was more influenced by calcium concentration.

A key focus of this dissertation was to examine the viability of the vegetative cells that were inoculated in cement paste. Viable *S. pasteurii* cells were found to be present in hardened cement paste samples that were as old as 330-days, and 50% of the viable cells detected were defined as vegetative cells. At last, the use of including internal nutrient reservoirs as a means to extend the viability of the bacterial cells within hardened cement paste was explored. The results showed that the percentage of vegetative cells remaining was affected when internal nutrient reservoirs was incorporated into the system.

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Chapter 1: Introduction

Portland cement-based materials are the most widely used construction materials. They are complex composite materials, which are strong in compression while weak in tension. However Portland cement-based materials are susceptible to cracking; internal stresses (due to mechanical loads, shrinkage, improper curing, and temperature changes) induces microcracks, which can propagate and coalesce to form macroscopic cracks upon the application of additional stresses. Not only do these cracks reduce the strength of Portland cement concrete (PCC)[1], but they also provide internal access to aggressive agents (e.g. chloride ions), which can affect the integrity of the concrete.

Recent research in the field proposes that it might be possible to develop a smart, cement-based material that is able to self-heal by leveraging the metabolic activity of microorganisms to provide biomineralization [1, 2]. Biomineralization is a biochemical process in which microorganisms stimulate the formation of minerals such as carbonates [3]. Microbial-induced calcium carbonate precipitation (MICCP) is an example of a biomineralization process, and the resultant precipitates could serve to bind particles (e.g., sand and gravel) together to form a composite material. Similar to the healing

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[1] A list of abbreviations can be found in Appendix A: Abbreviations.

process that occurs in bones when cracked, a "bio-calcified" concrete would autogenously heal the cracks formed in the matrix phase via the precipitation of the carbonate minerals.

1.1. INCORPORATING MICROORGANISMS IN CEMENT-BASED MATERIALS

Perhaps the simplest approach for using biomineralization in cementitious material is to suspend the bacterial cells in the mixing water. It is also possible to replace the mixing water with a bacterial culture and nutrient broth. This nutrient broth has the nitrogen, carbon, trace elements and vitamins that the microorganisms need for metabolic activity[4, 5]. However, the optimal amount of nutrients needed for metabolic activity should be considered. Furthermore, addition of these nutrients might also have an effect on the mechanical and chemical properties of concrete.

Another possible method is to encapsulate the microorganisms, synthetically or naturally, prior to mixing them into the concrete [2, 6–8]. This approach protects the microorganisms from the high pH environment of the cement paste and maintains their viability for an extended time for self-healing applications. The synthetic encapsulation method consists of immobilizing the bacteria in a protective covering, (e.g. porous aggregates, polymeric membrane, etc.) [2, 6, 7]. The natural encapsulation addition method consists of introducing the bacteria in the form of bacterial endospores [8]. Endospores are tough, dormant, non-reproductive structures that certain bacteria turn into

when they undergo stress (e.g. nutrient depletion, extreme temperatures, etc.). They enable the bacteria to be dormant for extended periods; when the endospores are exposed to a suitable environment again, this allows the bacteria to return to a vegetative state. The vegetative cell is a cell that can be capable of growing. Endospores can be advantageous in which the long term viability of the microorganisms required such as self-healing of old 'bacterial' concrete structures (e.g. 10-15 years old structures). Nevertheless for early age cracks such as shrinkage cracks during casting, vegetative cells can provide a faster response to crack formation. Therefore considering the time frame for crack formation, importance of metabolic state becomes more critical.

However, *a priori* encapsulation of the microorganisms might be unnecessary. With the proper selection of microorganism, nutrients, and inoculation approach, the inoculated microorganisms might survive for extended periods without undertaking a priori encapsulation. The inoculation of vegetative cells approach consists of suspending the bacterial cells in an aqueous solution and combining it with the remaining ingredients of the cement-based material.

1.2. KEY QUESTIONS

Current studies suggest that biomineralization could be used for healing the cracks on the surface of concrete, to reduce permeability, and to improve compressive strength and durability. However key questions, such as those listed below, remained unanswered:

- Can the microorganisms induce biomineralization, without any additional calcium source, in cement paste matrix?
- How are the mechanical properties, hydration, and microstructure development of Portland cement influenced by biomineralization?
- Can non-encapsulated microorganisms survive in cement-based materials for extended time periods?
- To what extent can internal reservoirs (e.g. lightweight aggregates) be used to extend the viability of non-encapsulated microorganisms?
- Can biomineralization be used as an approach to remediate internal cracks, rather than just surface cracks?

This dissertation presents the results of a comprehensive research program that provides answers to these questions and contributes to the fundamental understanding of the biomineralization process in cement-based materials and properties of the materials.

1.3. STATEMENT OF OBJECTIVES

The goal of this research was to investigate mechanisms influencing biomineralization in cement-based materials provided by inoculation of vegetative *Sporosarcina pasteurii* cells. Vegetative inoculation requires less processing than an encapsulation approach and therefore if this approach were successful it would be a simpler and more cost-effective method to inducing biomineralization in cement-based materials.

The specific research objectives of the work were to:

1. Examine the effects of calcium concentration and pH on calcium carbonate precipitation and morphology of precipitate.
2. Measure the effect of inoculated vegetative microorganisms on cement hydration and compressive strength of mortar.
3. Examine the influence of inoculate vegetative microorganisms on microstructure and chemical composition of cement paste.
4. Determine the metabolic state of the inoculated vegetative cells in aging cement paste and mortar (i.e. vegetative cells, endospores or dead cells).
5. Determine if bacterial metabolic activity and biomineralization can be improved by delayed release of nutrient medium in aging mortar.

6. Measure self-healing of internal microcracks of mortar modified by inoculated vegetative microorganisms.

1.4. ORGANIZATION OF DISSERTATION

This dissertation includes eight chapters including Chapter 1, Introduction. Chapter 2 is a summary of the literature of biomineralization in cement-based materials. Chapter 3 explains the microorganism selection process, the microbial culturing and growth process, and the inoculation of vegetative cells method. In addition calcium carbonate precipitation and morphology of the precipitate were investigated. Chapter 4 investigates the influence of biomineralization on hydration kinetics, chemical composition and mechanical properties. Metabolic state (i.e. viable, dead and endospore percentage) of inoculated vegetative bacterial cells over time is presented in Chapter 5. A novel method to extend the survival of microorganisms is proposed in Chapter 6. Self-healing of cracks measured by compressive strength and coda wave interferometry is summarized in Chapter 7, and Chapter 8 presents conclusions from this research study and recommendations for future work.

Chapter 2: Literature Review

Microbial induced calcium carbonate precipitation (MICCP) is an example of biomineralization process. Biomineralization is a process in which organisms influence the formation of minerals mainly carbonates, phosphates, iron oxides, and sulfides. More information regarding the biomineralization in cement-based materials can be found in Appendix B: Use of Biomineralization in Developing Smart Concrete Inspired by Nature.

2.1. CHEMICAL REACTIONS IN MICCP

Microorganisms possessing the urease enzyme can decompose urea into ammonia and carbon dioxide, tending to increase the pH of their environment. If dissolved calcium is present, then the pH increase from the urea decomposition triggers calcium carbonate precipitation [9–11]. Generally, one mole of urea is hydrolyzed to produce two mole of ammonia and one mole of carbonic acid (Equation 2.1).

$$CO(NH_2)_2 + H_2O \xrightarrow{urease} 2NH_3 + CO_2 \qquad (2.1)$$

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad pK_a = 9.3 \qquad (2.2)$$

$$H_2CO_3^* \leftrightarrow HCO_3^- + H^+ \qquad pK_a = 6.3 \qquad (2.3)$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \qquad pK_a = 10.3 \qquad (2.4)$$

Ammonia is a nitrogen source commonly used by bacteria. Bacteria possessing the urease enzyme can decompose urea ($CO(NH_2)_2$) into ammonia and carbon dioxide (Equation 2.1), and ammonia and carbon dioxide can participate in acid-base reactions (Equations 2.2-2.4). These reactions serve to increase the pH, such that calcium carbonate precipitation (Equation 2.5) becomes more favorable.

$$CaCO_3(s) \leftrightarrow Ca^{+2} + CO_3^{-2} \qquad K_{sp}= 3.8 \times 10^{-9} \qquad (2.5)$$

While urease-active microorganisms can decompose urea into ammonia and dissolved inorganic carbon (DIC) positively charged calcium ions are attracted by negative surface of the microorganism and creating a heterogeneous nucleation site for calcium carbonate precipitation. Calcium carbonate crystals start to nucleate on the cell wall and encapsulate the microorganisms.

Due to the complexity of the reactions, there is more than one factor influencing microbially induced calcium carbonate precipitation, such as calcium ion concentration, the concentration of DIC, pH of the environment and availability of nucleation sites for crystal growth [12]. To identify the factors that influence the amount of precipitates, Dick et al. [13] studied the influence of *Lysinibacillus sphaericus (*formally known as *Bacillus sphaericus)* and *Bacillus lentus*. The effects of urea degradation rate, zeta potential, pH, extracellular polymeric substances (EPS) and biofilm on morphology of the precipitate

were investigated. Results showed that the strains with a very negative zeta potential and capable of producing a continuous layer of dense calcium carbonate crystals.

2.2. MORPHOLOGY OF CALCIUM CARBONATE [2]

There are six known polymorphs of calcium carbonate [14]. Calcite and aragonite are more stable than vaterite and monohydrocalcite while ikaite and amorphous calcium carbonate (ACC) are rarely observed. Calcite and vaterite are the most common phases in MICCP applications [9, 15, 16]. As already mentioned, one of the main factors effecting morphology is microorganism type; thus the urease enzyme kinetics affect the precipitate [17]. In addition, the factors listed in previous section; calcium concentration, pH, availability of nucleation sites and DIC will influence the morphology [12].

Lian et al. [15] investigated the influence of having viable bacterial cells on precipitation mechanisms. Presence of viable or dead *Bacillus megaterium* cells at pH 7.0 stimulated calcite precipitation. When cells were excluded in the growth inoculum, vaterite was observed along with calcite in the supernatant solution. Therefore it could be concluded that when bacterial cells were extracted from the medium, the lack of nucleation sites for calcite, leads to the unstable polymorph vaterite [15].

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[2] See Appendix B: *Use of Biomineralization in Developing Smart Concrete Inspired by Nature* for further information.

The morphology of calcium carbonate crystals becomes more critical in self-healing applications such that bonding between precipitate and the substrate should be durable and strong. Considering this aspect, more stable calcite is more favorable than vaterite in self-healing of cementitious materials. On the other hand, formation of calcite rather than vaterite was found to be governed by affinity of the microorganisms with the substrates [18]. In case of bio-calcification process, negative surface charge of microorganisms became more pronounced and created a better attachment to calcitic substrate. The lower affinity of microorganisms with the substrate, leads to vaterite precipitation. Since there is a lack of structural bonding between the microorganisms and vaterite, this decreases the mechanical strength of binding material. Rodriguez-Navarro et al. [18] indicated that morphology of *Myxoccocus xanthus* induced calcium carbonate was governed by the attachment substrate mineralogy; calcium based substrates trigger calcite formation while vaterite was promoted by silica based minerals.

Another effect on morphology of calcium carbonate is the high alkalinity of cement-based materials. According to Rodriguez-Navarro et al. [18], vaterite could be more favorable than calcite in high pH environment (pH 8.5-9.0) even though the substrate is calcium based. Some specific strains, such as *Bacillus subtilis* were known to form precipitate even in high pH. However the reaction rate could take place at a slower rate compared to lower pH values. However morphology of calcium carbonate in cement

paste was generally defined as calcite, such that De Muynck et al. [19] has suggested calcite precipitated due to biocalcification is due to carbonation of portlandite phase and affects the durability of cement-based materials. It was also suggested that precipitation of vaterite was favored over calcite when the calcium source was provided as calcium acetate instead of calcium chloride [20].

2.3. BIOMINERALIZATION IN CEMENT-BASED MATERIALS

In a soil environment, successful cementation of sand particles with calcium carbonate precipitated via the bacterium *Sporosarcina pasteurii* (formally known as *Bacillus pasteurii*) has been shown to substantially improve the engineering properties of the sand [21, 22]. A Portland cement-based system is challenging due to the complex series of physio-chemical reactions occurring from the reaction of cement with water. Thus it is likely to be affected differently by the metabolic activity of microorganisms and the precipitates resulting from MICCP.

As introduced in **Section 2.2**, MICCP results from a chain of simultaneous metabolic reactions by certain species of bacteria. These metabolic reactions are initiated by hydrolysis of urea into carbonate and ammonia ions, which is governed by the urease enzyme. Thus, in order for MICCP to occur, the microorganisms should have high urease enzyme activity, which catalyzes the hydrolysis of urea into carbonate and ammonia. Due to its alkaliphilic nature and urease enzyme activity, *Sporosarcina pasteurii (S. pasteurii,*

formally known as *Bacillus pasteurii)* is the most common microorganism used for biomineralization applications in cement paste and concrete. In addition, these microorganisms might also induce calcite precipitation by serving as nucleation sites, which occur as a result of its negative surface charge that attracts positively charged calcium ions [23]. Ions are deposited on cell membranes and acts as nucleation sites for mineral deposition [24]. According to Dickson and Koohmaraie [25], species of *Bacillus* have a relatively large surface area compared to some other species that might be used in this application. Since the total charge of bacteria increases with increasing surface area, this suggests good bonding potential between the calcium ions and *S. pasteurii* cells.

However, *S. pasteurii* is not the only type of species that has been used in biomineralization applications in cementitious materials. Table 2.1 summarizes the list of microorganisms that have been used in cement-based materials. For instance, Achal et al. [4] used *B. megaterium* and they reported that this is another bacterial strain that is alkaliphilic, endospore-forming, and can also decompose urea. However one major drawback of this microorganism is that urease enzyme activity can be repressed under increasing ammonium and nitrogen concentration [26].

Ghosh et al. [27] introduced one strain of *Shewenella* species in a modified medium (pH 7.5) and then the bacteria were suspended in mixing water to achieve seven different cell concentrations from 10 to 10^7 per ml. Mercury intrusion porosimetry (MIP)

was used to test the porosity of mortar with and without bacteria at 28 days. The results showed that for *Shewenella* microorganism, the cubes with 10^5 CFU/mL exhibited the greatest reduction in porosity while the authors attributed the porosity reduction to the addition of the *Shewenella* microorganism, no explanation was provided on why the samples containing the 10^5 CFU /mL concentration exhibited the greatest reduction of porosity.

Microbial calcium carbonate precipitation can be either microbially induced, controlled or microbially influenced [28]. Both of these processes are referred as biomineralization, however the role of the microorganisms in these processes differs. Microbially induced precipitation is a direct result of the microbial activities that generates the biochemical conditions favorable for precipitation [23, 28], whereas microbially controlled precipitation results from cellular activity directs the nucleation and growth of the minerals [28]. Microbially influenced precipitation is a term introduced by Dupraz et al [28] and refers to a passive precipitation process, in which an interaction between the microorganism and the surrounding geochemical environment promotes precipitation. In microbially influenced precipitation the presence of living organisms is not required. In cement-based materials it is still not clear that if the calcium carbonate that is precipitated by the microorganisms is due to urea hydrolysis by the microorganisms (i.e., microbially induced) or due to the electrostatic attraction between

the negative surface charge of the microorganisms and ions (e.g. calcium and carbonate ions) in the pore solution (i.e., microbially controlled) or can result from interactions of extracellular substances produced by the microorganisms (microbially influenced) or some other mechanism. Thus in this dissertation the general term “biomineralization” will be used.

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

Bacteria Type	Introduction approach	Viability in cement-based materials	Reference
Shewanella	*Shewanella* spp. cells were suspended in tap water	Survived up to 6-7 days in mortar	Ghosh et al.[29]
Bacillus megaterium	*B. megaterium* cells were incorporated in mortar and concrete by vegetative inoculation method	Approximately 0.1% remained viable of the initial cells retained both in mortar and concrete	Achal et al.[4]
Bacillus cohnii	Naturally encapsulated *B. cohnii* suspended in mixing water of cement stone	2% spores survived after 10 days of mixing	Jonkers and Schlagen [1]
		1% spores survived after 9 days of mixing and can detect active spores for 4 months	Jonkers et al. [8]
	Spores of *B. cohnii* incorporated in concrete by organic capsules with calcium lactate	Spores remained viable for several months after mixing of concrete	Wiktor and Jonkers [7]
Sporosarcina pasteurii (Bacillus pasteurii)	*S. pasteurii* cells were suspended in mixing water	Viability was not reported	Jonkers and Schlagen [1] Ramachandran et al. [30]
	S. pasteurii cells were embedded in polyurethane membranes and glass beads	Urease enzyme activity was enhanced but viability was not reported	Bang et al. [2, 6]
Bacillus subtilis	Suspended in mixing water	Viability was not reported	Vempada et al.[31]

Table 2.1: Type of microorganisms used in biomineralization applications in cement-based materials (cont'd)

Bacteria Type	Introduction approach	Viability in cement-based materials	Reference
Escherichia coli	Cells were suspended in mixing water in mortar	Viability was not reported	Ghosh et al.[27] Vempada et al.[31]
Lysinibacillus sphaericus (Bacillus sphaericus)	Cells were immobilized in diatomaceous earth (DE) in mortar	Higher urease activity was observed in DE but viability was not reported	Wang et al.[32]
Bacillus halodurans	Spores of *B. halodurans* and *B. pseudofirmus* were suspended in mixing water in cement stone	2% spores survived after 10 days of mixing	Jonkers and Schlagen [1]
Bacillus pseudofirmus		7% spores survived after 10 days of mixing	
Pseudomonas aeruginosa	Suspended in mixing water	Viability was not reported	Ramachandran et al. [30]

2.4. INCORPORATING MICROORGANISMS IN CEMENT PASTE

As mentioned in **Section 1.1**, there are several encapsulation methods, both synthetic and natural, for incorporating microorganisms in cement-based materials. Bang et al. [6] proposed using a polyurethane (PU) barrier as a synthetic encapsulation approach. The PU membrane protected the microorganisms from the high pH environment within the cement paste matrix and enhanced the efficiency of urease enzyme activity. The PU matrix also acted as a nucleation site for calcite precipitates;

consequently improving the biomineralization capacity of the cell [6]. However a main disadvantage of PU is limited diffusion capability, which may limit the calcium carbonate precipitation induced by microorganisms. Further investigation of encapsulated microorganisms has also been conducted with microorganisms encapsulated in Siran™ Glass beads [2]. Cell suspensions were grown in nutrient broth media and centrifuged prior to immobilization. Compared to PU encapsulation method, the glass beads encapsulation method was better at improving strength and crack healing capability. *S. pasteurii* cells were immobilized in glass beads. When immobilized cells were submerged in an urea-$CaCl_2$ solution, significant calcium carbonate precipitation was observed both on cells and on glass beads [2]. With respect to the natural encapsulation approach, researchers at Delft Technological Institute have been the most active in this approach and have proposed the using the resilient nature of endospores themselves to protect the microorganisms. *B. cohnii* endospores were washed and suspended in mixing water without any additional nutrients provided. This procedure resulted in up to 4 months survival of the bacterial endospores in mortar [8]. Following this application, the researchers investigated using a natural-synthetic hybrid approach in which lightweight organic spheres were developed and *B. cohnii* endospores were embedded into these porous aggregates with lactate [7]. This application method was provided to keep endospores viable, thus the self-healing ability of the system is extended for years.

2.5. NUTRIENT MEDIUM SELECTION

Efficiency of calcium carbonate precipitation depends on the microorganism type, availability of sites that microorganisms can attach to, concentration of cells and the accessibility of nutrient sources. To keep microorganisms active in a metabolic state, carbon and nitrogen source are required. For *S. pasteurii* ammonium sulfate is a common compound that is used as a nitrogen source [5, 8, 9]. Yeast extract is commonly used as a carbon source; in addition to providing a source of carbon, yeast extract has many amino acids and vitamins required for survival of bacteria [4, 9]. An alternative for yeast extract is lactose mother liquor (LML), which is dairy industry by-product. Achal et al. [5] conducted a comparison study in order to show the influence of three different carbon sources on urease enzyme activity and soil properties. Effects of addition of urea to nutrient broth, yeast extract and LML were investigated. With respect to calcite precipitation and bacterial growth, the results suggested that LML media performed better compared to yeast extract and nutrient broth [5].

While sources of nitrogen and carbon are essential for bacterial growth, it is important to bear in mind that in order for MICCP to occur, urea and a source of calcium are also required. The bacteria should have an urease enzyme to produce carbonate ions for calcium carbonate precipitation, and work by Whiffin [26] suggests that urease enzyme activity and thus MICCP is governed by urea concentration rather than calcium

concentration. Since urea contains nitrogen, this can be used as a nitrogen source instead of ammonium sulfate. One possible drawback of urea hydrolysis is that increasing the ammonia concentration can repress urease enzyme activity specifically on microorganisms [26]. There was no significant effect of ammonium concentration on *S. pasteurii* urease enzyme activity; however the effects of nitrogen production on cement paste properties is still unclear.

Although cement-based composites naturally have sources of calcium, additional calcium sources are often added [26, 33]. Reported calcium sources include calcium chloride, calcium nitrate, and calcium lactate [7, 26]. Calcium lactate was used by Wiktor and Jonkers [7], and they found that it did not have any adverse effect on compressive strength of concrete. Calcium chloride is also reported as being more effective at inducing biomineralization in cement-based materials than calcium nitrate [26]. However, calcium chloride is a concern for corrosion in cases where reinforcement is required since chloride ions interfere with the passive oxide layer surrounding steel reinforcement. In addition Ramachandran et al. [30], showed that presence of chloride ions decreased the compressive strength of Portland cement mortar. In addition calcium chloride is a very well known accelerator that can increase the early age strength when it is added below the defined limit 2% of cement weight [34].

2.6. VIABILITY OF CELLS IN CEMENT-BASED MATERIALS

Nonetheless, the limited research involving biomineralization in cement-based systems has shown promising results and suggests that microbial-induced calcium carbonate precipitation and self-healing of cracks can occur on the surface of concrete [7].

The list of microorganisms reported, as being capable of inducing calcium carbonate precipitation is extensive. However, since the microorganisms will be embedded within concrete, key challenges are finding a microorganism that can (1) tolerate the highly alkaline conditions (pH 12-13), (2) survive the mixing process used to prepare the concrete, and (3) survive with limited access to nutrients. While few bacteria can tolerate highly alkaline conditions [35], *Bacillus* and *Sporosarcina* species have been shown to survive in cement-based environments for limited time periods [4, 7, 8]. These bacterial species are alkaliphilic and endospore-formers. Endospores are dormant, non-metabolically-active, non-reproductive structures that certain bacteria develop when they encounter stress (e.g., nutrient depletion). Endospores enable the bacteria to remain dormant for extended periods; when endospores are exposed to a suitable environment, the spore can transform into a vegetative cell, capable of normal metabolic function. *Bacillus* and *Sporosarcina* tend to form endospores under conditions of high cell densities or nutrient limitations related to carbon, phosphate, and nitrogen [36, 37].

It has been shown that spores of *S. pasteurii*[3] can tolerate high pH, and that they can be viable up to 4 months [8]. Moreover, Wiktor and Jonkers [7] developed a porous organic system in which endospores and a calcium lactate source were combined and encapsulated into lightweight aggregates. Compared to the mixtures that only contained endospores, the viability of the microorganisms encapsulated with calcium lactate increased. Thus, the time available for the microorganism to precipitate calcium carbonate for crack healing purposes is extended. Ramachandran et al. [2, 6] showed that microorganisms could display prolonged crack healing ability when they are embedded in polyurethane based polymers and glass beads. Achal et al. [4] showed that species of *B. megaterium* could survive up to 28 days in both mortar and concrete. The concentration of microorganisms was found to be close to initial values when plate counts were conducted on 3-day specimens. Even though the percent of microorganisms that survived decreased from 7 to 28 days, they were not completely killed. According to the results obtained, fly ash addition in both mortar and concrete also increased the total number of viable microorganisms retained.

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3 Bacteria were incorporated into the cement-based matrix by replacing the portion of the mixing water with bacterial spore suspension.

2.7. EFFECTS OF BIOMINERALIZATION ON STRENGTH

Studies conducted up to this point suggest that compressive strength can be enhanced with addition of microorganism such as *S.pasteurii, B.megaterium* and *Shewenella* species [4, 29, 30, 38].

Achal et al. [4] used *B. megaterium* in cement paste mortar with different fly ash replacement ratios and specimens were cured in a nutrient-broth-urea (NBU) medium that contained calcium chloride. Compared to the control specimen, a 21% increase in 28-day compressive strength was seen when *B. megaterium* was added. However this increase was not observed at 7 days [4]. At 28 days, specimens with 10% fly ash replacement showed 19% strength increase relative to control specimen without microorganisms. It is believed that the increase in compressive strength is due to metabolic activity of bacterial cells and plugging of pores due to precipitation of calcite precipitation induced by microorganisms [4].

Jonkers and Schlagen [1] conducted experiments using three sets of concrete bars (16 x 4 x 4 cm), including one control set, one added with bacteria (4 different strains: *S.pasteurii, B. cohnii, B. pseudofirmus* and *B. halodurans*) and the other with amino acid. The specimens were cured in tap water at room temperature. There was no significant difference in three point bending strength and compressive strength among the three sets of concrete bars. It should be noted that the compressive strength of cement paste cubes

prepared with bacterial endospores was also measured. The result revealed that the specimens did not lose any strength compared to the control set for 3, 7 and 28 days cement stones with *S. pasteurii* cells suspended in mixing water.

Park et al. [38] investigated the influence of *S. pasteurii* ATCC 11859 and 4 other calcite-forming bacteria (CFB), which were isolated from 7 concrete structures on the compressive strength of mortar cubes. Using gene sequencing analysis, it was identified that the 4 CFB collected from concrete were *Sporosarcina soli* KNUC401, *Bacillus massiliensis* KNUC402, *Arthrobacter crystallopoietes* KNUC403, and *Lysinibacillus fusiformis* KNUC404. In this work, fresh medium with bacteria was suspended in mixing water to make mortar cubes, and the cubes were cured in a urea-$CaCl_2$ medium. The result showed that KNUC402 and KNUC404 achieved increased 7-day (13.2% and 11.7%) and 28-day (3.8% and 4.5%) compressive strength compared to control group. The author attributed the improvement of strength to the carbonate precipitates and EPS in mortar's pores. The cubes mixed with *S. pasteurii* did not show strength increase, which coincides with the result of Jonkers and Schlagen [1] discussed earlier. Hence, it is still questionable if the addition of calcifying bacteria can improve the compressive strength of concrete, or, it depends on the species of bacteria, as suggested by Park et al. [38].

The influence of *Pseudomonas aeruginosa (P. aeruginosa) and S.pasteurii* on compressive strength was studied by Ramachandran et al. [30]. One set of specimens was prepared by addition of both *P. aeruginosa and S. pasteurii*. Second set of specimens was prepared by using *S. pasteurii* pure culture. Both live and dead cultures were used in preparation of these two sets of specimens. Mortar cubes were cured in urea-calcium chloride solution at room temperature. The results also showed that *P. aeruginosa* had no significant impact on the 7-day compressive strength compared to the specimens containing only *S. pasteurii*. According to the authors, one possible reason could be the metabolic state of the bacterial cell since at earlier ages the mortar is more porous and the microorganisms have easier access to nutrients than at later ages. However, it is also possible that the high pH environment of the mortar caused the microorganism to die very early and that the strength gain was due to microorganisms acting as organic fibers. However, over time they decomposed and lost their efficiency [30]. As stated earlier, *S. pasteurii* is known to be MICCP capable microorganism since it exhibits urease enzyme activity, and thus is capable of decomposing urea into ammonia and carbonate.

Ghosh et al. [27, 29] used *Shewenella* species that was extracted from river water. Mortar mixes were batched with addition of microorganisms to mixing water at different concentrations and air cured at room temperature. Results of the tests suggested that addition of microorganisms enhanced the compressive strength, particularly at

concentration of 10^5 CFU/mL. In contrast to the previous study, the general trend of compressive strength increase became more predominant at later ages. Imaging analysis showed that fiber-like structures were observed in pores due to addition of microorganisms which decreases porosity of the cement past mortar.

Achal et al. [39] designed a special experiment to examine the role of nutrient medium and biomineralization on the pullout strength of reinforced concrete (RC). *Bacillus* sp. CT-5, isolated from commercial cement, was cultured in two different nutrient media. One medium was nutrient broth (NB) medium containing urea, and the other was named CSL (pH 8.0), which was actually an effluent from corn wet-milling industry. The specimens were cured in the same nutrient medium as the mixing medium. Concrete without bacteria in it was used as control group. The authors attributed this pullout strength increase to bacterial precipitation at 7 days.

In addition to containing nutrient broth media, bacterial suspensions typically contain a buffer. To understand how the buffer solution influences incorporation of *S. pasteurii* in cement paste, Ramachandran et al. [30] investigated the role of the buffer solution on compressive strength. This study was conducted with both killed and live *S. pasteurii* and *P. aeuruginosa* suspended in both saline and phosphate buffer. The results showed a decrease in compressive strength when a saline buffer solution was used. This was explained due to addition of chloride ions.

2.8. REMEDIATION OF CRACKS BY BIOMINERALIZATION [4]

Throughout the literature there are two main approaches to using biomineralization in crack healing applications for cement-based materials. First approach was to apply precipitates to fill the cracks. Ramachandran et al. [30] proposed a crack healing system for cubes and beams by filling external cracks with sand and *S. pasteurii* cells with urea-calcium chloride solution at different concentrations (10^7 -10^8 cells/cm^3). A similar procedure was applied to cracks having different depths on mortar beam and cube surface and bacteria concentration was kept constant at 3.9 x10^9 cells/cm^3 [30]. They also investigated the influence of biomineralization on strength of cubes with various concentrations of microorganisms. Results suggested that biomineralization could be applicable in surface crack healing; however efficiency of the application was limited by crack width such that increase in strength and stiffness was more evident in shallow cracks rather than deep cracks. In addition, it has been suggested that biomineralization was more efficient in crack healing rather than compressive strength improvement. In order to protect the microorganisms from alkalinity of cement paste, Bang et al [30] suggested encapsulation of *S.pasteurii* cells in polyurethane foams and applied immobilized cells on surface cracks of cubes. They observed insignificant increase in

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4 Further examples for bio-smart self- healing materials can be found in Appendix B: *Use of Biomineralization in Developing Smart Concrete Inspired by Nature*

tensile strength recovery even though the urease enzyme activity was reduced. This was also attributed to lack of chemical bonding between the substrate and calcium carbonate precipitated in the PU foam.

Bang et al. [2] developed the crack remediation technique by filling glass beads with *S. pasteurii* culture into mortar cracks. In the specimens, the artificial cracks with width and depth of 3.175 mm and 12.7 mm were made in beams (25.4 x 25.4 x152 mm), and with width and depth as 3.175 mm and 25.4 mm in cubes (50.8 x50.8x 508 mm). Bacteria with different concentrations (10^7 -10^9 cells/cm^3) were encapsulated in Siran™ glass beads and injected into the cracks with nutrient medium and calcium chloride. Compressive strength and stiffness were measured at 7 and 28 days. The results showed that compressive strength and stiffness increased with time for all bacteria concentrations compared to control samples with only glass beads, nutrient solution and calcium chloride. In addition glass bead immobilized with *S. pasteurii* cells provided an additional calcite protective layer on the surface of cement-based material, which could enhance the durability of the sample.

Next approach is the idea of self-healing developed by integrating microorganisms in cement-based materials, thus there would be autogenous healing with out external application of microorganisms. Jonkers et al. [8] incorporated *B. cohnii* endospores for crack remediation in cement stones, which was found to be efficient in sealing and

plugging of cracks. At last, Wiktor et al. [7] encapsulate *B. cohnii* spores in lightweight coarse aggregate in concrete. With the proposed system, biomineralization was able to seal the cracks on surface of concrete beams and improved the self-healing capacity compared to naturally encapsulated *B. cohnii* cells.

Chapter 3: Microorganism selection, growth and calcium carbonate precipitation

3.1. INTRODUCTION

This section explains the microorganism selection, microbial growth process, and capability of the selected microorganism to precipitate polymorphs of calcium carbonate ex-situ. Microorganism selection was done based on the resistance of the bacteria to restricted environmental conditions. Then the growth curve was obtained via correlating absorbance readings with cell concentration in batch cultures. After defining the growth curve of the microorganism selected, calcium carbonate precipitates were collected at different pH values with different calcium concentration additions.

3.2. MICROORGANISM SELECTION

The list of microorganisms reported as capable of inducing calcium carbonate precipitation is extensive. However, the main challenge was finding one that would be active in the highly alkaline environment of concrete. Furthermore, since the microorganisms would be embedded as vegetative cells within the microstructure of the concrete in this work, the microorganisms had to be able to survive in limited environmental conditions. As seen from Table 2.1, the longest reported time for inoculated vegetative *B. megaterium* cells was 0.02% viability retention at 28 days [4], whereas active viable *B. cohnii* endospores were detected in cement stone for 4 months

with a natural encapsulation method [40]. Moreover, the viability retention of *B. cohnii* endospores was extended with lightweight aggregate encapsulation [7].

Microbial selection plays a role in the type of mineral precipitated when bacteria are incorporated in cement paste. *B. megaterium* was reported to precipitate calcium carbonate [4] whereas the *Shewanella* species precipitates an aluminosilicate mineral [29]. However, both of these microorganisms were found effective in improving compressive strength of mortar [4, 27, 29]. Achal et al. [4] reported a 21% increase in 28-day compressive strength of mortar by inoculated vegetative *B. megaterium* cells with initial concentration of 5 x 10^7 CFU/mL. Relatedly, Ghosh et al. [27] determined the effects of the *Shewanella* species on compressive strength of mortar at various concentrations (10^1-10^7 CFU/mL). In comparison to the conclusions of Achal et al. [4], there was a 11% increase in compressive strength at 28 days due to the addition of *Shewanella* species with initial concentration of 10^7 CFU/mL [27].

S. pasteurii is the most commonly used bacteria for biomineralization applications in soil, which is likely due to high intercellular urease enzyme activity and its alkaliphilic nature [21, 41]. Therefore it has the potential to trigger calcium carbonate precipitation in cement paste. Several studies reported that *S. pasteurii* was capable of self-healing in cement-based materials without sacrificing the strength of mortar [1, 2, 6, 30].

Based on these findings, *S. pasteurii* was selected to study in this research and the strain was obtained from the American Type Culture Collection (ATCC 6453). *S. pasteurii* is a non-pathogenic, spore-forming microorganism, commonly found in soil.

In addition, similar to many other microorganisms, the cells of *S. pasteurii* have a negative surface charge. Thus, dissolved calcium cations in the cement paste may be electrostatically attracted to the negatively charged cell surface, thereby creating nucleation sites for calcium carbonate precipitation [18, 23, 42].

3.3. MICROORGANISM GROWTH

3.3.1. Urea-Yeast Extract (UYE) nutrient medium

To keep the microorganisms in an active metabolic state, carbon, nitrogen, and other nutrients are required. In MICCP applications, urea is commonly used as a nitrogen source and yeast extract is commonly used as a carbon source for *S. pasteurii* [9, 43, 44]. *S. pasteurii* ATCC 6453 was grown in Urea-Yeast Extract (UYE) medium; the UYE medium contained 0.13 M tris base, 10 g of urea and 20 g of yeast extract per liter of distilled deionized water (DDI). 20 g of agar were added to the liquid medium if a solid media was required. The pH of the medium was controlled by tris base; and 0.13M tris base was prepared in 1-liter DDI water. The pH was adjusted to 9 by adding 2 mL of hydrochloric acid. The tris base solution was divided into two equal volumes; urea and

yeast extract were added separately to these solutions. The mixtures were autoclaved separately[5]. After sterilization, samples were kept at room temperature (23°C) and mixed when they were cooled down.

3.3.2. Determination of bacterial concentration

S. pasteurii cells were grown aerobically at 30°C and 150 rpm shaking conditions in a 600mL UYE medium until the stationary growth period was reached. Stationary growth period starts when the growth of microorganisms slows down and growth rate drops to zero. 1 mL of sample was obtained at each time interval and serially diluted (10^0-10^{-4}) in test tubes. Then they were vortexed to provide homogeneity. Optical density measurement at 600 nm (OD_{600}) was measured by a BIO-TEK Synergy HT spectrometer (Winooski, VT, United States). The growth curve of *S. pasteurii* was generated by correlating the absorbance of the cells at OD_{600} with the viable plate counts at certain time intervals. The details of this procedure can be found in Appendix C: Correlating the cell concentration with absorbance readings at OD_{600}. Growth curve batch experiments were conducted in triplicate. Figure 3.1a and Figure 3.1b show representative growth curves for ATCC 6453 *S. pasteurii*, which was consistent with the other two sets. The lag period

[5] Tris base solution was divided into three equal volumes in a case of solid media and ingredients autoclaved separately.

is defined as the time frame prior to the exponential growth phase when cells can be metabolically active however they are not able to grow, while the exponential phase is the active growth period in which the cell concentration doubles within a fixed time period.

Using the results shown in Figure 3.1, the following cell concentration equation (Equation 3.6) was determined:

$$Y = 5x10^{8} X^{8.4714} \qquad (3.6)$$

Where, Y is the colony forming units per mL (CFU/mL) and X is the absorbance reading at OD_{600}. This equation was used in determining the initial concentration of the bacterial cells prior to mixing.

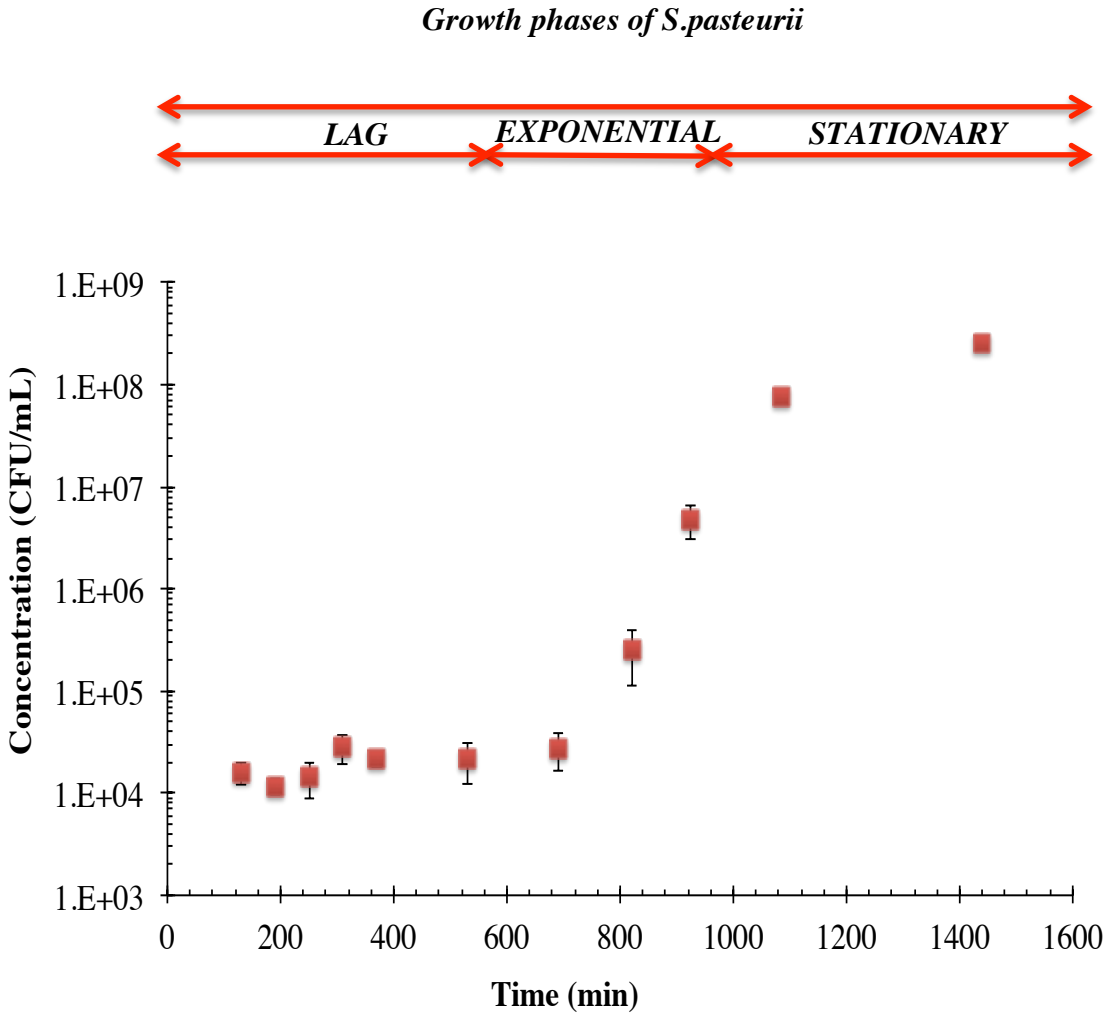

Figure 3.1a: Growth curve for ATCC 6453 *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*) in 600 mL Urea-Yeast extract medium. Error bars represent the standard deviations of concentrations of triplicate measurements.

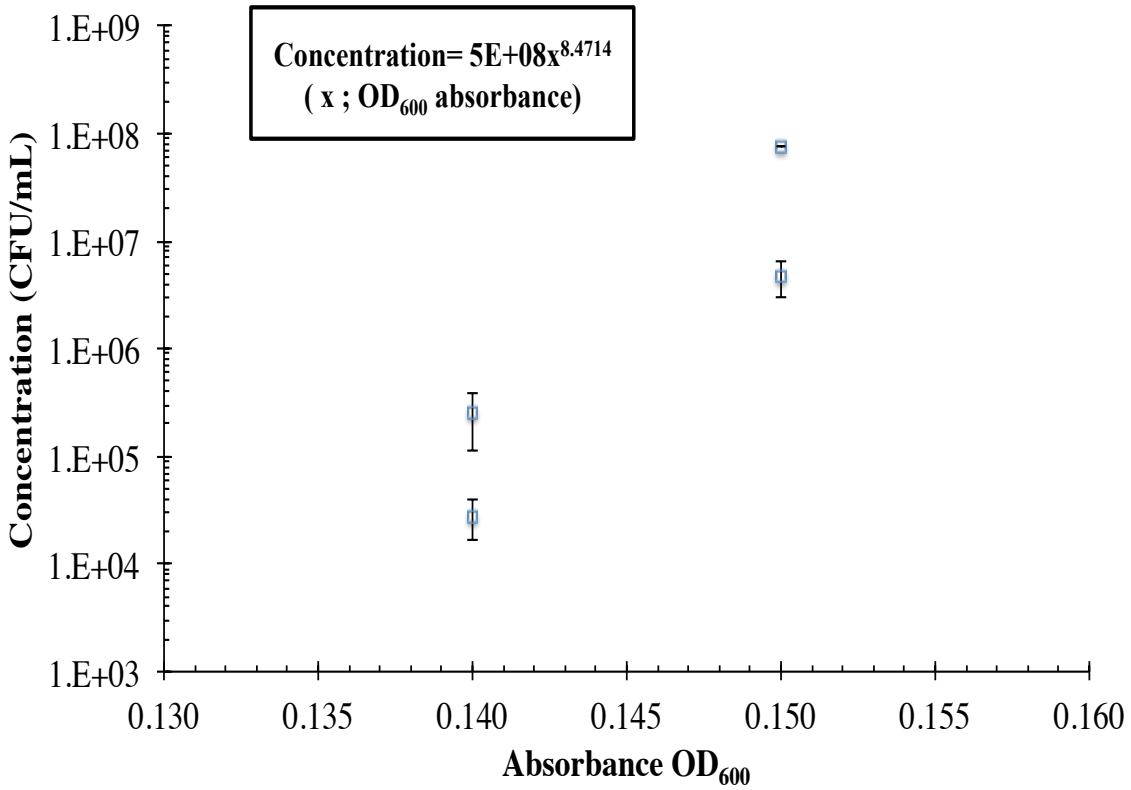

Figure 3.1b: Concentration and absorbance at OD_{600} correlation for ATCC 6453 *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*) in 600 mL Urea-Yeast extract medium. Error bars represent the standard deviations of concentrations of triplicate measurements.

3.4. OPTIMIZING CALCIUM CARBONATE PRECIPITATION IN UYE MEDIUM

3.4.1. Ex-situ culture preparation

To determine the influence of calcium concentration and pH on the morphology of calcium carbonate precipitated by *S. pasteurii*, the vegetative cells were prepared exposed to different calcium concentrations and pH values.

Four types of ex-situ culture experiments were prepared with 100 mL UYE medium in 250 mL flasks. To simulate the cement-paste pore solution alkalinity, the pH of the UYE medium was adjusted to 12 by addition of sodium hydroxide. For the first set, *S. pasteurii* cells were allowed to grow in a 100 mL UYE medium supplemented with 0.167M $CaCl_2$ until the desired cell concentration was obtained. The final cell concentration was determined based on the correlation of measured OD_{600} to cell concentration, as stated in **Section 3.2.1.2.** However the intrinsic calcium concentration within the cement pore solution is lower than what is required to precipitate all carbonate ions. Calcium ions in cement paste pore solution has been reported as ranging from 0.02M to 0.1M in fresh cement paste and 0.0005M to 0.002M in older samples [45]. Therefore another culture inoculated such as the low calcium concentration of cement paste pore solution was stimulated. The second 100 mL UYE medium was prepared with a lower calcium ion concentration of 0.03M $CaCl_2$ at pH 12. The third and fourth

solutions were prepared at pH 9 and 0.167M and 0.03M of $CaCl_2$ were added to these solutions respectively. Cells were grown aerobically with shaking at 150 rpm and 30°C for 24 hours. A triplicate of culture batches was prepared for each set.

3.4.2. X-ray diffraction analysis

X-ray diffraction (XRD) analysis was conducted using a Siemens Bruker X-ray Diffractometer (Madison, WI, United States) to determine the crystal structure of calcium carbonate obtained in ex-situ cultures. After 24 hours of inoculation with $CaCl_2$, a Fisher Scientific Centrific™ Centrifuge Model 225 (Industry Drive, Pittsburgh, PA) was used to centrifuge *S. pasteurii* and the precipitates in UYE medium at 2720 x g for 15 minutes. XRD analysis was conducted with the pellets collected from centrifuged solutions. Samples were placed and compacted in a sample holder and analysis was conducted at angles from 20-60° 2θ with 2 seconds dwell time. The diffractometer was operated at 40 keV and 30 mA, at a step size of 0.02° 2θ.

3.4.3. Results and discussion

Microbial calcium carbonate precipitation was observed in every culture regardless of the pH and calcium concentration. The XRD results in Figure 3.2 and Figure 3.3 indicate that the morphology of calcium carbonate in the ex-situ culture solutions was more affected by calcium concentration rather than pH when calcium chloride was added during the lag period. At a pH of 9, vaterite and calcite crystals were observed at 0.03M and 0.167M calcium chloride additions (Figure 3.2). In the high pH samples (see Figure 3.3), calcite peaks and short vaterite peaks appeared when the calcium concentration was 0.03M, while only vaterite peaks were observed in the high calcium concentration of 0.167M. Since there was not any internal standard used and there was a possibility of preferred orientation, the data obtained was used only for qualitative analysis to determine the phases present in the culture.

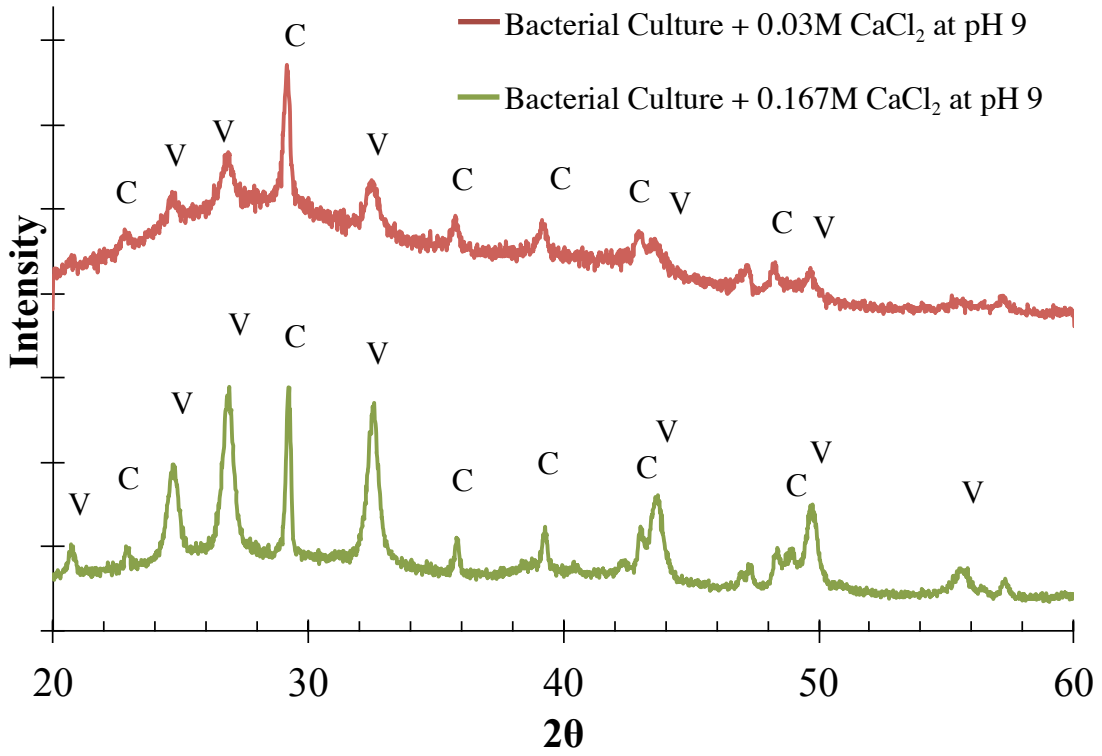

Figure 3.2: XRD pattern of precipitates obtained from *S. pasteurii* cultures in UYE medium at pH 9 with low [Ca^{+2}] (Bacterial Culture+0.03M $CaCl_2$) and high [Ca^{+2}] (Bacterial Culture + 0.167M $CaCl_2$) added during the lag period. C: Calcite peaks; V: Vaterite peaks

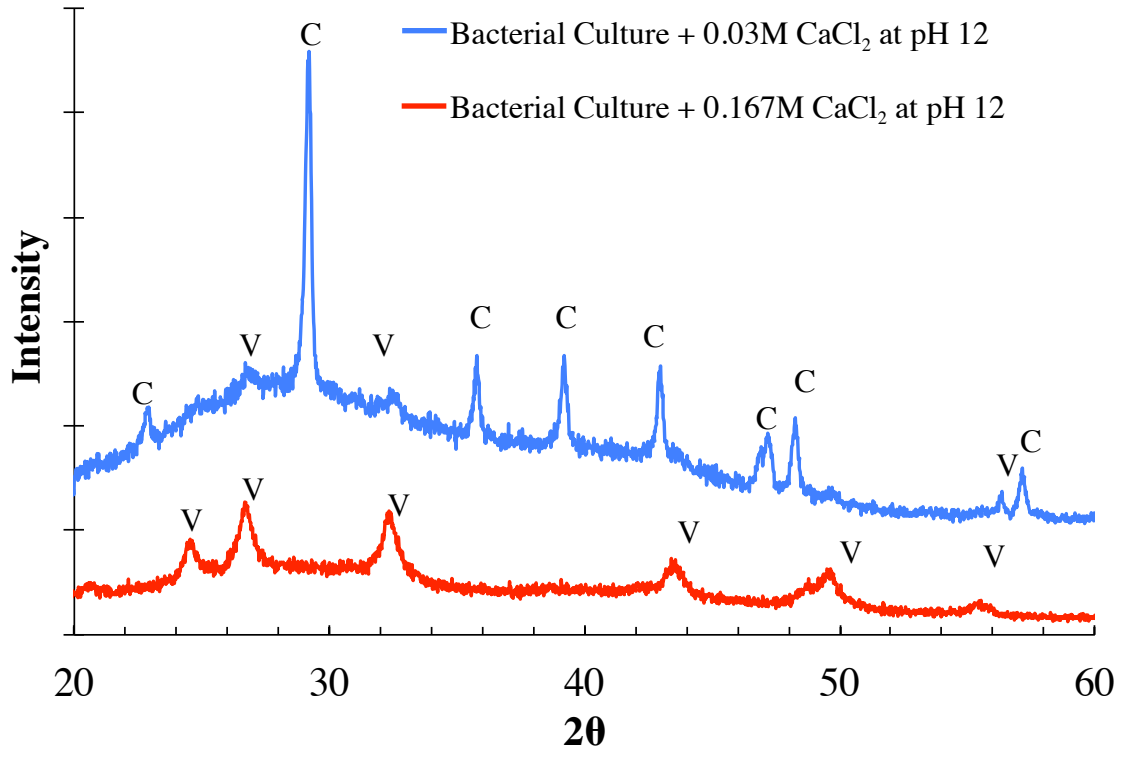

Figure 3.3: XRD pattern of precipitates obtained from *S. pasteurii* cultures in UYE medium at pH 12 with low [Ca^{+2}] (Bacterial Culture+0.03M $CaCl_2$) and high [Ca^{+2}] (Bacterial Culture + 0.167M $CaCl_2$) added during the lag period. C: Calcite peaks; V: Vaterite peaks.

In ex-situ cultures calcium carbonate precipitates according to Oswald's rule which defines the order of crystallization wherein the least stable polymorph precipitates first [18]. The least stable polymorph (ACC) has the highest solubility index and the most stable (calcite) has the lowest solubility index. Thus, the crystallization order for calcium carbonate is from the least stable polymorph to the thermodynamically most stable

polymorph. Considering the three non-hydrated crystalline polymorphs of calcium carbonate, the reaction should progress in the order of ACC to vaterite to aragonite or calcite [46]. However reaction kinetics may be influenced by parameters such as temperature, pH, ion concentration, etc. Aragonite is more favorable in cases when the temperature is above 40^0C and below pH 12; however at room temperature vaterite is more favorable than aragonite [47]. Since the ex-situ cultures were incubated at 30°C, vaterite and calcite were the expected final polymorphs of MICCP.

However, the calcium concentration also affects the calcium carbonate precipitation. Ogino et al. [47] determined that an increase in calcium and carbonate ion concentrations in ex-situ cultures triggers an increase in vaterite content at 25°C. As stated in **Section 3.3.1**, 10 grams of urea ($CO(NH_2)_2$) was added per liter of DDI water, which yielded 0.167M of urea. Mass balance of Equation 3.7 yielded 0.334M of ammonia and 0.167M of carbonate ions when 0.167M of urea was used as the molar concentration for urea.

$$CO(NH_2)_2 + H_2O \longrightarrow CO_3^{-2} + 2NH_4^{+} + 2H_2O \qquad (3.7)$$

Assuming that the microorganism can hydrolyze the urea completely, 0.167M of carbonate ions formed in a 1L solution. In order to precipitate calcium carbonate with all of the carbonate ions produced, 0.167M of calcium ions are required per liter for DDI water (Equation 2.5) when 0.167M of urea are present for full precipitation.

Even though the intrinsic calcium concentration within the pore solution of cement-based materials is theoretically lower than what is required for full calcium carbonate precipitation, it is possible that the natural calcium ion source in cement paste pore solution is adequate for MICCP applications. The intrinsic calcium concentration promotes the biomineralization process since precipitation can occur gradually with time as calcium sources (via internal reservoirs or CO_2 from the atmosphere) interact with the microorganisms.

Vaterite can readily transform to calcite due to its thermodynamic instability in aqueous solution [47]. Diaz- Dosque et al. [48] showed that in biopolymer (polymers produced by living microorganisms) applications for MICCP, calcite was the final product in biomineralization. Even though vaterite was observed as the preliminary precipitate, the transformation of crystals occurred in less than 24 hours [48].

Several hypotheses regarding transformation of vaterite to calcite have been proposed and it is still unknown whether vaterite precipitation is more favorable in high or low pH or if pH has an influence on calcium carbonate polymorph [46, 47]. Han et al. [49] observed that calcite precipitation was initiated with pH 11 and vaterite was observed at low pH (pH 7-8) due to increase in supersaturation of ammonia ions. However, Spanos and Koutsoukos [50] observed vaterite to calcite transformation was not influenced by pH value. The results of the ex-situ culture experiments conducted in

this study showed that the polymorph formation is in fact influenced by the pH value but is also influenced by the calcium concentration. MICCP produced stable calcite at low calcium concentration even at pH 12. However, similar to the work of Ogino et al. [47], an increase in calcium concentration favored vaterite precipitation and inhibited the transformation of vaterite to calcite.

These studies were conducted in ex-situ culture solutions, but when a substrate is introduced the resultant precipitates may be different compared to free cells suspended in the nutrient solution. Navarro-Rodriguez et al. [18] suggested that the morphology of calcium carbonate induced by microorganisms is mainly governed by mineralogy of the substrate that the bacteria was attached to rather than bacteria type. When they exposed *Myxoccocus xanthus* cells to silicate-based substrates, only vaterite was observed at high pH. This was due to the weak affinity of the microorganisms to the substrate and in this case precipitation was governed by Oswald's rule, which mandates that the least stable polymorph precipitates first. However when the cells were introduced to a carbonate-based substrate, calcite was the only polymorph observed because the attachment of microorganisms to the calcitic substrate was stronger than its attachment to a silicate substrate. In addition to the influence on the morphology of calcium carbonate, stronger attachment of the microorganisms to the substrate resulted in a higher metabolic activity due to increased resistance to external stresses (e.g. high pH, high ion concentration) [51,

52]. In some rare cases, it was observed that vaterite precipitated even if there was a calcitic substrate. It was suggested by Navarro-Rodriguez et al. [16] that this occurred due to the high pH (pH 8.5-10) interfering with the reaction kinetics. As a result, the polymorph with the lower solubility index was preferable. In our studies, high pH and high calcium concentration led to the suppression of calcite peaks and only vaterite peaks were observed. The type of calcium carbonate polymorph could be correlated to the calcium ion concentration.

It is likely that in a cement-based system, the order of crystallization should also be vaterite to calcite. However the kinetics that may influence the system are more complex than ex-situ culture studies. Cement paste matrix without any external calcium source has a calcium ion concentration as low as 0.03M [53] in its pore solution. The high alkalinity of the pore solution suggests that calcite should be a favorable polymorph of calcium carbonate in cement-based materials. However, the complexity of the cement paste substrate requires further investigation regarding crystal structure of microbial induced calcium carbonate, which will be discussed in **Chapter 4.**

Chapter 4: Influence of biomineralization on cement-based materials

The previous chapter examined the morphology of calcium carbonate precipitated ex-situ. This chapter examines the influence of **inoculated vegetative** *S. pasteurii* cells on mechanical and chemical properties of cement-based materials. Of particular interest is whether biomineralization can occur by using calcium ions that are already present in cement (i.e. no additional sources of calcium will be added).

4.1. INTRODUCTION

A key question is whether inoculated vegetative microorganisms in cement paste can precipitate calcium carbonate. Most applications of biomineralization in cement-based systems were conducted by adding calcium chloride either with mixing water or to the curing solution [11, 22]. Disadvantages of calcium chloride usage in the cement-based systems were summarized in **Section 3.2.3**. In my approach, ions in the pore solution of cement-paste matrix served as the calcium source for the inoculated vegetative bacterial cells. Considering the fact that intrinsic calcium ions are used for biomineralization, which can impact the composition of cement paste, some changes may be stimulated in properties of cement-based materials.

As seen in **Chapter 3**, the calcium concentration played a role on the precipitated polymorph. A low concentration of calcium and high pH induced precipitation of mainly

calcite and less of vaterite. The morphology of calcium carbonate is also crucial in cement paste because calcite is preferred over vaterite due to its lower solubility and better affinity with the substrate. The observations in the literature suggested calcite was the preferred polymorph when calcitic substrates were used. This suggests calcite might be the favorable polymorph of calcium carbonate in cement-based systems [18].

4.2. MATERIALS AND METHODS

4.2.1. Materials

Texas Lehigh Type I/II (Buda, TX) Portland cement was used for all cement paste mixtures, and Table 4.2 shows the mass percentage distribution for oxides. As shown in Table 4.2, Type I/II cement has 3.2% free lime content, and 77.3% of the free lime was found to be calcium carbonate. Deionized distilled (DDI) water was used to prepare the nutrient solution and bacterial growth medium; tap water was used as the mixing water for the neat paste and neat mortar.

A Mastersizer 2000 particle size analyzer with a Hydro MU 200 wet dispersion unit (Malvern, Worcestershire, United Kingdom) was used to determine the particle size distribution of cement. The refractive index (RI) and absorption of the cement particles were defined as 1.7 and 1.0 respectively while the specific gravity (SG) of cement was 3.15 [54]. The cement particles were dispersed in isopropyl alcohol (RI: 1.39) instead of

water to prevent hydration. The cement particles were sonicated for 30 seconds to provide homogenous dispersion in the alcohol solution before the measurement started. The particle size distribution determined is presented in Figure 4.4.

Table 4.2: Texas Lehigh Type I/II Portland cement oxide composition [55]. LOI: Loss of ignition.

Oxides	% (w/w) composition
CaO	65.0
SiO_2	20.5
Al_2O_3	4.5
Fe_2O_3	3.0
MgO	1.6
SO_3	2.6
LOI	2.4
C_3A	7.0
Na_2O Equivalent	0.79
Free Lime%	3.2

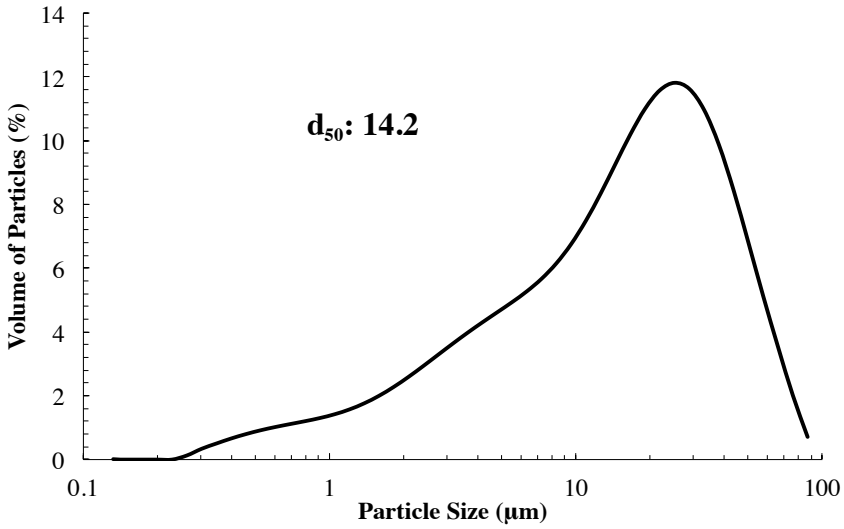

Figure 4.4: Particle size distribution for Texas Lehigh Type I/II Cement. The x-axis is the particle sizes logarithmically between 0.1 and 100 μm and the y-axis shows the volume of particles between these sizes.

The particle size distribution for Colorado River sand was determined according to ASTM C136 Standard Test Method for Sieve Analysis for Fine and Coarse Aggregate [56]. ASTM C128-07 Standard Test Method for Density, Relative Density (Specific Gravity) and Absorption of Fine Aggregate [57] was used to determine the absorption coefficient of the sand. The absorption capacity of the sand was determined as 0.65% while the specific gravity is determined as 2.62. Three hundred grams of oven dry fine sand was sieved through #4 to #200 (4.75 mm-75 μm) sieves by a mechanical shaker. Figure 4.5 shows the particle size distribution for Colorado River sand.

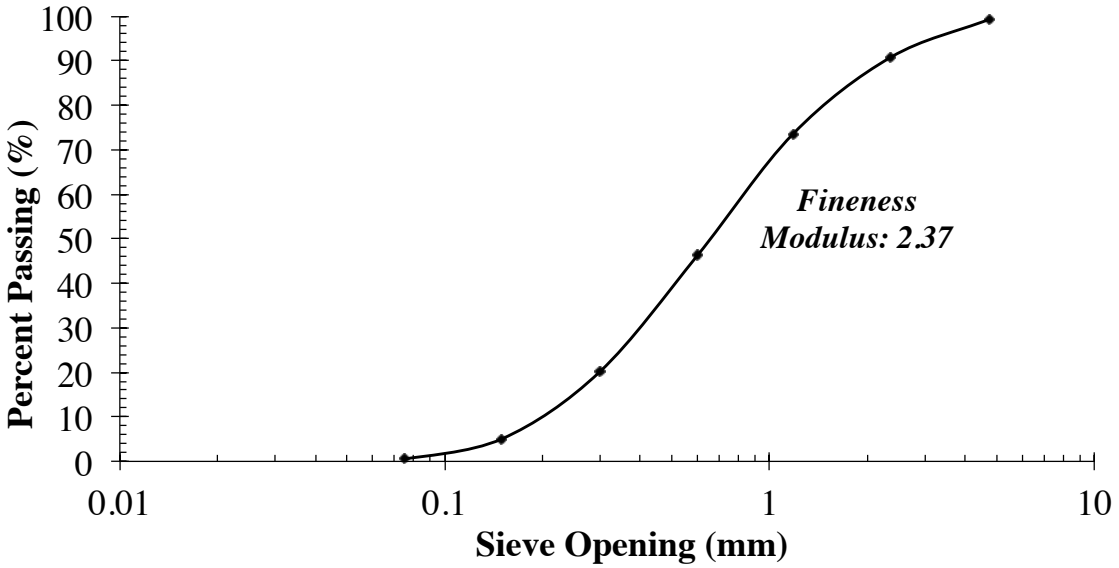

Figure 4.5: Particle size distribution for Colorado River sand.

4.2.2. Inoculation of vegetative cells

As stated in **Section 1.1**, there are several ways to incorporate microorganisms in cement-paste matrix, from suspending them in mixing water to encapsulating them in inorganic protective barriers. However forced encapsulation might not be necessary. Rather, with the proper selection of microorganism, nutrient and inoculation approach, the inoculated microorganism might provide biomineralization without undertaking the additional processing steps required for encapsulation.

Even though the limited literature suggests that viable microorganisms remain after mixing [4, 7, 8], these organisms must be capable of synthesizing urease such that calcium carbonate can be precipitated. To produce the urease enzyme, the microorganisms must be in a vegetative state (i.e., metabolically active) [41, 58]. However uncertainty exists regarding the degree to which vegetative cells are able to persist in a cement-based environment. While endospores are designed to persist in restrictive environments, the conditions (e.g., time, nutrient supply) needed to convert the endospores back to a vegetative state capable of triggering the production of biogenic calcium carbonate are not known. Therefore active vegetative cells might be able to facilitate continuous calcium carbonate precipitation in cement-based materials. In this case, introduction of vegetative cells and their resistance to the harsh cement paste environment becomes more critical. In the current work, the initial cell concentration

prior to mixing was selected in the range of 10^5 to 10^7 CFU/mL, where the cells were in the exponential growth phase (See **Section 3.3.2**).

In this study **vegetative cells (i.e., metabolically active cells)** were inoculated with cement paste with their growth medium. The vegetative *S. pasteurii* cells with their growth medium in cement paste might aid the bacteria in resisting the physico-chemical stressors inherent from being embedded in cement paste (e.g. high pH, nutrient limitation) that could limit calcium carbonate precipitation and/or cause cell death. The viability of *S. pasteurii* was tested via most probable number (MPN) method in aged cement paste and mortar samples, which is discussed in **Chapter 5**.

4.2.3. Preparation of pastes and mortar for compressive strength

The cement paste samples were prepared according to modified ASTM C305 Standard Practice for Mechanical Mixing of Hydraulic Cement Pastes and Mortars of Plastic Consistency [59]. In general 3 types of cement paste were prepared:

- Neat paste (Neat)[6] prepared with distilled water and cement;
- Nutrient paste (Nutrient) prepared with UYE medium and cement;

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[6] Throughout the text, samples are referred to by these names: neat, nutrient, and bacterial paste

- Bacterial paste (Bacterial) prepared with bacterial culture grown in an UYE medium and cement. The bacteria were cultured in the UYE medium at 30°C until the desired concentration was obtained (according to the procedure stated in **Section 3.2.2)** Then the bacterial culture suspension was mixed with cement. Table 4.3 summarizes the mixing proportions for neat, nutrient and bacterial pastes.

Table 4.3: Mixing proportions for Neat, Nutrient and Bacterial Paste. The w/c and s/c ratio of 0.5 was used.

	Weight of ingredients per mixture (g)				
	Cement	**Water**	**UYE Medium**	**Bacterial Culture**	**w/c or s/c**
Neat Paste	3207.50	1603.75	-	-	0.5
Nutrient Paste	3303.72	-	1651.86	-	0.5
Bacterial Paste	3303.72	-	-	1651.86	0.5

The water to cement (w/c) and solution to cement (s/c) ratios were kept at 0.50. Mixtures of neat, nutrient and bacterial paste samples were prepared and cast into 5.08 x 5.08 cm cube molds. The specimens were initially cured at 100% relative humidity at room temperature at 25°C for 24 hours. Day one testing was conducted right after the molds were removed. Additional curing was provided by submersion of the cubes in an UYE medium saturated with lime until the time of testing. The bacterial paste mixes were prepared with three different initial concentrations of *S. pasteurii* in the inoculum ($3x10^5$, $4x10^6$ and $2x10^7$ CFU/mL). A compressive strength test was conducted with triplicate

samples at 1, 7, 28 and 56 days according to ASTM C 109/C 109M-8 Standard Test Method for Compressive Strength of Hydraulic Cement Mortars (Using 2-in. or [50-mm] Cube Specimens) [60]. The loading rate was kept between 0.9 and 1.8 kN/s.

The mortar samples were also prepared according to a modified ASTM C305-Standard Practice for Mechanical Mixing of Hydraulic Cement Pastes and Mortars of Plastic Consistency [59] protocol, and the mortar samples were prepared using a w/c or s/c of 0.50. The sand to cement ratio was 1:4. The cell concentration of cells in the culture was calculated according to the correlation obtained in **Section 3.3.2** and the initial *S. pasteurii* concentration in the inoculum was kept constant for the bacterial mortar samples at 2.5 x 10^6 CFU/mL. Another set of samples was prepared using the same bacterial concentration and procedure, but in these samples 7.7 g of $CaCl_2$ (0.7% of cement weight) was added to the S. *pasteurii* inoculum (4 x 10^6 CFU/mL) right before mixing. Therefore the calcium ion concentration in the mix was calculated to be 0.167M based on the mass balance relationship discussed in **Section 3.4**. Theoretically this concentration should balance the carbon concentration produced via the urea hydrolysis, assuming that the microorganisms can decompose all the urea present. The samples were cured at 100% relative humidity (RH) at 25°C for 24 hours and submerged in an UYE medium saturated with lime until the time of testing. A compressive strength test was

conducted on triplicate of samples at 1, 7, 28 and 56 days. The loading rate was kept in between 0.9 and 1.8 kN/s.

Since the bacteria are metabolically active when inoculated to the cement paste, they might have time to produce by-products in the UYE medium. We will call such UYE medium "spent UYE". In order to determine whether the by-products of microbial activity influenced compressive strength, in addition to the traditional bacterial mortars (that were prepared with cells in spent UYE medium), two additional samples were prepared for examination, where the s/c ratio was kept at 0.50 for both samples:

- *Nutrient mortars prepared with spent UYE media: S. pasteurii* were grown in UYE medium until 2 x 10^6 CFU/mL was reached, and then the bacterial culture solution was filtered through a 0.2-micrometer filter so that the bacteria could be separated from the spent UYE medium. Afterwards, the spent UYE medium was mixed with cement and sand.
- *Bacterial mortars prepared with fresh UYE media:* To investigate the influence of bacterial cells on strength without spent UYE medium, *S. pasteurii* were grown in the UYE medium and were pelleted by centrifuging at 2720 x g for 15 minutes. Then the cells were washed with phosphate buffer (PBS) twice and resuspended in a fresh UYE medium immediately before

mixing with cement and sand. The cell concentration was determined to be 2.3 x 10^6 CFU/mL after suspension.

Additional samples were prepared to determine the effect of the presence of viable microorganisms on compressive strength. For these samples, *S. pasteurii* cells were grown in UYE medium until a concentration of 3.4 x 10^6 CFU/mL was reached. Then the cells were killed by autoclaving the bacterial-UYE medium for 35 minutes at 255 °C. There was not any growth on the plate inoculated with the sample obtained after the autoclaving process, which confirms the cells were not viable. The sterilized UYE medium with killed cells was mixed with cement paste and sand. Then the samples were cured at 100% RH at 25°C for 24 hours and submerged in UYE medium saturated with lime until the time of testing. A compressive strength testing was conducted on triplicate samples at 1, 7, 28 and 56 days. The loading rate was kept between 0.9 and1.8 kN/s.

4.2.4. Preparation of Pastes for Isothermal Calorimeter Analysis

To study the influence of bacteria and UYE medium on cement hydration, pastes were prepared by mixing 7 g of water (neat), 7 g of UYE medium (nutrient) or 7 g of bacterial culture solution and 14 g of cement by hand for 2 minutes. Twenty grams of cement paste samples were used in isothermal calorimeter analysis. Three different

bacterial concentrations in the initial inoculum ($4x10^5$, $2x10^6$ or $3x10^7$ CFU/mL) were used for the bacterial pastes.

Another set of samples was prepared to study how the individual components of the nutrient solution impacted hydration kinetics. For these samples, 0.13M tris solution (0.110 g), yeast extract (0.14 g), or urea (0.07 g) were dissolved in 7 mL DDI water, which presumably the same amount in the UYE medium. Then these solutions were mixed with 14 g of cement.

To investigate whether the byproducts of microbial activity affected cement hydration, another series of experiments was conducted. First, *S. pasteurii* was grown in 50 mL UYE medium until 10^7 CFU/mL was reached, and the culture was filtered through a 0.2-micrometer filter. Then 7 mL of spent UYE medium was mixed with 14 g of cement. Second, *S. pasteurii* cells were grown in 50 mL of UYE medium, and were pelleted by centrifuging at 2720g for 15 minutes. The cells were resuspended in a 7mL fresh UYE medium immediately before mixing with cement. The cell concentration was determined to be 10^7 CFU/mL after suspension. The effect of viable microorganisms on hydration behavior was examined by growing *S. pasteurii* cells in 50 mL of UYE medium until 10^7 CFU/mL was reached, and then killing the cells by autoclaving. 7 mL of sterilized UYE medium with killed cells was mixed with 14 g of cement. 20 g of

cement paste samples were used in the analysis. The s/c ratio was kept at 0.50 for both samples.

Twenty grams of cement paste samples were used in isothermal calorimeter analysis. Samples were placed into a 3114/3236 TAM Air Thermal, Thermometric AB Isothermal Calorimeter (Sweden) for 48 hours. The temperature was maintained at 23°C throughout the testing and PicoLog software was used to collect data to a computer. All of the sets were carried out by triplicate samples. Qualitative analysis was conducted by examining how the shape of the hydration curve (see Figure 4.6) evolved. The induction period is the "dormant" period, where the rate reaction slows down immediately after the initial rapid reaction and it is the period between the rapid dissolution and acceleration.

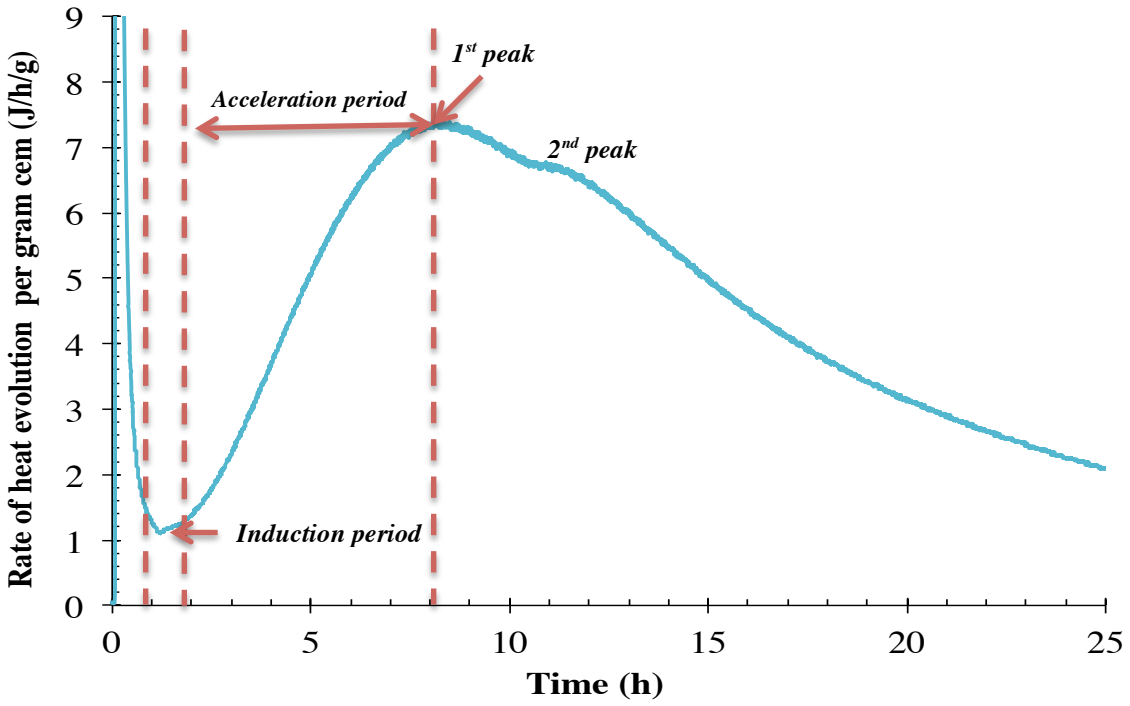

Figure 4.6: A typical hydration curve for neat paste. The y-axis is the rate of heat evaluation per gram cement. The w/c was 0.5.

4.2.4. Initial set

Vicat Needle tests were conducted to determine the setting time of the cement pastes. For the test, cement paste samples were prepared according to a modified ASTM C191-08 Standard Test Methods for Time of Setting of Hydraulic Cement by Vicat Needle [61]. Instead of determining the w/c ratio that will yield a "normal consistency" paste, the w/c and s/c ratios were maintained 0.50 to be consistent with the w/c and s/c ratios used throughout this study. Six hundred fifty grams of cement were mixed with

325 g of water for neat paste control mixes. Similarly, 650 g of cement were mixed with 325 g of UYE medium and bacterial culture (10^7 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.

To determine the influence of by-products of *S. pasteurii* on the initial set time, the cells were grown in a 600 mL UYE medium until 10^7 CFU/mL was reached, and the spent media obtained via filtering of the bacterial culture through a 0.2 μm mesh as stated in **Section 4.2.2**. Three hundred twenty five grams of the spent UYE medium was mixed with 650 g of cement (s/c ratio of 0.50). Similarly, *S. pasteurii* was grown in 600 mL UYE medium cells centrifuged and suspended in a 325 mL fresh UYE solution right before the mixing. The OD_{600} reading obtained after suspension was correlated with the cell concentration and the results yielded 10^7 CFU/mL as the final concentration in the suspension. The cell suspension in fresh UYE medium was mixed with 650g of cement (s/c of 0.50).

4.2.5. Preparation of pastes for Thermogravimetric analysis (TGA)

In order to analyze the calcium hydroxide and the calcium carbonate content, TGA was conducted on the paste samples with Mettler Toledo TGA/DSC Analyzer (Schwerzenbach, Switzerland). The neat, nutrient, bacterial and killed bacterial cement paste samples were prepared as it is described in **Section 4.2.3.** Triplicate of samples

were prepared. The *S. pasteurii* concentration in the mixing culture was determined via OD_{600} readings as 7.6 x 10^6 CFU/mL. For killed bacterial samples, *S. pasteurii* cells were grown in UYE medium until a concentration of 5 x 10^6 CFU/mL was reached and then the bacterial culture was autoclaved.

The w/c and s/c ratio were kept as 0.50. All samples were mixed by hand for 2 minutes. The cement paste samples were cured in the UYE medium saturated with lime until testing and TGA was done at 1, 7 and 28 days.

At the time of testing, the samples were removed from the UYE medium and immediately after removing the samples from the medium; they were crushed and pulverized such that they were finer than 53μm size. The powdered cement paste was ground with ethanol to stop hydration until it completely dried [62]. Prepared specimens were kept in a vacuum desiccator for 24 hours. The analysis was conducted by increasing the temperature from 40°C to 1200°C. Decomposition of calcium hydroxide (portlandite) was determined between 450°C-550°C and calcium carbonate decarbonation was measured between 700°C-900°C [63].

4.2.6. X-ray diffraction analysis

X-ray diffraction analysis was conducted in order to determine the morphology of calcium carbonate precipitated by inoculated vegetative *S. pasteurii* cells in cement paste. The neat, nutrient and bacterial cement paste samples were prepared as it is described in

Section 4.2.3. Two sets of bacterial cement pastes were prepared. One set of bacterial paste samples was prepared with mixing the inoculum (7×10^7 CFU/mL) with cement without any additional calcium source. For the second set of samples, 1.23 g of $CaCl_2$ (0.7% of cement weight) was added during mixing to the S. *pasteurii* inoculum (8×10^7 CFU/mL), therefore the calcium ion concentration in the mix was calculated as 0.167M. Control samples were prepared mixing Portland cement with tap water with and without addition of calcium chloride. Control samples were prepared as neat and nutrient paste. The w/c and s/c ratio were kept as 0.50. All samples were mixed by hand for 2 minutes.

Representative samples were obtained from the core of the cement paste specimens and the samples were crushed and pulverized; such that the samples were finer than 53μm size. Then the pulverized material was ground with ethanol to stop hydration. The prepared samples were kept in a vacuum desiccator until they were tested, at which point the samples were placed and compacted into the sample holder. A Siemens Bruker X-ray Diffractometer (Madison, WI, United States) was used for analysis; X-ray scan was generated at angles from 10-60° 2θ with 2 seconds dwell time. The diffractometer was operated at 40 keV and 30 mA, at a step size of 0.02° 2θ.

4.2.7. Porosity and electrical resistivity measurement

The porosity of neat and bacterial mortar samples was estimated using two methods: ethanol exchange and electrical resistivity. For the ethanol exchange method, 7- and 28-day old neat and bacterial mortar samples (3 cm in diameter and 2 cm in thickness) were cured in an UYE medium saturated with lime until the time of testing. Then saturated samples were removed from the curing solution and submerged in ethanol for pore solution exchange. Periodically, the samples were removed from ethanol, dried to a saturated surface dry (SSD) state, and weighed using a digital analytical balance (OHAUS Explorer digital scale, Parsippany, New Jersey). The sample mass was recorded until the equilibrium mass was obtained. Porosity was calculated by considering the weight change and the specific gravities of pore water (SG: 1) and ethanol (SG: 0.789). Triplicate samples were processed for neat and bacterial mortar at each time period.

For the electrical resistivity method, neat and bacterial mortar samples were prepared and cured in UYE medium saturated with lime until the time of testing. Upon removal from the medium, the samples were dried to an SSD state. Then, the impedance of the samples was measured using a resistivity meter (Giatec Scientific RCON^{TM} Concrete Resistivity, Ontario, Canada). The frequency was maintained at 1kHz during the measurement. To calculate the resistivity (kΩ.cm), the impedance value (Ω) was multiplied by the cross sectional area of the samples and divided by the length of the

sample. The resistivity was used as an indication of the porosity; resistivity is inversely proportional to conductivity. It has been found to be correlated with hydraulic permeability [64, 65], and permeability decreases as porosity and conductivity decreases.

4.3. RESULTS AND DISCUSSION

4.3.1. Effects of biomineralization on hydration kinetics and setting time:

Figure 4.7 shows the rate of heat evolved during hydration of cement paste samples with w/c of 0.50. From Figure 4.7, it can be seen that the induction period of the cement paste was considerably extended when the nutrient medium and bacterial culture solutions were used instead of water in the mixing process.

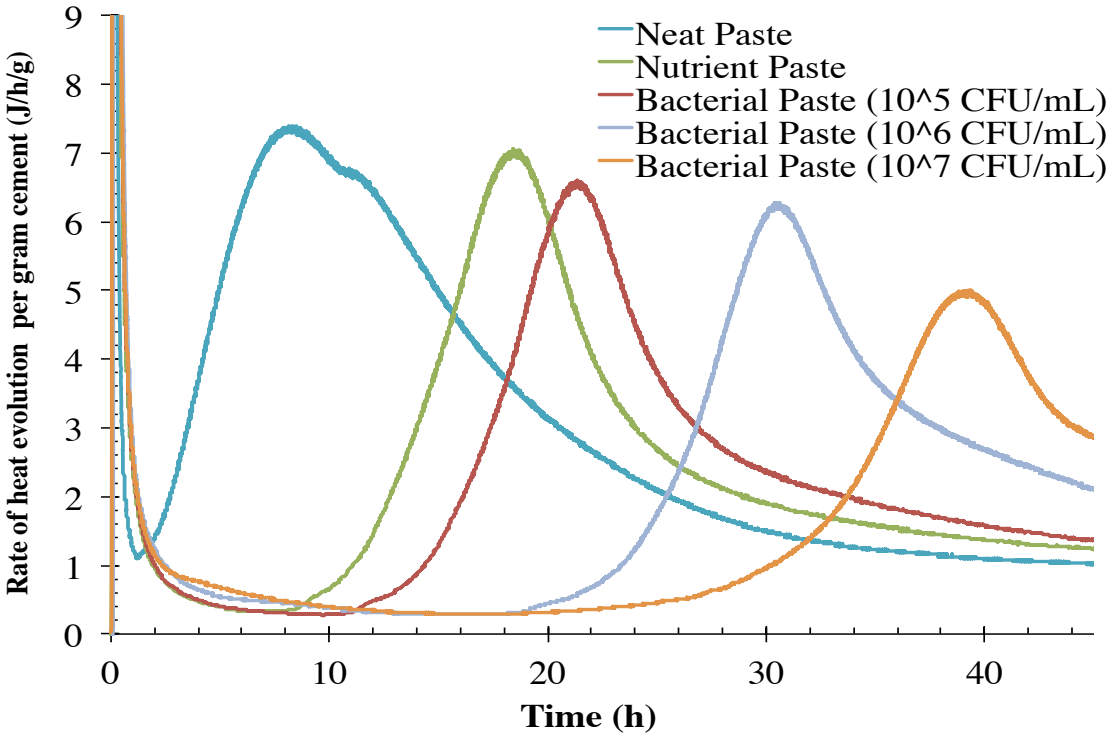

Figure 4.7: Heat of hydration of neat, nutrient and bacterial pastes with various concentrations of cells (10^5,10^6 And 10^7 CFU/mL) in UYE Medium. The w/c and s/c of 0.50 was used.

The nutrient medium primarily caused the delay in the initiation of acceleration period. However, with the addition of bacteria, the delay became more pronounced. Since the nutrient medium is composed of various components, a calorimetry analysis was conducted to elucidate the effects of the individual components on the hydration kinetics.

Figure 4.8 shows the hydration behavior of pastes prepared using each constituent of the nutrient solution. It was readily apparent that the yeast extract, which comprises 0.67% of the total sample weight and 2% of the UYE medium weight,

governed the hydration behavior of the nutrient paste. The yeast extract contained sugar and carbohydrates, which are known to be good retarders extending the induction period, thus delaying the acceleratory period of rapid calcium silicate hydration, which results in the first peak [66]. Similar retardation effect was observed significantly when 0.5% of yeast extract by cement weight was added to cement paste [67].

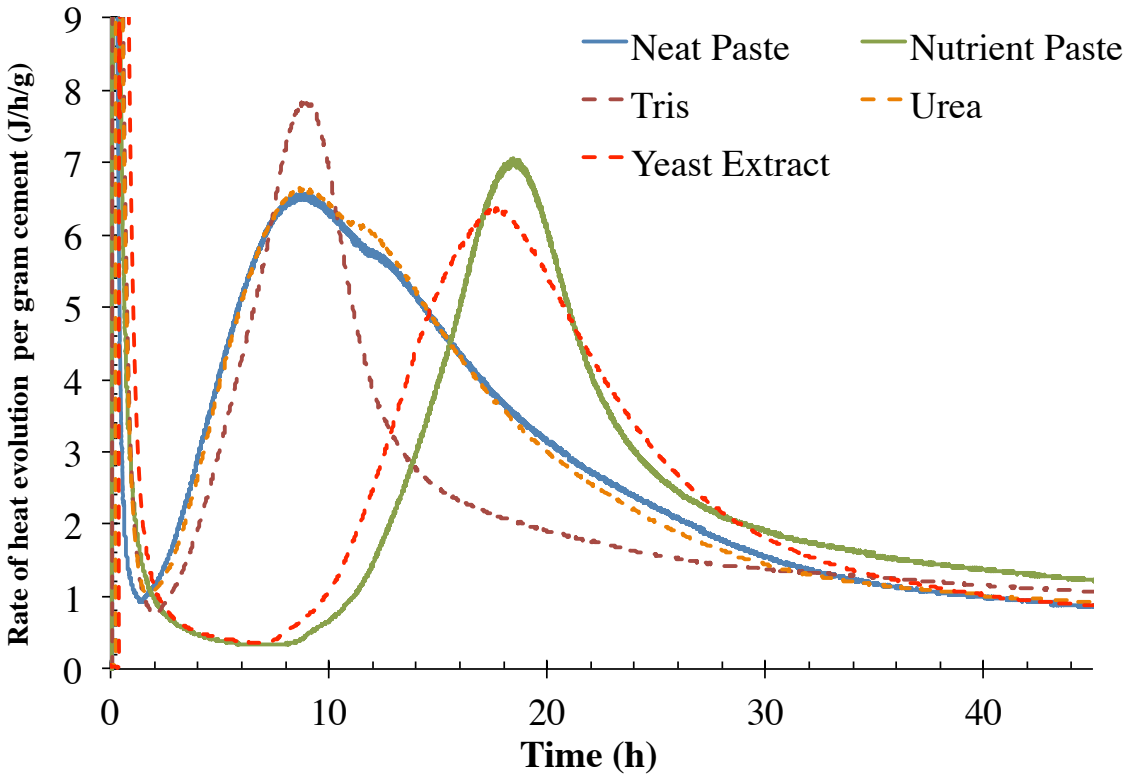

Figure 4.8: Effects of UYE medium compounds on hydration kinetics. Tris: Tris solution and cement, Urea: Urea solution and cement, Yeast Extract: Yeast Extract solution and cement, Neat Paste: Water and cement, Nutrient Paste: UYE medium and cement. The w/c and s/c of 0.50 was used.

However, nutrient solution does not contain only yeast extract that may interfere with the hydration behavior, it also has other ingredients that can influence the hydration behavior. Mwaluwinga et al. suggested that the presence of urea in concrete depressed the heat of hydration and retarded the initial setting of concrete [68]. Interestingly, the urea did not play a significant effect on induction period; this is inconsistent with the results obtained in this research according to Figure 4.8. Addition of urea at pH 7 showed no change in the end of the dormant period compared to neat paste. While the tris buffer did not significantly influence the point at which the induction period ended, the acceleration stage and deceleration stage were affected. The first peak is caused by the rapid hydration of calcium silicate (primarily C_3S) phases in cement [69]; and therefore the change in the first peak with tris was caused by a change in the dissolution of C_3S and/or nucleation of hydration products.

Besides the fact that the yeast extract acts as a retarder, consumption of UYE medium would increase the ionic concentration particularly from the generation of ammonium, carbonates, and hydroxyl ions. Theoretically, calcium carbonate precipitation is more preferable as the ratio of calcium ions to carbonate ions decreases. This is because of the relative solubility difference between calcium hydroxide, K_{sp} =5.02 x 10^{-6} [70] and calcium carbonate, K_{sp}= 3.8 x10^{-9}, which suggest calcium carbonate is more stable than calcium hydroxide. Reaction of calcium and carbonate ions was determined as

instantaneous [26]; therefore precipitation might occur before nucleation of calcium hydroxide. As carbonation occurs, calcium ion concentration in the pore structure decreases and leads to a higher hydroxide saturation in the system.

In order to determine whether the long induction period was exacerbated to the presence of microorganisms or microbial by-products, spent nutrient solution pastes and centrifuged pellet pastes were prepared as discussed in **Section 4.2.3**. Figure 4.9 displays the results of these experiments. The retardation in the onset of the acceleration stage for the spent UYE medium paste sample suggested that by-products due to hydrolysis of nutrient solution influence the hydration reaction. This is possible considering the fact that that spent medium consists of various ions hydrolyzed as a result of bacterial metabolic activity and it is highly probably that these ions influenced the rate of dissolution and precipitation processes. However, comparison of the spent UYE medium curve and the bacterial paste 10^7 CFU/mL curve showed that that when vegetative *S. pasteurii* cells are inoculated into cement paste with a spent media (i.e. as that done with the bacterial paste) that the delay in the induction period was much more severe than that seen when just the spent UYE medium alone. The exact reason for this behavior is not fully understood but could be due to a compounding effect caused from the metabolic activity of the *S. pasteurii* cells producing further by-products to interact with the already produced products. Interestingly, bacterial paste-Fresh UYE medium curve showed that

hydration behavior could be restored to behavior similar to that of just a nutrient paste. Since the bacterial concentration for bacterial paste-Fresh UYE medium and bacterial paste were the same (10^7 CFU/mL), the key difference between the two samples was that the bacteria had longer time to grow and metabolize medium components outside the cement paste in the bacterial paste sample than in the bacterial paste-Fresh UYE medium sample.

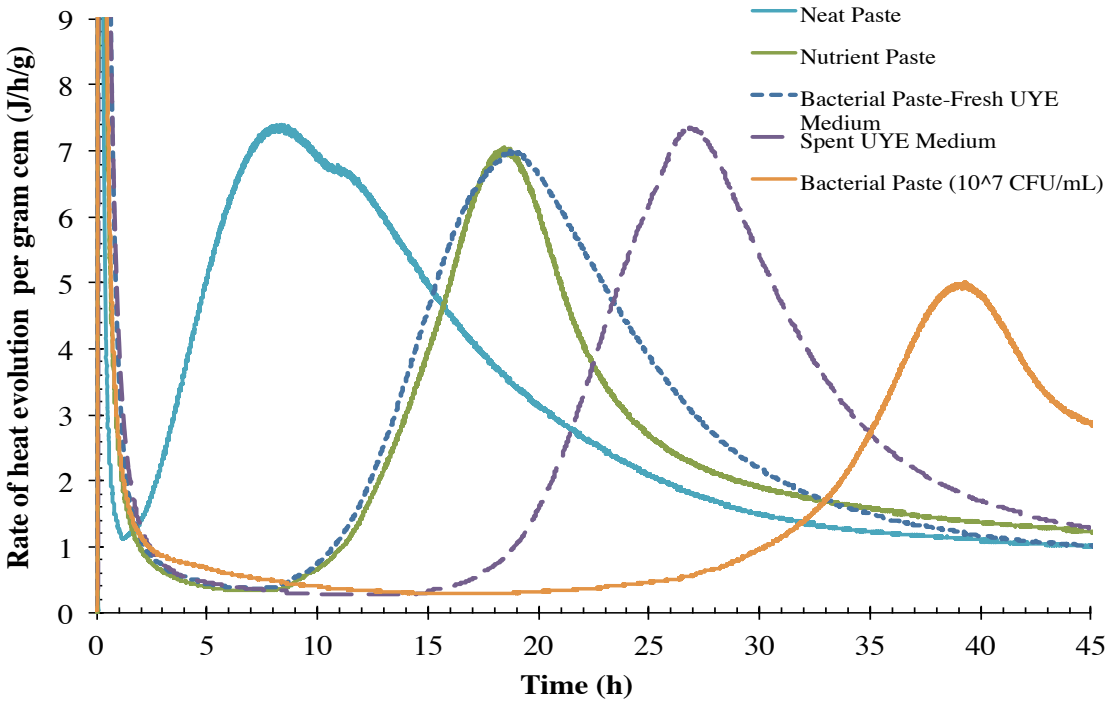

Figure 4.9: Influence of spent medium on hydration kinetics. *Neat paste:* water and cement; *Nutrient paste:* UYE medium and cement; *Bacterial paste- Fresh UYE Medium*: *S. pasteurii* cells centrifuged from spent UYE medium and resuspended in fresh nutrient solution and cement; *Spent UYE Medium:* spent solution obtained from filtration of *S. pasteurii* and cement; *Bacterial paste: S. pasteurii* cells in spent UYE medium. The y-axis is the rate of heat evaluation per gram cement. The w/c and s/c ratio were kept at 0.50.

In comparison to the nutrient paste, the bacterial paste (10^7 CFU/mL) curve shows that the hydration kinetics was significantly delayed when the bacteria were added (Figure 4.7 and Figure 4.9). The retardation in the spent UYE medium paste suggested that by- products due to hydrolysis of nutrient solution might influence the hydration reaction. This could be possible considering the fact that that spent medium consists of various ions hydrolyzed as a result of bacterial metabolic activity, which might have influenced the rate of dissolution. Comparison of the spent UYE medium curve and the 10^7 CFU/mL curve suggested that the presence of *S. pasteurii* cells (and by-products) in the mixture prevented the dissolution of phases in cement and caused a delay in induction period. Interestingly, bacterial paste-fresh UYE medium paste curve showed that hydration behavior could be restored to behavior similar to that of just a nutrient paste when the bacteria were suspended in a fresh nutrient medium. One other thing should be pointed out is that the concentration of microorganisms were kept at 10^7 CFU/mL after centrifuging and suspending. The key difference between the bacterial paste and bacterial paste-fresh UYE medium paste sample and the 10^7 CFU/mL sample was that the bacteria had longer time to grow and metabolize medium components outside the cement paste. Besides, the metabolic activity of the microorganisms might be slowed down due to

external stresses, therefore the influence of the extracellular compounds produced might not be pronounced during the first 72 hours.

The difference between the bacterial paste-fresh UYE medium and bacterial paste at the same concentration arose the question for whether the cells were killed when they were mixed with cement. In order to evaluate the influence of incorporating killed cells, an autoclaved bacterial culture was mixed with cement. As it is shown in Figure 4.10, there was no change in hydration behavior of bacterial paste compared to the nutrient paste when killed microorganisms were added. Thus, the results from this phase of the research suggested that the retardation in the hydration kinetics occurs when **vegetative** *S. pasteurii* cells were inoculated in cement paste and that the cells do not all immediately die when mixed into cement paste.

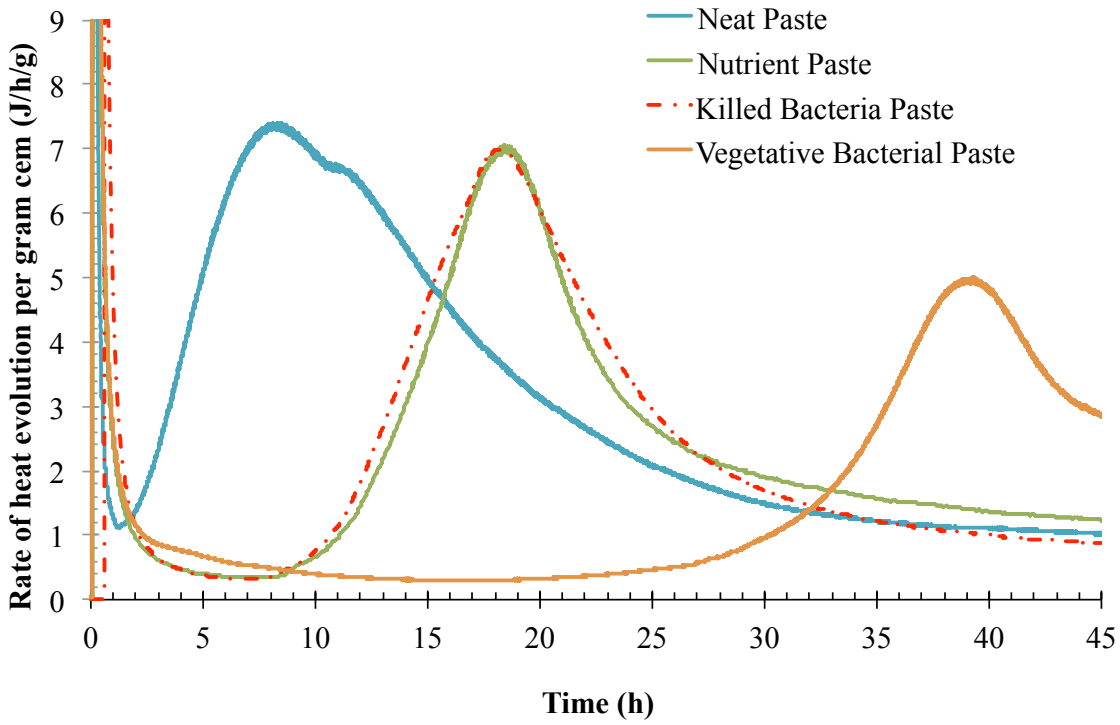

Figure 4.10: Influence of killed bacteria on hydration kinetics. Neat paste: water and cement; Nutrient paste: UYE medium and cement; Killed Bacterial Paste: *S. pasteurii* cells were grown in UYE medium and autoclaved solution was mixed with cement; Vegetative Bacterial paste: *S. pasteurii* concentration of 10^7 CFU/mL. The y-axis is the rate of heat evaluation per gram cement. The w/c and s/c of 0.50 was used.

As shown in Figure 4.7, the rate of heat evolution was found to be a function of the bacterial concentration. As the bacterial concentration increased the total heat evolved decreased and there was a significant delay in the onset of the acceleration period of hydration. This might be due to an increase in by-products produced as a result of a higher consumption of nutrients as the bacterial concentration is increased. Two other

possible explanations are that the delay in the onset of the acceleration period might be related to (a) the effective yeast concentration (b) surface charge of the bacteria. One other possible reason can be the consumption of urea might occur at a faster rate than the consumption of the yeast extract. This would result in the effective concentration of the yeast extract in the solution increasing and as shown in Figure 4.8 the yeast extract played a significant role on retarding the hydration reaction.

4.3.2. Effects of biomineralization on initial set:

The modified Vicat needle test results are summarized in Table 4.4 and showed that the same trends as indicated from the calorimetry curves. Addition of the UYE medium delayed the initial setting significantly compared to the neat paste. Similarly, the addition of microorganisms made this delay much more pronounced and the spent medium cement paste between the set time of the nutrient paste and the bacterial paste. Suspension of cells showed a similar trend as the nutrient solution, and is consistent with the results obtained from the calorimeter tests.

Table 4.4: Vicat needle initial set results; Neat paste: water and cement; Nutrient paste: UYE medium and cement; Bacterial paste: *S. pasteurii* culture (concentration of 10^7 CFU/mL) and cement; Spent UYE Medium: spent UYE medium and cement; Bacterial paste-Fresh UYE Medium: *S. pasteurii* cells resuspended in fresh UYE medium and cement. The w/c and s/c ratio of 0.50 were used.

Sample	Vicat time (avg± st.dev, hr.)
Neat paste	3.0 ± 0.3
Nutrient paste	13.3 ± 0.9
Bacterial paste (10^7 CFU/mL)	36.5 ± 1.1
Spent UYE Medium (initially 10^7 CFU/mL)	27.9 ± 3.8
Bacterial paste- Fresh UYE Medium (10^7 CFU/mL after suspension)	12.7 ± 0.2

4.3.3. Effects of biomineralization on calcium carbonate content:

TGA results indicate that there was a considerable increase in calcium carbonate content at every age of testing in the bacterial paste specimens when compared to the neat and nutrient paste specimens. Figure 4.11 and Figure 4.12 show the mass percentages of calcium hydroxide (portlandite) and calcium carbonate (calcite) calculated from the mass losses obtained from TGA. The values presented were obtained by averages of triplicates of samples and the error bars present standard deviations. The mass change at 450°C to 550°C is due to H_2O loss from calcium hydroxide and constitutes 24% calcium hydroxide mass. While mass loss at 700°C to 900°C is due to CO_2 loss from calcium carbonate and it is 44% of calcium carbonate weight.

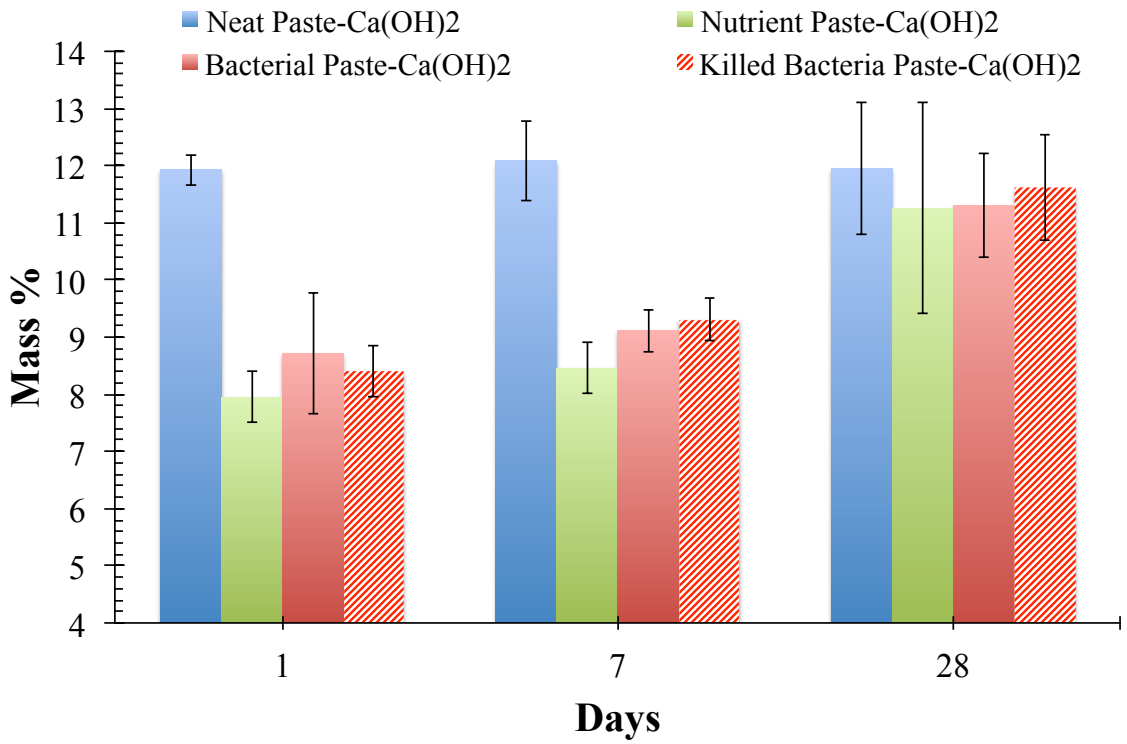

Figure 4.11: Calcium hydroxide mass percentages in neat, nutrient, bacterial (Inoculation of vegetative cells) and killed bacterial pastes samples at 1, 7 and 28 days. Columns show average values obtained from triplicates of samples and error bars represent the standard deviation. The w/c and s/c of 0.50 was used.

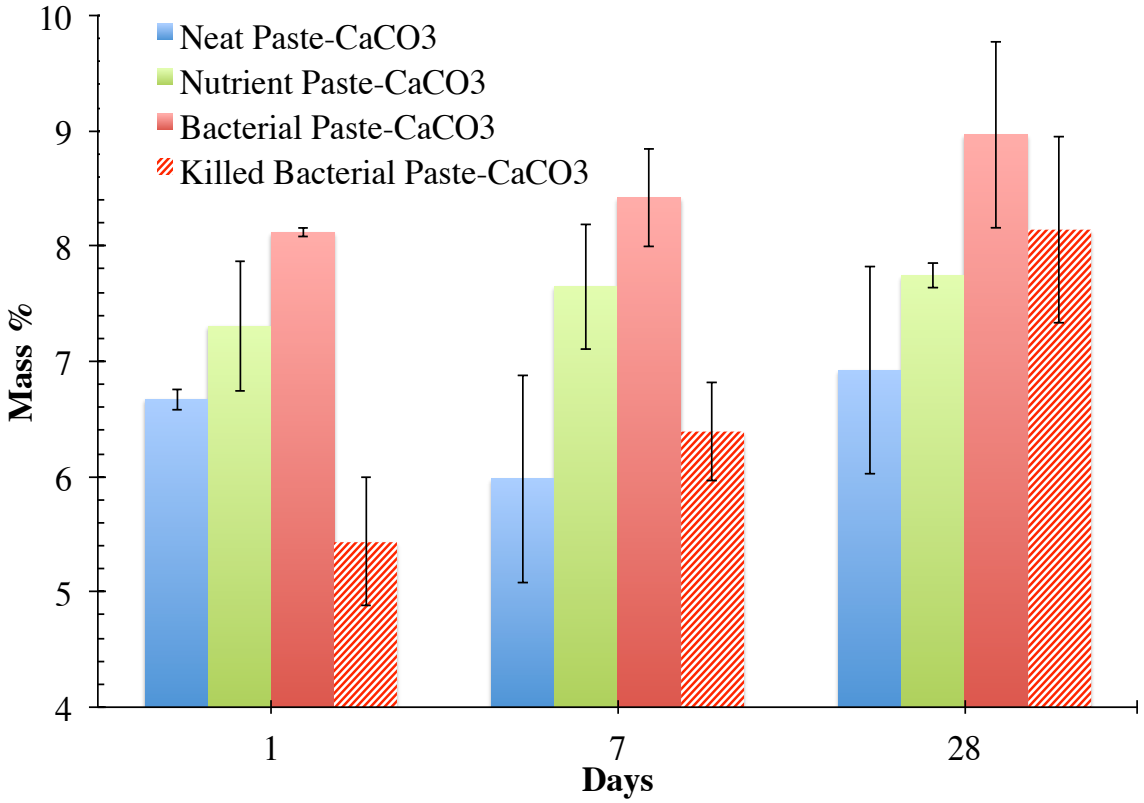

Figure 4.12: Calcium carbonate mass percentages in neat, nutrient, bacterial (Inoculation of vegetative cells) and killed bacterial pastes samples at 1, 7 and 28 days. Columns show average values obtained from triplicates of samples and error bars represent the standard deviation. The w/c and s/c of 0.50 was used.

At 1-day, the portlandite content decreased considerably in bacterial and nutrient paste samples compared to neat paste, which could be attributed to the extended induction period in these samples. In contrast, the opposite trend was observed in the calcium carbonate content such that it was noticeable higher in bacterial pastes compared

to the nutrient and neat pastes. The inoculated vegetative *S. pasteurii* cells improved the calcium carbonate mass by 22% compared to the neat paste.

At 7-days the portlandite content in the bacterial paste has slightly increased compared to 1-day bacterial paste. However it was still considerably lower comparison to 7-day neat paste. There was not any increase in portlandite content in the nutrient paste such that the relative difference compared to neat paste did not change. Compared to the neat paste, there was a 42% increase in the calcium carbonate content in the bacterial paste. At both 1 day and 7 days, the influence of UYE medium on carbonation was substantially lower compared to bacterial paste. Thus the results prove that calcium carbonate content was increased with inoculation of **vegetative** *S. pasteurii* cells. Vegetative inoculation method provides extracellular carbonate ions due to hydrolysis of urea, thus when mixed with cement, *S. pasteurii* cells initiated the calcium carbonate precipitation. An important point that must be noted is that the same influence was not observed when autoclaved (killed cells) bacterial culture was mixed with cement (Figure 4.12). The increase in calcium carbonate contents at 1 and 7 days was initiated by introducing vegetative cells. At 1 day, incorporation of autoclaved cells in UYE medium did not influence the calcium carbonate; even the neat paste had higher calcium carbonate content. At 7 days, addition of killed cells did not influence the calcium carbonate content relative to neat paste such that the values were with in the 1% range. In contrast at 1 and

7 days, the calcium hydroxide content in the killed bacterial paste was similar to that in the bacterial paste, which could be de to the longer induction period comparison to neat paste. Moreover, in comparison to the nutrient paste, the calcium carbonate content in the killed bacterial paste was lower at 1 and 7 days. As shown in Figure 4.12 after 28 days, the calcium carbonate content was as high as the bacterial paste containing vegetative cells; this might be due to the presence of carbonate ions in the spent nutrient solution. Therefore it could be concluded that even though by-products produced during the growth contains required carbonate ions, vegetative cells were required in order to induce calcium carbonate in a faster rate at early ages.

It could be concluded that the influence of microorganisms on calcium carbonate precipitation was particularly observed at 1-day. Even though the reaction continued up to 28 days, the rate decays and initial precipitation rate governs the mass percentage of calcium carbonate. Carbonation of calcium hydroxide in cement-based materials is inevitable when the material is exposed to carbon dioxide. However this rate is very slow in the nature. As a result of TGA, it could be concluded that the calcium carbonate mass percentage increased in aged bacterial paste whereas this percentage did not increase in neither neat nor nutrient paste with time. This indicates that introducing vegetative *S.pasteurii* cells could be able to produce calcium carbonate within cement paste at a faster rate in comparison to nutrient paste and cement paste with killed cells.

4.3.4. Morphology of microbial induced calcium carbonate

The results of thermogravimetric results showed that the addition of *S. pasteurii* cells and the UYE medium substantially affected calcium carbonate and calcium hydroxide content. The ex-situ studies presented in **Chapter 3** suggested that calcite precipitation would be more favorable than vaterite in low calcium concentration and high pH environment. However, there are many factors affecting "bio-calcification" of cells in the cement paste such as high pH environment (>pH 12), lower calcium concentration, lack of nutrients and calcitic substrate. Figure 4.13-4.15 shows the X-ray diffractograms for neat, nutrient and bacterial pastes samples at 1, 7 and 28 days. Since there was not any internal standards added and possibility of preferred orientation, only qualitative analysis was conducted with the XRD data. The results of the XRD analysis revealed that the calcium carbonate was calcite rather than vaterite in the bacterial paste. This is in agreement with the ex-situ culture considering the alkaliphilic environment and low calcium ion concentration (0.03M); calcite was the expected polymorph by calcium carbonate in cement paste matrix.

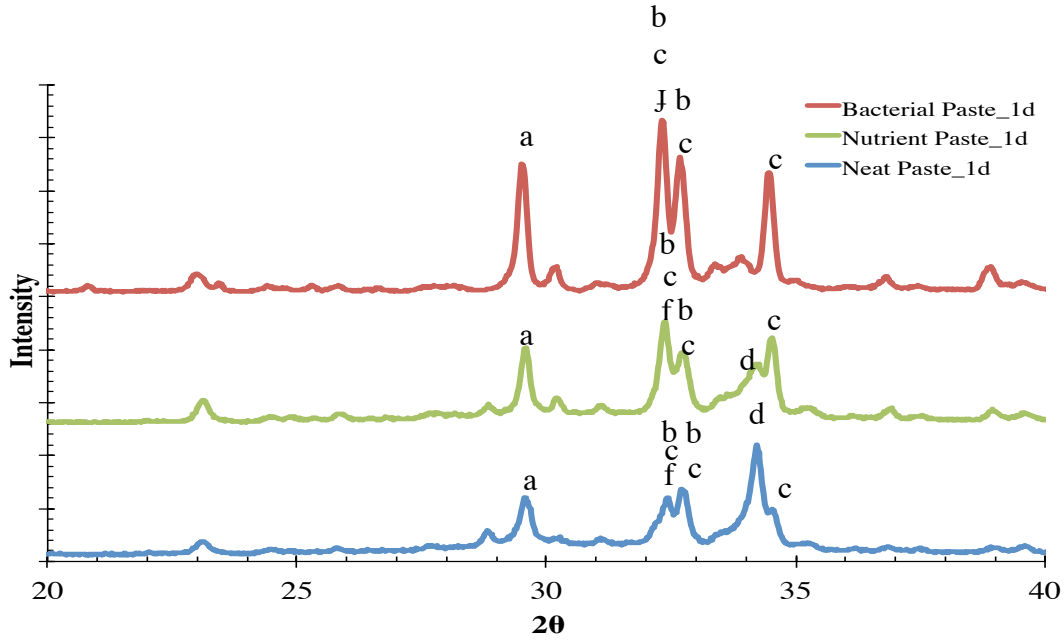

Figure 4.13: XRD pattern for neat, nutrient and bacterial pastes samples at 1 day. The w/c and s/c were 0.5: Calcite "a"; C_2S and C_3S: "b" and "c"; Portlandite: "d", f: Ettringite.

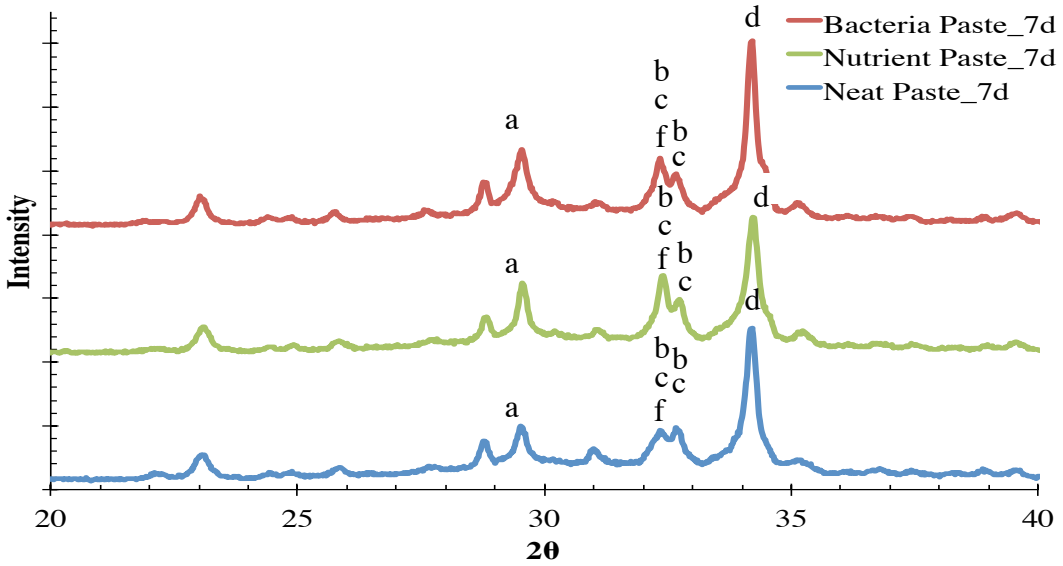

Figure 4.14: XRD pattern for neat, nutrient and bacterial pastes samples at 7 day. The w/c and s/c of 0.50 was used. Calcite: "a"; C_2S and C_3S: "b" and "c"; Portlandite: "d", f: Ettringite.

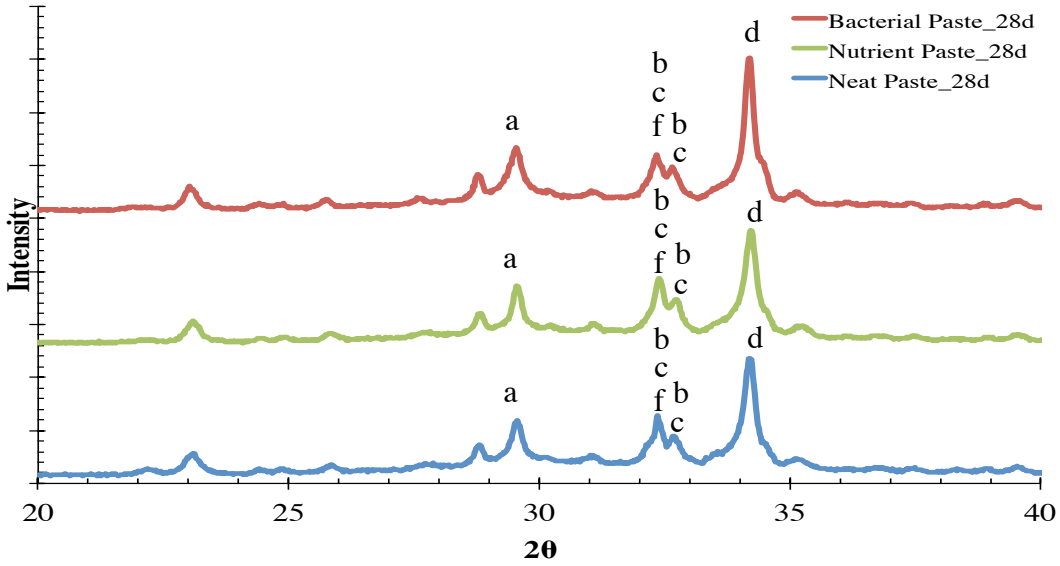

Figure 4.15: XRD pattern for neat, nutrient and bacterial pastes samples at 28 day. The w/c and s/c of 0.50 was used. Calcite: "a"; C_2S and C_3S: "b" and "c"; Portlandite: "d", f: Ettringite.

Figure 4.16 represents the XRD pattern for cement paste with 0.12M calcium chloride additions so that the final concentration of calcium ions reached 0.167M. One point that should be noted is since calcium chloride is an accelerator; calcium silicate peaks were determined to be lower when it was added (0.7% of cement paste). Interestingly, calcite is the polymorph in cement paste even when the calcium content has increased. No change in the peaks was determined in bacterial paste with addition of calcium chloride. However, the calcite peak was lower when additional calcium ions were introduces, which could suggest that presence of high calcium ion concentration might inhibit the precipitation of calcite. This suggests the influence of substrate on

calcium carbonate precipitation is a critical influence in the biomineralization in cement-based materials and that the calcitic nature of cement paste promotes the formation of calcite at the calcium concentrations examined in this research. This is in agreement with work conducted by De Muynck et al. [19] who showed that when a calcitic substrate was used that mainly calcite precipitation can be induced even in a high pH condition. It was proposed that this was due to a strong affinity of the microorganisms and calcitic substrate accelerating metabolic activity. Relatedly, Jonkers et al. [7] observed mainly calcite crystals with addition of 0.4M of calcium lactate, however needle-like aragonite crystals were observed as well. However, it could be concluded that vaterite was rarely observed in cement-based materials even when additional calcium ions were provided [19].

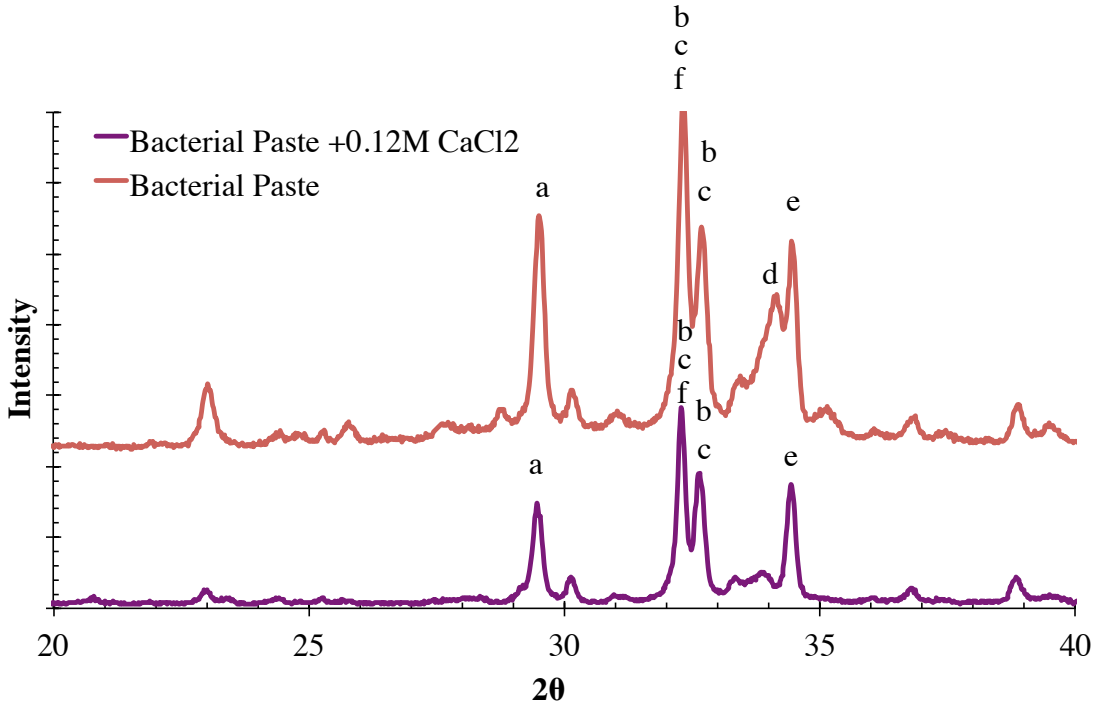

Figure 4.16: XRD pattern for bacterial paste with addition of 0.127 M of [Ca^{+2}] at 1-day. The s/c of 0.50 was used. Calcite: "a"; C2S and C3S: "b" and "c"; Portlandite: "d", f: Ettringite.

4.3.5. Effects of biomineralization on porosity and conductivity

As shown in Table 4.5 higher electrical resistivity values were obtained in the bacterial mortar samples versus the neat mortars. For 7-days mortar samples, the resistivity increased by 28% with addition of microorganisms and by 42% for 28-days mortar samples. The resistivity of the nutrient solution, bacterial solution and tap water was also measured and it was found that the ions present in nutrient and bacterial solution lead to a decrease in the resistivity compared to that of water. The resistivity of water was

found to be 570 Ω.cm while it was determined as 62.8 Ω.cm and 50.3 Ω.cm in nutrient solution and bacterial solution. Assuming that the influence of the nutrients on the electrical conductivity of the mortar will remain constant with time, then the increase in resistivity in bacterial pastes might be directly related to reduction in porosity due to calcium carbonate precipitation. One thing should be noted that this carbonation process was due to the influence of microorganisms (microbially induced or influenced) rather than carbon dioxide dissolving in the pore solution, thus the carbonation occurred even though the porosity was reduced. This modification in conductivity of sample may lead to a reduction in chloride ingress and corrosion risk in cement-based materials, and the increase in resistivity are attributed to the precipitation of calcium carbonate that fills the pores. This is consistent with the results obtained from the ethanol exchange method experiments (see Table 4.6). Microorganisms reduced the mass loss after ethanol submersion, which suggests a reduction in porosity. The 7-day old neat mortar samples had 31.5% porosity by volume while 7-day bacterial mortar samples had 24.9% porosity. In the 28-day mortar samples, the ethanol porosity of neat mortar and bacterial mortar were was determined as 19.5% and 14.8%, respectively. A similar trend was observed with cement paste samples. The inclusion of aggregates tends to increase the bulk porosity of the cement-based materials due to the interfacial transition zone (ITZ) that forms between the aggregates and paste matrix. Consistent with this tendency, it was

determined that the porosity of the neat mortar was higher than the porosity of the neat paste (see Table 4.6). However in the bacterial samples, the decrease in porosity was more pronounced in mortar samples compared to cement paste sample. This could be attributed to higher metabolic activity of the microorganisms, and is discussed more in **Chapter 5.**

Table 4.5: Electrical resistivity of the cement paste and mortar samples at 7 and 28 days, kΩ.cm (average ± st.dev). Results presented are average of triplicates of samples.

Sample	**Electrical Resistivity (kΩ.cm)(average ±st.dev)**					
	Days		**Change (paste vs. mortar)**		**Change (Neat vs. Bacterial)**	
	7	**28**	**7**	**28**	**7**	**28**
Neat Paste	1.4 ± 0.22	1.5 ± 0.06				
Neat Mortar	2.1 ± 0.10	3.8 ± 0.10	35%	62%		
Bacterial Paste	1.9 ± 0.02	1.9 ± 0.10			-37%	-24%
Bacterial Mortar	3.0 ± 0.10	4.9 ± 0.45	59%	61%	-42%	-28%

Table 4.6: Ethanol Porosity Volume (%)of the cement paste and mortar samples at 7 and 28 days (average ± st.dev %). Results presented are average of triplicates of samples.

Sample	**Ethanol porosity, % (average ±st.dev)**					
	Days		**Change (paste vs. mortar)**		**Change (Neat vs. Bacterial)**	
	7	**28**	**7**	**28**	**7**	**28**
Neat Paste	24.9 ± 1.4	14.4 ± 2.9				
Neat Mortar	31.5 ± 3.7	19.5 ± 1.8	27%	35%		
Bacterial Paste	21.4 ± 1.9	13.1 ± 0.5			-17%	-9%
Bacterial Mortar	24.9 ± 0.8	14.8 ± 1.7	17%	13%	-21%	-24%

4.3.6. Effects of biomineralization on compressive strength

The compressive strength of the cement paste and mortar cubes with *S. pasteurii* cells was measured and Figure 4.17 displays the results of these tests. Regarding 1-day strength results, the nutrient and bacterial pastes showed lower strength values as compared to the neat paste regardless of the bacteria concentration. This could be correlated with the delay in the hardening process due to the retarding effect of the nutrient medium. At later ages, 7, 28 and 56 days, the compressive strength increased regardless of the concentration. The increase in compressive strength in presence of the *S. pasteurii* cells particularly at 7-days could be correlated to the calcium carbonate precipitation. As mentioned in **Section 4.3.3**, calcium carbonate precipitation was found to be greatest in the bacterial paste specimen versus the neat and nutrient specimens. In addition reduction in calcium hydroxide at early age might influence the strength. At 28 days, the strength values of the bacterial pastes were greater than the nutrient and neat pastes at any concentration.

The rate of strength gain was significantly greater in the presence of *S. pasteurii*, compared to neat and nutrient paste samples, which showed the development of strength from one measurement period to another. Considering the rate of strength gain from 1 to 7 days, even though addition of microorganisms resulted with lower 1-day strength, it stimulated a higher strength development within the 7 days. The neat and nutrient

samples were in the same range of strength except day 1, which could be attributed to the delay in hardening. Bacterial samples showed a better development in strength than the samples without bacteria and the 7-day to 1-day strength ratio was 5.1 for 10^5 CFU/mL and 8.1 for 10^6 CFU/mL. However, at later ages the rate of strength gain in the presence of *S. pasteurii* decreases, and similar strength values were achieved between neat and bacterial paste by Ramachandran et al. [30] and was believed to be due to a reduction in the porosity of the older pastes and the increased tortuosity of the pore structure network, which restricted microbial access to nutrients.

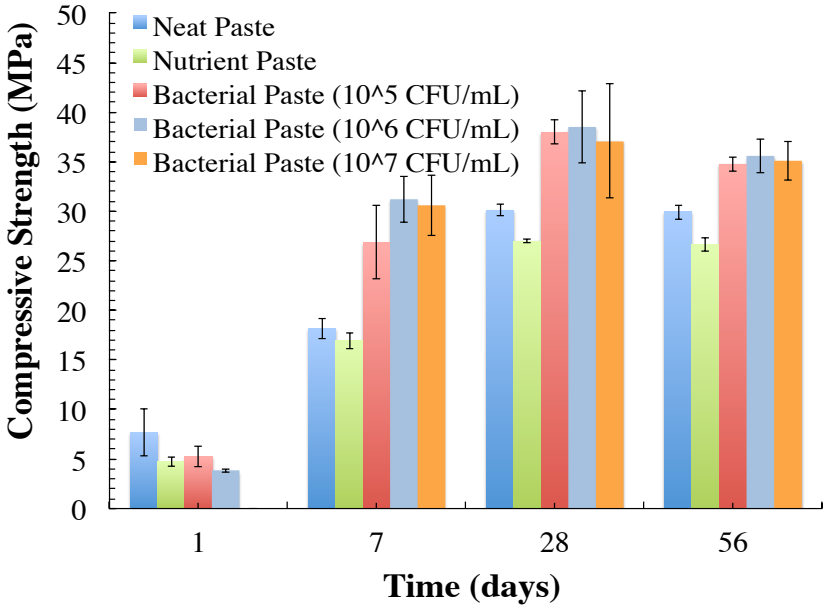

Figure 4.17: Compressive strength of neat, nutrient and bacterial paste with various concentrations. Solid bars show average compressive strength and error bars show the standard deviation of triplicates of samples. W/c and s/c of 0.50 was used.

Similar trend has been observed in mortar samples, the addition of UYE medium decreased the compressive strength, while addition of microorganism led to an increase in strength. Figure 4.18 shows the influence of vegetative-inoculated *S.pasteurii* cells on compressive strength of mortar.

Similar to the hydration reactions kinetics the compressive strength could be influenced by consumed nutrients by microorganisms or from the very presence of bacterial cells influencing the mechanical properties. Addition of spent UYE medium (Figure 4.18) did not generate any change in the compressive strength compared to the nutrient mortar. Furthermore, the addition of cells in fresh UYE medium did not create any difference compared to the nutrient mortar at 1 and 7 days, however at 28 days compressive strength was found to be as high as the bacterial mortar. Addition of killed cells resulted in a strength higher than solely the spent UYE medium but the impact was less then addition of viable cells. Thus, it can be concluded that presence of cells plays a significant role on the compressive strength of the mortar, perhaps even more so than the presence of required carbonate ions in the nutrient solution. Spent medium might have free carbonate ions but without any cells present strength development is hindered. The lower strength obtained by the addition of killed cells suggests that, the reaction was not only due to surface charge attraction. The suspended cells in fresh UYE samples did not show a noticeable increase in compressive strength as the bacterial mortar in which *S.*

pasteurii was introduced by vegetative inoculation method; rather the strength was on par to that of the nutrient mortar. This is likely due to the difference in the metabolic activity of the bacterial cells. *S. pasteurii* cells can hydrolyze urea and precipitate calcium carbonate when they are metabolically active, thus by adding the cells when they are in their exponential growth phase urea hydrolysis occurs during the bacteria most active growth period. Whereas, the cells that were suspended in fresh UYE before mixing were introduced to cement when they were in their stationary phase, which is the stage that the growth rate declines to zero. Therefore, the carbonate ion precipitation for the bacteria suspended in the bacterial paste-Fresh UYE might not be adequate enough to result in as much improvement in compressive strength as the bacterial paste in which the bacteria was added during the exponential phase. With respect to the effect of introducing an additional calcium source, the results showed that even though the resultant calcium carbonate polymorph produced was still calcite the additional calcium ions did the compressive strength was relatively similar to the mortar in which no additional calcium source was added. Thus, it could be concluded that intrinsic calcium content in cement paste was adequate enough to improve the compressive strength of mortar.

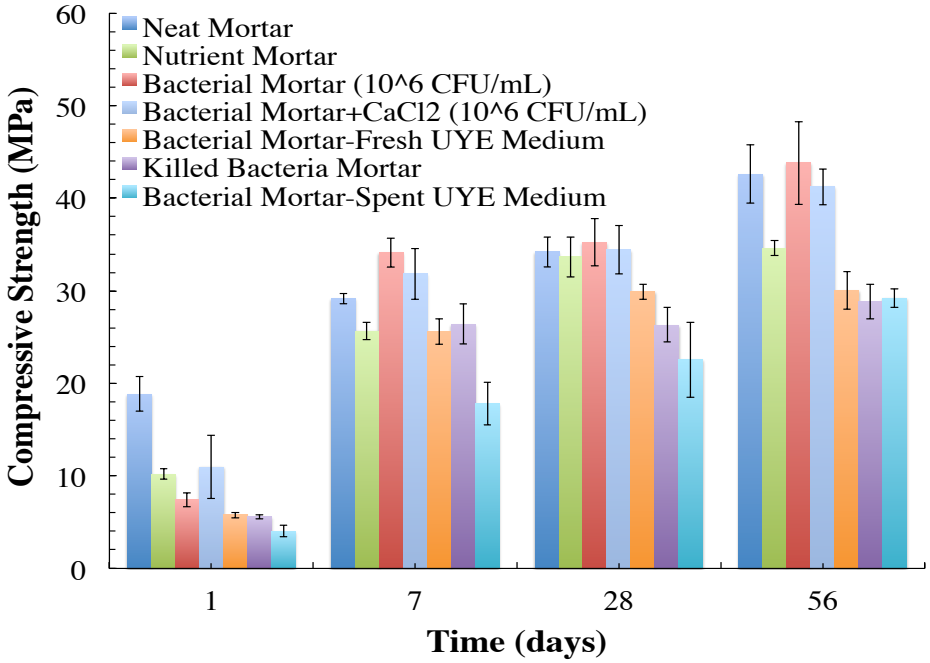

Figure 4.18: Influence of spent UYE medium, suspended cells in fresh UYE medium, vegetative and killed cells on the compressive strength of mortar. The w/c and s/c ratio were kept at 0.50. Solid bars show average compressive strength and error bars show the standard deviation of triplicates of samples.

The influence of inoculated vegetative *S. pasteurii* cells on mineral admixture modified mortar strength was also investigated. In these mixtures, 20% (by mass) of the cement was replaced with limestone filler (LSF) or Class C fly ash (C-FA). The results of these tests are summarized in Appendix D: Influence of biomineralization on LSF and C-FA modified mortar.

Chapter 5: Biomineralization in cement-based materials: Metabolic state and morphology of *S. pasteurii*

This section presents the results of a study that explores the metabolic state (i.e., vegetative cells versus endospores) of vegetative *S. pasteurii* cells inoculated to cement-based matrix.

5.1. INTRODUCTION

One of the main challenges in biomineralization applications in cement- based materials is the restrictive environment (*e.g.*, high pH, low oxygen concentration, low nutrient concentrations), which may result in cell death or insufficient metabolic activity. Thus calcium carbonate precipitation is restricted due to cell deficiencies.

Concerns about the microorganisms surviving in the restrictive environment of cement-based materials have led researchers to propose encapsulation of the microorganisms as introduced in **Chapter 1**. In order to explain the mechanisms of the biomineralization process, it is important to state whether the viable microorganisms found after mixing are vegetative cells or endospores; this is an important point because continued urease production will only be possible with metabolically active (i.e., vegetative) cells [5, 41, 71]. While endospores are able to survive in harsh environments, the conditions (e.g., time and concentration of nutrients in the cement paste) needed to convert the endospores to vegetative cells capable of triggering microbial induced

calcium carbonate precipitation are unknown. Thus the metabolic activity of vegetative cells, specifically for continued urease production, is critical for continuous calcium carbonate precipitation.

5.2. MATERIALS AND METHODS

5.2.1. Preparation of cement paste and mortar samples

S. pasteurii cells were grown in UYE medium (pH 9) to exponential phase as it was explained in **Section 3.3**. This inoculum was used to prepare bacterial paste and mortar. The concentration of bacteria in the inoculum was $1x10^6$ MPN/mL and mortar specimens (5.08 x 5.08 x 5.08 cm) were prepared by mixing this solution with cement and sand. This equates to 0.0002% by volume concentration of bacteria in the mortar; assuming a single *S. pasteurii* cell is approximately 4×10^{-9} microliter [72]. Nutrient mortar samples were prepared by mixing UYE medium (pH 9) with cement and sand. Neat mortar samples were prepared by mixing tap water with cement sand.

The samples were prepared according to the ASTM C305 mixing protocol [59] and cured at 100% RH at 25°C for 24 hours. Molds were removed and the samples were submerged in UYE medium saturated with lime for subsequent curing at 23°C until testing.

The w/c and s/c ratio were held constant at 0.50 by weight for all samples. The cement-to-sand ratio was maintained at 1:4 for the mortar mixtures.

Most probable number (MPN) analysis was conducted on samples in order to determine the metabolic state of cells and solvent-exchange method (see **Section 4.3.4**) was conducted in order to determine the porosity of samples

In addition, three 330-day old samples were interrogated via MPN method in order to quantify the viable cell concentration and endospore percentage. These samples were extra samples from the compressive strength testing conducted in **Chapter 4**. The initial concentration of the bacterial culture prior to the mixing process was determined to be 2.5 x10^6 CFU/mL and details of the sample preparation for this sample can be found in **Section 4.2.3.**

5.2.2. Viability testing

5.2.2.1.Most Probable number (MPN)

Viable *S. pasteurii* was enumerated via MPN analysis at 0 (initial inoculum), 1, 7, 28 and 330 days after mortar mixing. The MPN technique provides an estimate of the number of viable cells, which includes vegetative cells and endospores. The samples were removed from their curing environment and then ground into a powder with a mortar and a pestle. The mortar and pestle were sterilized with ethanol prior to use. The powder was then suspended in fresh UYE-medium at pH 12 to minimize possible pH

shock since an immediate change in pH may induce cell death. Then the resulting suspension was sonicated (MISONIX XL 2020 probe sonicator, Farmingdale, New York) for 2 minutes to release bound cells from the mortar substrate. The suspension was allowed to settle for 10 min, and the supernatant was transferred into a sterile tube and vortexed for 1 min. Triplicate serial dilutions were prepared with UYE medium (pH 12) in test tubes. The tubes were incubated at 30°C for 3 days, and growth of the cells in the test tubes was monitored via OD_{600} after the incubation period was completed. Cell concentrations were estimated from the duplicate MPN values by statistical analyses [73].

5.2.2.2.Quantifying endospores

To determine the relative percentages of endospores and vegetative cells in cement paste and mortar, a portion of the supernatant described in **Section 5.2.1.1** was pasteurized at 80°C for 15 minutes at 0, 1, 7, 28 and 330 days to inactivate vegetative cells. The pasteurized samples were interrogated via the MPN technique to estimate endospore concentrations.

5.2.3. Scanning Electron Microscopy

A JEOL JSM 6490-LV Scanning Electron Microscope (Pleasanton, California) was used. The accelerating voltage was kept at 20 kV while the working distance was held at 9-11 mm at various magnifications. The cement paste samples prepared with

bacteria (herein referred to as bacterial pastes) were prepared with *S. pasteurii* grown in 100 mL UYE medium (pH 9) to a concentration of 2 x 10^8 CFU/mL. Neat pastes were prepared by mixing tap water with cement while nutrient samples were prepared by mixing UYE medium with cement. The cell concentration was kept high to increase the volume fraction of cells to 0.02%. The samples were kept in a UYE medium saturated with lime solution until the samples were fractured to obtain specimens for imaging. Fracture surface specimens were obtained from crushed 7-day old neat, nutrient, and bacterial paste samples collected from the cores of crushed coupons. The fractured specimens were placed in a vacuum desiccator for 24 hours before testing.

5.2.4. Optical Imaging

Planktonic *S. pasteurii* cells were grown as described in **Section 3.1** in UYE medium (pH 9 and 12) and examined via Eclipse 80*i* Optical Microscope, (Nikon, Tokyo, Japan) at 600X magnification.

5.3. RESULTS AND DISCUSSION

5.3.1. Metabolic activity of *S. pasteurii* in cement paste and mortar

Figure 5.19 and Figure 5.20 display the results regarding the viable cell concentration and vegetative cell percentage in mortar and paste samples. The viability percentage was obtained by dividing the viable cell concentration at time *t* by the viable cell concentration at time 0. Endospore percentage was calculated by dividing the cell concentration after the pasteurization process, at time *t* by the viable cell concentration. Similarly, vegetative cell concentration was estimated by subtracting the endospore percentage from the total viable cell concentration at time *t* and vegetative cell percentage remaining was determined by dividing this value by the initial vegetative cell concentration at time 0. Control samples of the nutrient and neat samples, both the paste and mortar, did not yield any detectable turbidity, and the OD_{600} readings were close to UYE medium without any microorganisms. This means that if there were any microorganisms present, the concentration was lower than the detectable limit, $3x10^2$ MPN/mL, and thus it could not be enumerated. The same initial concentration (i.e. $t = 0$ in Figure 5.19) of viable cells was obtained for bacterial mortar and bacterial paste. Table 5.7 summarizes the estimated viable cell concentration and endospore percentage within the viable cell content. The upper and lower limits are determined the upper and lower

concentration values with in the 95% confidence interval. A detailed explanation of MPN analysis can be found in Appendix E: MPN Analysis cement paste and mortar samples.

As shown in the Table 5.7, 0.2% endospores were detected in the initial inoculum before the cells were mixed with paste. Thus the quantity of endospores was negligible compared to the initial percentage of vegetative cells that were mixed with cement paste. It was assumed that a similar trend existed for the mortar specimen.

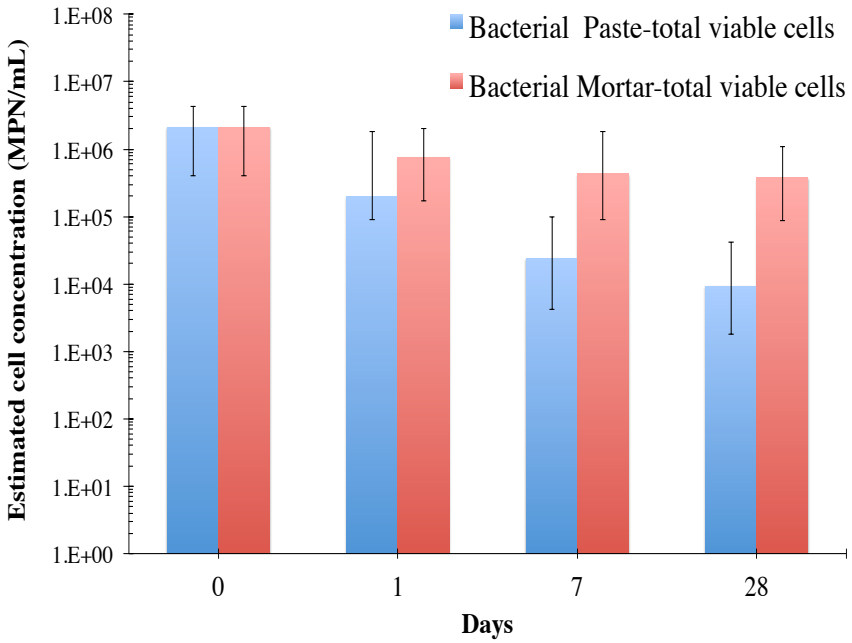

Figure 5.19: Viability of *S. pasteurii* in cement paste and mortar. The w/c and s/c ratio were 0.50. Samples were cured in UYE medium.

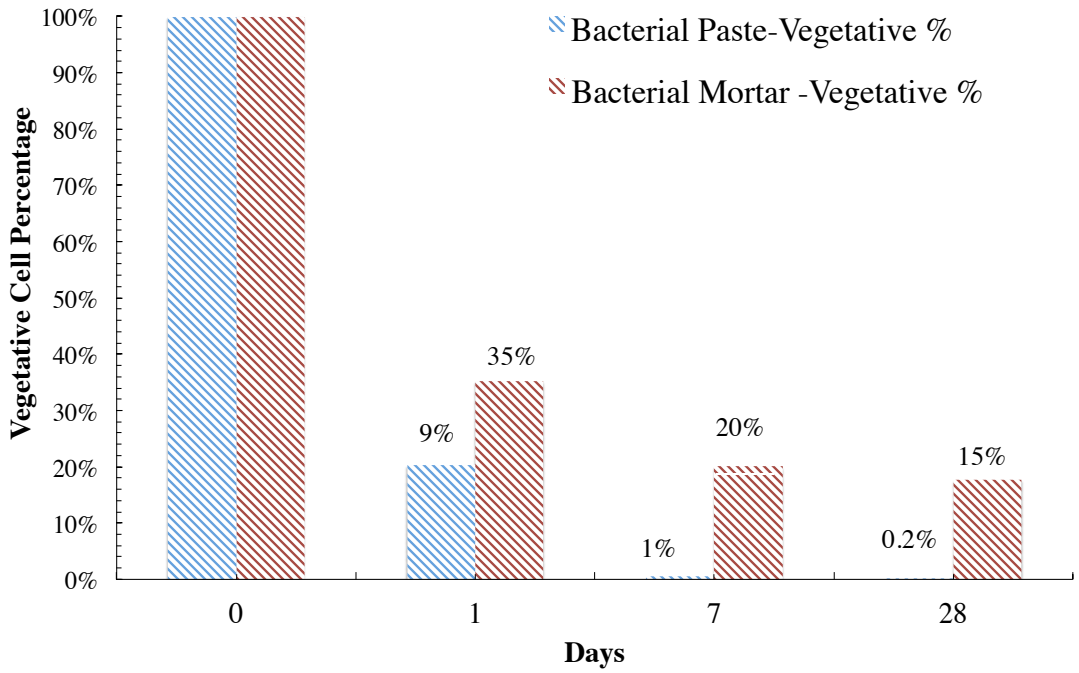

Figure 5.20: Vegetative cell percentage with respect to initial vegetative cell concentration in cement paste and mortar after mixing to cement paste (Initial vegetative cell percentage at t=0; 99.8%). The w/c and s/c ratio were 0.50. Samples were cured in UYE medium.

Figure 5.21 represents the vegetative cell percentage with in total viable cells calculated in the bacterial cement paste and mortar. As it is shown from Figure 5.21, the vegetative cell percentage with in the viable cells was determined similar in the bacterial cement paste and mortar at 1 and 7 days. However, at 28 days the vegetative cell percentage of the viable cells was higher in bacterial mortar at 28 days after mixing.

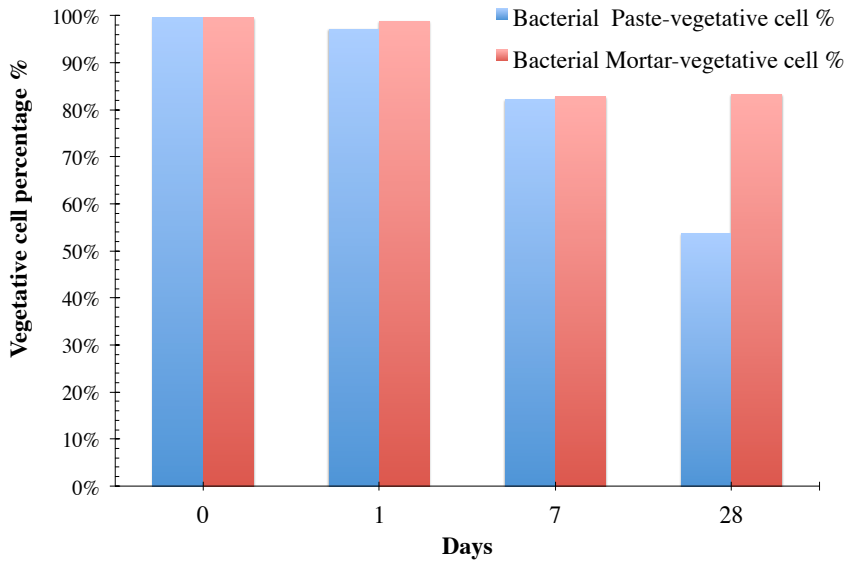

Figure 5.21: Vegetative cell percentage of viable cells in cement paste and mortar after mixing to cement paste. The w/c and s/c ratio were 0.50. Samples were cured in UYE medium.

Table 5.7: Viability and endospore percentage *of S. pasteurii* in bacterial paste

Days	Total Viable Cell Concentration (MPN/mL)				Endospore % of total viable cells
	Estimated Concentration	Lower Limit	Upper Limit	Viability % (with respect to Day 0)	
0	$2x10^6$	$4x10^5$	$4x10^6$	100	0.4
1	$4x10^5$	$4x10^4$	$4 x10^5$	20	1
7	$2x10^4$	$4 x10^3$	$1 x10^5$	1	28
28	$9 x 10^3$	$2 x10^3$	$4 x10^4$	0.4	46

20% of the initial concentration of cell remained viable observed in the bacterial paste between day 0 and day 1; and this retention became less pronounced at 7 days and 1% of cells remained viable. This demonstrates that cells tend to die rather than form endospores during the hardening process. At 28 days, the viable percentage decreased to 0.4%, however the percentage of endospores present in the total number of viable cells increased considerably from 28% to 46%. This could be due to the limitation of the nutrients; wherein the remaining vegetative cells could not be metabolically active and were thus forced to induce endospores. Furthermore, space limitations due to decrease in pores in aged cement paste may also play a role. It should be noted that, even though the viability percentage determined was as low as 1% at 7 days, the remaining cells were sufficient enough to increase the calcium carbonate mass percentage in the bacterial cement paste (see **Section 4.3.3**).

From 0 to 1 day, the remaining viable cells 35% of what was initially added in bacterial mortar while this percentage was determined as low as 20% in bacterial paste. Consequently, the vegetative cell percentage remained considerably higher in 1-day mortar sample than that of in 1-day cement paste sample. In general it can be concluded that regardless of whether the examined composite was cement paste or mortar, vegetative cells were killed during initial hardening period, rather than forming endospores. At 7 –days, the viability percentage remained as high as 20% in bacterial

mortar while this value was as low as 1% in the bacterial paste. At 28 days, the viability percentage decreased to 18% in the mortar sample while the vegetative cell concentration was determined to be 15%. Achal et al. [4] determined 0.1% viability for *B. megaterium* with a vegetative-inoculation approach (Initial inoculum before mixing was determined as 5 x 10^7 CFU/mL)after 28 days of curing in nutrient medium, the remaining viable cell concentration was 3 x 10^4 CFU/mL in bacterial mortar. Even though the viability percentage detected was lower than what we obtained, the remaining viable cell was in a similar range. However, the percentage of vegetative percentage of the viable cells was not examined. When Jonkers *et al*. [8] added a naturally encapsulated *B. cohnii* inoculum (2.4 x 10^8 spores cm^{-3}consisting of mostly endospores) to cement paste stone, they found that 1% of the initial concentration of *B. cohnii* was present at 9 days. However, after 135 days, the authors found that the viable cell concentration was below the detection limits (5 x 10^2 MPN/mL) of the MPN analysis [8]. This is interesting because even though endospores are supposed to be more resistant to harsh environments than non-sporulated bacteria, the percent survival of endospores determined by Jonkers et al. [8] was lower to the percentage of viable cells that remained as to with the vegetative-inoculation method used in this research. This is even more interesting to note considering that different microorganisms were used in that study than what was used in this study.

It is readily apparent that in comparison to bacterial cement paste samples, the concentration of the viable cells was determined to be higher in bacterial mortar. The higher viable cell concentration and the vegetative cell percentage in the bacterial mortar samples may be correlated to the relatively higher porosity of the mortar samples compared to the cement paste samples. Referring to the **Section 4.3.5**, Table 4.6 represents the ethanol porosity of cement paste and mortar samples. Compared to cement paste, mortar has higher porosity both for the neat and bacterial samples. The influence of *S. pasteurii* cells on the porosity of mortar materials was discussed in **Section 4.3.5** and the efficiency of microorganisms on porosity reduction was found to be less pronounced in the paste samples compared to the mortar samples. Thus, due to the lower amount of vegetative cells in the cement paste samples, less calcium carbonate might be precipitated. Relationship between the porosity and viability might be a mutual balance such that an increase in pore space increased the retained vegetative cell concentration and it is these vegetative cells that are metabolically active and producing the calcium carbonate that is reducing the porosity. Considering the metabolic activity of the cells, it could be concluded that application of biomineralization was found to be more effective in mortar comparison to cement paste. These results are promising for production of bacterial concrete because the porosity of concrete is higher than mortar, which may

increase the percentage of metabolically active cell that can enhance the efficiency the biomineralization in concrete.

However it must be stated that the actual magnitude of viable cells may be under predicted using the MPN method. MPN was used versus agar plates because MPN is more suitable for samples that have granular particles, since granular particles restrict growth on agar plates [73]. However the MPN method assumes that the cells are randomly distributed, viable, and that they exist as single cells [74]. If this single cell assumption is violated, then the MPN estimation method can cause under prediction of the true cell count. In a restrictive environment, such as cement paste, microorganisms might not occur as single cells. Figure 5.22 shows the micrographs of bacterial cells suspended in a UYE medium. Filamentous forms and a larger amount of linked chains were seen when *S. pasteurii* was exposed to a high pH environment (Figure 5.22-a), whereas in a low pH environment, single cells predominated (Figure 5.22-b). Due to the high pH environment of cement paste, it is likely that filamentous, aggregated forms of the bacterial cells occur in cement paste. If this were true, then viability results reported in this section might increase.

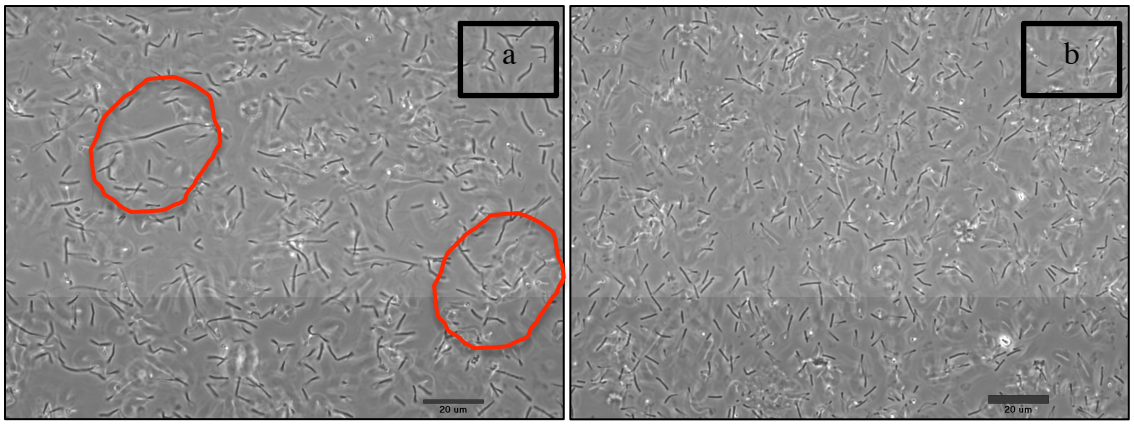

Figure 5.22: Optical microscope images of *S. pasteurii* cells in UYE medium (a) pH 12, Circled region depicts longer cells (b) pH 9 (Scale bar: 20 micrometer)

Another factor that might be affecting the enumeration of cells in the supernatant obtained from the samples is the sonication process such as if it was sufficient enough to remove all the attached cells from the supernatant. Even though preliminary tests were conducted on pure culture to determine a time frame for sonication process suggested a slight decrease in cell concentration when the sonication was applied more than 3 minutes, the full detachment of cell might not be provided with a shorter sonication. Therefore, if it was the case in which all the cells were not detached from the substrate, then, the viable cell concentration determined might be lower than the actual value.

5.3.2. Metabolic activity of *S. pasteurii* in mortar: 330-days old

One of the key questions with respect to biomineralization in cement-based materials is "what is the extended period of viability of the bacteria ***in*** cement-based materials? Furthermore, the metabolic state would be critical to know since it is the metabolically active bacteria that are able to hydrolyze urea. Compared to the 1% viability retention seen at 4 months when a naturally encapsulated approach was used [8] and the 0.1% viability after 28 days when vegetative *B. megaterium* was introduced in mortar [4], the *S. pasteurii* vegetative-inoculation approach used in this study showed extended microorganism viability even though the starting cell concentration in our mixes was lower than those used is reference [4], and [8]. MPN analysis done on 330-days old mortar showed that approximately 42% of the total viable cells were vegetative (0.84% with respect to initial inoculum), and that a total of 2% of the added $2x10^6$ CFU/mL cells (Initial concentration was determined by using the growth curve and OD_{600} readings) were still viable. This corresponds to 4 $x10^4$ MPN/mL, which is a relatively high number of cells especially considering that the samples were cured in water after 56 days and were not exposed to any additional nutrient sources (other than what was originally added) during this period. The considerable increase in endospore ratio to viable cells could be explained by restriction of nutrients. In *Bacillus* and *Sporosarcina* species, the main reason of endospore formation is the nutrient limitation in the

environment [37, 75]. It has to be mentioned that this is the first time that extended long-term viability of the bacteria in cement-based materials was determined. Furthermore, not only the viability, this is the first time the metabolic state of the microorganisms was reported as the percentage of vegetative cells.

5.3.3. Visual detection of *S. pasteurii* cells in cement-based materials

S. pasteurii cells are reported as ranging in size of 1.3 to 4.0 μm in length and 0.5 to 1.2 μm in diameter [72]. As shown in Figure 5.23, the rod-shaped feature in the bacterial paste could be *S. pasteurii* given its size and morphology. The rod-shaped feature was approximately 0.8 μm in diameter and 3.2 μm in length. Examination of the control pastes indicates that these rod-shaped structures were not visible (Figure 5.24-Figure 5.25).

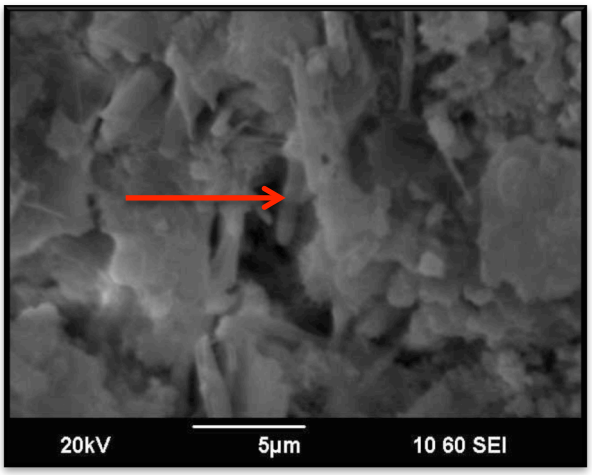

Figure 5.23: SEM images of cement paste samples 7-d old Bacterial paste (s/c=0.50)

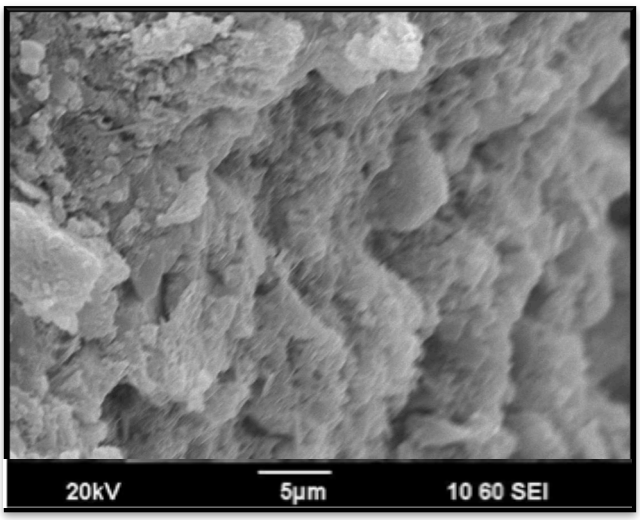

Figure 5.24: SEM images of cement paste samples 7-d old nutrient paste (s/c=0.50)

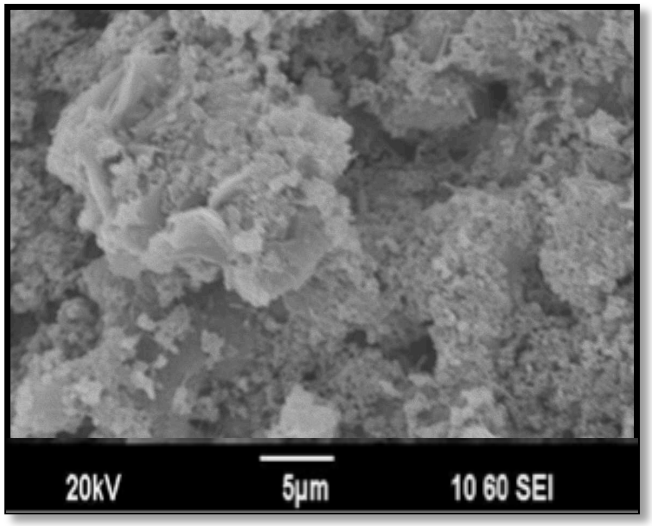

Figure 5.25: SEM images of cement paste samples 7-d old neat paste (w/c=0.50)

Chapter 6: Lightweight expanded shale as internal nutrient reservoir

This chapter introduces a novel approach to increasing the percentage of remaining vegetative cell percentage in mortars by providing internal nutrient reservoirs via pre-wetted lightweight expanded shale fine aggregates, wherein the delayed nutrient release in the hardened mortar can be provided. Compressive strength testing and metabolic state analysis of *S. pasteurii* cells are presented as techniques to determine whether this approach is successful.

6.1. INTRODUCTION

One of the main challenges to using a biomineralization approach for self-healing applications is that to have a system that is able to heal cracks over the lifetime of the structure then a long survival period for the vegetative microorganisms is needed. *We hypothesize that providing nutrients for bacteria at increased concrete ages can increase the percentage of vegetative (metabolically active) cells*. One possible approach is to provide nutrients internally, and an internal curing concept was used to provide nutrients at later ages for bacteria incorporated in cement-based matrix. Internal curing is the "*process by which the hydration of cement continues because of the availability of internal water that is not part of the mixing water* [76]." One of the possible ways to provide this internal water supply is by using pre-wetted lightweight aggregates.

Bentz et al. [77] defined the difference between external and internal curing concept with the Figure 6.26. As shown in Figure 6.26, the internal curing application provides internal water with in cement paste matrix, which is distributed more homogenously than that of the water provided by external surface curing. In our case, the mortar cubes were submerged into UYE medium during testing period, is a form of external curing. However in reality, it would not be possible to keep the concrete fully saturated for extended time period, thus even though the nutrient source for the microorganism are provided during the mixing, the availability of these nutrients will decrease during hydration as the water is consumed. However, if the nutrients are provided via an internal curing reservoir then it is likely that the microorganism will have access to the nutrients as they are desorbed from the aggregates. Internal curing may provide a nutrient reservoir to microorganisms to keep them metabolically active.

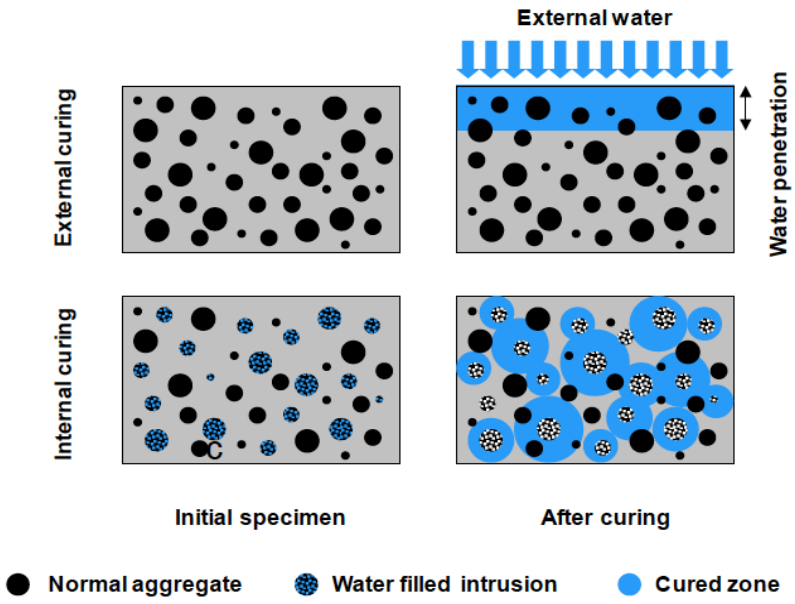

Figure 6.26: Penetration difference between external curing and internal curing [77].

While internal curing is generally useful for applications such as increasing in the hydration of cement paste with a low w/c, reducing shrinkage and to increase durability of cement-based materials [78]. In our application, the main goal of using internal curing reservoirs was to increase the percentage of vegetative cells at later ages. Therefore, the release of nutrients was targeted after seven days of external curing, rather than during the initial hardening period, which was suggested for adequate desorption of internal curing water [77–79].

6.2. MATERIALS AND METHODS

6.2.1. Materials

Colorado River sand with properties shown in **Section 4.2.1** was replaced partially with expanded shale fine aggregate with a fineness modulus of 0.92. ASTM C128-07- Standard Test Method for Density, Relative Density (Specific Gravity) and Absorption of Fine Aggregate [57] was used to determine the absorption coefficient of the expanded shale. The absorption capacity of the expanded shale was determined as 24.2% while the specific gravity is determined as 1.16. The particle size distribution for the expanded shale was determined according to ASTM C136- Standard Test Method for Sieve Analysis for Fine and Coarse Aggregate [56]. Three hundred grams of oven dry fine sand was sieved through #4 to #200 (4.75 mm-75 μm) sieves by a mechanical shaker. Figure 6.27 shows the sieve analysis for the expanded shale. The particle size distribution is an important factor for internal curing applications, fine aggregates (e.g. sand) were found to be more efficient in protecting the paste volume rather than large coarse aggregates due to increase in surface area with introducing finer particles [77, 78]. However, Zhutovsky et al. [79] determined that very fine particles (~400 μm) showed adverse effect on internal curing efficiency due to their limited porosity and suggested the use of larger fine aggregates. Therefore expanded shale that was retained between the #4 (4.75 mm) and #30 (600 μm) sieve were used in the mixes.

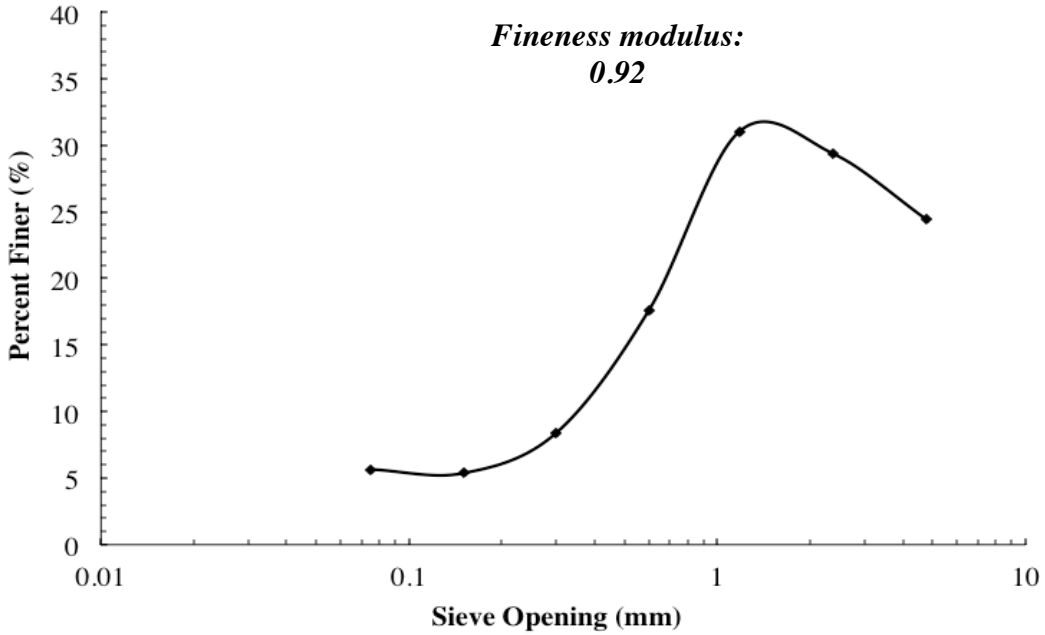

Figure 6.27: Sieve analysis for fine lightweight expanded shale.

Lehigh type I/II cement (Buda, Texas) described in **Section 4.2.1** was used in the mixes. Similar to the previous mixes in **Section 4.2.1,** tap water was used for the neat paste mixes, while DDI water was used in the nutrient and bacterial pastes with the UYE medium.

6.2.2. Preparation of mortar mixes

Colorado River sand was partially replaced with expanded shale by 25% and 50% of its volume. For the bacterial mortar samples, the expanded shale was fully submerged in the UYE medium in a sealed container at 25°C for 24 hours. Prior to the mixing, the aggregates were removed from the UYE medium and the excess UYE medium on the lightweight aggregates (LWA) was calculated by subtracting the

absorbed UYE medium in the aggregates from the total UYE medium content on the aggregates. The absorbed UYE medium was determined by multiplying the mass of LWA with the absorption capacity, which was determined as 24.2% according to ASTM C128-07a. The excess UYE media on the surface of the LWA was accounted for in the mix design by subtracting this excess content from the aqueous portion of the initial bacterial mixing solution required for the bacterial paste (see Table 6.8). The mix proportion for bacterial mortar samples are summarized in Table 6.9.

Table 6.8: Corrected bacterial solution content for mortar mixing

LWA %	Absorbed UYE medium (g)	Excess UYE Medium (g)	Initial Bacterial Solution Required (g)	Corrected Bacterial Solution (g)
0	0	0	589.88	589.88
25	122.80	75	589.88	507.85
50	245.45	53	589.88	522.60

Table 6.9: Mix Design For Bacterial Mortar With Partial Expanded Shale Replacement (0,25 And 50% By Volume Of Colorado River Sand)

LWA%	Expanded Shale Weight (g)	Bacterial Solution (g)	Cement (g)	Colorado River Sand (g)
0	0	589.88	1122.2	4461.86
25	494.17	507.85	1122.2	3345.71
50	987.74	522.60	1122.2	2230.93

S. pasteurii cells were grown in the UYE medium until concentration values reached a range of 10^5 CFU/mL, which was determined to be within the exponential growth phase. The concentration was determined by using the equation obtained from the growth curve in **Section 3.3.2**. Bacterial mortar specimens (5.08 x 5.08 x 5.08 cm) were prepared by mixing this solution with cement and aggregates. Neat and nutrient mortars were also cast. The nutrient mortar samples were prepared by mixing the UYE medium with cement, pre-wetted expanded shale (in UYE medium) and sand. Prior to mixing, the expanded shale aggregates were pre-wetting by submerging them a sealed container containing UYE medium for 24 hours. The temperature of the UYE medium was maintained at 25°C by placing the container in a temperature-controlled room. Similarly, the neat mortar samples were prepared by mixing tap water with cement, pre-wetted expanded shale and sand. For the pre-wetted aggregates used in the neat mortar samples, the aggregates were pre-wetted by submerging them in tap water versus submerging them in UYE media, as was done for the bacterial and nutrient mortars.

The mixes were prepared according to the ASTM C305 mixing protocol [59] and cured at 100% RH at 25°C for 24 hours. After the molds were removed, the mortar samples were submerged in a room temperature solution of UYE medium for 7 days. After 7 days of curing in the UYE medium, the samples were removed from the UYE medium and placed in an environmental chamber. The temperature and RH of the

environmental chamber was maintained at 23°C and 50% RH, respectively. The samples were kept in the environmental chamber until testing.

The w/c and s/c of the mixtures in Table 6.9 were all 0.50. In order to examine the influence w/c ratio neat and bacterial mortars were also prepared using w/c and s/c of 0.30. For these mixtures, the Colorado River sand was partially replaced with expanded shale by 25% of its volume and the control samples were prepared without any lightweight aggregate replacement. The samples were cured at 100% RH at 25°C for 24 hours. After removing the molds, the samples were placed in the environmental chamber (temperature = 23°C, RH = 50%) until testing.

6.2.3. Determination of mechanical properties and viability

Compressive strength tests were conducted as according to the protocol described in **Section 4.2.3**. MPN analysis was conducted in order to determine the mechanical properties and metabolic state of microorganisms. The procedures for determining viable cells and endospores are previously described in **Section 5.2.2**.

6.3. RESULTS AND DISCUSSION

6.3.1. Influence of delayed nutrient release on metabolic state of *S. pasteurii*

Table 6.10 summarizes the results obtained from the MPN analysis conducted on the bacterial mortar without any lightweight aggregates and kept at 50% RH after 7 days of curing in UYE medium. Since the samples were submerged in an UYE medium during the first 7 days, desorption of internal curing nutrients should not occur during this time period. However, as the sample dries then the internally embedded nutrient reservoirs should occur as the samples begin to dry. The viability percentage at a given time, *t*, was calculated by dividing the viable cell concentration at time t by the viable cell concentration at time 0. The percentage of endospore at a given time, *t*, was calculated by dividing the cell concentration after the pasteurization process, at time t by the viable cell concentration. Since only endospores would survive the pasteurization process the cell concentration after the pasteurization process represents the amount of endospores in the sample. The vegetative cell concentration was estimated by subtracting the endospore percentage from the total viable cell concentration at time *t*, and the vegetative cell percentage at time *t* was determined by dividing the vegetative cell concentration by the initial vegetative cell concentration at time 0. The control samples did not yield any detectable turbidity, which means either there was not any microorganisms detected or if

there was any, the concentration was lower than the detectable limit, $3x10^2$ MPN/mL for the analysis.

As shown in the Table 6.10, endospores were not detected in the initial inoculum before the cells were mixed with mortar, which indicates that only vegetative cells were mixed in the cement paste matrix. The upper and lower limits are determined the upper and lower concentration values with in the 95% confidence interval. "ND" stands for the samples wherein the endospore percentage was not determined for the samples. 57% of the initially inoculated vegetative cells were determined as viable in the bacterial mortar between day 0 and day 1, after which the viable cell concentration decayed slowly while the endospore concentration increased. These values were found to be slightly higher than the estimated cell concentrations determined in **Section 5.3.1**. The main difference between the samples examined in Table 6.10 versus those examined in **Section 5.3.1** are that initial concentration of the inoculated bacteria examined in the samples for **Section 5.3.1** were higher than the samples in Table 6.10. Referring back to the results obtained in **Section 5.3.1**, the initial bacterial concentration in the inoculum was $1x10^6$ MPN/mL and 36% cell viability ($2x10^5$ MPN/mL) was determined after the first day. In comparison, the initial concentration of the inoculum for the mortar reported in Table 6.10 is $1x10^5$ MPN/mL and 57% cell viability was found after the first day. Interestingly, this also corresponds to a bacterial concentration of $2x10^5$ MPN/mL. This suggest that

there might be threshold value for the remaining viable cells, and it is likely that this threshold value is dependent on the interrelationship among the bacterial concentration, access to nutrients, and space availability.

After 28 days, approximately 20% ($8x10^4$ MPN/mL) of the bacteria remained viable (i.e., vegetative cells or endospores) and 6% of viable cells were endospores ($5x10^3$ MPN/mL). Similar to the results in **Section 5.3.1**, it appears that the cells tend to die rather than form endospores as the mortar ages.

Table 6.10: Viability of *S. pasteurii* in bacterial mortar with 0% expanded shale and cured in UYE medium saturated with lime for 7 days then kept In 50% RH (s/c: 0.50), ND: Not Determined

Days after mixing	Total Viable Cell Concentration (MPN/mL)				Endospore % of total viable cells	Days at 50% RH
	Estimated Concentration	Lower Limit	Upper Limit	Viability % (with respect to Day 0)		
0	$4x10^5$	$9 x10^4$	$1 x10^6$	100	0	0 (specimens in the moist room)
1	$2x10^5$	$5 x10^4$	$9 x10^5$	57	ND	0 (specimens in UYE medium)
7	$1x10^5$	$5 x10^4$	$9 x10^5$	28	ND	0 (specimens placed in environmental chamber where RH=50%)
28	$8x10^4$	$2 x10^4$	$2 x10^5$	20	6	21
56	$4 x10^4$	$2 x10^4$	$2 x10^5$	10	6	49
90	$5 x10^3$	$2 x10^3$	$2 x10^4$	1	46	83

From Table 6.10, it can be seen that even at 90 days, a substantial number of viable cells ($5 x10^3$ MPN/mL) were detected. At 90 days, 54% of the total viable bacterial cells were estimated to be vegetative cells, thus proving that vegetative cells can survive in the cement-paste matrix without any additional pre-treatment to encapsulate them.

Figure 6.28 shows the time evolution of the viable cell concentration once the specimens were allowed to dry. The viable cell concentration at 0 days was expected to

be the same since the sample was not allowed to dry yet; thus, the internal reservoirs should not play a role at that point. However, it can be seen that the viable cell concentration for the 25% pre-wetted expanded shale replacement and the sample that did not contain any pre-wetted expanded shale was essentially the same at all the time periods considered in this study. Yet, the same trend was not observed in the samples with 50% LWA replacement. The remaining viable cell concentration in the samples with 50% LWA replacement was lower than viable cell concentration of the samples with that of it was in 0% and 25% replacement of LWA.

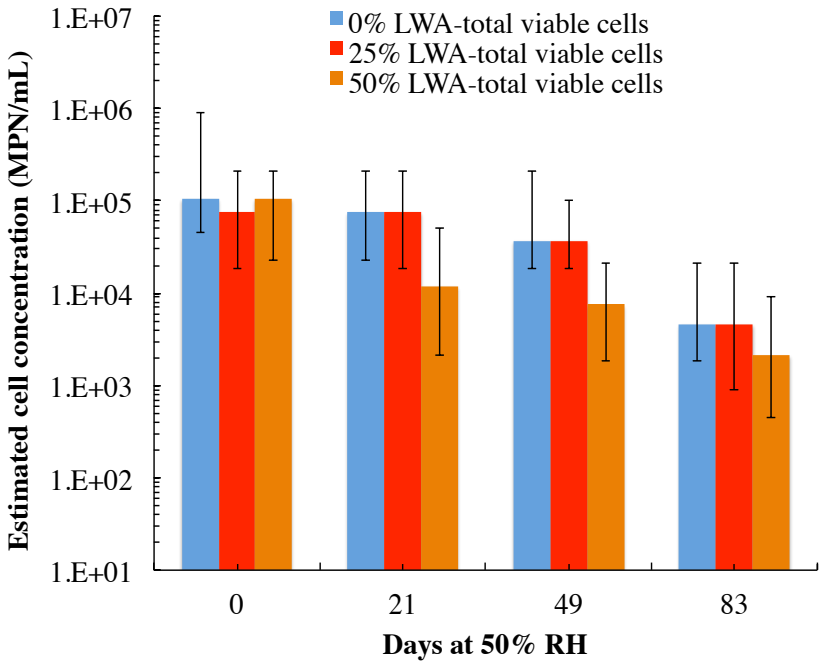

Figure 6.28: Viability of *S. pasteurii* in mortar with 0% 25% and 50% LWA replacement. The w/c and s/c ratio were 0.50. Samples were cured in UYE medium for 7 days, followed by curing at 50% RH curing (t=0). Error bars show the upper and lower limits for cell concentration.

Interestingly, even though the viable cell concentration of the 25% pre-wetted expanded shale replacement and the sample that did not contain any pre-wetted expanded shale were similar vegetative cell percentage of these mixtures differed (see Figure 6.29). After 21 days of 50% RH curing, the vegetative cell percentage was determined, as 19% in the sample did not contain any pre-wetted expanded shale. However this retention was increased to 33% with introduction 25% pre-wetted expanded shale. In contrast, with 50% replacement, only 1% of initial inoculated cells were remained vegetative. The 25% expanded shale replacement was effective in keeping the vegetative cell fraction higher than that of in 0% LWA mortar after 49 days curing. After 83 days of being subjected to 50% RH curing, the vegetative cell percentage decreased considerably along with the reduction the total viable cell concentration.

A possible reason for the decrease in vegetative cell percentage at later ages might be the limitation in desorption of aggregates. As calculated by Equation 6.8 [78] lightweight aggregates with an absorption capacity(ϕ_{LWA}) of 24.6% absorption would require more than 120 kg/m^3 of internal curing water,

$$Wcuring = \frac{C \times \alpha \max \times CS}{\phi LWA} \tag{6.8}$$

Where W_{curing} is internal curing water required and C stands for the cement content (kg/m^3), which is 430 kg/m^3 in our case. The term α_{max} is the degree of hydration, which

is s 1 for a sample with a w/c of 0.50 (as is the case for our mixtures). CS is chemical shrinkage (kg water /kg cement hydrated) which is assumed to be 0.07 [78].

However with 25% expanded shale replacement by the volume of sand, only 50 kg/m^{3}internal curing water was provided, assuming that 494.17 g of LWA in a 2.6 L batch absorbs at 24.6% and desorbs all the absorbed water.

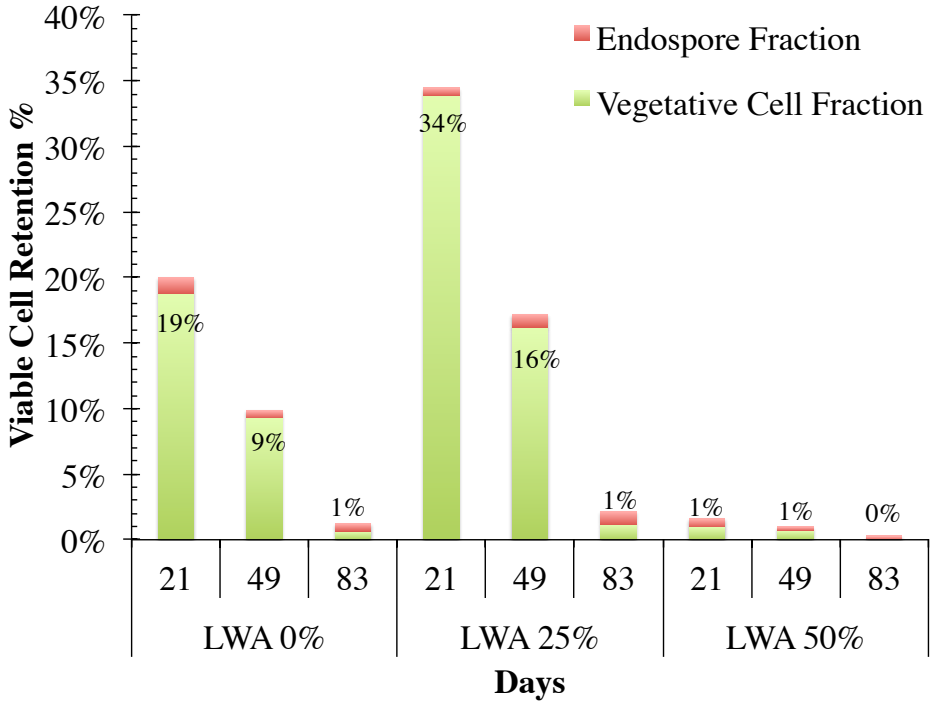

Figure 6.29: Vegetative cell percentage in total remaining *S. pasteurii* in mortar with 0% 25% and 50% LWA replacement after 21, 49 and 83 days of 50% RH curing. The w/c and s/c were 0.50. (Initial vegetative cell percentage at t=0; 99.8%).

This content might be insufficient to keep the vegetative cells active. At 50% expanded shale replacement, the internal curing water estimation was 100 kg/m^3, which was closer to the value required for full saturation. However, at this percentage of replacement, both the viable cell concentration and the vegetative cell percentage decreased even though more nutrient source was provided. An important point that might be related to loss of efficiency in this batch is the limited absorbance time (24 hours) provided for the LWA to absorb the nutrients during the pre-wetting conditioning period. Although the rate of absorption is fastest during the first 24 hours, LWA could continue to absorb the internal curing source for several days with a decaying rate [77]. In this case the absorption time was limited to 24 hours, in which the fine aggregates with limited porosity might not be fully saturated. While it is unclear exactly why a decrease in viable cell count occurred, one possible reason for possible reason to explain why the internal reservoirs were insufficient for improving viability when a higher content of LWA was used could just be related to the variable nature of LWA. Variance in the internal porosity of the LWA can affect the absorption and desorption capacity of the aggregate as well as microbial attachment. Regardless, based on the results of this work, it can be concluded that 25% replacement volume of LWA was noticeably more effective in increasing the vegetative cell percentage a cement-based composite than the 50% replacement volume

of LWA. It was determined to be noticeably efficient compared to 50% replacement due to higher vegetative cell percentage up to 2 months.

6.3.2. Influence of delayed nutrient release on metabolic state of *S. pasteurii* with lower w/c at 50%relative humidity

The samples discussed in **Section 6.3.1** were not subjected to drying until 7 days and the s/c ratio of the samples was 0.50. However, internal curing agents are known to be more beneficial in providing sources of water for mixtures prepared at lower w/c (< 0.42). As such, this portion of the work examined samples prepared with lower s/c ratios in order to see whether viability will increase since aggregate desorption should be higher at the lower s/c. In addition, instead of submerging the sample for 7 days in a UYE medium, the samples were immediately placed in the curing chamber after demolding since based on results conducted regarding curing regimes (See **Chapter 7**) full bacterial viability was better when the sample was subjected to air curing or spray curing vs. full water submersion curing. The initial concentrations of the bacterial inoculum were for the mortar samples discussed in this section and those discussed in the previous section were both within the same range (1-4x10^5 MPN/mL).

Table 6.11 summarizes the viability results obtained at 1,7, 28 and 56 days. The control neat paste samples did not yield any detectable turbidity at any age of testing.

Table 6.11: Viability *of S. pasteurii* in bacterial mortar with 0% expanded shale and cured in 50% RH (bacterial solution/cement: 0.30)

Days after mixing	**Total Viable Cell Concentration (MPN/mL)**				**Endospore % of total viable cells**	**Days at 50% RH**
	Estimated Concentration	**Lower Limit**	**Upper Limit**	**Viability % (with respect to Day 0)**		
0	$4x10^5$	$9\ x10^4$	$1\ x10^6$	100	0	0 (specimens in the moist room)
1	$7x10^4$	$5\ x10^4$	$9\ x10^5$	20	3	0 (specimens placed in environmental chamber where RH=50%)
7	$4x10^4$	$7\ x10^3$	$1\ x10^5$	10	6	6
28	$5x10^3$	$9\ x10^2$	$2\ x10^4$	1	46	27
56	$1x10^3$	$9\ x10^2$	$4\ x10^3$	0.3	65	55

Reducing the s/c from 0.50 to 0.30 resulted in a reduction in the viable cell concentration (compare Table 6.10 and Table 6.11). At one day after mixing, the total viable cell concentration was $2x10^5$ (see Table 6.10) for the s/c=0.50 sample, whereas the total viable cell concentration was $7x10^4$ (see Table 6.11) for the s/c=0.30 sample. Since

both samples were cured in the same condition, this shows that the s/c ratio affects viability. The water content of concrete has a significant effect on the internal porosity of the composite, and as the w/c ratio increases the porosity of the sample also increases [80]. A reduction in the s/c ratio would result in a mortar sample with decreased porosity and thus the space available for bacterial growth decreases. This is consistent with the results discussed in **Section 5.3.1** in which the cell viability was higher in the sample that had a higher degree of porosity. Normally high porosity in a concrete is undesirable since for strength and durability considerations, and biomineralization can be a technique to consider in these high porosity concrete mixes wherein the reduction in porosity can be obtained via calcium carbonate precipitation.

As summarized in Figure 6.30 in general introducing 25% pre-wetted LWA to the bacterial mortar with an s/c of 0.30 did not influence the total viable cell concentration compared to the sample prepared without LWA. At 56 days, the viable and vegetative cell percentages in both mortar mixes were determined to be less than 1%. Although the concentration of viable cells determined at 56 days can be considered a low/moderate level (1-2x10^3 MPN/mL), this value is much less than the viable cell concentration (approximately 4 x10^4 MPN/mL) retained in the 330-day old mortar samples cured by water (see **Section 5.3.2** for the discussion about the 330-day old specimen). The s/c ratio of the 330-day old sample was 0.50. Thus, this further supports the premise that the initial

porosity of the samples has a major influence on viability. Even though the s/c=0.50 sample was cured under water (see **Section 5.3.2**) and was more than 8 months older than the sample prepared at a s/c=0.30, the viable cell concentration for the 330-day old sample was greater than the s/c=0.30 sample at 56 days.

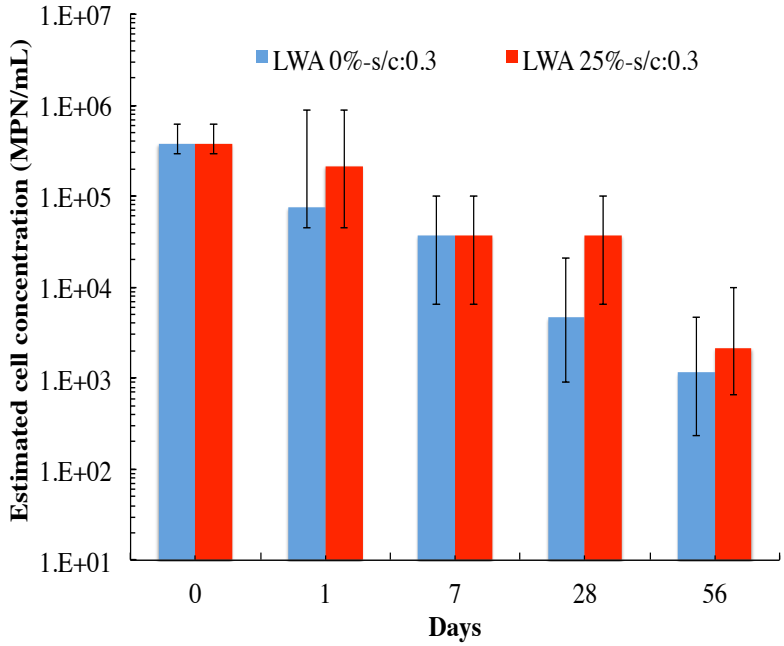

Figure 6.30: Viability of *S. pasteurii* in mortar with 0% and 25% LWA replacement. The w/c and s/c ratio were 0.30.Samples were cured at 50% RH curing after t=1.

But perhaps the true interesting result rests in the significant increase in the percentage of vegetative cells that occurred when the LWA was introduced (Figure 6.31). At 1 day, more than half of the initially inoculated vegetative *S.pasteurii* cells were able to remain vegetative. This is the largest percentage of vegetative cells that has been detected at 1 day for any of the samples examined in this research.

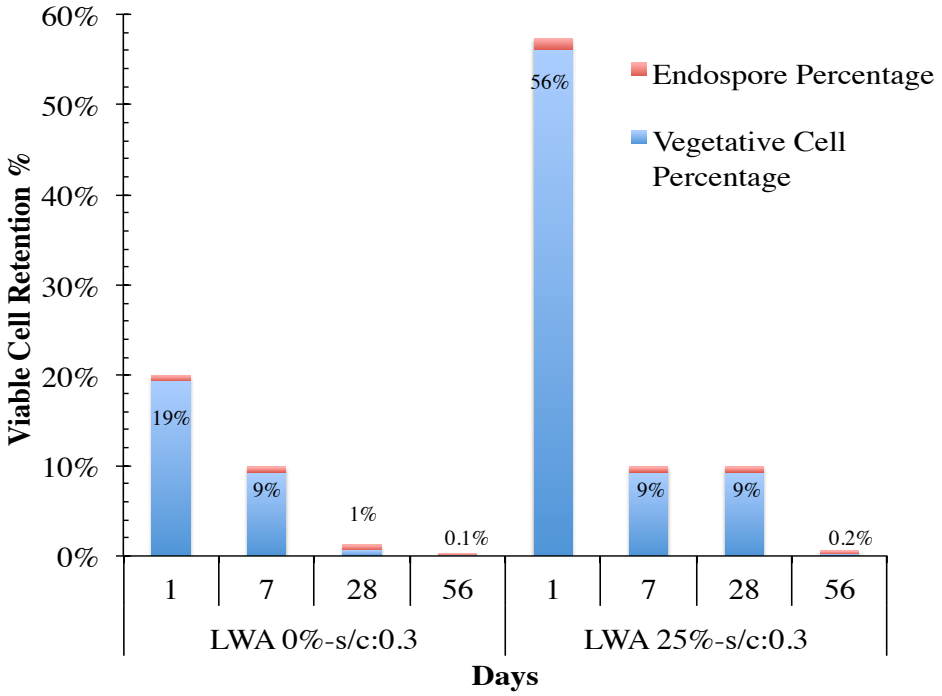

Figure 6.31:Vegetative cell and endospore percentage of *S. pasteurii* in mortar with 0% and 25% LWA replacement. (Initial vegetative cell percentage at t=0; 99.8%). Percentages marked are vegetative cell % with respect to initial vegetative cell concentration. The w/c and s/c ratio were 0.30.Samples were cured at 50% RH curing after t=1.

Furthermore, similar as to what occurred when pre-wetted LWA was added to mortars that had a s/c=0.50, the introduction of the internal curing reservoirs in the s/c =0.30 mortars extended the time period that the cells remained in vegetative form. The viable cell concentration and the vegetative cell percentage in the bacterial mortar with the internal nutrient source at 28 days were essentially the same as those obtained at 7 days. Thus, the introduction of the internal reservoirs provided nutrients to the bacteria so

that even at 28 days were the same values at 7 days. In contrast, the viable cell concentration (and thus the vegetative cell percentage) decreased considerably with bacterial mortar samples without any additional internal nutrient source.

In summary, internal curing reservoirs were found to be an effective way to promote vegetative cell occurrence in cement-based materials. Unfortunately, the use of internal curing reservoirs did not result with significantly improving the long-term viability of microorganisms in mortar.

6.3.3. Influence of internal curing reservoirs on strength

Figure 6.32 and Figure 6.33 represent the influence of the pre-wetted expanded shale reservoirs on the compressive strength of neat, nutrient and bacterial mortar samples with an s/c of 0.50. Since the samples were cured in the UYE medium saturated with lime for 7-days, the influence of internal reservoirs is expected to be observed during drying of the samples after removal from the curing medium.

As shown in Figure 6.32, the strength of neat mortar samples decreased with incorporation of the lightweight internal reservoirs. Following, 21 days of curing in 50% RH, the strength development in the neat mortar samples was roughly the same as the mortar samples that had 25% pre-wetted LWA (expanded shale) replacement. However, increasing the lightweight aggregate volume to 50% of sand decreased the compressive

strength. A likely reason is that the lightweight aggregates were softer and weaker compared to the sand used, and thus replacement of the sand with the expanded shale directly influenced the strength of mortar. The same trend was observed from 21 days to 49 days. Considering that the neat mortar also decreased the decrease in strength that is seen from 49 to 81 days is believed to be an artifact of the test. Based on the results of **Chapter 4**, the decrease in the compressive strength that occurred in the nutrient mortar sample was expected since yeast extract was used in the nutrient medium.

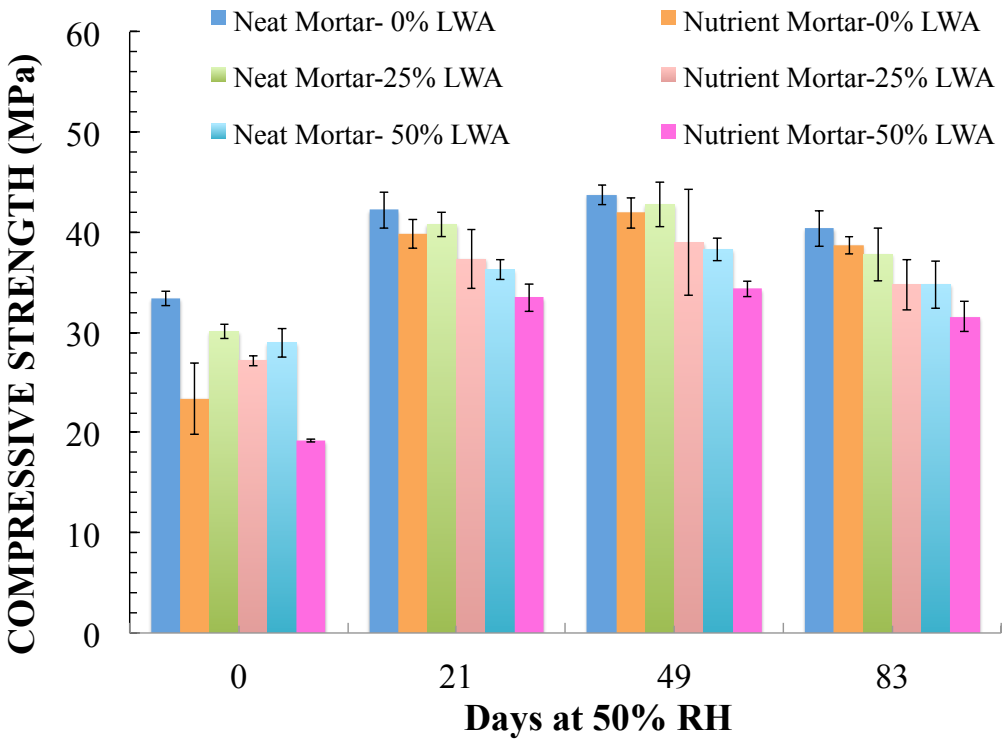

Figure 6.32: Influence of delayed nutrient release on compressive strength of neat and nutrient mortar (0%, 25% and 50% by volume of river sand). The w/c and s/c were 0.50. Samples were cured in 50% RH at 23°C (After 7 days of submersion in UYE medium saturated with lime) Bars represents average strength obtained from triplicates of samples, error bars indicates the standard deviation.

Introducing vegetative *S. pasteurii* cells to the mortar increased the compressive strength, even when no internal curing reservoirs were provided (Figure 6.33). However, this relative increase in the strength compared to the neat mortar was lower than the increase observed with the mortar samples cured in the UYE medium until testing (**Section 4.3.6**). However, in comparison to the bacterial mortar without any expanded

shale, there was no notable decrease in the strength of bacterial mortar with 25% expanded shale replacement of sand volume.

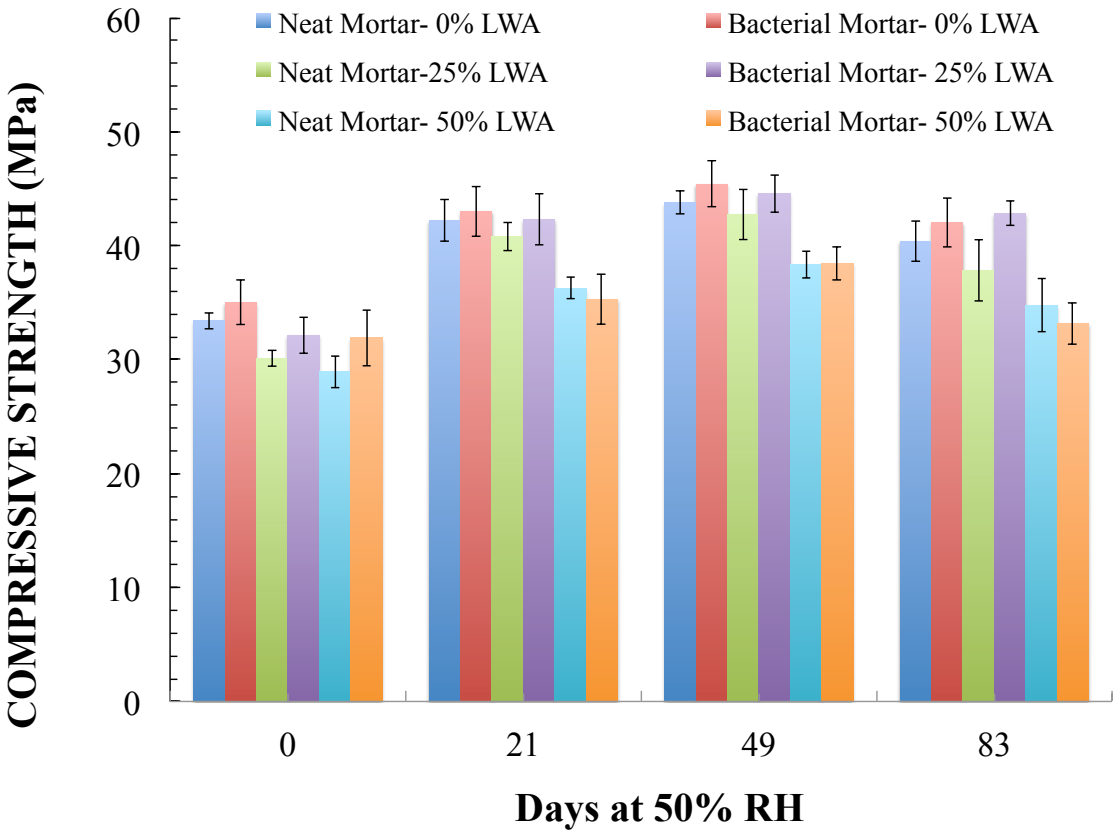

Figure 6.33: Influence of delayed nutrient release on compressive strength of neat and bacterial mortar (0%, 25% and 50% by volume of river sand). The w/c and s/c were 0.50. Samples were cured in 50% RH at 23°C (After 7 days of submersion in UYE medium saturated with lime) Bars represents average strength obtained from triplicates of samples, error bars indicates the standard deviation.

The influence of the internal curing reservoirs at a lower w/c (0.30) at 50% RH curing is summarized in Figure 6.34. The effects of microorganisms on the compressive strength of mortar were more pronounced when the s/c was decreased to 0.30 and an internal curing source was provided, especially during the first day of hydration. The incorporation of *S. pasteurii* cells with an additional internal nutrient source (25% pre-wetted LWA) resulted in the general trend of increasing the compressive strength when compared to the bacterial mortar without any internal reservoirs. Note, that the benefit of incorporating pre-wetted LWA into bacterial mortar was observed at lower s/c while no change was observed at high s/c (Figure 6.33). Also, autogenous shrinkage is more prominent at w/c < 0.42 and thus this could be due to increased desorption from the LWA caused by the self-desiccation of the cement paste.

The increase in the compressive strength at 1-day could be correlated with considerable increase in vegetative cell percentage in bacterial mortar samples. As discussed previously, metabolically active cells can produce urease enzyme, which might lead to an increase in calcium carbonate content. Thus the increase in calcium carbonate content could result in an increase in compressive strength due to reduction in porosity, healing of microcracks, or possibly another mechanism.

At 7 days, there were no changes observed in strength of the neat mortar samples modified by 25% lightweight aggregate replacement compared to the neat mortar without

any internal curing supplied. Similar trend was also observed at 28 days, which could suggest that the internal water supply was played a more substantial role on the microorganisms during the first day after hydration. Compared to the bacterial mortar samples without any internal curing supplied, the increase in compressive strength was less pronounced in the bacterial mortar in which 25% of the sand was replaced with the pre-wetted LWA by volume. Similarly, at 28 days there was no improvement in the strength of bacterial mortar due to addition of internal nutrients.

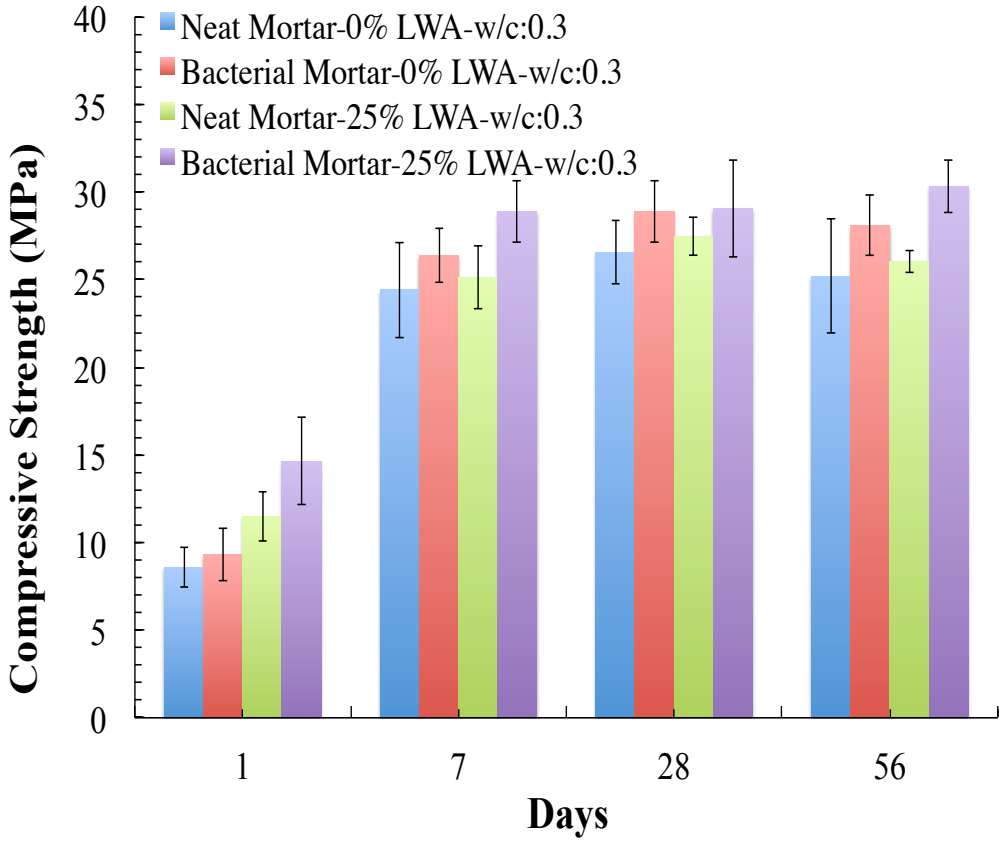

Figure 6.34: Influence of delayed nutrient release on compressive strength of neat and bacterial mortar (0% and 25% by volume of river sand). The w/c and s/c were 0.30. Samples were cured in 50% RH at 23ºC. Bars represents average strength obtained from triplicates of samples, error bars indicate the standard deviation.

Chapter 7: Self-healing ability of inoculated vegetative *S. pasteurii* cells

In the previous chapters it has been shown that calcium carbonate precipitation can be increased in cement paste matrix without introducing any additional calcium source or without protecting the microorganisms through encapsulation. Incorporation of vegetatively inoculated *S. pasteurii* in a UYE medium led to an increase in the compressive strength and a decrease in the porosity. Furthermore, even after 330-days, inoculated vegetative *S. pasteurii* cells were found to be viable with a noticeably high vegetative cell percentage. These findings lead to the development of a self-healing agent, which does not have any adverse effect on mortar properties and the self-healing ability can be applicable for long term. This section summarizes the self-healing ability of the inoculated vegetative *S. pasteurii* cells particularly for the self-healing ability of internal micro cracks due to applied external stresses.

7.1. INTRODUCTION

Self-healing can be described as the capability of the material to repair the cracks or fissures without any additional external processing [81]. Previous work discussed in **Section 2.8** summarized some examples, which were self-healing applications for external cracks by applying encapsulated microorganisms on the surface of the cement-based material [2, 7]. However a limitation determined so far was the width of the surface

crack that can be healed, where a maximum crack width of as 0.18 mm was reported for the self-healing applications [33]. In addition, the depth of the cracks may have an influence on crack healing ability of microorganisms; as such, the current research on using biomineralization for self-healing applications in cement-based materials has been focused on surface cracks. However, internal microcracking in cement-based materials is also a concern, since these cracks may eventually coalesce and cause detrimental effects to the durability of the material. The self-healing of internal microcracks due to microbial activity is more challenging since access to nutrients and oxygen becomes more restricted farther from the surface of concrete.

One of the key factors for in self-healing ability is the metabolic state of the microorganisms. Previous chapters in this dissertation have shown that inoculation of vegetative *S. pasteurii* cells can induce calcium carbonate precipitation in cement paste and that metabolically active cells were detected in specimens as old as 330-days (11 months). The state of the microorganisms is critical in the case of self-healing applications. Vegetative cells may provide immediate response to crack healing, but they may be more sensitive to environmental stressors than endospores. This may make vegetative cells more prone to death than endospores. Whereas endospores are more resistant to environmental conditions than are vegetative cells, but they may require some time to convert back into a vegetative form. Thus, endospores may be more useful for

long-term self-healing applications. In this phase of the work we will examine the self-healing capability of the cement paste matrix, as well as the metabolic state of the inoculated vegetative *S. pasteurii* cells.

Coda wave interferometry (CWI), an ultrasonic nondestructive testing method was employed in order to observe the self-healing process in mortar samples. The use of CWI provides for one to monitor the continuous development of self-healing of the sample. Strength recovery is also another aspect that has not been answered fully in the literature, especially with using the vegetative inoculation method. While sealing of cracks can be inferred by CWI, CWI does not indicate if the strength can be recovered. Therefore, a compressive strength test was accompanied along with the CWI analysis.

7.2. MATERIALS AND METHODS

7.2.1. Mixing and initial curing

Mortar mixes for neat and bacterial samples were prepared as explained in **Section 4.2.3.** The w/c and s/c for all the samples were 0.50. The bacterial paste mixes were prepared with concentrations of *S. pasteurii* varying from 2-6 x 10^7 CFU/mL (or 1-3 x 10^6 MPN/mL) and the cells were grown in a pH 9 UYE medium. Mixtures of neat and bacterial mortar samples were prepared and cast into 5.08 x 5.08 x 5.08 cm cube molds. The specimens were initially cured at 100% RH and at 25°C for 24 hours, followed by

UYE medium-saturated lime curing until 7 days. The viable *S. pasteurii* was enumerated via a most probable number (MPN) analysis at 0 (initial inoculum) and 35 days after mortar mixing (detailed description can be found in **Section 5.2.1.1**).

7.2.2. Microcrack initiation

In mortar specimens, as the applied axial load is increased, microcracks are induced, initiating from the weaker points within the specimen. At approximately 50% of ultimate strength of mortar sample, the bond between the aggregates and cement paste matrix weakens and cracks are introduced [82]. When the applied external loading increases to about 70% of the ultimate strength, cracks are initiated in the paste matrix [82]. It has been also suggested that the permeability of the concrete is not influenced by the microcracks induced until the load reaches 75% of the maximum strength [83].

A compressive strength test was conducted on triplicates of samples at 7 days of age in order to determine the ultimate strength of the samples. After the ultimate strength of the sample was determined, then the remaining samples were loaded to 70% of their ultimate compressive strength for each mix. In total three mixes were prepared with the same composition and initial curing conditions for both the bacterial and neat mortar. Initial coda wave measurements after loading proved that loading to 70% of compressive strength was adequate to induce internal microcracks. Each set of bacterial and neat mortars were kept at different curing regimes.

7.2.3. Exposure conditions

The neat and bacterial mortar samples were cured in a UYE medium saturated with lime for 7 days and then subjected to loading to initiate microcracks. After cracks were initiated on 7-days old mortar samples, they were exposed to three different regimes. One set of the samples was submerged in an UYE medium. While this condition is ideal for having the UYE medium penetrate into the sample and thus be used by the bacteria, this UYE submersion is not realistic for the field (although UYE ponding could be possible in certain applications). When a concrete product is placed into service it may be exposed to periods of rewetting (e.g. pavement) or it may be protected from the elements and thus only exposed to air (e.g. internal columns). Ideally, the preferred situation for a self-healing material is that no additional external treatments or human intervention is needed to initiate the self-healing process. Thus additional exposure conditions that could are more realistic for field applications, such as spray curing and air curing, were evaluated. Two additional sets of the neat and bacterial mortars were prepared and one set of samples were kept at 23°C in air, while the other set of samples were sprayed with the UYE medium until the full saturation obtained in the samples, spraying was conducted in 3-day time intervals after microcracks were induced. The saturated mortar samples exposed to UYE medium submerging were dried to SSD condition before testing.

7.2.4. Coda wave interferometry

CWI is a recently developed analysis technique used to obtain the relative velocity change in sound waves from diffuse fields, which are obtained from a fixed source and a receiver at two different moments of time. It exploits the increased sensitivity of multiple scattered elastic sound waves with long travel-times for monitoring weak changes in a medium before and after the perturbation [84, 85]. Sound waves propagate through the medium within a defined time period and whenever a change develops, such as cracks and pores, the velocity of the waves is correlated with the variance in the environment. When the relative velocity of a wave changes from time zero in a definite time frame, then this suggests that a change in the path of the waves have occurred; thus indicating the medium has changed. Table 7.12 shows the velocity of sound waves in different environments. In presence of a disturbance (e.g. cracks), the propagated waves scatter and alter their path, thus the velocity changes with respect to the initial velocity.

Table 7.12: Velocity of sound waves in water, air and mortar (m/s).

Medium	Velocity (m/s)
Water	1500
Air	3400
Mortar	3000

Considering the analysis of the time domain, there are two different CWI techniques: the doublet technique and the stretching technique. In this work, analysis has been conducted via the stretching method, wherein the axis of a time domain signal is stretched (or compressed) by the relative velocity change factor so that that the signal has much in common with the reference time signal.

Ultrasonic signals were measured on the cubes before loading and continuously measured during the pre-loading and after the loading for 28 days. For the measurement 7 mm diameter x 0.2 mm thick piezoelectric sensors were mounted to two opposite surfaces of the cubes with waterproof epoxy. Figure 7.35 shows the test set up for CWI analysis. The input signal was a 100V, 500 kHz square wave pulse generated by an Olympus Panametrics 5077PR pulser- receiver (Houston, TX, United States). A square pulse is known to be a periodical non-sinusoidal wave, wherein the amplitude alternates at a steady frequency in a fixed time frame. Samples were cured at different curing regimes after pre-loading. For the samples exposed to a liquid curing medium (i.e., the submerged sample and the sprayed sampled) the samples were brought to an SSD condition before the measurements were conducted.

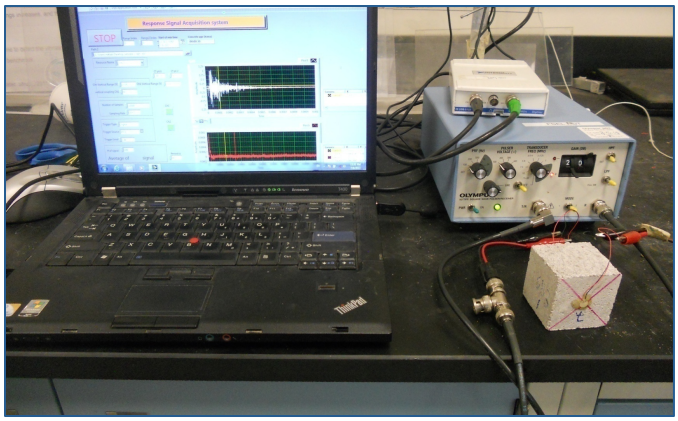

Figure 7.35: Test set-up for CWI analysis. Olympus Panametrics 5077PR pulser attached to sensors and to the cube.

Uncracked control samples for coda wave velocity and the same measurements were taken along with cracked samples, and the control samples were cured in different curing regimes.

7.3. RESULTS AND DISCUSSION

7.3.1. Impact of biomineralization on crack healing: Coda Wave Interferometry Analysis

Coda wave interferometer analysis showed that the relative velocity change in the cracked bacterial mortar samples was higher than the cracked neat samples and both of uncracked mortars. Figure 7.36 represents the difference in velocity in uncracked

bacterial and neat mortar samples cured in three various conditions after prior curing in the UYE medium saturated with lime for 7 day.

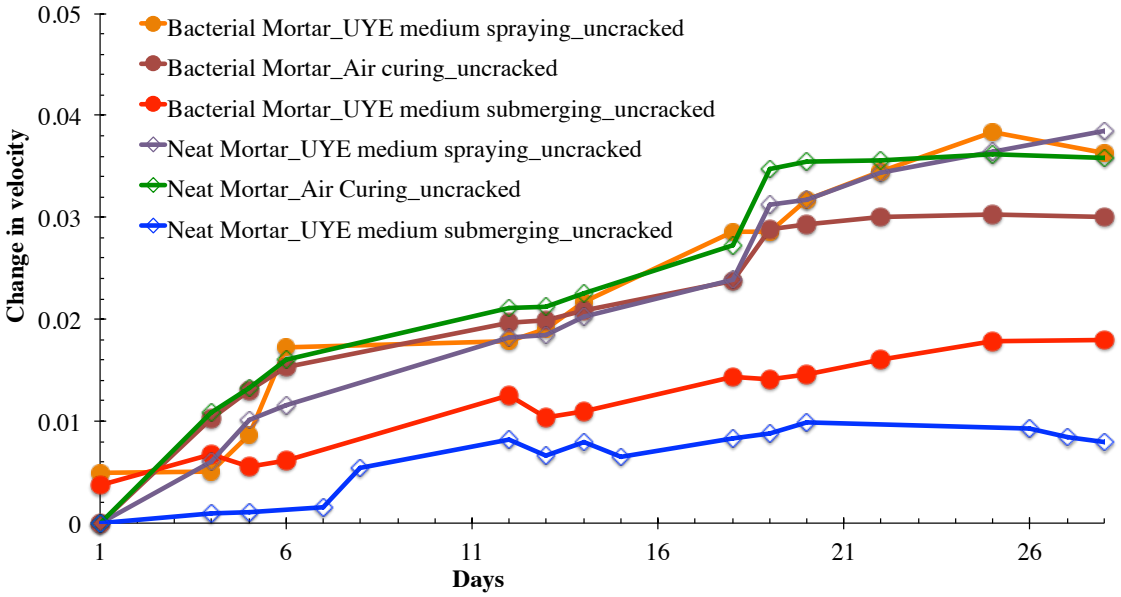

Figure 7.36: Relative velocity change in uncracked bacterial and neat mortar cured by UYE medium submerging, air curing and UYE medium spraying (t=1, 8th day after mixing). The w/c and s/c ratio were 0.50.

It can be seen that the CWI profiles of all the samples display a positive slope. It is expected that the CWI profile for these samples should not be horizontal line; rather the CWI profile should change with time to reflect the higher degree of wave scattering that is resulting from the changes that are occurring as hydration products form. Both the uncracked neat and bacterial mortar samples cured in air and UYE medium spraying showed a similar trend in velocity change, which suggests that the microstructural

development in both sample were similar. The neat and bacterial mortar samples submerged in the UYE medium showed considerably lower changes in velocity compared to the samples cured in other conditions (i.e. air and spraying). This is attributed to the difference in saturation level of the samples. If the cracks are fully saturated, the waves could be transmitted through the crack even though the velocity has changed with respect to initial velocity shown in Figure 7.36 [86]. Thus the velocity change in samples that were submerged in UYE medium showed lower velocity change profiles compared to spraying UYE medium and air curing due to changes in the wave path.

Figure 7.37 presents the velocity change in the cracked bacterial and neat mortar samples cured in a UYE medium. The cracks were induced by loading the samples up to 70% of their compressive strength after 7 days of curing in a UYE medium saturated with lime. It is seen that the velocity profiles of the cracked neat mortar samples display a similar degree of change as compared to the velocity profiles of the uncracked neat mortar sample. This is attributed to (1) higher amount of scattering that occurs due to the presence of the microcracks, and (2) due to the changes in the microstructure that is being provided from autogenous healing. Autogenous healing is a natural phenomenon occurring in cement-based material, and it has been reported that cracks even up to 0.3 mm can be sealed in presence of moisture [81]. The results from this phase of the

research suggest that the microcracks could be plugged and/or sealed via calcium carbonate precipitation more than the plugging/sealing provided by autogenous healing.

The change in the CWI profile of the cracked mortar samples in comparison to the uncracked samples indicates that there was a change in the microstructure due to initiation of cracks. The cracked bacterial mortar displayed a higher relative velocity change than the control cracked neat mortar. This suggests that there is a greater amount of change occurring within the cracked bacterial mortar over time than the cracked neat mortar, and we attribute this change to formation of products from the biomineralization products that are plugging/sealing the microcracks. The decrease in the relative velocity that occurred in the cracked bacterial mortar might be attributed to an error due to detachment of sensors from mortar surface such that the pulse waves were not transmitted properly.

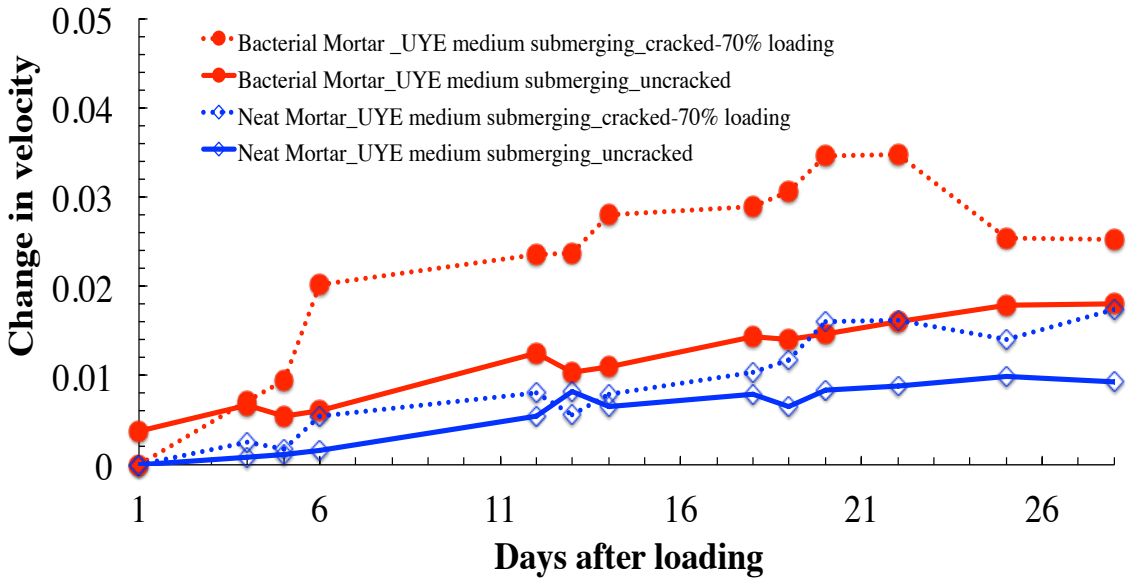

Figure 7.37: Relative velocity change in cracked (loaded to 70% of its compressive strength) and uncracked mortar cured in UYE medium after loading. The w/c and s/c ratio were 0.50.

The difference between the cracked bacterial and the cracked neat mortar samples that were sprayed with the UYE medium showed a similar trend (see Figure 7.38) as the UYE medium cured samples in which the time evolution of the CWI relative profile displayed the highest relative velocity change for the cracked bacterial sample. However, the velocity profile of the cracked neat mortar with spraying did not show any difference in comparison to the uncracked samples. Therefore any changes in the bacterial mortar profile for the sprayed sample is likely more due to healing of the cracks by deposition of

the microbial induced calcium carbonate rather than the influence of the autogenous healing.

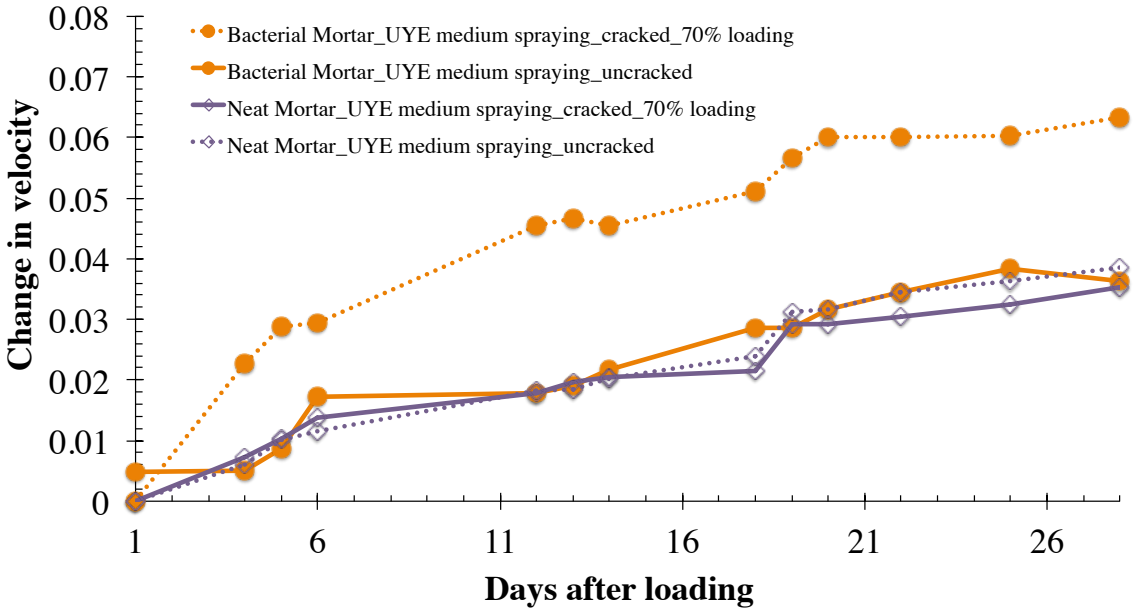

Figure 7.38: Relative velocity change in cracked (loaded to 70% of its compressive strength) and uncracked mortar cured by spraying UYE medium after loading. The w/c and s/c were 0.50.

Figure 7.39 depicts the coda wave profile for the uncracked and cracked mortar samples cured in air. Similarly the cracked bacterial mortar cured in showed the highest velocity change in comparison to other samples cured in air. This result was in a correlation with the mortar samples cured by UYE medium submerging and air curing. The cracked neat mortar showed the same trend with the uncracked samples up to first 6 days after cracking. However at later ages, the autogenous healing was slower than the

hydration of the mortar, and may not be adequate to heal the cracks. As it was suggested earlier, autogenous healing requires moisture to progress and in case of air curing no additional water was provided to the mortar samples, which would inhibit to regenerate the hydration.

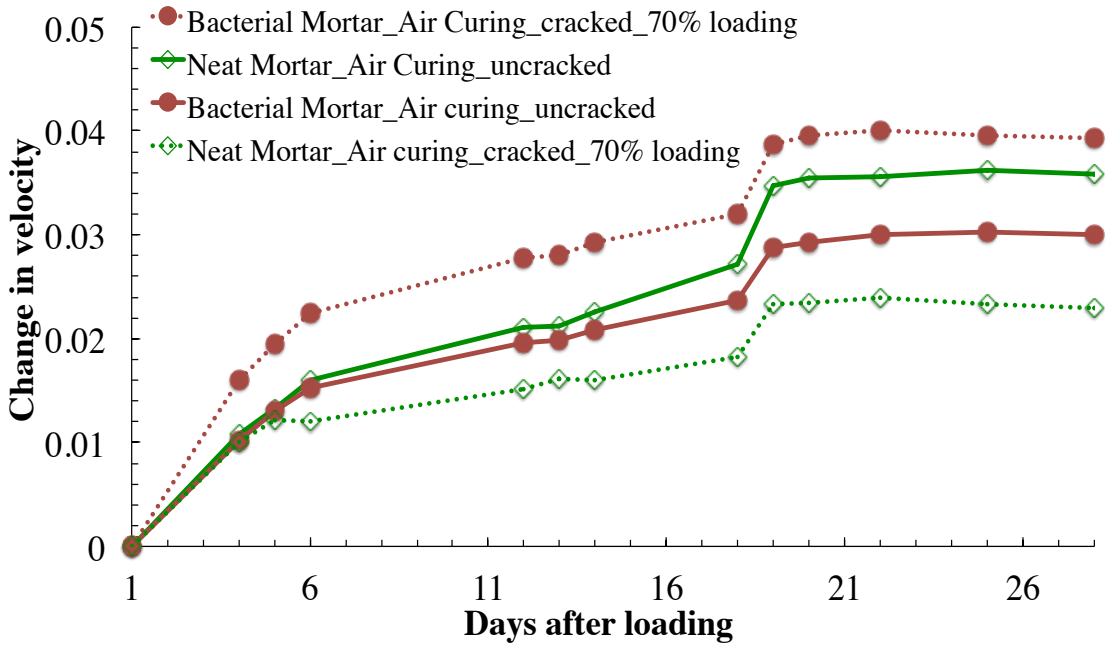

Figure 7.39: Relative velocity change in cracked (loaded to 70% of its compressive strength) and uncracked mortar cured in air after loading. The w/c and s/c ratio were 0.50.

Considering the relative velocity change in bacterial mortar samples cured at different conditions after loading to 70% of their compressive strength, the highest relative velocity change was observed in the samples exposed to spraying with UYE medium spraying (Figure 7.40). The lowest relative velocity change among the three

bacterial mortar sample was observed in the sample that was cured in an UYE medium. This is attributed to the sample being fully saturated. Simple air curing was adequate for the bacterial mortars to provide a change in the medium, thus indicating the possibility of having crack sealing with calcium carbonate precipitate without the need to apply additional moisture. Even though air curing provides self-healing without any external intervention, it would likely be suitable for freshly cast concrete that requires moisture for hydration to prevent shrinkage. Moreover, the applicability of air curing to an old structure (older than a year) is questionable. The results presented showed the bacteria require additional nourishment to provide more efficient self-healing. Therefore, considering crack healing applications, spraying UYE medium could be the most effective way to remediate an old bacterial concrete structure.

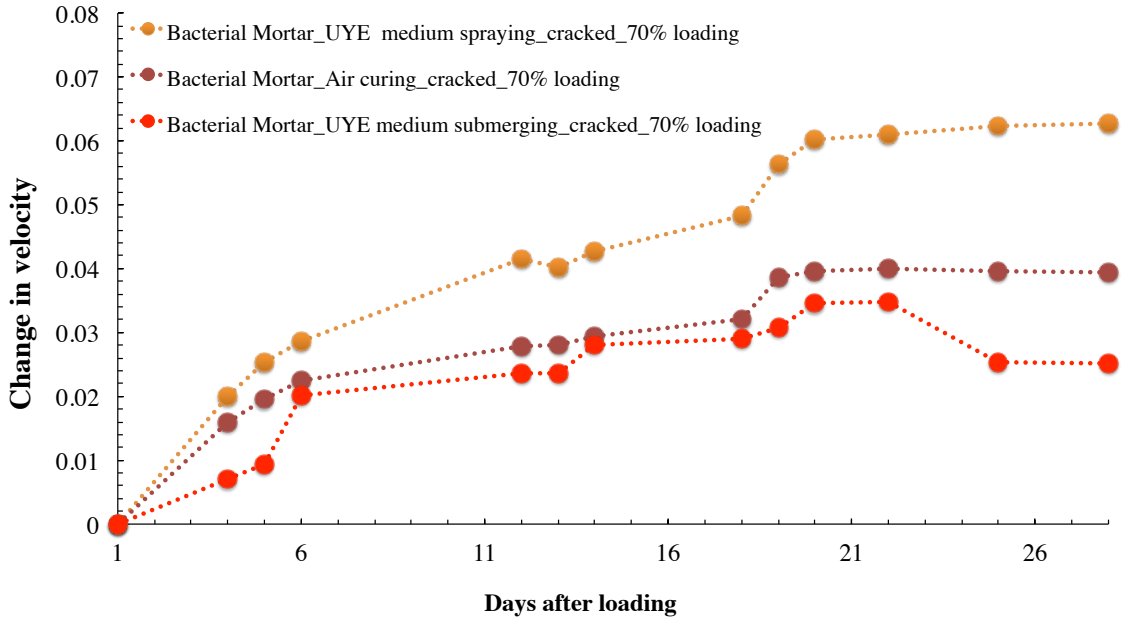

Figure 7.40: Relative velocity change in cracked (loaded to 70% of its compressive strength) bacterial and neat mortar cured in UYE medium, air and UYE medium sprayed after loading. The s/c ratio was 0.50.

7.3.2. Impact of biomineralization on self-healing: Compressive strength recovery

While CWI provides an indication of the changes occurring in microstructure, it does not provide information about the mechanical properties. Thus compressive strength testing was conducting to determine whether the change in the microstructural development suggested by the CWI analysis is reflected in the macroscopic mechanical behavior of the mortars. According to the CWI analysis, the bacterial mortar samples would be expected to recover strength more than the neat mortar samples. Figure 7.41 presents the results of the compressive strength tests, and the results of the compressive

strength test shows that inoculation of vegetative *S. pasteurii* cells heals the microcracks. The strength values of bacterial mortars were higher compared to the neat mortar samples at 7 and 28 days after loading regardless of curing regime.

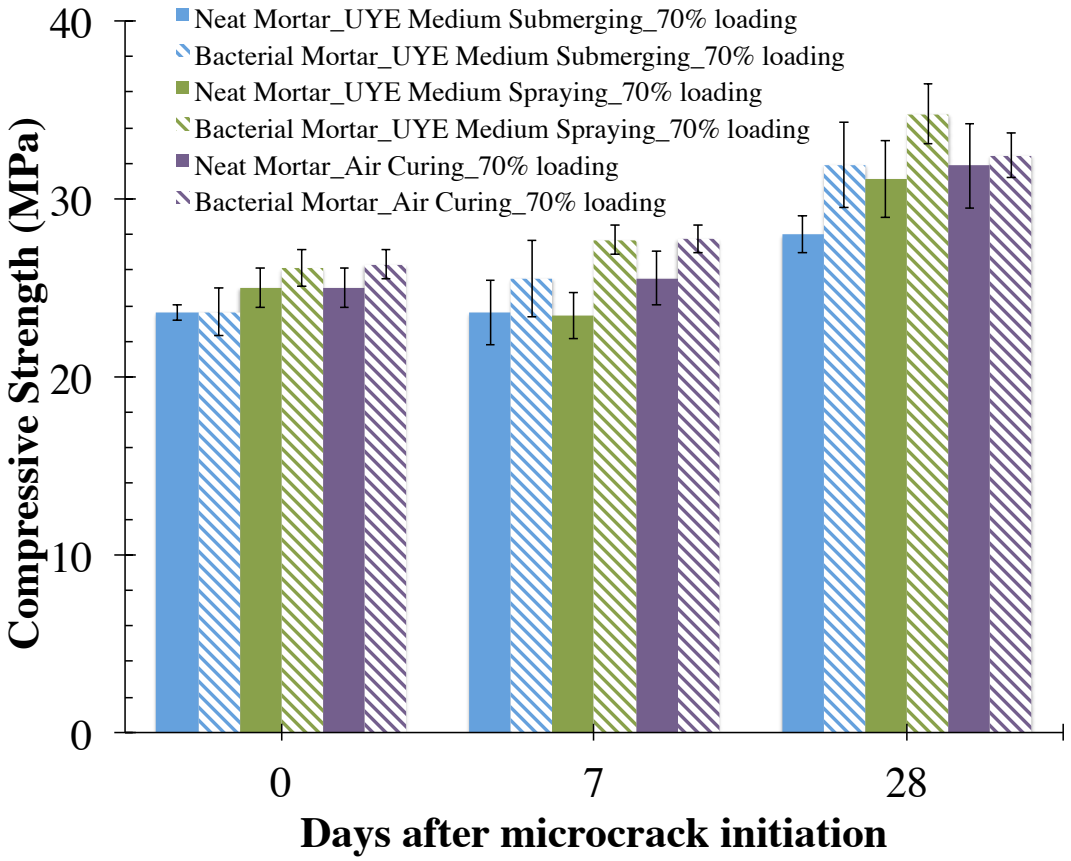

Figure 7.41: Compressive strength in cracked bacterial and neat mortar (loaded to 70% of its compressive strength at time 0) cured by UYE medium submerging, air curing and UYE medium spraying. The w/c and s/c ratio were 0.50. The bars show average compressive strength and error bars show the standard deviation of triplicates of samples. The compressive strength values at 0 days refers to the strength of the pristine sample (i.e. prior to microcracks initiation).

At time 0, the compressive strength of the 7-day old bacterial and the neat mortar were determined within the same range for all the samples. Note that the mix design of the bacterial and the neat mortar and the initial curing conditions were the same. After the cracks were initiated, the strength recovery in the bacterial mortars exposed to UYE spraying and UYE submersion was higher than their companion neat mortar specimens at 28 days. In general, the mortar samples submerged in UYE medium showed a lower strength than the samples exposed to UYE medium spraying and air curing. This was expected due to the fact that the saturated samples generally exhibit lower strength values in comparison to dry samples [87]. Compared to the compressive strength values of the pristine bacterial mortar samples (i.e., strength values at 0 days in Figure 7.40), at 7 days after the microcracks were initiated an increase in compressive strength was obtained in all the bacterial mortar samples, regardless of their exposure conditions. However, the strength values for the neat mortar samples at 7 days after microcrack initiation were similar to or even slightly lower than the compressive strength values of the pristine neat mortars. Table 7.13 shows the percent change in compressive strength for the samples with respect to their uncracked control samples. A 0% change means the strength of bacterial mortar recovered completely while a positive change indicates an increase in compressive strength in cracked mortar samples in comparison to its uncracked control sample. A negative change represents a partial recovery in the strength. At t=28 days of

curing after loading, none of the cracked samples showed a positive change in the compressive strength. However, the percent change cracked bacterial mortar sprayed with UYE medium was in the range of 0%, which could be considered to be a full strength recovery. The crack neat mortar cured via UYE medium spraying has a negative change of approximately 10%, which suggests providing moisture only provided a partial recovery.

Table 7.13: The percent recovery in compressive strength of bacterial mortar in comparison to control uncracked mortar after 28 days of curing

UYE Medium Submerging		UYE Medium Spraying		Air	
Neat	Bacterial	Neat	Bacterial	Neat	Bacterial
-18.1±0.9%	-9.4±0.1%	-9.2±2.0%	-1.1±2.5%	-26.7±1.8%	-24.5±1.6%

Even though, submerging the cracked bacterial mortar samples in UYE medium provided a partial recovery, the cracked bacterial mortar showed a more effective improvement in strength compared to cracked neat mortar samples cured by the same method. Unfortunately, the cracked bacterial mortar samples cured in air without any external curing process applied did not show any improvement in the strength in comparison to that of the cracked neat mortar samples. Therefore it could be suggested that to provide strength recovery in bacterial mortar where internal microcracks are present, additional nutrients should be supplied to system, preferably by spraying.

Considering the recovery in the compressive strength, the bacterial mortar samples that were exposed to UYE medium spraying showed the best performance at all-time intervals. This is in direct agreement with the velocity profiles obtained from CWI analysis, such that spraying of the nutrients resulted in CWI profiles that display a high degree of velocity change. This was believed to be due to the biomineralization process forming products that plug/seal the cracks. The results from this portion of the work provides an indication that not only do the calcium carbonate products that results from the presence of vegetatively inoculated *S. pasteurii* cells plug/seal the cracks, but that also ***healing*** of the cracks can occur. The bacterial mortars showed a better performance in compressive strength (and thus crack remediation) vs. their companion neat mortar specimens at 7 and 28 days regardless of the exposure conditions (see Figure 7.41). The overall trend of enhanced crack remediation could be directly related to the metabolic activity of *S. pasteurii* cells. Referring back to **Section 5.3.1,** after 7 days of curing in the UYE medium, the estimated viable *S. pasteurii* concentration in the mortar samples was $2x10^5$ MPN/mL, which was 20% of the initial concentration[7] of the inoculum. Furthermore, 83% of the total viable cells were identified as vegetative cells. Since the spray curing resulted in better self-healing results than UYE submerged curing, it is

[7] Initial concentration refers to the concentration of the bacteria in the inoculum and it was determined from samples of the inoculum that were taken immediately before mixing the cement with the inoculum. This was determined via MPN analysis. See **Section 5.2.2.1** for further details.

hypothesized that the samples subjected to spray curing have a higher percentage of vegetative cells than the UYE submerged specimens. If this is true, then crack-healing process might be directly correlated with metabolic state of the cells in in the mortar. In the following section, we will examine whether this hypothesis is true.

7.3.3. Influence of metabolic state of *S. pasteurii* on self-healing

In **Section 6.3.3** it was showed that the increase in compressive strength was mostly influenced by the viable cell concentration particularly the vegetative cell percentage within the cement paste matrix (see Figure 6.33 and Figure 6.34). This is important because the metabolic state of the microorganisms could affect the time frame for the crack remediation, such that endospores would require additional nutrients and a recovery period to activate the intercellular urease enzyme. However, the remaining vegetative cells after the mixing process could produce the urease enzyme and might not require a recovery period for calcium carbonate precipitation. Thus there could be a time lag between the point at which calcium carbonate precipitation is initiated with endospores and the point at which calcium carbonate precipitation is initiated with vegetative cells. Consequently, the vegetative cells could provide an immediate remediation for the microcracks. The MPN analyses of the neat and bacterial mortar at 35 days after mixing (28 days after loading) are summarized in Figure 7.42. The control neat mortar cured in the same conditions did not yield any detectable turbidity in the tubes,

thus indicating that there is few to no viable cells present in the neat mortar samples. In contrast, there was a substantial viable cell concentration in the bacterial mortars.

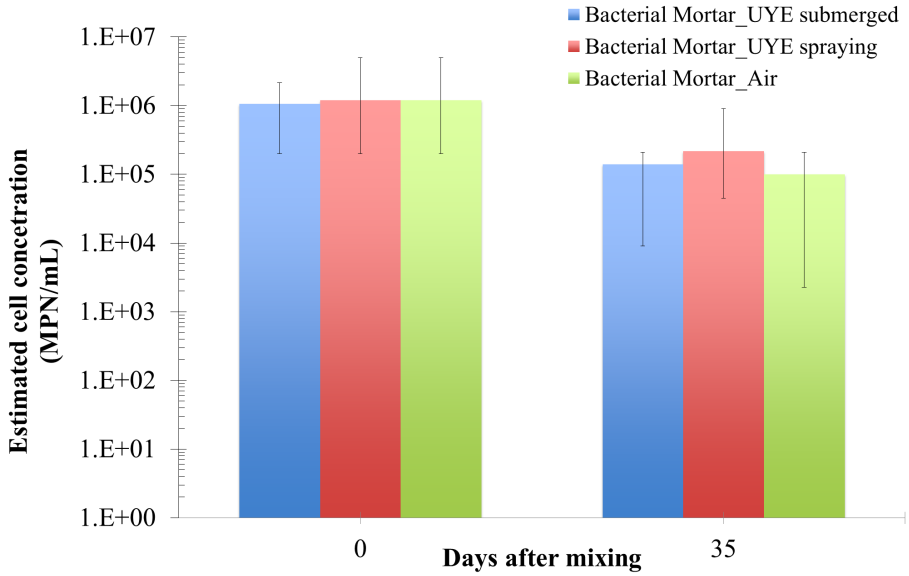

Figure 7.42: Viability of *S. pasteurii* in cracked bacterial mortar was cured in UYE medium, air and UYE spraying. The s/c was 0.50. The error bars show the upper and lower limit for the viable cell concentration. The initial bacterial concentration in the inoculum before mixing is shown at day 0.

The vegetative cell concentration in the 35-day old samples (see column 1 in Table 7.14) was calculated by subtracting the endospore concentration from the total viable cell concentration at 35 days. The vegetative cell percentage (column 2 in Table 7.14) was calculated by dividing the vegetative cell concentration (column 1 in Table 7.14) by the initial vegetative cell concentration in the mixing inoculum (see the bars at 0

days in Figure 7.42[8] and footnote 7) and multiplying that result by 100%. This vegetative cell percentage can be considered the worst-case scenario in which cells that were not detached are considered to not be vegetative. Note, it is not known whether all the cells were detached or the metabolic state of any cells that remained in the sample. However, the vegetative cell percentage of the viable cells that were able to be detached from the cement paste matrix can be calculated. The vegetative cell % of the detached viable cells (see Column 3 in Table 7.14) was determined was calculated by dividing the vegetative cell concentration (column 1 in Table 7.14) by the viable cell concentration at 35 days (see bars at 35 days Figure 7.42).

Table 7.14: Vegetative *S. pasteurii* concentration and remaining vegetative cell % in bacterial mortar after 35 days mixing. Samples were exposed to UYE medium submerging, UYE medium spraying and air curing. The s/c was 0.50.

Curing Condition	Vegetative Cell Concentration (MPN/mL)	Vegetative Cell % (compared to vegetative cell concentration detected in the mixing inoculum)	Vegetative Cell % of detached cells (compared to total viable cells detected at t=35 days)
UYE medium submerging	$1x10^5$	13%	99%
UYE medium spraying	$2x10^5$	18%	98%
Air curing	$9x10^4$	8%	93%

[8] These values are stated in **Section 7.2.1**.

At 0 days, the *S. pasteurii* concentration for all the samples was estimated to be $1x10^6$ MPN/mL, and the percentage of endospores was negligible (0.04%). Thus, the initial inoculum was primarily composed of vegetative cells. At 35 days, the remaining viable cell concentration was found to be in the same range for all curing conditions; however the vegetative cell percentage was influenced by the environmental conditions of the sample. $1x10^5$ MPN/mL of the initially inoculated $1x10^6$ MPN/mL vegetative *S. pasteurii* were remained viable in the mortar that was cured in the UYE medium. Moreover, 99% of total viable cells were identified as vegetative cells. This may be attributed to the lack of oxygen availability when the samples are submerged. *S. pasteurii* is an aerobic bacteria and oxygen is a critical nutrient for aerobic bacteria [72]. Results obtained in this study suggested that submersion as curing technique might not be the best way to keep the bacteria viable, rather spraying of UYE medium periodically provided both oxygen and required nutrients, thus keeping more cells viable.

The highest compressive strength recovery and the relative coda wave velocity change were observed in the bacterial mortar that was periodically sprayed with the UYE medium. As hypothesized, the MPN analysis conducted on samples cured with UYE medium submerging and UYE spraying yielded higher vegetative cell concentrations, $2x10^5$ and $1x10^5$ MPN/mL respectively, when compared to air cured bacterial mortar samples.

With respect to the bacterial mortar that was air cured, approximately 9 x 10^4 MPN/mL of vegetative cells were remained. This is a relatively high concentration, however a lower degree of strength recovery at 35 days was obtained for these samples. This may be due to the fact that additional nutrient sources were not provided to the system. The result suggests that the portion of vegetative cell and their availability to nutrients play a role on crack remediation. As stated earlier (See Section 5.1) since the vegetative cells are capable of continuous urease enzyme production; it is the vegetative cells, and not the endospores that are capable of producing carbonate ions for calcium carbonate precipitation.

Chapter 8: Conclusion and suggestions for further research

8.1. CONCLUSION

This study investigated a novel approach providing microbial induced calcium carbonate precipitation within the cement paste matrix by incorporating the vegetative *S. pasteurii* cells. Results presented herein provide a better understanding of biomineralization in cement-based materials. Thus, this research advances the development of a bio-smart material, which can self-heal internal microcracks rather than solely surface crack remediation as was suggested in literature. The following section in this chapter summarizes the key findings of this research.

8.1.1. Key findings

- **Polymorphs of calcium carbonate was influenced by calcium concentration and pH**

Results of the work presented showed that at the ATCC recommended optimum pH for *S. pasteurii* growth (a pH of 9), the calcium carbonate morphology was not affected by the calcium concentration. Both vaterite and calcite were observed in XRD analysis, regardless of the calcium concentration. To stimulate cement paste pore solution conditions, a culture with low calcium concentration (0.03M) at high pH was prepared and calcite was the favorable polymorph. On the other hand, at high calcium

concentration (0.167M), vaterite precipitation was observed. Moreover, in cement paste, calcite was found to be the favorable polymorph of calcium carbonate induced by microorganisms. Opposed to what was determined in ex-situ cultures, the calcium concentration did not have any influence on morphology of calcium carbonate in cement paste. While vaterite was observed in ex-situ growth cultures when the calcium ion concentration increased to 0.167M, calcite was observed in cement paste even though the calcium concentration increased to 0.167M.

- **Inoculation of vegetative *S. pasteurii* delayed the initial set of cement paste**

The hydration kinetics and setting behavior of cement paste were highly influenced by the addition of urea-yeast extract medium; the initial setting was delayed due to incorporating yeast extract to the mix. The isothermal calorimeter analysis and modified Vicat needle test indicated that the delay in hardening process was longer in spent nutrient UYE paste than in nutrient paste. Presence of *S. pasteurii* cells made this delay more pronounced, which may be due to the presence of the cells influencing the pore solution by precipitating calcium carbonate and producing by-products.

- **Inoculation of vegetative *S. pasteurii* was able to precipitate calcium carbonate in cement paste without any additional calcium source provided**

Results of TGA suggested there was a considerable increase in calcium carbonate content with the addition of viable cells to the cement paste. This suggests that biomineralization could be obtained within the cement paste matrix without any additional calcium source.

- **Biomineralization decreased the porosity and increased the compressive strength of cement paste and mortar**

Increase in calcium carbonate content resulted in a decrease in porosity as measured by ethanol replacement of water, which was also correlated with the increase in electrical resistivity in both bacterial pastes and mortar in comparison to neat paste and mortar. The change in porosity was more noticeable in mortar samples than in cement paste samples. At 28 days, a 24% reduction was observed in bacterial mortar in comparison to neat mortar, while this change was as low as 9% in cement paste samples.

The decrease in porosity correlated to an increase in compressive strength of both cement paste and mortar. The compressive strength of cement-based materials increased considerably with the addition of vegetative *S. pasteurii* cells while the urea-yeast extract medium decreased the compressive strength. The influence of microorganisms on

compressive strength was attributed to the addition of viable cell with urea-yeast extract medium.

- **Vegetative *S. pasteurii* cells were detected viable in up to 330-day old mortar samples**

Vegetative *S. pasteurii* cells, which were inoculated without any protection, were found to be viable up to 330-days in mortar with approximately 2% viability retention. Moreover 50% of these viable cells were detected as vegetative cells, which could be metabolically active. It should be noted that this is the first time that viability of *S. pasteurii* in cement-based systems was investigated and it has been the longest survival period recorded for microorganisms in cement-based materials without any encapsulation. In addition, porosity of the cement-based material might have been influential on viability when vegetative *S. pasteurii* cells were inoculated in mortar such that, the viability retention of the cells was increased.

- **Delayed nutrient release provided via internal curing reservoirs increased the vegetative cell retention in bacterial mortar.**

Estimated viable cell concentration and the vegetative cell percentage were found to be higher with 25% expanded shale replacement of river sand by volume with both s/c ratios of 0.50 and 0.30. Increase in vegetative cell concentration also improved the

compressive strength of bacterial mortar at later ages. At 90 days, control samples with bacterial mortar (s/c=0.50) showed a decrease in strength without any lightweight aggregates, while this decrease was less pronounced when 25% internal nutrient reservoir was provided. Results obtained from the compressive strength test were found to be influential with viable cell concentration, particularly vegetative cell percentage. The increase in compressive strength in bacterial mortar samples with s/c of 0.30 was even observed at 1 and 7 days, since the samples were initially placed in drying conditions.

- **Vegetative *S. pasteurii* cells were able to remediate internal microcracks and recover compressive strength**

The compressive strength and coda wave interferometry (CWI) results indicated that when internal microcracks were induced at early ages, the presence of vegetatively inoculated *S. pasteurii* cells in a UYE medium might remediate internal cracks. A higher velocity change was observed in the cracked bacterial mortar compared to the uncracked samples and the cracked neat mortar samples. In addition, a greater strength recovery was observed in the bacterial samples that were cracked versus the neat cracked samples. The compressive strength recovery and CWI results were also dependent on the exposure condition (e.g. air cured, UYE cured, or UYE sprayed) of the sample, and the samples sprayed with a UYE medium showed the highest recovery in compressive strength and CWI. Coupled together the results suggest that crack remediation is influenced not only

by the presence of the vegetative cells, but also the exposure condition of the sample. Samples in which nutrients were supplied (i.e., UYE submerged samples and UYE sprayed samples) had higher compressive strength recovery values than the samples in which nutrients were not supplied (i.e., air cured samples).

8.2. FUTURE WORK

The current research introduced the possible use of biomineralization by investigating the outcome of incorporating the vegetative microorganisms without any protective barrier and their behavior in cement-based systems. However there are still several unanswered questions, which would provide a better understanding to the approach and lead to a possible material that will be used in the field. Suggestions for future work are provided here:

- One of the main concerns is the bonding ability of microbially induced calcium carbonate with the cement-based material. According to our results, calcite was favored over vaterite in cement paste. One of the advantages is of precipitating calcite via MICCP is that it is a more stable and stronger polymorph than vaterite. However the bonding strength between the microbially induced calcium carbonate and cement has not been investigated yet. The results of a study that investigates the bonding strength between the precipitate and the cement-based

substrate will provide the information regarding healing ability of the microorganism and durability of the application.

- According to Jonkers [88], a "true self-healing material" should be able to continuously provide self-healing in repeated cycles, and that the self-healing material should not be consumed during the process. Even though, our results suggested crack healing was observed when cracks were initiated at 7 days, it is not clear that inoculation of vegetative microorganisms can heal cracks multiple times if the cracks reoccur. To determine the influence of biomineralization on repeated cracks, cycled loading should apply to the already biomineralization treated mortar samples.
- The viability results obtained in this research were promising for the self-healing applications. The inoculated vegetative *S. pasteurii* cells could be metabolically active up to 330-days. This period is still shorter than the service life of common buildings. Self-healing experiments including CWI and compressive strength tests should be conducted on older samples with various curing regimes.
- From this research it is apparent that some of the vegetative-inoculated cells die, some form endospores, and some are able to remain in a metabolically active state. Further research is needed to understand the factors, such as calcium carbonate precipitation on the cells that can influence the long-term survival of

the microorganisms. According to Zamarreño et al. [89] even the calcium carbonate precipitation influences the viability of microorganisms such that the cells embedded in calcium carbonate crystals were found to be viable up to 300 days. The impact of the MICCP process on the viability of the bacteria within cement paste matrix needs to be examined in further detail. It is not known whether the cells become fully embedded in the calcium carbonate crystals, and if they do whether they remain in vegetative state or become endospores. In addition it is not clear the influence that the surface charge of the bacteria plays on calcium carbonate precipitation. Can calcium carbonate precipitation occur from attraction of positively charged calcium ions to the bacterial cells that have negative surface charge? If so, what is the surface charge of vegetative cells and endospores? If endospores have a positive surface charge, can this be a means to attract calcium ions and cause calcium carbonate to be produced, even though the endospores are not metabolically active?

- The endospores require a recovery time to turn into metabolically active vegetative cells, which can create a delay in healing process. The conditions required providing recovery of endospores and the delay time due to reformation are still not clearly understood. Moreover, the influence of endospores on the properties of cement-based materials is not fully known.

- Detailed investigation regarding the extended induction period should be conducted and use of an alternative carbon source, such as lactose mother liqueur, instead of yeast extract should explored in order to overcome the impact of yeast extract on hydration kinetics.
- The hydrolysis of urea leads to production of a noticeably high concentration of ammonia. During mixing, the ammonia ions are also added to cement paste. The presence of ammonia ions secretes a strong smell. For the design of environmental friendly material, generation of ammonia is undesired. Thus, capturing the ammonia or adding another ingredient to interact with the ammonia could be examined. In addition, another nitrogen source that produces carbonate ions without the production of ammonia would be favored. Alternatively, investigations regarding the ability to inoculate different vegetative bacterial cells should be explored; these bacteria should produce carbonate ions yet still be viable when embedded in cement-based materials. An example for alternative microorganisms is *B. cohnii*, which is capable of decomposing lactate and produce carbonate ions; therefore urea is not required in this system. Jonkers et al. introduced in cement paste stone by natural encapsulation [8] and synthetic encapsulation [7], however it encapsulation might not be necessary to keep this microorganism viable in cement paste. Concerns with respect to the practical

application also hinder the application of biomineralization technique in building material. One aspect is the quantity of cement paste produced was limited to mortar samples. Incorporation of *S. pasteurii* in concrete with vegetative inoculation requires production of a larger volume of inoculum. Design of bacterial concrete will bring more advanced questions regarding to the efficiency of the biomineralization, such as influence of mixing and admixtures on metabolic state of microorganisms.

Appendix A: Abbreviations

ATCC: American Type Culture Collection

ASTM: American National Standards Institute

ACC: Amorphous calcium carbonate

C-FA: C types fly ash (Parish)

CH: Calcium hydroxide, portlandite

CFB: Calcite forming bacteria

CFU: Colony forming units

CWI: Coda wave interferometry

d_{50}: where 50% of the total powder volume consists of particles with effective diameter smaller than this indicated diameter

DIC: Dissolved inorganic carbon

DDI: Distilled deionized water

EDS: Electron diffraction X-ray spectrometer.

EPS: Extracellular polymeric substances

K_{sp}: Solubility constant

LSF: Limestone filler

LML: Lactose mother liquor

LWA: Lightweight aggregate

LOI: Loss on ignition

MPN: Most probable number

MICCP: Microbial induced calcium carbonate precipitation

MIP: Mercury intrusion porosimetry

NBU: Nutrient broth- Urea (8g nutrient broth- 2% urea)

OD_{600}: Optical density at 600 nm

θ: X-ray incident angle

PBS: Phosphate buffer solution

PCC: Portland cement concrete

PU: Polyurethane

RH: Relative humidity

RI: Refractive index

SEM: Scanning electron microscopy

SG: Specific gravity

SSD: Saturated surface dry

St.dev: Standard deviation

TGA: Thermogravimetric analysis

UYE: Urea-Yeast extract

XRD: X-ray diffraction

Appendix B: Use of Biomineralization in Developing Smart Concrete Inspired by Nature[9]

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Abstract

Recently, interest has focused on leveraging the biological functions of microorganisms to develop smart cement-based materials. This paper provides an overview of the calcium carbonate biomineralization process in nature and presents a review of the work conducted by various groups around the world on biogenic calcium carbonate formation as it relates to the hydration, microstructure, properties, and performance of cement-based materials. Promises and concerns of applying biomineralization in cement-based materials are also discussed, and directions for future research are explored.

Key words: Biomineralization, Self-healing, Bio-inspired, Smart Concrete

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[9] This paper was submitted to *International Journal of Materials and Structural Integrity* on August 2012 and accepted on March 2013. It has not been published yet.

1. Introduction

Biomineralization is a process, in which organisms influence the formation of minerals and almost 60 different biogenic minerals, mainly carbonates, phosphates, iron oxides, and sulfides, have been reported. According to some early research, many microorganisms show calcification mechanism, absorbing calcium cations on their cell surface and extracellular glycocalyx (a polyanionic polysaccharide matrix) (Costerton, Irvin, and Cheng, 1981; Beveridge and Fyfe, 1985). The calcium cations which accumulate on the cell surface may then act as nucleation sites for both gypsum and calcite precipitation (Thompson and Ferris, 1990a).

Interest in incorporating biomineralization in Portland cement concrete was prompted from questions regarding whether this type of mechanism could be used to develop a concrete that is able to repair itself when cracked. Internal stresses, due to shrinkage, improper curing, temperature changes etc., may induce microscopic cracks which will continue to propagate upon the application of additional stresses, and these cracks can provide pathways for harmful chemicals to ingress, and lead to loss of strength and integrity. Conventional methods of repairing cracks include patching with sealants, application of various coatings, or embedding the concrete with fibers to prevent the cracks from widening. However, the bonding strength between the new patching material and the old material is often a concern. Furthermore, with regards to repairing cracks using externally applied compounds, the time lag between initiation of the microcracks and their detection often increases the severity of the damage and may demand demolition and/or full replacement of the structure. In addition to self-healing applications, the possibility of using biomineralization as an approach to create a "natural concrete", (one with little to no portland cement) in which the biogenic materials serve as the cementing compound, has prompted research in this area.

2. Biogenic Calcium Carbonate Precipitation Mechanism

Calcium carbonate precipitation can be either biologically induced or biologically influenced (Dupraz et al., 2009; De Muynck, De Belie, and Verstraete, 2010). Biologically induced precipitation is a direct result of the microbial activities that generates the biochemical conditions favorable for precipitation, whereas biologically influenced precipitation is a passive process, in which an interaction between extracellular polymeric substances (EPS) of the microorganism and the surrounding geochemical environment promotes precipitation. Calcium carbonate precipitation by ureolytic microorganisms is a direct result of bacterial activities since the precipitation occurs due to the urea-hydrolysis by the microorganisms and hence belongs to the first category.

2.1 Biomineralization Reaction Overview

Many researchers have summarized the chemical reactions involved in the biomineralization process (Ferris et al., 1996; Warren et al., 2001; Mitchell and Ferris, 2006; Tobler et al., 2011). Generally, one mole of urea is hydrolyzed to produce one mole of ammonia and one mole of carbonic acid (equation 1). The ammonia reacts with one mole of water and produces one mole of ammonium and one mole of hydroxide ions (equation 2). The carbonic acid formed earlier can further react with hydroxide ions to form carbonate ions and water (equation 3).

$$CO(NH_2)_2 + 2H_2O \rightarrow 2NH_3 + H_2CO_3 \quad (1)$$

$$H_2COOH + H_2O \rightarrow NH_3 + H_2CO_3\, NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \quad (2)$$

$$2OH^- + H_2CO_3 \leftrightarrow CO_3^{2-} + 2H_2O \quad (3)$$

Hence the total reaction of urea hydrolysis is summarized as below:

$$CO(NH_2)_2 + 2H_2O \rightarrow 2NH_4^+ + CO_3^{2-} \quad (4)$$

In presence of calcium ions, the carbonate ion in equation 4 reacts with the free calcium ion and precipitate calcium carbonate.

$$CaCO_3(s) \leftrightarrow Ca^{2+} + CO_3^{2-} \quad (5)$$

The decomposition of urea to produce ammonia and carbon dioxide proceeds in chemical equilibrium, and to precipitate calcium carbonate the concentrations of carbonate and calcium ions must exceed saturation conditions (Tobler et al., 2011).

Many parameters can influence the precipitation process, however the following are often considered as the four key factors: (1) the calcium concentration, (2) the concentration of dissolved inorganic carbon (DIC), (3) the pH and (4) the availability of nucleation sites (Hammes and Verstraete, 2002). To identify the factors, which influence the amount of precipitates, Dick et al. (2006) studied five different strains of the *Bacillus sphaericus* group and one strain of *Bacillus lentus*. In their research, five parameters, including urea degradation rate, zeta potential, production of extracellular polymeric substances (EPS) and biofilm, and morphology of the precipitate were tested for different strains. The influence of varying pH value was also investigated. The result showed that the strains with a very negative zeta potential and high initial urea degradation capability were able to produce a continuous layer of dense calcium carbonate crystals.

The relationship between the calcium carbonate precipitation and microbial activity was summarized in a review papers by Hammes and Verstraete (2002). In this paper, the

researchers proposed that in a calcium rich environment, extracellular calcium concentration is generally 10^3 times higher than the intracellular concentration. This is due to energy being consumed by the microorganisms to pump calcium ions out of the cells, against the calcium concentration gradient. Hence, carbonate precipitation helps ureolytic microorganisms maintain their normal activity as it reduces the intracellular calcium concentration.

2.2 Morphology of Mineral Formed and Role of Substrate

To understand the biomineralization process and to investigate how different factors influence the calcium carbonate precipitation, the morphology of the precipitated crystals were studied by several researchers. Calcite, aragonite, vaterite, monohydrocalcite, ikaite and amorphous calcium carbonate (ACC) are six known forms of calcium carbonate, among which the first five have definite crystal structure (Addadi, Raz, and Weiner, 2003). Calcite and aragonite are more stable than vaterite and monohydrocalcite while ikaite is rarely produced biologically. The crystal structure of ACC has only short-range order and is generally formed as precursor of other more stable forms in chemical process. However, multiple polymorphs including ACC are known to coexist and often transform between different polymorphs.

The phase transformation process is well established in relation to the chemical synthesis of calcium carbonate. Rieger et al. (2007) chemically synthesized calcium carbonate by mixing solutions of sodium carbonate and calcium chloride. It was reported that amorphous calcium carbonate spheres form as precursors, which then aggregates to form micrometer sized vaterite spheres. At the end of the process, vaterite recrystallizes further to form calcite. Zhou et al. (2010) also studied the influence of pH and temperature on the polymorphs of the precipitate. They reported that the formation of vaterite is a result of aggregation of oriented vaterite microcrystals and the morphology of vaterite varies with pH and temperature (Figure 1). With time, rhombohedral calcite growth is reported among irregular aggregates of vaterite. This indicates dissolution of vaterite and reprecipitation as irregular calcite as calcite is favored in a chemical synthesis as the final product. In aqueous solution vaterite rapidly transforms into calcite or aragonite (Kralj, Brečević and Kontrec, 1997; Spanos and Koutsoukos, 1998). Zhou et al. (2010) mentioned that at room temperature and under normal atmosphere, orthorhombic aragonite and hexagonal vaterite are metastable polymorph and usually transform to calcite.

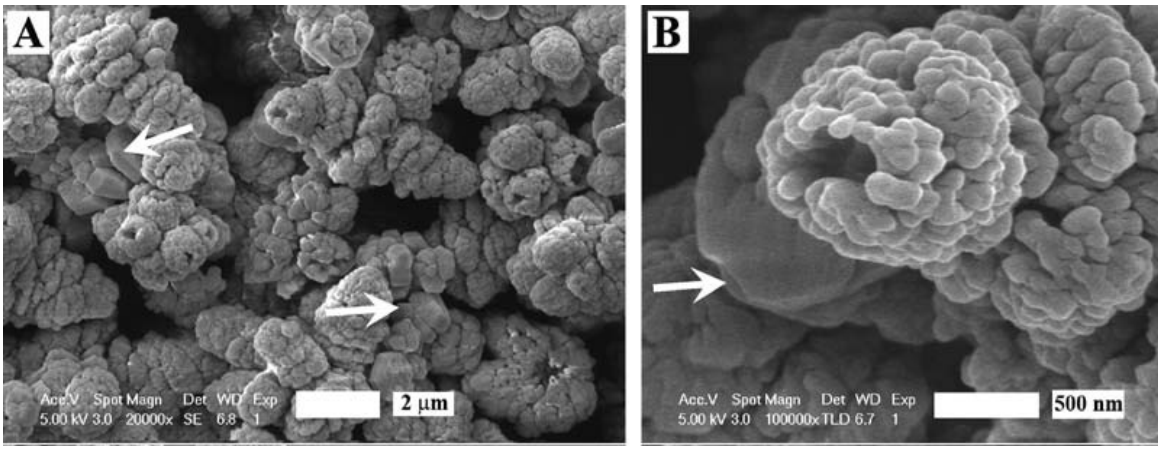


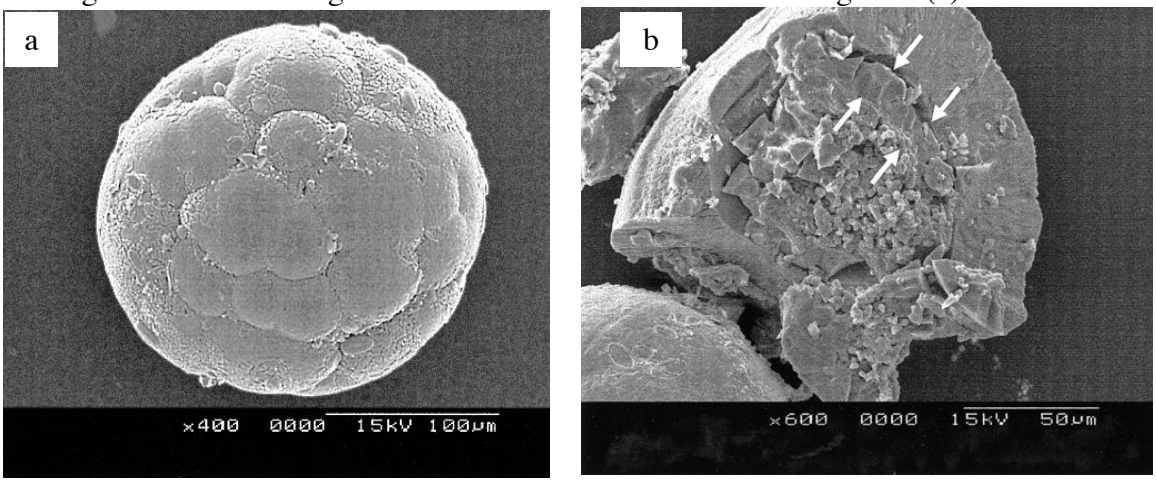
Figure 1. Field emission scanning electron microscope (FESEM) images of precursor vaterite (at two different magnification) aged for 5 h at 37°C at pH value of 1.5 (Zhou et al. 2010)

As the stability and bonding capacity of different polymorphs varies, understanding the transformation between different polymorphs in a biomineralization process will be important for its engineering application. Hammes et al. (2003) collected microbial strains from four different places: garden soil, land fill soil, concrete surface and a biocatalytic calcification reactor. By comparing the morphology of the precipitated crystals, DNA sequences and urease activity of sampled microorganisms, they concluded that for all strains, morphological differences of the precipitate come from different microbial urease enzymes; for some special strains, besides the enzymes, the influence of calcium on urease activity also caused morphological differences.

While studying biomineralization for removal of inorganic contaminants from ground water, Warren et al. (2001) observed that the morphology of the precipitates was altered by the presence of different contaminants. Based on scanning electron microscope (SEM) and atomic force microscopy (AFM) imaging, they reported that the precipitates in the presence of copper (Cu) group were dumbbell, whereas in presence of strontium (Sr) group, the shape changed to pseudopolyhedral.

Microorganisms sampled from natural environments, such as caves and soil, are reported to precipitate a mixture of different calcium carbonate polymorphs in laboratory conditions. Cacchio et al. (2003) reported that some calcifying strains collected from a cave could produce mixture of calcite and vaterite when cultured in laboratory. Mixture of different calcium carbonate polymorphs has also been found in natural environment such as lakes and caves (Giralt, Julia and Klerkx, 2001; Cacchio et al., 2003; Rusznyák et al., 2012).

Though calcite is the most chemically stable form, Rodriguez-Navarro et al. (2007) indicated that the existence of vaterite was more common in natural environments as a metastable polymorph. They suggested that the microorganisms and organic by-products from bacterial activity were critical for stabilizing the vaterite structure. Rodriguez-Navarro et al. (2007) studied *Myxococcus xanthus* induced vaterite formation using transmission electron microscopy (TEM) imaging. They reported that vaterite formed as small spherical mass (spherulite) in which branch-like vaterite crystals form radially outward from the microorganism cell wall. As the microorganism cell wall acts as nucleation site, the orientation of vaterite was reported to be governed by the electrostatic affinity between the crystal planes and the negatively charged functional groups of organic molecules on the cell wall. Giralt, Julia and Klerkx (2001) divided the crystal structure of vaterite biscuits forming in lake into four sections from inner core to the outer part based on the SEM result as shown in Figure 2 (a) and (b): (i) the core is a mixture of hundreds of bacteria, covered by a thin carbonate layer; (ii) the second section is a 7-8μm carbonate coating, that forms by the calcification of the mucilaginous sheath of the central bacterial mixture; (iii) the third section is a lamina formed by 30 μm-long radial vaterite crystals , and (iv) the outer part is a thin (2-3 μm) and discontinuous coating. A schematic diagram of the four sections is shown in Figure 2 (c).



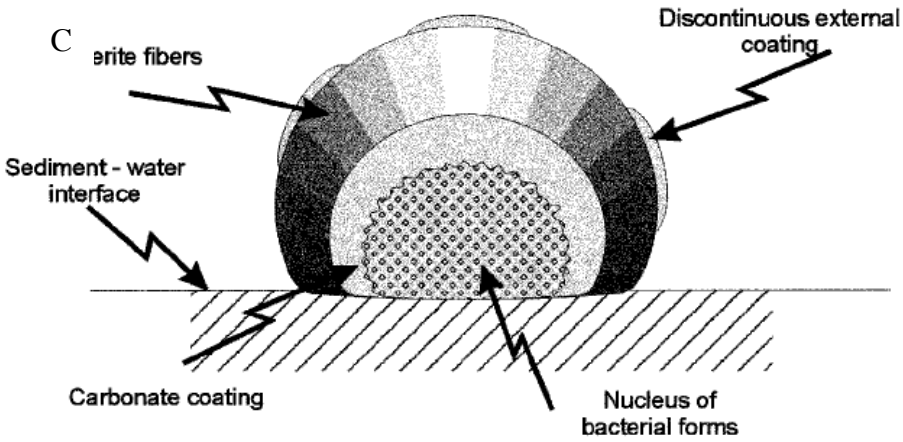


Figure 2. (a) Overview of single vaterite biscuit; (b) internal structure of vaterite biscuit; (c) Schematic diagram of the four sections of vaterite (Giralt, Julia and Klerkx 2001)

Since the orientation of precipitated crystals was observed to be affected by the negatively charged organic groups on the cell wall, the role of extracellular polymeric substances (EPS) or biofilm attracted interest of many researchers (Decho, 2010). EPS is reported to influence both the mm-scale properties of the minerals and the nanoscale structure of the carbonate crystals (Dupraz et al., 2004; Dupraz et al., 2009). On the mm-scale, the gel-like properties of EPS is suggested to influence both the diffusion process and adsorption/complexation of calcium ions, which subsequently affects the types of produced minerals. EPS matrix initially inhibits the precipitation process by trapping calcium ions into its porous structure from external solution. This inhibition is due to the acidic amino acids and carboxylated polysaccharides on EPS matrix, which can bond calcium ions. In contrast, the hydrolysis of EPS caused by bacterial metabolism and the decarboxylation of EPS caused by UV light, can lead to the release of the calcium ions. This increases the calcium ion concentration and can promote carbonate precipitation. On nano-scale, the final morphologies of the minerals were proposed to be affected by different enzyme kinetics during urea hydrolysis (Hammes et al., 2003).

3. Biomineralization in Nature

Many researchers have investigated biomineralization in soil and marine environment. Based on the research performed by Boquet, Boronat and Ramos Cormenzana (1973), which is later summarized by Hammes and Verstraete (2002), biomineralization is a common phenomenon as all bacteria isolated from soil were capable of inducing carbonate precipitation. Cacchio et al. (2003) isolated 31 calcifying strains from a limestone cave of central Italy and a loamy soil of northern Italy. Among these strains,

64% belonged to genus *Bacillus*, the others from genus *Arthrobacter, Kingella* and *Xanthomonas*. By examining the precipitates induced by these strains in laboratory condition using X-ray diffraction (XRD), it was reported that all strains precipitated rhombohedral calcite. In addition, two strains isolated from cave (incubated at 4°C and 22°C) and three strains isolated from soil (incubated at 32°C) precipitated vaterite as well as calcite. To study the microbial urease activity, Swensen and Bakken (1998) collected samples from a lysimeter trench in a field test and incubated the microorganisms collected from soil in laboratory. By measuring the ammonium concentrations, they demonstrated that urease activity existed in all soil samples.

Ample literature exists on the study of lake sediments in order to understand the nature and mechanism of the mineral precipitation by microorganisms in aquatic environment (Thompson and Ferris, 1990b; Giralt, Julia and Klerkx, 2001; Pérez et al., 2002). Thompson and Ferris (1990a) isolated *Synechococcus* sp. from Fayetteville Green Lake, New York and observed in the laboratory that this species could deposit gypsum and calcite. Based on the results, they concluded that this microorganism could also form sediment in the lake. Similarly, Giralt, Julia and Klerkx (2001) found vaterite biscuits formation in Lake Issyk-Kul, Republic of Kyrgyzsta as a sign of biomineralization mainly by cyanobacteria *Synechococcus* microorganism. Rusznyák et al. (2012) studied biominerals in Herrenberg Cave in Germany, and reported that two species of microorganisms, *Arthrobacter sulfonivorans* and *Rhodococcus globerulus*, found in the cave were able to produce EPS and precipitate mixtures of calcite, vaterite, and monohydrocalcite.

4. Precursors to Biomineralization in Cementitious Materials

The cementing capacity of microbial induced calcite precipitation (MICP) observed in nature inspired researchers to investigate its applicability for engineering application such as sand and soil consolidation. Biomineralization was also used to heal cracks and fill pores in ornamental stones for restoration of buildings and improving durability. DeJong, Fritzges and Nüsslein (2006) pumped a mixture of *Bacillus pasteurii* (renamed recently as *Sporosarcina pasteurii*), nutrient and calcium chloride into cylindrical containers filled with sand to study the cementing property of MICP. Shear wave velocity was measured through microbially treated and untreated specimens. The test exhibited that both the shear stiffness and elastic capacity of bacteria-treated specimens increased comparing to the untreated specimens. SEM imaging also revealed existence of grainy precipitate in the treated specimen, which was cementing the sands.

Rodriguez-Navarro et al. (2003) immersed porous limestone blocks in tubes filled with *M. xanthus* and liquid medium. The tubes were shaken for various time intervals and the

difference in weight of the stone blocks before and after immersion were measured. Comparing to the stone blocks without bacterial inoculation, the bacterially treated ones showed increase in weight with time. The weight gain due to the mineral precipitation occurring on the stone surface was confirmed by SEM observation. They also immersed larger porous limestone slabs (2.5×4.5×0.5 cm) in flasks filled with the same bacteria and medium but kept in stationary condition. The increase in weight was also observed in this case. However, in SEM images, EPS film was found to form on the stationary samples' surface, which was not seen on the samples shaken continuously. The authors concluded that the MICP could consolidate decaying stone and the shaking hinders the formation of EPS film. Success of these studies prompted Civil Engineers to investigate if MICP can be effectively introduced in cementitious materials.

5. Biomineralization in Cementitious Materials

5.1 Type of Bacteria

As introduced in Section 2.1, MICP results from a chain of simultaneous metabolic reactions by certain species of bacteria. These metabolic reactions are initiated by hydrolysis of urea into carbonate and ammonia ions, which is governed by urease enzyme. Thus, in order for MICP to occur, the microorganisms should have high urease enzyme activity, which catalyzes urea hydrolysis into carbonate and ammonia. Due to its alkaliphilic and extremophilic nature and urease enzyme activity, *B. pasteurii* is the most common microorganism used for MICP application in cement paste and concrete. In addition, these microorganisms may also induce calcite precipitation by serving as nucleation sites, which occur as a result of its negative surface charge that attracts positively, charged calcium ions (De Muynck, De Belie and Verstraete, 2010). Ions are deposited on cell membranes and acts as nucleation for mineral deposition (Zhong and Islam, 1995). According to Dickson, Koohmaraie (1989), species of *Bacillus* have a relatively large surface area compared to some other species that may be used in this application. Since the total charge of bacteria increases with increasing surface area, this suggests good bonding potential between the calcium ions and *B. pasteurii* cells.

However, *B. pasteurii* is not the only type of species that has been used in biomineralization applications in cementitious materials. For instance, Achal, Pan and Özyurt (2011) used *Bacillus megaterium* and they reported that this was another bacterial strain that was alkaliphilic, endospore forming, and could also decompose urea. However one major drawback of this microorganism is that urease enzyme activity can be repressed under increasing ammonium and nitrogen concentration [26].

Ghosh et al. (2005) cultured one strain of *Shewenella* species in a modified medium (pH 7.5) and then the bacteria were suspended in water to achieve seven different cell concentrations from 10 to 10^7 per ml. The suspensions were mixed with sand and cement

to cast mortar cubes and then the cubes were cured in air at room temperature. Mercury intrusion porosimetry (MIP) was used to test the porosity of mortar with and without bacteria at 28 days. The results showed that for *Shewenella* microorganism, the cubes with 10^5 cells/ml exhibited the greatest reduction in porosity. While the authors attributed the porosity reduction to the addition of the *Shewenella* microorganism, no explanation was provided on why the samples containing the 10^5 cells/ml concentration exhibited the greatest reduction of porosity.

5.2 Method of Bacteria Addition

Perhaps the simplest approach to use biomineralization in cementitious material is to suspend the bacterial cells in the mixing water. Note, the cells should be centrifuged and washed prior to placing them in the mixing water. It is also possible to replace the mixing water with a bacterial culture and nutrient broth. This nutrient broth has nitrogen, carbon, protein and vitamins that microorganisms need for metabolic activity (Achal et al., 2009; Achal, Pan and Özyurt, 2011); however, the optimal amount of nutrients needed for metabolic activity should be considered. Furthermore, addition of these nutrients might also have an effect on the mechanical and chemical properties of concrete.

Another possible method is to encapsulate the microorganisms, synthetically or naturally, prior to mixing them into the concrete (Bang, Galinat and Ramakrishnan, 2001; Bang et al., 2010; Wiktor and Jonkers, 2011). This approach protects the microorganisms from the high pH environment of the cement paste and keeps them alive for extended time for self-healing applications. The synthetic encapsulation method consists of immobilizing the bacteria in a protective covering, e.g. porous aggregates, polymeric membrane, etc (Bang, Galinat and Ramakrishnan, 2001; Bang et al., 2010; Wiktor and Jonkers, 2011). Bang, Galinat and Ramakrishnan (2001) used polyurethane-based (PU) encapsulation to immobilize bacteria. The polyurethane membrane protected the microorganisms from the high pH environment within the cement paste matrix and enhanced the efficiency of urease enzyme activity. The PU matrix also acted as a nucleation site for calcite precipitates; consequently biomineralization capacity of the cell improved (Bang, Galinat and Ramakrishnan, 2001). However one main disadvantage of PU is limited diffusion capability, which may limit the calcium carbonate precipitation induced by microorganisms. Further investigation of encapsulated microorganisms has been conducted with microorganisms encapsulated in Siran Glass beads (see Figure 3) (Bang et al., 2010). Cell suspensions were grown in nutrient broth media and centrifuged prior to immobilization. Compared to PU encapsulation method, the glass beads encapsulation method was better in improving strength and crack healing. Glass beads shown in Figure 3 (A) were submerged into 20 mL cell suspension and incubated for 1 hour. *B. pasteurii* cells were immobilized in glass beads as shown in Figure 3 (B). When immobilized cells

were submerged in urea-$CaCl_2$ solution, significant calcium carbonate precipitation was observed both on cells and glass beads. Figure 3 (C) shows enlarged view of calcium carbonate crystals obtained with embedded cells (Bang et al., 2010).

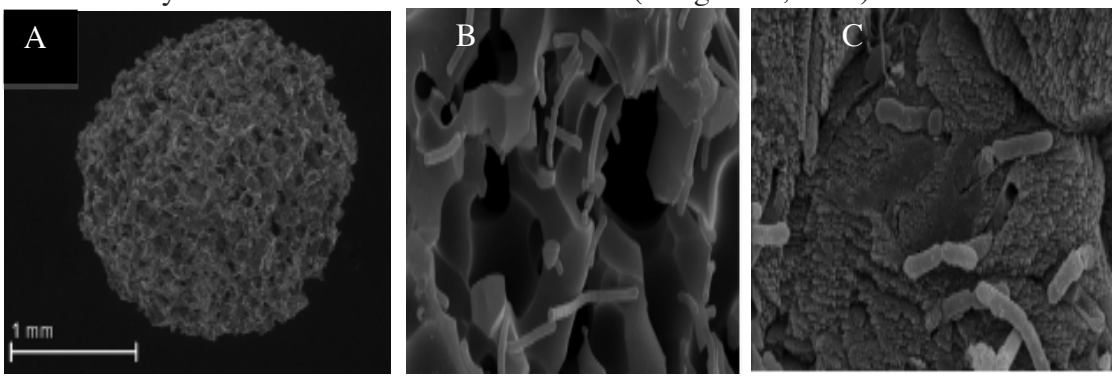


Figure 3. Encapsulation of *Bacillus pasteurii* in siran glass bead. (A) Microstructure of siran glass bead; (B) cells attached to glass bead pores; (C) calcite precipitation on the cells (Bang et al. 2010)

The natural encapsulation addition method consists of introducing the bacteria in the form of bacterial endospores. Endospores are tough, dormant, non-reproductive structures that certain bacteria turn into when they undergo stress (e.g. nutrient depletion, extreme temperatures, etc); they enable the bacteria to be dormant for extended periods and when the endospores are exposed to a suitable environment again, this reactivates the bacteria into a metabolic state. Researchers at Delft Technological Institute have used this particular approach. *B. pasteurii* endospores were washed and suspended in mixing water without any additional nutrients provided. This procedure resulted up to 4 months survival of the bacterial endospores in mortar (Jonkers, et al., 2010). Following this application, lightweight organic spheres were developed and *B. cohnii* endospores were embedded into these porous aggregates with lactate (Wiktor and Jonkers, 2011). This application method was provided to keep endospores active, thus self-healing ability of system is extended for years. While the encapsulation shows promise, one possible drawback of using this application method (both for natural and synthetic encapsulations) is the bonding between the encapsulation constitute and the matrix.

5.3 Use of Nutrient Medium

Efficiency of calcium carbonate precipitation depends on the microorganism type, availability of sites that microorganisms can attach to, concentration of cells and the nutrient sources accessible. In order to keep microorganisms active in a metabolic state, carbon and nitrogen source are required. For *B. pasteurii* ammonium sulfate is a common compound used as a nitrogen source (Stocks-Fischer, Galinat and Bang, 1999; Achal et al., 2009; Jonkers et al., 2010). Yeast extract is commonly used as carbon source;in addition to providing a source of carbon, yeast extract has many amino acids and vitamins required for bacteria (Stocks-Fischer, Galinat and Bang, 1999; Achal et al., 2009). However, one side effect of yeast extract is the retardation of initial setting (see Figure 4) since yeast extract contains sugar. An alternative for yeast extract is lactose mother liquor (LML), which is dairy industry by product. Achal et al. (2009) conducted a comparison study in order to show the influence of three different carbon sources on urease enzyme activity and mortar properties. Effects of nutrient broth, yeast extract and LML added urea solutions were investigated. With respect to calcite precipitation, compressive strength and bacterial growth, the results suggested that LML media performed better compared to yeast extract and nutrient broth.

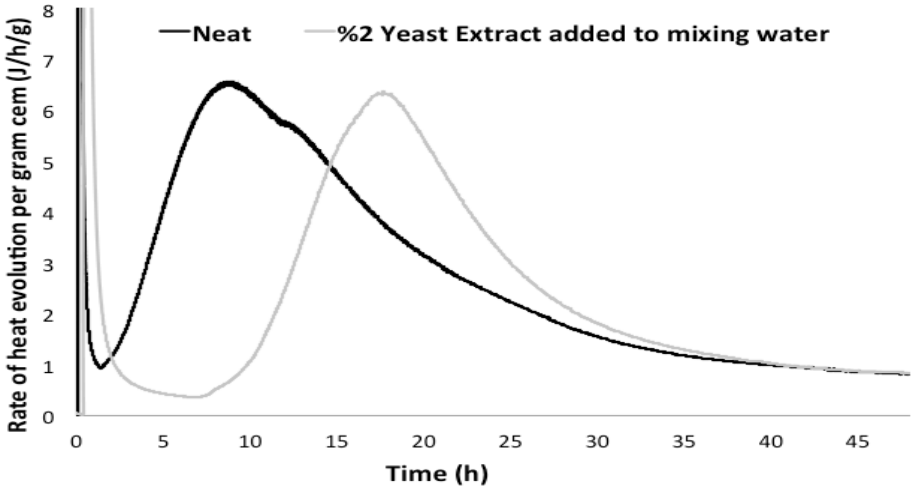


Figure 4. Effects of yeast extract on cement paste hydration- neat paste: cement paste with water to cement ratio of 0.5. Cement paste w/ ye: cement paste with 2% yeast extract added to mixing water (w/c=0.5) (Basaran, Douglas and Kirisits, 2012)

While sources of nitrogen and carbon are essential for bacterial growth, it is important to bear in mind that in order for MICP to occur, urea and a source of calcium are also required. The bacteria should have an urease enzyme to precipitate calcium carbonate, and work by Whiffin (2004) suggests that urease enzyme activity and thus MICP is governed by urea concentration rather than calcium concentration. Since urea contains nitrogen, this can be used as a nitrogen source instead of ammonium sulfate. One possible drawback of urea hydrolysis is that increasing the ammonia concentration can repress urease enzyme activity specifically on microorganisms [26].There was not any significant effect of ammonium concentration on *B. pasteurii* urease enzyme activity, however the effects of nitrogen production on cement paste properties is still unclear.

Although cement-based composites naturally have sources of calcium, additional calcium sources is often added (Jonkers and Schlangen, 2008; Whiffin, 2004). Reported calcium sources include calcium chloride, calcium nitrate, and calcium lactate (Whiffin, 2004; Wiktor and Jonkers, 2011). Calcium lactate was used by (Wiktor and Jonkers, 2011), since it did not have any adverse affect on compressive strength of concrete. Calcium chloride is also reported as being efficient at inducing MICP precipitation in cement-based materials than calcium nitrate (Whiffin, 2004), showed that. However, calcium chloride is a concern for corrosion in cases where reinforcement is required since chloride ions interfere with the passive oxide layer surrounding steel reinforcement.

5.4 Viability of Bacteria

Considering the viability of the bacteria, portland cement based systems are challenging systems due to the complex series of physio-chemical reactions occurring from the reaction of cement with water. High pH (>12) and temperatures (>50 degrees Celsius) during hydration and lack of nutrients are limiting the applicability of mineral precipitation in cement paste. Nonetheless, the limited research involving biomineralization in cement-based systems has shown promising results and suggests that microbial-induced calcium carbonate precipitation and self-healing of cracks can occur on the surface of concrete (Wiktor and Jonkers, 2011). It has been shown that spores of *B. pasteurii* can tolerate high pH, and that they can be viable up to 4 months, when it was incorporated into the cement-based matrix by replacing the portion of the mixing water [8]. Moreover, Wiktor and Jonkers (2011) developed a porous organic system in which endospores and calcium lactate source were combined and encapsulated into lightweight aggregates. Comparing to the mixtures that only contained endospores, the viability of the microorganisms encapsulated with calcium lactate increased. Thus, the time available for the microorganism to precipitate calcium carbonate for crack healing purposes is extended. Researchers show that microorganisms can display prolonged crack healing ability when they are embedded in polyurethane based polymers and glass beads (Bang,

Galinat and Ramakrishnan, 2001; Bang et al., 2010). Achal, Pan and Özyurt (2011) showed that species of *B. Megaterium* could survive up to 28 days in both mortar and concrete. The concentration of microorganisms was found to be close to initial values when plate counts were conducted on 3-day specimens. Even though the percent of microorganisms that survived decreased from 7 to 28 days, they were not completely killed. One other thing should be pointed out is there was not any significant change in concentration of microorganisms from 7 days to 28 days. According to the results obtained, fly ash addition in both mortar and concrete also increased the number of the microorganisms.

6. Effects of Bacteria Addition on Strength

Studies conducted up to this point suggest that compressive strength can be enhanced with addition of microorganism such as *B. pasteurii, B. megaterium* and *Shewenella* species (Achal, Pan, and Özyurt, 2011, Ghosh et al., 2005b; Ramakrishnan, Ramesh, and Bang, 2001; Ramachandran, Ramakrishnan, and Bang, 2001).

Achal, Pan and Özyurt (2011) used *B. megaterium* in cement paste mortar with different fly ash replacement ratios and specimens were cured in a nutrient-broth-urea (NBU) medium that contained calcium chloride. Compared to the control specimen, a 21% increase in 28-day compressive strength was seen when *B. megaterium* was added. However this increase was not observed at 7 days (Achal, Pan, and Özyurt, 2011). At 28 days, specimens with 10% fly ash replacement showed 19% strength increase relative to control specimen without microorganisms It is believed that the increase in compressive strength is due to metabolic activity of bacterial cells and plugging of pores due to calcite precipitation induced by microorganisms [4].

Jonkers and Schlangen (2006) conducted experiments using three sets of concrete bars (16 x 4 x 4 cm), including one control set, one added with bacteria and the other with amino acid. The specimens were cured in tap water at room temperature. No significant difference in three-point bending strength and compressive strength among the three sets of concrete bars were found. However, images obtained from environmental scanning electron microscopy (ESEM) revealed that copious amounts of mineral crystals precipitated on the surface of the specimens. It should be noted that the compressive strength of cement paste cubes prepared with bacterial endospores were also measured. The result revealed that the specimens had strength decrease comparing to control set for 3, 7 and 28 days curing.

Park et al. (2010) investigated the influence of *B. pasteurii* ATCC 11859 and 4 other calcite-forming bacteria (CFB), which were isolated from 7 concrete structures on the compressive strength of mortar cubes. Using gene sequencing analysis, it was identified that the 4 CFB collected from concrete were *Sporosarcina soli* KNUC401, *Bacillus*

massiliensis KNUC402, *Arthrobacter crystallopoietes* KNUC403, and *Lysinibacillus fusiformis* KNUC404. In this work, fresh medium with bacteria suspended in it was used as mixing water to make mortar cubes, and the cubes were cured in a urea-$CaCl_2$ medium. The result showed that KNUC 401 and KNUC 404 achieved increased 7-day (13.2% and 11.7%) and 28-day (3.8% and 4.5%) compressive strength compared to control group. The author attributed the improvement of strength to the carbonate precipitates and EPS in mortar's pores. The cubes mixed with *B. pasteurii* did not show strength increase, which coincides with the result of Jonkers above. Hence, it is still questionable if the addition of calcifying bacteria can improve the compressive strength of concrete, or, it depends on the species of bacteria, as said by Park et al. (2010).

The influence of *Pseudomonas aeuruginosa* and *B. pasteurii* on compressive strength was studied by Ramachandran and Bang (2001) . One set of specimens was prepared by addition of both *P. aeuruginosa and B. pasteurii*. Second set of specimens was prepared by using *B. pasteurii* pure culture. Both live and dead cultures were used in preparation of these two sets of specimens. Mortar cubes were cured in urea-calcium chloride solution at room temperature. The results also showed that *P. aeuruginosa* had no significant impact on the 7-day compressive strength compared to the specimens containing only *B. pasteurii*. According to the authors, one possible reason could be the metabolic state of the bacterial cell since at earlier ages the mortar is more porous and the microorganisms have easier access to nutrients than at later ages. However, it is also possible that the high pH environment of the mortar caused the microorganism to die very early and that the strength gain was due to microorganisms acting as organic fibers. However, over time they decomposed and lost their efficiency (Ramachandran and Bang, 2001). As stated earlier, *B. pasteurii* is known to be MICP capable microorganism since it exhibits urease enzyme activity, and thus is capable of decomposing urea into ammonia and carbonate. However, it should be noted that improvement in compressive strength has also been reported in cases in which high urease enzyme bacteria was not used. Ghosh et al. (2005a) used *Shewenella* species that was extracted from hot spring. Mortar mixes were batched with addition of microorganisms to mixing water at different concentrations and air cured at room temperature. Results of the tests suggested that addition of microorganisms enhanced the compressive strength, particularly at concentration of 10^5 CFU/ml. In contrast to the previous study, the general trend of compressive strength increase becomes more predominant at later ages. Imaging analysis showed that fiber-like structures were observed in pores due to addition of microorganisms which decreases porosity of the cement past mortar

Achal et al. (2012) designed special experiment to examine the role of nutrient medium and biomineralization on the pullout strength of reinforced concrete (RC). *Bacillus* sp. CT-5, isolated from commercial cement, was cultured in two different nutrient mediums.

One medium was nutrient broth (NB) medium containing urea, and the other was named CSL (pH 8.0), which was actually an effluent from corn wet-milling industry. Steel rebar reinforced concrete prisms were prepared by using medium with bacteria suspended in it, rather than using mixing water. Then the specimens were cured in the same nutrient medium as the mixing medium. Concrete without bacteria in it was used as control group. After 7 days, a pullout test was conducted. The pullout strength of the concrete specimen containing the NB medium was 35kN and that of the CSL medium was 31kN, which were both higher than control group of 26 kN. The authors attributed this pullout strength increase to bacterial precipitation.

In addition to containing nutrient broth mediums, bacterial suspensions typically contain a buffer medium. To understand how the buffer solution influences incorporation of *B. pasteurii* in cement paste, Ramachandran, Ramakrishnan and Bang (2001) investigated the role of the buffer solution on compressive strength. This study was conducted with both killed and live *B. pasteurii* and *P. aeuruginosa* suspended in both saline and phosphate buffer. The results showed a decrease in compressive strength when a saline buffer solution was used. This was explained due to addition of chloride ions.

7. Effects of Bacteria Addition on durability

Jonkers (2011) studied effects of MICP on permeability of concrete. They made two groups of concrete slabs; one with cement paste mixed with clay and *Bacillus* strain B2-E2-1 while the control group was with only cement and clay. 8 cm long and 0.15mm wide crack was created through the 1.5 cm thick concrete disk by controlled splitting tension test. After that the disks were submerged in tap water for 14 days. The authors reported that cracks were filled in all the disks with bacteria, while only two out of six were healed from the control group.

Jonkers et al. (2010) also used the mercury intrusion porosimetry (MIP) method to investigate changes in pore size distribution of cement paste cubes. Specimens were made by mixing ordinary portland cement with washed spore suspensions of *Bacillus pseudofirmus*. Additional control specimens were made by mixing cement with tap water. The w/c ratio was 0.5 and the fresh cubes were cured in gas sealed environment at room temperature. Jonkers et al. (2010) concluded that the pore sizes in cement paste affect the viability of bacteria. Comparing MIP results at 3, 7 and 28-days, he reported that larger size pores (0.8–1μm) became fewer with time and this will adversely affect bacterial viability as the larger pores are more likely to accommodate bacterial spores.

De Muynck et al. (2008a) studied the effect of bacterial treatment on water absorption of mortar cubes and compared the water-repellence effect of bacterial treatment with other conventional treatments such as application of acrylates surface coatings, silanes and silicones water repellents. Bacteria *B. sphaericus* LMG 225 57 were chosen for this

treatment. Mortar cubes with w/c ratio of 0.5, 0.6, 0.7 were immersed into 1 day-old medium with bacteria for 1 day, and then submerged in medium with calcium chloride and calcium acetate which work as different calcium source or brushed with conventional materials. The results showed that compared to untreated specimens, the bacterial treated specimens had lower water absorption, but the water absorption of high w/c (w/c =0.7) bacterial treated sample was higher than the specimens with w/c ratio of 0.5 and 0.6, due to incomplete filling of larger pores by the precipitates. However, some coatings and sealants treated specimens had significantly lower water absorption compared to some bacteria treated samples for all three w/c ratios. De Muynck et al. (2008b) also investigated the role of *B. sphaericus* on the strength of mortar slabs after freeze-thaw damage. In the experiment, plain mortar slabs were cast and cured for 28 days and then immersed for 24 h in the bacterial solution. The slabs were taken out and immersed into calcium chloride and calcium acetate solutions for 3 days. After these treatments, specimens experienced 21 freezing and thawing cycles, and then the tensile strength of the specimens was measured by splitting tests. The test results showed that biomineralization significantly improved the tensile strength comparing to untreated specimens, and hence reduced freeze-thaw damage.

As degradation of concrete to freezing and thawing is a major concern, Bang et al. (2010) also measured the expansion and weight change of bacterially treated mortar beams together with control beams after 300 freeze-thaw cycles. Mortar beams were prepared by adding *B. pasteurii* and were cured in urea-$CaCl_2$ medium for 7 days and in air for 14 days along with control beams without bacteria. The expansion and weight of the beams were recorded every 30 freeze-thaw cycles till the cycles reached 300. After 300 cycles, the mean expansion of beams with bacteria was 0.03% while it was 0.054% for the control beams. Regarding the weight change, bacterially treated beams remained at 98% of original weight while the control beams were only at 69%. The result indicated that the layer of calcite precipitation on mortar surface can effectively prevent degradation of concrete.

Achal, Mukherjee and Reddy (2011) tested water absorption of bacterially treated mortar cubes. *Bacillus* sp. CT-5, isolated from commercial cement, was grown in nutrient broth, and then the bacterial culture was used to mix mortar with water/cement ratio 0.47. To implement the water absorption test, one side of each cube was immersed into water and the four adjacent sides were treated by polysiloxane and silicon paint. The weight of the cubes was measured at regular interval to calculate the amount of absorbed water. The results showed that after 168 hours, bacterially treated cubes absorbed nearly one sixth of the water absorbed by the control group. It indicates that the precipitation of calcium carbonate, as observed by SEM, can effectively lower the water sorptivity of mortar surface.

In another paper, Achal, Pan and Özyurt (2011) reported effects of *B. megaterium* ATCC 14581 on the water absorption of mortar and the water permeability of concrete. Bacteria were incubated in nutrient broth containing urea and then the mixture with bacterial concentration of 5×10^7 CFU/ml were added into cement to make mortar cubes and beams, while blank mortar and concrete were used as control samples. Fly ash was used to replace part of the cement in four different dosages: 0%, 10%, 20% and 40%. The de-molded cubes were cured in nutrient medium for 28 days, then dried till constant weight and then immersed in water for different time intervals. The weight gained after immersion was used to calculate water absorption. The result showed that for all sample sets with varying amount of fly ash, when cured for the same period, cubes containing bacteria had lower water absorption than the control group. It can be explained that the calcium carbonate precipitated on mortar surface appeared to seal the pores in mortar. Following similar method, effect of bacteria treatment on water absorption of concrete was also studied. During absorption test, atmospheric pressures of 1, 3 and 7 bars were applied for 24 h on each concrete specimen, and then the specimens were cut into halves to measure the depth of water penetration. The result revealed that the bacterially treated specimens had less than half of penetration depth of the control groups. This result was explained as an effect of possibly denser interfacial zone of calcite between aggregates and concrete matrix in the bacteria treated specimens.

As the use of recycled aggregates in concrete is often limited by its high water absorption, Grabiec et al. (2012) inoculated *B. pasteurii* into recycled aggregates and cultured for 5 days to achieve calcium carbonate layer on aggregates surface and then the aggregates were used in concrete to improve the high water absorption. The result showed that the concretes with two different water/cement ratios and two different aggregate fractions all appeared to have lower water absorption than concrete with untreated aggregates. It means that the precipitated calcium carbonate can plug the pores of recycled aggregates and reduce water absorption.

8. Biomineralization for developing Smart Self-healing Concrete

Self-healing of concrete is a process that can occur via various mechanisms, and traditionally there are 3 different approaches have been suggested. The first approach involves hydration of remaining unhydrated cement in concrete near the crack to fill up the crack. However, the capacity of this mechanism is limited by the availability of favorable environment for hydration (presence of water), amount of unhydrated cement left, and the width of crack. Another approach is to use special cementitious materials such as calcium sulfoaluminate-based expansive additives. The idea here is that calcium sulfoaluminate prompts the release of calcium ion from mortar specimens which lead to the precipitation of calcium carbonate on the surface of specimens. The formation of

ettringite inside the cracks was also observed. The precipitates may fill the cracks partially; however, overdose of expansive additives is an issue as it could cause unexpected expansion and cracking (Sisomphon, Copuroglu, and Koenders, 2012). Finally, the third approach is to seal the cracks by impregnating of cracks with epoxy or other synthetic fillers (Jonkers et al., 2010). However, the fillers cannot continuously work on healing the cracks after they are used up. In contrast to the above three traditional crack-healing techniques, biomineralization appears to be another choice which not only shows promises for repeated healing action at the same location, but also has advantage with respect to the environmental protection and economy (Van Der Zwaag et al., 2009). If biogenic minerals replace cementitious material as filler or sealant to heal cracks, significant amounts of clinker can be saved and therefore the output of green-house gasses can be reduced. If bacteria-integrated-concrete autonomously and continuously decrease permeability of the material and heal cracks, the manual inspection and repair work can be reduced.

Van Tittelboom et al. (2010) used two methods to create cracks in concrete, in order to simulate the cracks forming naturally: one was a standardized method, in which cracks were created by inserting copper chips; the other was called "realistic cracks", created by splitting test. After cracks were created in concrete cylinders by these two methods, bacteria strain LMG 222 57 from *B. sphaericus* was cultured and then the cracked concrete cylinders were placed in the culture solution for 1 day. Cylinders were then transferred to urea–calcium solution for another 3 days treatment. In order to protect bacteria from hostile high alkaline environment of concrete, silica gel was used to encapsulate the bacteria. $CaCl_2$ or $Ca(NO_3)_2$ or $Ca(CH_3COO)_2$ was added during treatment to supply calcium externally. After treatment, permeability of the specimens was tested. The results showed that the bacteria treatment with silica gel in the presence of externally added calcium compounds, $CaCl_2$ or $Ca(NO_3)_2$ or $Ca(CH_3COO)_2$, reduced water permeability of concrete cylinders. It indicted that the precipitates could fill the cracks partially or completely, or at least reduces the crack width.

Bang et al. (2010) developed the crack remediation technique by filling glass beads with *B. pasteurii* culture into concrete cracks. In the specimens, the artificial cracks with width and depth as 3.175 mm and 12.7 mm were made in 25.4×25.4×152mm beams, and with width and depth as 3.175 mm and 25.4 mm in 50.8×50.8×50.8mm cubes. In this study, glass beads were used to encapsulate the bacteria. Bacteria in different concentrations were added into the cracks of mortar cubes and beams along with nutrient medium, $CaCl_2$ while the control group did not include any bacteria. All specimens were cured for 7 and 28 days. Compressive strength and stiffness were measured at the end of the treatment. The results presented in Figure 5 shows that compressive strength increased with time for all bacteria concentrations. Bacteria treated specimens also had higher stiffness than

blank specimen. But at bacteria concentration higher that 6.1×10^8, stiffness did not increase further.

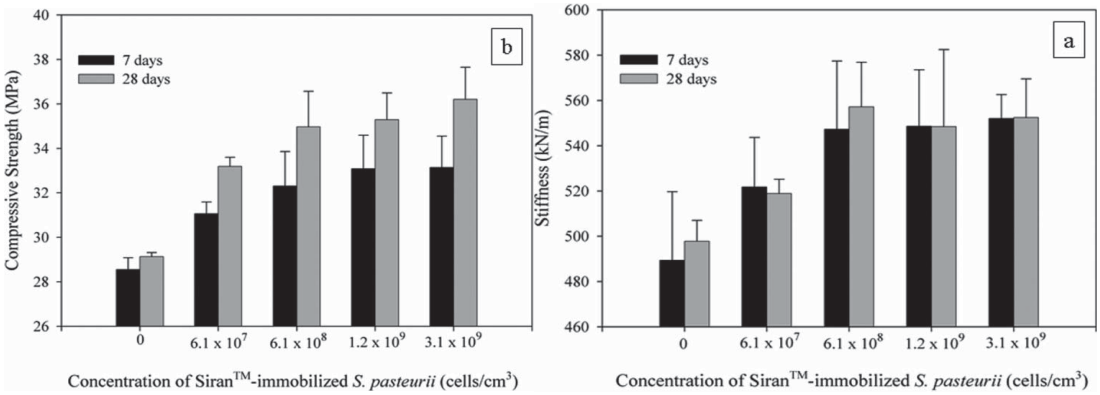


Figure 5. (a) compressive strength of mortar cubes (b) stiffness of mortar beams. Both for 7 days and 28 days (Bang et al., 2010).

9. Promises and Further Challenges

While biomineralization in cement-based materials is in its infancy, the current work shows that the addition of bacteria into cement paste has improved concrete strength and reduces the permeability of concrete. With more research and resources expended in this area, the potential exists for using a biomineralization approach to develop strong, energy-efficient, self-healing, and durable concrete for construction. Continuing research in the exploration of biomineralization promotes interdisplinary research and education and will also contribute other research areas such as biology and material science. Below areas of concerns and considerations for future research directions are discussed:

- Structure-Property Relationships: By analyzing the critical factors, which affect the metabolic activity of the microorganisms, it may be possible to control the morphology and amount of the biogenic precipitates such that the performance of the concrete is optimized. Further study in this aspect has potential to establish a link between the micro-scale structures of the precipitated crystals and the mechanical property improvement of large-scale construction materials.

- In general, a bacterial concentration of 10^4 to 10^8 CFU/mL has been found to yield improvements in compressive strength [19, 22, 64].However, definitive relationships regarding bacterial concentration and strength/durability have not been defined. Considering the relatively infancy of this area, more research is needed to establish the parameters governing the quantity of bacteria needed for improved strength and durability.

- Environmental Considerations: One major possible application of this MICP is to reduce portland cement content in concrete. This is beneficial from an environmental standpoint since cement manufacturing is a CO_2 emitting intensive process, but also from a economic standpoint since portland cement is often the most expensive component of a traditional concrete mixture. Research pertaining to leveraging the MICP process so that cement content is significantly reduced will have important implications from environmental, economic, and social perspectives, and is an area that warrants further attention.

- Interaction with the ecosystem: The impact of biomineralization to the carbon cycle and nitrogen cycle is an area that requires further research. As shown in equation 4, the ammonia produced during the metabolic activity of ureolytic bacteria contains nitrogen, and the environmental impact to the nitrogen load of the environment is an area that has been discussed as a potential drawback to this method (Jonkers, 2007). Furthermore, since the biomineralization process requires carbonate ions in order to precipitate calcium carbonate, leveraging this technology for carbon sequestering applications is a topic of interest.

- Fresh State Properties: To date, the majority of the research has focused on the hardened state properties, but understanding the role the bacterial suspension play on the fresh state performance of concrete will be critical to its implementation in the field. Microbial suspensions in which the flagella located at the back exert force at their tail and are called pushers, whereas pullers exert a thrust force at their head. Pushers, such as bacteria, are also known as "tensile swimmers" and they decrease the viscosity of the solution. Whereas, pullers are called "contractile swimmers" and they increase the viscosity of the solution (Rafai, Jibuti, and Peyla, 2010). The fresh state properties of the concrete are likely to be influenced by the type of microorganisms, and further research is needed to understand how the dynamics and rheological parameters of the concrete are influenced. Further study is also needed in understanding the relationship among bacterial motility, spatial distribution of the biogenic precipitates in the composite matrix, rheological properties of the concrete/cement paste, and processing.

- Self-Healing Efficiency: Considering the self-healing applications, crack width and depth are main limitations (Jonkers and Schlangen, 2006). Based on the research so far, biomineralization remediation has been limited to narrow and shallow surface cracks; this is likely a result of the ease of nutrient migration and its availability. Furthermore, most of the reported research shows that MICP can

effectively reduce permeability by filling cracks (which can be actually referred as self-sealing). However, further research is necessary to understand if this method can promote strength recovery which will mean true healing of cracks. Hence, research focusing on the bonding between the biogenic material and concrete is critical.

- Transferring to Field: The concerns with respect to practical application also hinder the application of biomineralization technique in building material. This technique currently seems not feasible due to the large demand of labor work for culturing bacteria and supplying medium continuously. Another concern is about whether bacteria will be able to remain viable for a prolonged time and can be active in the process of sealing the cracks. Current research estimates the life span was up to 4 months (De Muynck, De Belie and Verstraete, 2010), which was still shorter than the service life of common buildings. Research regarding extending the viability of the bacteria is critical to the self-healing aspect of biomineralization.

Appendix C: Correlating the cell concentration with absorbance readings at OD_{600}

The growth curve of *S. pasteurii* in pH9 UYE medium was represented in Figure 3.1. The equation 3.6 was determined by correlating the absorbance of the cells at OD_{600} and viable plate counts at time intervals corresponding cell growth (exponential stage). A viable cells is able to divide and form endospores, simply they are live cells. Therefore viable cell counts is a method to enumerate live cells in a culture. 1mL of sample obtained at each time interval serially diluted (10^{0}-10^{-4}) with phosphate buffer solution (PBS) in test tubes and vortexed. 0.1mL of samples from each dilution was cultured on UYE-medium agar plate by a spread plate method. Triplicate of plates were prepared for each dilution. Plates were incubated for 72 hours and surface colonies were counted. Results represented as colony forming units per mL (CFU/mL).

The absorbance readings at OD_{600} were obtained from the same 1mL sample obtained from the batch. 0.2mL of samples obtained from the each dilution were transferred to a 96-well plate and placed into a BIO-TEK Synergy HT spectrometer (Winooski, VT, United States). For control blank readings, 0.2mL of PBS and UYE medium were used. Absorbance measurements were obtained at a wavelength of 600 nm. Triplicate of readings were obtained from each control and dilution.

The average values obtained from the plate counts and the absorbance readings were plotted as represented in Figure 3.1b and the exponential correlation was obtained in the exponential growth interval.

Appendix D: Influence of biomineralization on LSF and C-FA modified mortar

As discussed in **Section 4.3.5**, the influence of inoculated vegetative *S.pasteurii* cells on mineral admixture modified mortar strength was also investigated. In these mixtures, 20% (by mass) of the cement was replaced with limestone filler (LSF) or Class C fly ash (C-FA). In order to determine if the inoculated vegetative bacterial cells can compensate the reduction in compressive strength typically seen when these mineral admixtures are added, a weight replacement of cement content was preferred rather than addition to these minerals. Table C.15 shows the chemical oxide analysis for the fly ash. Compressive strength was monitored up to 90 days and the results are shown in Figure D. 43and Figure D. 44.

Table D.15: Oxide composition for Parish C type fly ash

Oxides	Mass %
Silicon Dioxide (SiO_2)	33.14
Aluminum Oxide (Al_2O_3)	18.12
Iron Oxide (Fe_2O_3)	6.65
Calcium Oxide (CaO)	27.49
Magnesium Oxide (MgO)	5.45
Sulfur Trioxide (SO_3)	2.71
Sodium Oxide (Na_2O)	1.91
Potassium Oxide (K_2O)	0.30
Total Alkalis (as Na_2O)	2.11
Loss on Ignition	0.4

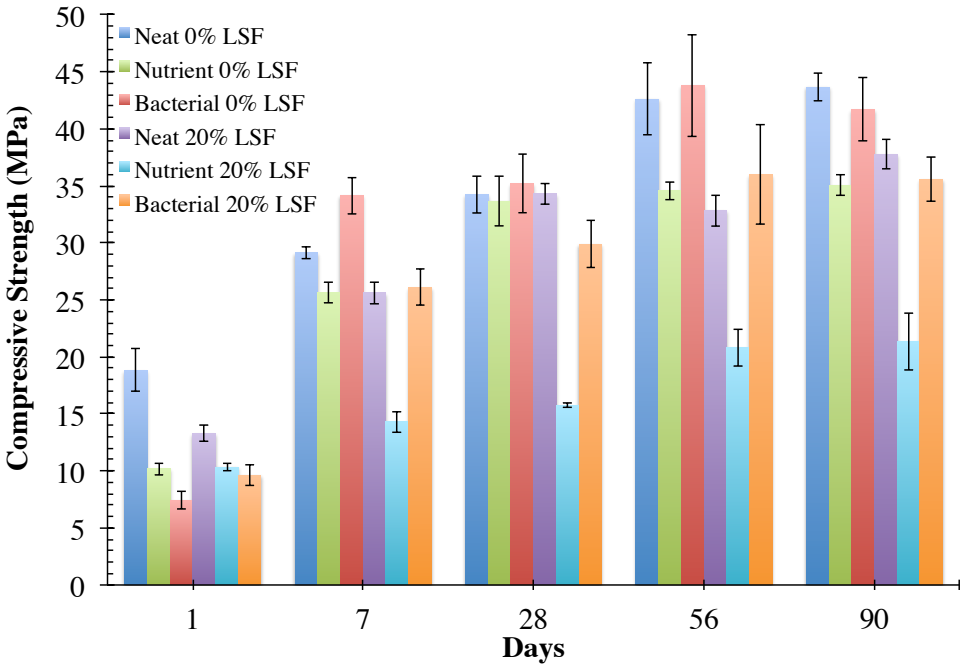


Figure D. 43:Effects of limestone powder replacement (0 and 20% by mass of cement) and *S.pasteurii* ($3x10^6$ CFU/mL) on mortar compressive strength. The w/c and s/c were 0.50.

Samples were prepared using neat mortar and with limestone powder to investigate whether the biomineralization could employ as a means to replace significant portions of the cement (Figure D. 43). As expected, in the neat mortar, the compressive strength was inversely proportional to the limestone powder concentration.

Furthermore, as seen in **Section 4.3.5**, the UYE medium had a negative effect on compressive strength, and, at every age, samples containing the UYE medium exhibited lower strength values compared to neat mortar samples. However it is interesting to note that the bacterial mortar overcame the reduction in compressive strength due to addition

of the UYE medium; in some cases, the compressive strength of the bacterial mortar actually exceeded that of the neat mortar.

Considering 1-day compressive strength results, both nutrient and bacterial mortar exhibited lower strength values compared to neat mortar. Moreover, addition of cells was unable to recover the reduction in strength due to addition of UYE medium, which could be related to the delay in hardening behavior. At 7-days, the bacteria-amended samples were able to recover the strength lost due to the addition of the UYE medium; furthermore they enhanced the strength of cement mortar samples. Same trend was also observed at later ages.

As shown in Figure D. 44, similar trend was observed with 20% FA replacement. Even though the strength values were lower in bacterial nutrient mortar in comparison to neat mortar at day 1. At 7 days and later ages, bacterial mortar overcame the reduction in compressive strength due to addition of the UYE medium and reached to neat mortar strength.

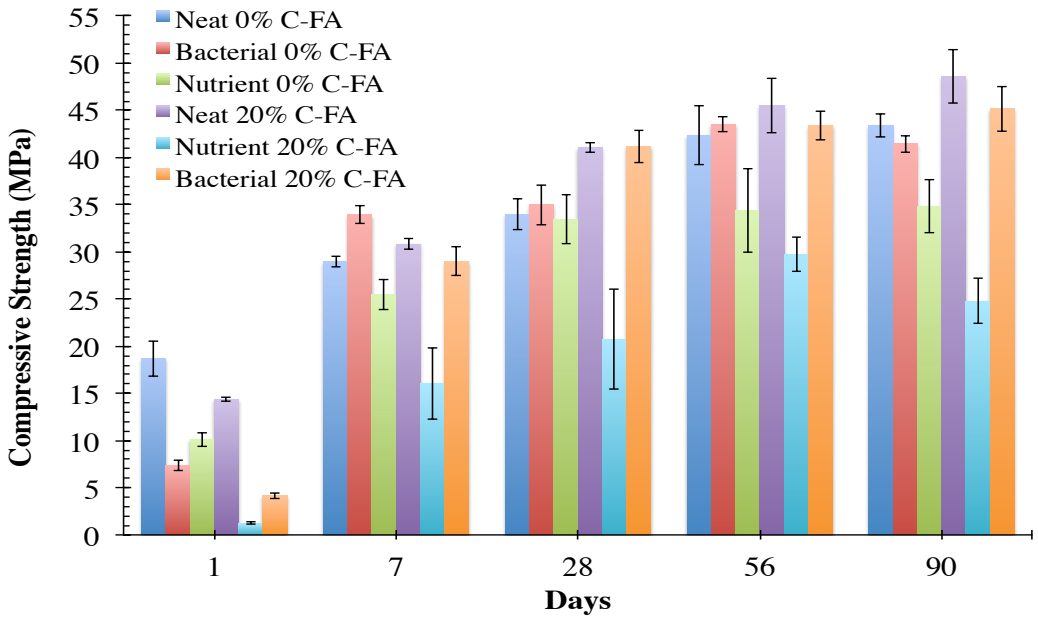


Figure D. 44: Effects of fly ash (0 and 20% by mass of cement) and *S.pasteurii* ($3x10^6$ CFU/mL) on mortar compressive strength. The w/c and s/c were 0.50.

Appendix E: MPN Analysis cement paste and mortar samples

As stated in **Section 5.2.2,** the viability of inoculated vegetative *S.pasteurii* cells within cement paste and mortar was interrogated via MPN method. An example of serial dilution tubes with and without turbidity shown in Figure E. 45.

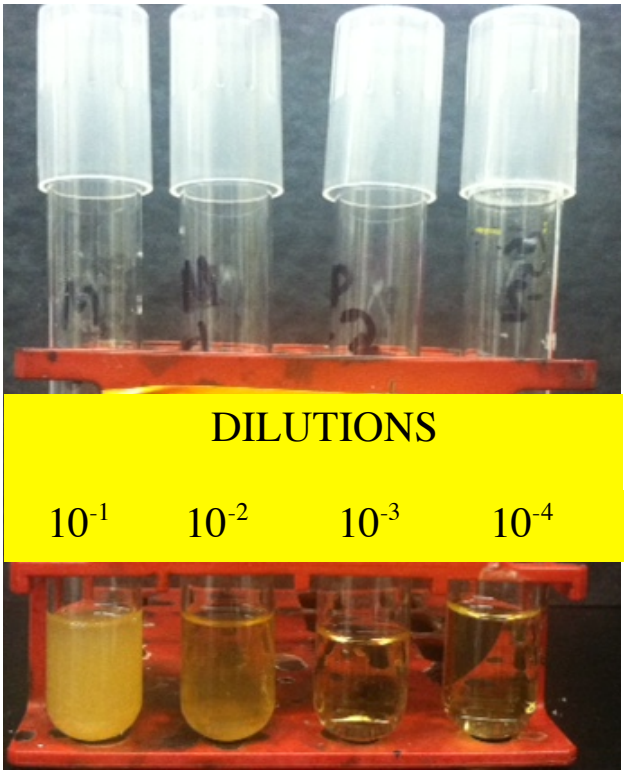


Figure E.45: An example of turbid vs. non-turbid tubes in MPN method. The dilutions 10^{-1} and 10^{-2} are turbid while the dilutions 10^{-3} and 10^{-4} are not turbid

Table E.16 and E.17 summarizes the results obtained from triplicates of tubes from duplicates of samples. The cell concentration was determined by using MPN indices and 95% Confidence Limits for 3 tube dilution series [73, 90].

Table E.16: MPN Index and 95% Confidence Limits of positive tubes in bacterial paste with a 3 tube dilution series

TIME	SAMPLE	DILUTIONS (mL)				95% Confidence Limits (MPN)			Cell Concentration
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	MPN	Lower	Upper	MPN/mL
0 days	Viable	3	**3**	**2**	**2**	**2100**	400	4300	$2x\ 10^{6}$
	Endospores	**3**	**2**	**0**	0	**93**	18	420	$9x\ 10^{3}$
1 day	Viable	3	**3**	**1**	**0**	**430**	45	420	$4x\ 10^{5}$
	Endospores	**3**	**1**	**0**	0	**43**	9	180	$4x\ 10^{3}$
7 days	Viable	**3**	**2**	**1**	0	**150**	46	940	$2x\ 10^{4}$
	Endospores	**3**	**1**	**0**	0	**43**	9	180	$4x\ 10^{3}$
28 days	Viable	**3**	**2**	**0**	0	**93**	18	420	$9x\ 10^{3}$
	Endospores	**3**	**1**	**0**	0	**43**	9	420	$4x\ 10^{3}$

Table E.17: MPN Index and 95% Confidence Limits of positive tubes in bacterial mortar with a 3 tube dilution series

TIME	SAMPLE	DILUTIONS (mL)				95% Confidence Limits (MPN)			Cell Concentration
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	MPN	Lower	Upper	MPN/mL
0 days	Viable	3	**3**	**2**	**2**	**2100**	400	4300	$2x\ 10^{6}$
	Endospores	**3**	**2**	**0**	0	**93**	18	420	$9x\ 10^{3}$
1 day	Viable	3	**3**	**1**	**1**	**750**	170	2000	$7x\ 10^{5}$
	Endospores	**3**	**2**	**0**	0	**93**	18	420	$9x\ 10^{3}$
7 days	Viable	3	**3**	**1**	**0**	**430**	90	1800	$4x\ 10^{5}$
	Endospores	**3**	**1**	**1**	0	**75**	17	200	$7x\ 10^{3}$
28 days	Viable	3	**3**	**0**	**1**	**380**	87	1100	$4x\ 10^{5}$
	Endospores	**3**	**1**	**1**	0	**75**	17	200	$4x\ 10^{3}$

A sample calculation for the cell concentration fro 1 –day mortar sample was shown below. The combination for three positive tubes was selected from the four dilution such as the highest dilution was chosen that gives the positive results in all four dilutions testes and the two next succeeding higher dilutions.

DILUTIONS (mL)				Combination for positive tubes
10^{-1}	10^{-2}	10^{-3}	10^{-4}	
3	**3**	**1**	**1**	**3-1-1**

For 1-day bacterial mortar sample the MPN index was determined as 750 from the table listed in Most Probable Number Procedure and Tables in United States Department of Agriculture Report [90]. 3-1-1 defined with an MPN index of 750. In order to calculate the estimated cell concentration per mL, the MPN index was divided by the highest dilution for the combination and multiplied by 10.

$$\text{MPN/mL} = \frac{\text{MPN value from the table [86] x 10}}{\text{largest dilution series used in MPN determination}}$$

Therefore,

$$\text{MPN/mL} = \frac{750 \text{ x } 10}{0.01} = 7.5 \text{ x } 10^5 \text{ MPN/mL}$$

The MPN detection limit was determined assuming there was not any detectable turbidity observed in the testing tubes such that the combination will be 0-0-0 yielding an MPN index is less then 3 with a highest dilution of 0.1.

$$\text{MPN/mL} = \frac{3\text{x } 10}{0.1} = 3 \text{ x } 10^2 \text{ MPN/mL}$$

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