SCREENING OF MICROORGANISMS, CALCIUM SOURCES, AND PROTECTIVE MATERIALS FOR SELF-HEALING CONCRETE

by

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A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Biological Sciences

Hammond, Indiana

May 2019

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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ACKNOWLEDGMENTS

I would first like to thank my thesis advisor Prof. W. T. Evert Ting of the Biological Sciences Department at Purdue University Northwest for the continuous support of my graduate study and research. The door to Prof. Ting office was always open whenever I ran into a trouble spot or had questions about my research or writing. She consistently allowed this paper to be my own work, but steered me in the right the direction whenever she thought I needed it.

I would also like to thank the experts and my thesis committee who were involved in this research project: Assistant Professor Chien-Chung Chen and Prof. Scott Bates. Without their passionate participation and input, the study could not have been successfully conducted. I am gratefully indebted to their very valuable comments on this thesis.

This research project was funded by an Interdisciplinary Research Grant (2017-2018) and PNW Graduate Research Grant (2018-2019) from Purdue University Northwest, Indiana, USA. I would like to thank Expanded Shale, Clay and Slate Institute for providing the expanded shale and expanded clay used in this research.

I thank my colleagues: Hsin-Wen Liang for the stimulating discussions, for the time we were working together, and for all the fun we have had in the past years.

Last but not the least, I am profoundly grateful to my family: my family, Ming-Hsiang Chiu, Shu-Mei Liu, Yu-Huai Chiu, and my friends in the United States: Luke Dalach, Linda Dalach, Caleb Dalach, David Dalach, Elliana Dalach, and Matthew Hess for supporting me spiritually and providing happy distraction to rest my mind outside of my research..

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ABSTRACT

Author: Chiu, Chen-Hsuan. MS
Institution: Purdue University
Degree Received: May 2019
Title: Screening of Microorganisms, Calcium Sources, and Protective Materials for Self-healing Concrete.
Committee Chair: W. T. Evert Ting

To make bacterial-based self-healing concrete, alkaline-resistant bacterial spores, nutrient sources, and a calcium source are incorporated into a concrete matrix. Two ureolytic spore-forming bacteria, *Sporosarcina pasteurii*, *Lysinibacillus sphaericus*, and two non-ureolytic spore-forming bacteria, *Bacillus cohnii*, and *Bacillus pseudofirmus*, which have been used in previous studies as bacterial concrete healing agents, were compared in this study. The four bacteria were compared for their (1) sporulation rates on different sporulation agar plates, (2) growth in five liquid media, (3) survival rates in light weight aggregates (LWA) and in mortar samples, and (4) calcium carbonate precipitation rates from either calcium lactate or calcium nitrate. Sporulation was successfully induced after three-day incubation at 30°C on an appropriate sporulation medium. High sporulation rates of *B. cohnii*, and *B. pseudofirmus* (93% and 99% respectively) were found on alkaline R2A medium (AR2A). A sporulation rate (89%) of *S. pasteruii* was observed on tryptic soy agar supplemented with 2% urea (TSAU). The highest sporulation rate (60%) of *L. sphaericus* was found on R2A medium supplemented with 2% urea (R2AU). In the growth study, tryptic soy broth supplemented with 2% urea (TSBU) was a positive control which supported rapid growth of all four bacteria. *Sporosarcina pasteurii* and *L. pasteurii* showed rapid growth rates in alkaline yeast extract broth (AYE) and yeast extract with 2% urea broth (YEU) respectively. In contrast, *B. cohnii*, and *B. pseudofirmus* grew poorly in all media except in the positive control. Viable counts of the four bacterial spores reduced (1.8–3.3 logs) during the first 24 h in mortar samples and then remained stable for next 27 days testing period. Among the four, *S. pasteurii* showed the smallest reduction of viable counts (1.8–2.5 logs) in mortar after one day of incubation. Both *S. pasteurii* and *L. sphaericus* showed high $CaCO_3$ productions (>80%) after 24 h incubation at 30°C in YEU containing either calcium nitrate or calcium lactate. However, *B. pseudofirmus* and *B. cohnii* showed low calcite recovery rates (<11%) in AYE containing either calcium nitrate or calcium lactate under the same incubation condition. Overall, *S. pasteurii* was the best bacterial concrete

healing agent of the four. This bacterium had (1) rapid growth rate in AYE, (2) about 90% sporulation rate within 3 days, (3) highest survival rates after 24 h in mortar samples and, (4) high $CaCO_3$ precipitation rates, 82 or 98%, in broth containing calcium nitrate or calcium lactate respectively.

In addition, two different lightweight aggregates (LWA), expanded shale (ES) and expanded clay (EC), which were used as bacterial carriers and protective materials, were compared in this study. Each type of LWA was separated into three sizes (<0.85 mm, 0.85– 2.0 mm, and >2.0 mm) and immobilized with spores of *B. cohnii* or *B. pseudofirmus*. Viable counts recovered from EC and ES reduced <1.0 log after the immobilization process and remained stable during the 150 days testing period. Neither the type nor the particle sizes of the two LWA significantly affected the survival rates of the bacterial spores. This result showed that both EC and ES could be used as carriers for bacterial healing agents. It was also found that when the spores were immobilized with nutrients in LWA, their survival rates in mortar samples can be improved slightly (<1.0 log).

CHAPTER 1. INTRODUCTION

Concrete is one of the most commonly used material in constructions because of its durability, availability, ease of preparation, and low cost as compared to other construction materials [1]. However, concrete has a tendency to form cracks when it is exposed to environmental factors causing shrinkage and expansion. Unrepaired concrete cracks cause concrete deterioration, decreased performance, and lower lifespan. Significant amounts of money are required to repair damaged concrete. It was reported that the direct cost for concrete production is between \$65/m^3 and \$80/m^3; whereas maintenance of concrete and repairing cracks was approximately \$147/m^3 [2, 3].

Concrete can be repaired by either passive or active treatment. In passive treatment, sealers are applied to the areas where cracks occur. These sealing agents are usually made of chemical polymers such as epoxy resins and polyurethane, which are applied directly to the surface of the cracked concrete. The disadvantages of passive treatments include: (1) inability to seal cracks located in the interior portions of the concrete, (2) degradation of chemical sealers over time due to environmental impacts such as hot and/or cold temperatures, moisture, etc.,(3) potential environmental hazards caused by chemical sealers, and (4) human efforts required to inspect the concrete structure and apply the treatment [4–7].

Active treatments is a repair process that begins immediately after cracks in the concrete are formed. Such treatments do not require labor works for inspection and repairing as mentioned in the passive treatments. The active treatments are, therefore, considered as a self-healing process. The repair process can be the results of different chemical reactions that occur naturally in concrete in the presence of water. Such healing effects are also called autogenous healing. This occurs when water enters through the cracks of unhydrated cement resulting in the formation of new concrete to fill the cracks. Another autogenous healing mechanism is hydration of calcium oxide (CaO), which leads to the production of calcium hydroxide ($Ca(OH)_2$). Calcium hydroxide can further react with dissolved carbon dioxide (CO_2) to form calcium carbonate ($CaCO_3$) which can fill the cracks. The efficiency of autogenous repair mechanisms depends on the amount of unhydrated cement and CaO in concrete. The amount of the former can be controlled by lowering the water to cement ratio during concrete preparation. However, the workability of the concrete during the concrete preparation and concrete performance will be affected. A different way to achieve active

treatment is to incorporate chemical healing agents during concrete preparation to repair interior cracks. Due to the above mentioned limitations of existing active treatments, researchers are looking for alternative methods that are more cost-effective, not harmful to the environment, and that have little impact on the workability or performance of the concrete [8]. One alternative method for autogenous repair of concrete is to add bacteria-based "healing" agents to the concrete mix during preparation. These agents contain alkaline resistant bacterial spores, nutrients, and a calcium source. To achieve concrete healing, the agents are mixed with sand, cement, and water. After concrete curing, healing agents are thoroughly distributed inside the concrete. When the concrete cracks, water seeps in through the cracks allowing the bacterial spores to germinate and catalyze $CaCO_3$ formation, which then fills the crack. Since this process occurs naturally once bacterial healing agents are activated, such preparations are also known as "self-healing" concrete (Figure 1). This method can overcome the drawbacks in the existing active treatments [1].

To make self-healing concrete, it is important to find an ideal mix of self-healing agents, which include (1) a bacterium which can produce numerous spores, survive in concrete, and efficiently produce $CaCO_3$, (2) appropriate nutrients that support bacterial growth in the concrete after spore germination, and (3) an appropriate calcium source for the biomineralization process.

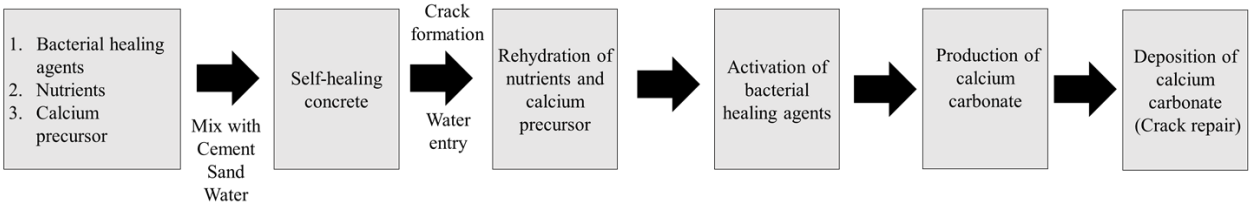


Figure 1 Preparation and repair mechanism of self-healing concrete.

1.1 Microbially Induced Carbonate Precipitation (MICP)

Biomineralization is a natural occurring mineral formation process induced by living organisms. For example, physiological developments in human such as teeth and bone formation are a biomineralization process. Biomineralization can also be accomplished by microorganisms, including bacteria, fungi, algae, and protozoa. It is known that many different inorganic minerals derived from cations (Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Na^{+}, *etc*.) and anions (OH^{-}, O^{2-}, SO^{2-}, CO^{2}, PO_4^{3-}, *etc.*) are formed in the biomineralization process. Among different biominerals, $CaCO_3$ is the most

common mineral produced [9]. Eight polymorphs of $CaCO_3$ that have been identified. Seven polymorphs are crystalline, and one is amorphous. Pure $CaCO_3$ are known as calcite, aragonite, and vaterite polymorphs [8–9]. Different microorganisms can induce biomineralization under specific conditions. For example, several types of microbial metabolisms can modify calcium sources and induce carbonation, in which $CaCO_3$ is produced. This process is called microbially induced $CaCO_3$ precipitation (MICP). Since $CaCO_3$ is the most common minerals formed in biomineralization, and it is highly compatible with cementitious compositions, it is a good candidate filler for concrete cracks [8].

Microbially induced $CaCO_3$ precipitation can be categorized as occurring into two pathways: autotrophic and heterotrophic. In autotrophic pathway, carbonate (CO_3^{2-}) is converted from CO_2 by microorganisms in three distinct ways: non-methylotrophic methanogenesis, oxygenic photosynthesis, and anoxygenic photosynthesis [10]. Each of these metabolic pathways can only be carried out by specific groups of microorganisms (*e.g.,* archaea, cyanobacteria, and purple bacteria) that are able to use CO_2 as a carbon source in the environment. During methanogenesis, CO_2 is converted into methane (CH_4), and the oxidation of CH_4 leads to the production of CO_3^{2-}. Dissolved CO_3^{2-} will precipitate as $CaCO_3$ if there is sufficient calcium available in the environment [8]. In both oxygenic and anoxygenic photosynthesis pathways, CO_2 is converted into methanol by using different types of electron donors. Water serves as the electron donor in oxygenic photosynthesis whereas hydrogen sulphite (H_2S) is the electronic donor in anoxygenic photosynthesis. It was reported that depletion of CO_2 in a bicarbonate solution from microorganisms leads to the production of $CaCO_3$.

In heterotrophic pathway, the mineralization occurs when bacterial metabolites are introduced into an environment where calcium sources are present [8]. For example, the degradation of urea ($CO(NH_2)_2$) in aerobic environments by bacteria using urease enzyme will lead to the production of ammonium (NH_4^+) and CO_3^{2-}. In the presence of Ca^{2+}, CO_3^{2-} are able to precipitate into $CaCO_3$. Some microorganisms are able to metabolize organic calcium compounds such as calcium lactate to produce CO_2 and Ca^{2+}. Dissolved CO_2 in solution becomes HCO_3^-, which will also interact with Ca^{2+} to produce $CaCO_3$ precipitate.

1.2 Concrete Healing Microorganisms

Different microorganisms, including bacteria and fungi, have been used for making self-healing concrete. The internal environment of concrete is dry and alkaline (pH >13). Therefore, an ideal microbial healing agent should be able to tolerate such conditions. Bacterial spores are dormant form of spore-forming bacteria that are able to withstand environmental stress such as high and low temperatures, desiccation, extreme pH, etc. The structure of a typical bacterial spore includes exosporium, spore coat, cortex, bacterial cell wall, and a core (Figure 2). Exosporium and spore coat are made of proteins, whereas cortex and bacterial cell wall are made of peptidoglycan. Exosporium, spore coats, and cortex form layers and protect the core, which contains spore genetic materials. These unique outer structural components allow bacterial spores to resist environmental stresses [11].

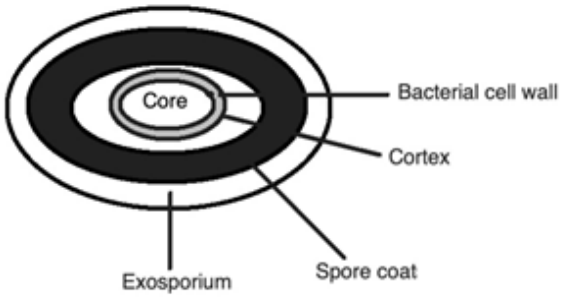


Figure 2 A representation of a bacterial spore (Structures are not drawn to scale).

Spore-forming bacteria form spores when the cells are stressed or starving. Sporulation process of *Bacillus* spp. can be divided into 7 stages: (1) asymmetric cell division, (2) prespore formation, (3) engulfment of the prespore, (4) formation of cortex, (5) formation of spore coats, (6) maturation, and (7) release [11]. A mature spore is able to withstand external stresses. When surrounding environments become ideal, in which water and nutrients are available, the spore can be re-activated and show microbial activity. The re-activation process includes activation, germination, and outgrowth. Activation can be triggered when the spore coat is damage by heat, chemicals, UV lights, *etc*. After germination, the cell will undergo a series of metabolisms, including protein synthesis, and eventually grow into a vegetative cell. In these three phases, only

activation is a reversible process. A bacterial spore loses its dormancy once it commits to germinate [11].

Alkaline resistant spore-forming bacteria with MICP ability are potential candidates for making self-healing concrete. *Sporosarcina pasteurii, Lysinibacillus sphaericus, Bacillus cohnii, and Bacillus pseudofirmus* are the most studied concrete healing microorganisms [1–8]. Among these, *S. pasteurii* and *B. sphaericus* are ureolytic bacteria which form $CaCO_3$ through urea decomposition; whereas *B. cohnii* and *B. pseudofirmus* are non-ureolytic bacteria [1, 3, 7, 8]. They produce $CaCO_3$ by converting organic calcium salts into CO_3^{2-} [3, 8].

1.3 Sporulation Media

Several sporulation media have been described in previous self-healing concrete studies. A liquid minimal basal salts medium, containing (g/L): 6.8 KH_2PO_4, 0.3 $MgSO_4 \cdot 7\ H_2O$, 0.02 $MnSO_4$, 0.02 $Fe_2(SO_4)_3$, 0.02 $ZnSO_4 \cdot 7\ H_2O$, 0.2 $CaC1_2$, supplemented with 1% tryptose and 0.2% yeast extract was used to produce spores of *L. sphaericus* (Table 1) [15,22]. The bacteria were incubated in this medium for 14 to 28 days at 28°C on a shaker at 100 rpm before harvesting the spores. Jonkers *et al.* compared sporulation rates of *B. cohnii, B. pseudofirmus,* and *S. pasteurii* in a liquid minimal basal salts medium containing (g/L) 0.2 NH_4Cl, 0.02 KH_2PO_4, 0.225 $CaCl_2$, 0.2 KCl, $MgCl_2$, 0.2 $MgCl_2 \cdot 6H_2O$, 1.0 ml trace element solution SL12B, 0.1 yeast extract, 6.45 citric acid trisodium salt, 8.4 $NaHCO_3$ (pH 9.2) on a shaker at 150 rpm [23]. The sporulation rates of *B. cohnii and B. pseudofirmus* were 75% and 25%, respectively, whereas *S. pasteurii* did not produce any spores in this mineral medium. The length of incubation period used in this study is not known. Jonkers *et al.* used an alkaline mineral medium (pH 10), containing (g/L) 0.2 NH_4Cl, 0.02 KH_2PO_4, 0.225 $CaCl_2$, 0.2 KCl, 0.2 $MgCl_2 \cdot 6H_2O$, 1.0 ml trace element solution SL12B, 0.1 yeast extract, 5.16 citric acid trisodium salt, 0.01 g $MnSO_4 \cdot 2H_2O$, 4.2 g $NaHCO_3$ and 5.3 g Na_2CO_3 to produce spores of *B. cohnii and B. pseudofirmus*. However, the sporulation rates were not reported [24]. A recent study claimed that a 98% sporulation rate of *B cohnii* and 100% sporulation rates of *B. pseudofirmus* occurred after 6 h and 26 h respectively in the same alkaline mineral medium supplemented with manganese [25]. Sporulation media mentioned above were made of numerous chemicals with minimal amounts, and some ingredients are not common. These media are not commercially available and making these media are very time-consuming. Therefore, searching

for alternative sporulation media that are commercially available and can be easily prepared may help in the future studies.

R2A is a commercially available medium recommended for enumeration and isolation bacteria from environmental samples. This medium contains less nutrients than the commonly used plate count agar to allow stressed bacteria to recover from environmental stresses. Composition of R2A is shown in Table 2. Some *Bacillus* species isolated from soil samples were found to sporualte vigorously on R2A agar in our lab. Therefore, this medium was chosen for testing sporulation of the four bacterial concrete healing agents in this study. Sporulation agar (SA) was recommended by the culture provider. It contains manganese which is known to promote bacterial sporulation.

Table 1 Overview of media which have been used to grow *Lysinibacillus sphaericus*, *Sporosarcina pasteurii*, *Bacillus pseudofirmus*, and *Bacillus cohnii* for self-healing concrete

Microorganism	Nutrients	References
L. sphaericus	Yeast extract and urea	[1, 13, 15, 16, 22]
	Minimal basal salts (MBS) medium	[15, 16, 26]
	Peptone, glucose, yeast extract	[8]
	Nutrient broth (NB), urea	[27]
S. pasteurii	NH_4-YE	[17]
	Peptone, meat extract, urea	[28]
	ATCC 1832 medium	[29]
	Brain heart infusion (BHI)	[30]
	Tryptic Soy broth, urea	[31]
	Yeast extract	[14]
B. pseudofirmus	Luria-Bertani (LB)	[25]
	Peptone, meat extract, alkaline solution	[23, 24]
B. cohnii	Luria-Bertani (LB)	[25]
	Peptone, meat extract, alkaline solution	[24]

Table 2 Media composition for nutrient broth (NB), trypticase soy broth (TSB), brain heart infusion (BHI), Luria-Bertani broth (LB), sporulation agar (SA), and R2A.

Media	Nutrients composition (g/L)
NB	Yeast extract 3.0, glucose 1.0, peptone 15.0, NaCl 6.0
TSB	Tryptone 17.0, soytone 3.0, NaCl 5.0, K_2HPO_4 2.5
BHI	Brain heart infusion 8, peptic digest 5.0, casein 16.0, NaCl 5.0, glucose 2.0, Na_2HPO_4 2.5
LB	Tryptone 10.0, NaCl 10.0, yeast extract 5.0
SA	Peptone 5.0, meat extract 3.0, $MnSO4{\cdot}H_2O$ 0.01, agar 15.0
R2A	Casein acid hydrolysate 0.5, yeast extract 0.5, dextrose 0.5, soluble starch 0.5, K_2PO_4 0.3, Na pyruvate 0.3, casein peptone 0.25, peptic digest of animal tissue 0.25, $MgSO_4$ 0.05

1.4 Culture Media

As shown in Table 1, both complex media, such as nutrient broth (NB), brain heart infusion (BHI), tryptic soy broth (TSB), Laureia broth (LB), yeast extract (YE), and synthetic media have been used in previous studies to cultivate the four concrete healing bacteria. Ingredients of these media are either listed in Table 2 or described in the previous paragraph.

Bacteria used in previous studies were cultivated at 30°C overnight before use. Urea was supplemented into media to promote growth of ureolytic bacteria such as *S. pasteurii* and *L. sphaericus*. The final pH of media was close to 9 due to the hydrolysis of urea during autoclaving. To cultivate non-ureolytic bacteria, alkaline media, prepared by adjusting the base media with filter sterilized Na-sesquicarbonate solution containing 4.2 g $NaHCO_3$, 5.3g Na_2CO_3, 100 mL dH_2O to achieve final pH 9.5, were used [12]. Despite the fact that different media could be used to cultivate microbial healing agents, comparisons of microbial growth in different media have not been reported previously, and the results may contribute to the selection of embedded nutrients to support bacterial growth in self-healing concrete. Yeast extract was selected as a culture medium for bacteria preparation in previous studies [1, 8, 12–17]. Yeast extract was also a nutrient source incorporated into self-healing concrete [19–21]. The concentration of YE to supported microbial growth was tested and found to be between 0.4% to 2% [18].

1.5 Survival of Bacterial Strains in Cement

The number of microorganisms in a sample can be determined through direct and indirect methods. A direct method such as viable plate counts is achieved by inoculating a diluted bacterial sample on agar plates and enumerating bacterial colonies formed on the plate after incubation. On the other hand, indirect methods are measurements of a microbial activity such as oxygen consumption or urease activity.

In a bacteria-based self-healing concrete system, the ability of the bacterial healing agent to survive in the concrete is critical. The concrete environment is known to be dry and extreme alkaline (pH 10–13). Most bacteria do not survive in this harsh environment. Alkaline resistant spore-forming bacteria have been a preferred choice as concrete healing agents because bacterial spores can remain dormant and survive in dried environment for many years. A previous study showed that bacterial healing agents survived for less than 135 days when the spores were added

to concrete mix directly [24]. Death of the spores may be caused by the decreased pore sizes in aged cement matrix. It has been shown that pore sizes in concrete decreased due to concrete hydration. Young (3 and 7 day cured) cement stone samples had more large pores (0.1–1 μm) whereas aged (28 day cured) cement stone samples contained more small pores (0.01–0.1 μm). The sizes of bacterial spore range from 0.8 to 1.2 μm [32]. Bacterial spores would be crushed when pore sizes drop below 0.8 μm [24]. Therefore, it is expected that immobilization of bacterial healing agents in a carrier prior to cement mix can enhance the survival.

1.6 Protective Materials

Shorten lifespan and low healing effects of a self-healing concrete may result from the low survival of bacterial healing agents. Therefore, it is necessary to provide protection to microbial healing agents. Researchers have tried different means to protect bacteria, including immobilizing healing agents in different protective materials (Table 3). An ideal protective material should be cost efficient and extend lifespan of self-healing agents in self-healing concrete without compromising the concrete performance.

1.6.1 Encapsulation in Polyurethane and in Silica Gel

Protection to microorganisms can be achieved by different means, including encapsulation using polyurethane and microcapsules [1, 15, 29]. Polyurethane (PU) are porous, mechanically strong, and stable materials that have been used to protect enzymes or cells. During the process of polymerization, CO_2 is released from the PU, and voids are created. The formation of voids not only increases the surface areas for substrates or products of an enzyme reaction but also solves the diffusion limitation. [29]. Bang *et al.* studied $CaCO_3$ precipitation induced by *S. pasteurii* immobilized in PU [29]. In that study, a suspension of *S. pasteurii* was mixed with PU components and embedded in cement mix. Mortar samples with bacterial-immobilized PU exhibited increased tensile strength and calcite precipitation after 7 days in a calcium sufficient solution. Although reduced microbial enzymatic activity and calcite precipitate rates were reported, authors concluded that PU could be a potential carrier that might stabilized microbial activity for extended period.

In another study, PU and silica gel were compared for both protective effects on microorganisms and healing effects on concrete [1]. Silica gel is a common carrier for microorganisms or enzymes based on the inert characteristics of thermal and mechanical stability

it provides. The porous surface on silica gel molecule makes it suitable for carrying different agents. In the past, bacteria were mixed with silica sol and injected into concrete cracks artificially [7]. Reduced water permeability was seen in treated samples. The same research group further modified previous work by immobilizing healing agents (bacteria, nutrients, and calcium sources) in different carriers (silica gel and PU), and then the carriers were placed in glass tubes during casting cements. All the healing agents were designed to be released once glass tubes were broken due to compression in concrete, and the capillary effect would allow healing agents to flow to the cracks. Specimens were then tested for microbial activities and healing effects. According to the results, higher ureolytic and carbonatogenesis activities were seen from samples made with bacteria immobilized in silica gel. However, concrete prepared with bacteria immobilized in PU showed increase in strength and lower water permeability. These results suggested that silica gel provided higher protective effects than did PU; whereas, PU had more potential to serve as a bacterial carrier in self-healing concrete [1].

1.6.2 Encapsulation in Hydrogel and in Melamine-Based Microcapsules

Water is an essential element for microbial activity. Having the ability to retain water, hydrogel was proposed to carry and protect microbial healing agents. It was found that mortar samples with hydrogel-encapsulated microbial healing agents showed superior healing results than the ones without hydrogel [16].

In another study [15], melamine-based microcapsules were used as a carrier for *L. sphaericus* spores. Each microcapsule particle is approximately 5 μm in size with characteristics of humidity sensitive and resistance to the high pH. Encapsulated microcapsules are expected to withstand the mixing process and distributed evenly in concrete. Upon cracks formation and water entry, exposed microcapsules in cracks are able to release healing agents. In the study, microcapsules containing approximately 10^9 spores, nutrients, and calcium sources were prepared and used in mortar samples preparation, and specimens were tested for crack healing ratio and water permeability. Specimens with healing agents in microcapsules showed better healing effects with 0.5 mm healed cracks and 68% decrease in the water permeability. The spores released from microcapsules were remained viable after 3 days. Therefore, the authors suggested that bacterial spores could be protected by microcapsules in cement.

1.6.3 Diatomaceous Earth

Diatomaceous earth (DE) is a siliceous sedimentation from fossilized remains of diatoms with average 10 to 200 μm particle sizes [33, 34, 35]. It is highly porous, light in weight, and chemically and mechanically stable. Application of DE in cementitious environment to serve as a carrier or a protective material for *L. sphaericus* was studied [13]. In the study, 24-h old vegetative cultures were immobilized in DE, and microbial enzymatic activities were tested. Microbial viability was determined based on the ureolytic activities from immobilized bacteria under different pH conditions. It was found that bacteria immobilized in DE showed a high ureolytic activity in high pH environments than did non-immobilized bacteria. Mortar specimens containing DE-immobilized bacteria showed crack-healing effects and reduced water penetration. However, DE was found to reduce compressive strength of mortar samples in another study [38].

1.6.4 Light Weight Aggregates

Light weight aggregates (LWA), such as expanded clay (EC), are light and porous materials used to prepare lightweight concrete. Jonkers *et al*. [36] immobilized bacterial spores and organic compounds in EC prior to mixing into mortar and found the immobilized spores remained stable up to six months after incorporation. Wiktor and Jonkers [12] immobilized *Bacillus alkalinitrilicus* spores and organic compounds in EC particles and showed that incorporating both nutrients and bacterial spores could enhance self-healing effects. Therefore, it was reported that EC could act as a reservoir for self-healing agents.

1.6.5 Graphite Nano Platelets

In a recent study, graphite nano platelets (GNP) were used as a carrier to protect microorganisms in cement mix and prepared into concrete samples [37]. Crack-healing effects between concrete prepared with bacteria immobilized in GNP and bacteria immobilized in EC were compared. It was found that among the concrete samples that were cracked after a short-term curing (3 and 7 days), samples contained bacteria immobilized in GNP showed better healing than did the samples contained bacteria immobilized in EC. On the other hand, among the concrete samples that were cracked after a long-term curing (14 and 28 days), the samples containing bacteria immobilized in EC showed better healing effects than did samples contained bacteria immobilized in GNP. It was concluded that EC provided long-term healing effects than GNP.

1.6.6 Negative Impact of Protective Materials on Mortar

Although many materials have been studied to enhance microbial survival in concrete or mortar samples, some of them may negatively impact concrete performance. In a study, the compressive strength of mortar samples made with five different protective materials (DE, EC, granular activated carbon, metakaolin, and zeolite) immobilized with *L. sphaericus* spores were compared [38]. Metakaolin is a dehydroxylated form of the clay mineral kaolinite particle with 5 μm to 200 μm size. Granular activated carbon and zeolite, similar to EC, are materials with sizes between 0.5 mm to 2 mm. According to their results, mortar samples made with zeolite and mortar samples with DE showed reduced compressive strength. The rest three protective materials increased the compressive strength. The authors also suggested that the negative effects caused by microorganisms on compressive strength could be avoided by using porous materials such as EC and granular activated carbon [24, 25].

Table 3 Overview of microorganisms and protective materials used in concrete matrix

Microorganisms	Protective materials	References
Ureolytic		
B. sphaericus	Polyurethane	[29]
	Silica gel	[7]
	Silica gel and polyurethane	[1]
	Microcapsules	[15]
	Diatomaceous earth	[13]
B. subtilis	Diatomaceous earth	[35]
	Light weight aggregates, graphite nano platelets	[37]
Non-ureolytic		
B. cohnii	Light weight aggregates	[39]
B. alkalinitrilicus	Light weight aggregates (Expanded shale)	[21]

1.7 Calcium Compounds as Calcium Sources

Calcium carbonate ($CaCO_3$) can be produced through MICP process in the presence of a calcium source. Carbonate ions can be produced through many different pathways, including hydration of unhydrated cement, reactions between CO_2 and dissolved calcium hydroxide ($Ca(OH)_2$), hydrolysis of urea, heterotrophic growth of bacteria on different organic nutrients such as lactate, *etc*.

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3$$

Calcium chloride ($CaCl_2$) was used as a calcium source for calcite production in several previous studies [14, 29, 40, 41]. Calcium carbonate precipitation outside cementitious environment was observed when ureolytic *Bacillus* sp. bacteria were mixed with calcite precursor solutions composed of urea and $CaCl_2$ [41]. Calcium carbonate production induced by *S. pasteurii* was observed by Stocks-Fischer *et al.* in a sand column [14]. After a sand column mixed with microorganisms continuously fed with urea-$CaCl_2$ solution, crystals were observed and identified as $CaCO_3$. Same crystals formation in cementitious conditions was seen in other studies and proved to enhance durability of concrete [29, 42]

However, a study showed that chloride ions released from $CaCl_2$ may gradually degrade steel reinforcement bars [17]. Therefore, alternative calcium sources such as calcium nitrate ($Ca(NO_3)_2$), calcium acetate ($Ca(C_2H_3O_2)_2$), and calcium lactate ($CaC_6H_{10}O_6$), were used to replace $CaCl_2$ in self-healing concrete [8, 17, 26, 28, 43]. Xu *et al.* compared calcium lactate and calcium nitrate as a calcium source for $CaCO_3$ precipitation, and results showed that the

precipitation rate of $CaCO_3$ from calcium lactate was over twice of that from calcium nitrate [28]. In another study three different calcium sources, $CaCl_2$, $Ca(NO_3)_2$, and $(Ca(C_2H_3O_2)_2$ were incorporated in concrete and their impacts on compressive strength, splitting tensile strength, and water absorption were compared. It was found that mortars treated with calcium acetate showed the highest compressive and splitting strength [17]. Comparison of two calcium sources showed that the maximum yield of $CaCO_3$ precipitation was achieved from $CaCl_2$, followed by calcium nitrate [43]. Another concern was the ammonium production during MICP process when ureolytic bacteria decompose urea into NH_4^+. The excess NH_4^+ production problem can be resolved by using non-ureolytic bacteria. Jonkers *et al*. [12, 21, 36, 44, 45] studied non-ureolytic *B. cohnii*, and *B. pseudofirmus* that are able to use calcium containing organic compounds such as calcium lactate to produce both Ca^{2+} and CO_2, which form $CaCO_3$.

Various concentrations of calcium sources were used in previous studies (Table 3). Calcium chloride concentrations between 0.025 M–1.000 M were tested previously as the calcium source for $CaCO_3$ precipitation [8, 14, 29, 35, 41, 46, 47]. Concentrations of other calcium salts ranged between 0.025 M-0.253 M were also tested [8, 25, 28, 31, 38, 46]. In some studies, the amounts of calcium sources were reported as the weight percentage of cement instead of exact concentrations [35]. In one study, concentrations of both calcium ions and urea were studied for optimum conditions for microbial carbonate precipitation [30]. Three different concentrations of $CaCl_2$ solutions (2.5, 25, and 250 mM Ca^{2+}) and two concentrations of urea (333 and 888 mM) were tested, and maximum $CaCO_3$ production (13 mg) was observed under concentrations of 250 mM Ca^{2+} and 666 mM urea. The authors also concluded that the amount of $CaCO_3$ precipitation would be proportional to the concentrations of urea and Ca^{2+}.

Table 4 Overview of calcium sources and microorganisms which have been used to produce calcium carbonate

Calcium sources	Concentration of calcium sources	Microorganisms	References
Calcium chloride	25 mM	*Bacillus* sp.	[46]
	12.6, 25.2, 50.4 mM	*S. pasteurii*	[14, 29]
	1 M	*Bacillus* sp.	[41]
	25, 40 g/L	*L. sphaericus*	[8]
	20 g/L	*Bacillus* sp.	[47]
	0.22 cement wt%	*B. subtilis*	[35]
Calcium oxide	40 mM, 50 mM	*Bacillus* sp.	[46]
Calcium acetate	25 mM	*Bacillus* sp.	[46]
	2.5 g/L	*B. cohnii, B. pseudofirmus*	[25]
	13.5, 9 g	*L. sphaericus*	[38]
	25, 40 g/L	*L. sphaericus*	[8]
Calcium nitrate	25 mM	*Bacillus* sp.	[46]
	25 mM	*S. pasteurii*	[28]
	25, 40 g/L	*L. sphaericus*	[8]
	13.5, 9 g	*L. sphaericus*	[38]
Calcium lactate	25 mM	*S. pasteurii*	[28]
	91.7 mM	*S. pasteurii*	[31]
	25, 40 g/L	*L. sphaericus*	[8]
	0.5 cement wt%	*B. cohnii, B. pseudofirmus*	[24]
	18 kg/m^3	*B. subtilis*	[37]

1.8 Calcium Carbonate Quantification and Identification

In order to evaluate the effectiveness of microbially induced carbonate, it is necessary to quantify the amount of $CaCO_3$ formed in the medium using either direct or indirect measurements. Direct quantification is to measure the weights of $CaCO_3$ produced by bacteria in the presence of a calcium source [30, 31]. In this method, $CaCO_3$ precipitation is separated from the liquid culture medium with a filter paper and then the dry weight of the precipitate is measured. Indirect measurement does not measure the amount of $CaCO_3$ directly, instead, it converts $CaCO_3$ into different elements. For example, the chemical reaction between $CaCO_3$ and hydrochloric acid (HCl) will generate $CaCl_2$, CO_2, and H_2O. By measuring the amount of CO_2 generated can help determine the amount of $CaCO_3$ in a sample [3]. Ethylenediaminetetraacetic acid (EDTA) titration is another indirect method that were used to quantify $CaCO_3$ in a sample [42]. When EDTA is introduced into a solution containing calcium, it combines with the calcium. Therefore, the concentration of the calcium can be determined directly. Atomic absorption spectroscopy (AAS) can be used to measure dissolved Ca^{2+} concentration [26]. Atomic absorption spectroscopy is used for analysis of many trace elements in food, pharmaceutical, and industrial products with high sensitivity. Samples are vaporized and decomposed into gaseous atoms in AAS. The absorptions of these atoms under specific wavelength of radiation showed unique absorption bands that help the identification. Quantification can be achieved by comparing the amount of absorption from a sample with a standard curve.

Different equipment can be used to identify $CaCO_3$. Scanning electron microscope (SEM) is a widely used in previous studies to image the $CaCO_3$ crystals in a sample [12, 17, 23, 29, 37, 43, 47]. Instead of light, electron beams are the source and used to scan over samples to create images in SEM. Although SEM provides a high resolution, quantification of $CaCO_3$ in a sample cannot be achieved. X-ray diffraction (XRD) analysis is commonly used to determine the crystallinity of a compound. The X-ray is generated and aims directly toward samples. Each crystalline sample perform unique diffraction pattern and different intensities of the peaks. Therefore, it was used to identify and quantify $CaCO_3$ in mortar samples previously [14, 17, 18, 28, 43, 47].

In some self-healing studies, $CaCO_3$ was not quantified. Instead, the strength or the water permeability of cracked concrete were measured. Concrete strength is expected to increase when cracks are repaired as a result of MICP. Water permeability test can test the tendency and the speed of water to permeate through concrete. Higher water permeability is expected in concrete with

wider cracks since the water can permeate easily. Therefore, a decreased water permeability of a self-healing concrete suggests crack healing due to MICP.

1.9 Objectives

Both ureolytic and non-ureolytic alkaline resistant spore-forming bacteria have been used for making self-healing concrete. However, there is lack of study comparing their performance as concrete crack healing agents. In the study, two ureolytic bacteria, *L. sphaericus* and *S. pasteruii* and two non-ureolytic bacteria, *B. pseudofirmus*, and *B. cohnii* were compared for the following four aspects: (1) their sporulation rates on various sporulation agar plates, (2) their growth rates in various liquid media, (3) their survival in mortar samples, and (4) their $CaCO_3$ production rates using calcium lactate or calcium nitrate as a calcium precursor in liquid media. The best of the four bacteria based on results of above tests was prepared in mortar samples for the visual evaluation of crack repair.

Additionally, two different lightweight aggregates, expanded clay (EC) and expanded shale (ES) in three different size ranges,: small (S, <850 μm), medium (M, 850 μm– 2.00 mm), and large (L, 2.00 mm– 4.75 mm), were compared for their ability (1) to serve as a carrier for bacterial spores for making self-healing concrete, (2) to enhance survival of bacterial spores in mortar samples.

CHAPTER 2. EXPERIMENTAL METHODS

2.1 Bacteria Cultures

Bacillus pseudofirmus LMG 17944 and *L. sphaericus* LMG 22257 were obtained from Belgian Coordinated Collections of Microorganisms (Ghent, Belgian). *Bacillus cohnii* ATCC 51227 and *S. pasteurii* ATCC 11859 were obtained from American Type Culture Collection (Manassas, VA, USA). *Lysinibacillus sphaericus* and *S. pasteurii* were maintained on tryptic soy agar (CriterionTM) supplement with 2% urea (CriterionTM) slants (TSAU). *Bacillus cohnii* and *B. pseudofirmus* were maintained on nutrient agar (CriterionTM) supplemented with filter-sterilized Na-sesquicarbonate solution (4.2 g $NaHCO_3$, 5.3 g Na_2CO_3, 100 mL dH_2O) (alkaline nutrient agar, ANA). Stock cultures were stored at 4°C.

2.1.1 Working Cultures Preparation

Working cultures of *L. sphaericus* and *S. pasteurii* were prepared by inoculating stock cultures into tryptic soy broth (CriterionTM) supplement with 2% urea (TSBU) and cultivated at 30°C overnight prior to experimentation. Working cultures of *B. cohnii* and *B. pseudofirmus* were prepared by inoculating stock cultures into nutrient broth (CriterionTM) supplemented with filter-sterilized Na-sesquicarbonate solution (alkaline nutrient broth, ANB) and cultivated at 30°C overnight prior to experimentation. To prepare a bacterial suspension for inoculation, 1 mL of an overnight culture was centrifuged at 13,000 rpm for 2 min and the pellet was resuspended in 1 mL of sterile dH_2O. This procedure was repeated for three times.

2.1.2 Spore Suspension Preparation

To prepare a spore suspension for sporulation study and survival study, a bacterial suspension was spread on a sporulation agar. *Bacillus cohnii* and *B. pseudofirmus* were inoculated on AR2A; *L. sphaericus* and *S. pasteurii* were inoculated on R2AU and TSAU respectively. Cultures were incubated at 30°C for 3 days. Each culture was washed from the plate with sterile dH_2O and heated at 70°C for 20 min to kill vegetative cells. The viable counts of spores were determined by pour plate method using TSAU or ANA.

2.2 Media Preparation

Six sporulation agar plates were prepared for the sporulation test: TSAU, sporulation agar supplemented with 2% urea (SAU), R2A (CriterionTM) supplemented with 2% urea (R2AU), sporulation agar supplemented with filter-sterilized Na-sesquicarbonate solution (ASA), ANA, and R2A supplemented with filter-sterilized Na-sesquicarbonate solution (AR2A).

Media containing 2% urea (TSBU, SAU, YEU, and R2AU) were first adjusted to pH 6 and then autoclaved at 121°C for 15 min. The final pH values were around 8.0. Alkaline media, which included ANA, ANB, ASA, AYE, and AR2A, were prepared by rehydrating ingredients of each base medium using 90% (v/v) of dH_2O required by the formula (Table 2) and then adding (100 mL/L) filter-sterilized Na-sesquicarbonate solution (4.2 g $NaHCO_3$, 5.3 g Na_2CO_3, 100 mL dH_2O) to the base medium after sterilization to achieve final pH 9.5.

Five different broth were prepared for the growth rate study: ANB, TSBU, 2% yeast extract (YE) (BD Bacto™), YE supplemented with 2% urea (YEU), YE and YEU supplemented with filter-sterilized Na-sesquicarbonate solution (alkaline yeast extract, AYE, and AYEU respectively).

Calcium lactate (CL, $CaC_6H_{10}O_6$) and calcium nitrate (CN, $Ca(NO_3)_2$) solutions (366.8 mM) were prepared and sterilized by using a filter system (0.22 μm Bottle-top vacuum filter system, Corning, NY). Four percent of YEU and AYE were prepared as mentioned above. To prepare the media for the calcite production test, each calcium solution was mixed with 4% YE solutions (YEU and AYE) in 1:1 ratio to become YEUCN, YEUCL, AYECN, and AYECL. The final Ca^{2+} concentration in each medium was 183.4 mM with 2% YE. Both calcium sources decreased the pH values of the media, and the pH values of media were recorded in Table 5. The supplementation of calcium nitrate caused pH values dropped 0.73– 1.47 in TSBU, YEU, and AYE; whereas, the supplementation of calcium lactate caused the pH values dropped approximately 0.40– 0.94 in these media.

Table 5 pH values of tryptic soy broth with 2% urea (TSBU), yeast extract with 2% urea (YEU), and alkaline yeast extract (AYE) supplemented with calcium nitrate or calcium lactate. (Ca source 183.39 mM)

Medium Base	Supplement	pH
ANB	-	9.6
TSBU	-	7.5
	Calcium nitrate	6.2
	Calcium lactate	6.7
YEU	-	6.8
	Calcium nitrate	6.1
	Calcium lactate	6.4
AYE	-	8.9
	Calcium nitrate	7.4
	Calcium lactate	7.9

2.3 Sporulation Study

Working cultures of the four bacterial strains were individually inoculated on the six different agar plate, TSAU, SAU, R2AU, ANA, ASA, and AR2A with sterile cotton swabs and incubated at 30°C. Bacterial growth was compared after incubation for 24 h. Grown bacterial samples on each medium were collected after 1, 3, and 7 days of incubation and stained using Bartholomew and Mittwer's endospore staining method. A total of 300 cells from each sample were examined for spore formation under a microscope. Mean sporulation rates of three trials were used for comparison.

Sporulation rates (%) = (number of spores/ total number of cells examined) x 100%.

2.4 Growth Study

Bacterial growth of each microorganisms was tested in ANB, TSBU, YE, YEU, and AYE. Bacterial suspension of each strain was prepared and adjusted to 0.3 optical density ($OD_{540\ nm}$) which contained approximately 10^6 CFU/mL. Twenty µl of a bacterial suspension and 180 µl of a test medium were added to each of triplicate wells in a 96 well plate (Tissue Culture Plate 96 wells Flat Bottom, Fisher, Pittsburgh, PA) and incubated in a plate reader (SpectraMax 13, Molecular

Devices, Sunnyvale, CA) at 30°C for two days. Bacterial growth in each well was measured by determining OD_{540nm} periodically. The plate was shaken 5 secs before taking each OD reading. Growth curves were generated based on the $OD_{540\ nm}$ readings.

2.5 Survival Study

In this part, survival of the four bacteria in LWA and in mortar samples were compared. The experimental design was shown in Figure 3. Bacterial spores of each microorganism were harvested from sporulation media that induced the maximum spore production of each culture after incubation at 30°C for 3 days. Two different lightweight aggregates, expanded clay (EC) and expanded shale (ES), were used in this study for comparing their effectiveness in protecting bacteria. Such goal can be achieved by evaluating the viable plate counts of immobilized spores in both LWA and in mortar samples.

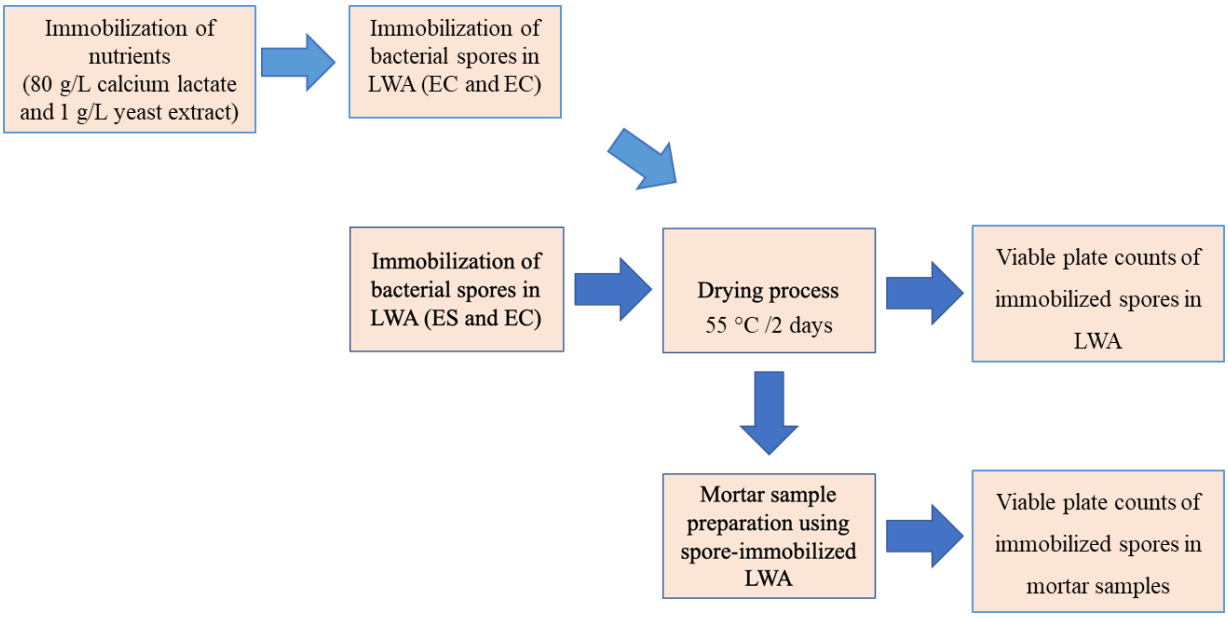


Figure 3 Experimental design of survival test

2.5.1 Immobilization of Bacteria or Immobilization of Nutrients and Bacteria into LWAs and Mortar Samples

Both lightweight aggregates (EC and ES) were sieved into three different sizes: small (S, <850 μm), medium (M, 850 μm– 2.00 mm), and large (L, 2.00 mm– 4.75 mm). All the materials were sterilized by heating in a hot air oven at 180°C for 24 h before mixing. Immobilization of spores into LWA was accomplished by soaking 50 g of sterile sieved LWA in 40 ml of spore suspension in a beaker and then applying a negative pressure (-0.8 bar) for 20 min and a positive pressure (+0.6 bar) for 10 min. After immobilization, samples were dried at 55°C for 2 days. The final concentration of bacterial spores on LWA was approximately 10^6 spores/g particle. The day which samples finished drying process was considered day 0. The LWA with immobilized spores were stored in sterile containers at room temperature before testing. To determine the effect of nutrients on the survival of immobilized spores in mortar samples, calcium lactate (80 g/L) and yeast extract (1 g/L) were first immobilized and dried in LWA using the same procedure above, followed by the immobilization of bacterial spores.

2.5.2 Preparation of Mortar Samples for Survival Tests

River sand and type I Portland cement were sterilized in a hot air oven at 180°C for 24 h before use. Mortar samples were prepared by mixing LWA (ES or EC) with immobilized bacterial spores, sand, cement, and sterile dH_2O in a ratio of 1:3:2:1 (by weight) and the mixtures were allowed to harden at room temperature for 24 h. Control mortar samples were prepared by non-immobilized bacterial spores.

2.5.3 Survival of Immobilized Bacterial Spores in LWA and in Mortar Samples

Viable counts of spores in LWA were determined on day 0, 7, 14, 21, 90, and 150 using a plate count method. Viable counts of spores in mortar samples were enumerated on day 0, 7, 14, 21, and 28. To recover spores from LWA, 30 mL of sterile dH_2O was mixed with 3 g LWA and agitated with a vortex for 30 sec. To recover spores from a mortar sample, 30 mL of sterile dH_2O was mixed with 6 g crushed mortar sample and agitated with a vortex for 30 sec. The wash solutions were serially diluted with sterile dH_2O, plated on ANA or TSAU, and incubated for 48 h at 30°C. The numbers of viable cells were recorded as Log CFU/ g of LWA or mortar sample.

2.6 Calcium Carbonate Production Study

Microbially induced $CaCO_3$ precipitation from two different calcium sources, calcium lactate and calcium nitrate, by the four bacteria was compared. Each bacterial suspension was adjusted to 1.0 OD_{540nm} and 1 mL of a bacterial suspension, which contained approximately 10^8 CFU, was inoculated in a 100 mL medium. The media used for $CaCO_3$ precipitation by ureolytic bacteria, *L. sphaericus* and *S. pasteurii*, were YEUCN and YEUCL, whereas the media for $CaCO_3$ precipitation by non-ureolytic bacteria, *B. cohnii* and *B. pseudofirmus*, were AYECN and AYECL. Samples were incubated at 30°C for 24 h by shaking at 200 rpm.

It was noted that YEUCN and YEUCL remained clear after mixing with calcium lactate or calcium nitrate. If 100% of the 183.4 mM calcium source was converted into $CaCO_3$ by the inoculum, 1.834 g of $CaCO_3$ would be recovered from 100 mL broth. On the other hand, AYECN and AYECL became turbid immediately after combining with calcium lactate or calcium nitrate, and the precipitation was 0.355 and 0.282 g/100 mL respectively (Figure 4). Therefore, theoretically 100% recovery of $CaCO_3$ from AYECL and AYECN were expected to be 1.479 and 1.552 g/100 mL respectively.

Precipitants in each culture medium were collected by filtering the culture medium through a filter paper (Whatman filter No. 44) using a vacuum filter system (Figure 5) and then washed with 1 mL dH_2O thrice. Filters with samples were dried at 105°C for 2 h before measure the dry weight. Recovery rates were calculated as follows:

Net weight (g) of $CaCO_3$ precipitate
= weight of precipitate collected from culture – weight of precipitate collected from control
Recovery rates (%)
= Net weight (g) of $CaCO_3$ precipitate / theoretically 100% recovery weight (g) x 100%

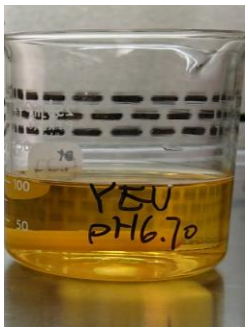
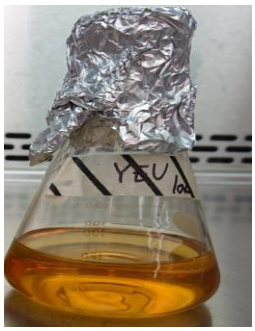
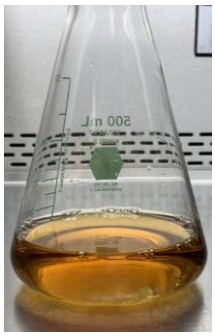
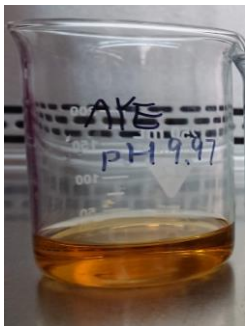
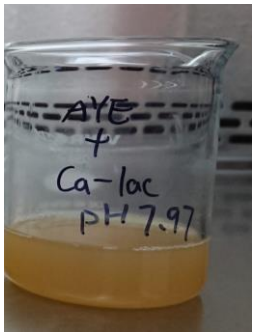
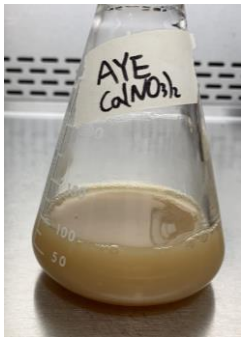
		
YEU	YEUCL	YEUCN
		
AYE	AYECL	AYECN

Figure 4 Calcite precipitation media without microorganisms

Figure 5 Vacuum filter system

2.7 Visual Evaluation of Crack Repair

To prepare six round-shaped mortar samples (60 mm × 15 mm), 238.5 mL water, 264.0 g ES, 775.6 g sand and 477.0 g cement were used (Table 6). Expanded shale was first immobilized with *S. pasteurii* spores and dried using the preparation method mentioned previously. Table 7 shows the concentrations of urea, yeast extract, and $Ca(NO_3)_2$ used to prepare mortar samples. All the nutrients were weighted and dissolved in 238.5 mL of water. Mortar paste was transferred into six Petri dishes (60 mm × 15 mm, Fisher, Pittsburgh, PA). A control mortar sample was prepared without incorporating any self-healing ingredients into the mixture. After 72 h of curing at room temperature, the prepared mortar specimens were cracked using a clamp and cured in water at room temperature until the test day.

Table 6 Mix proportions of mortar samples

Ingredient	Quantity (g)
Cement	447.0
Sand	775.6
LWA (Expanded shale)	264.0
Water	238.5

Table 7 Concentrations of nutrients in water used for mortar sample preparation

Ingredient	Concentration (g/L)
Urea	20
Yeast extract	20
$Ca(NO_3)_2$	70

2.8 Statistical Analysis

Mean results from sporulation tests, survivability tests, and calcite recovery rates were individually compared using analysis of variance (ANOVA) followed by Tukey HSD test at one significant level ($P < 0.05$).

CHAPTER 3. RESULTS

3.1 Sporulation Media Study

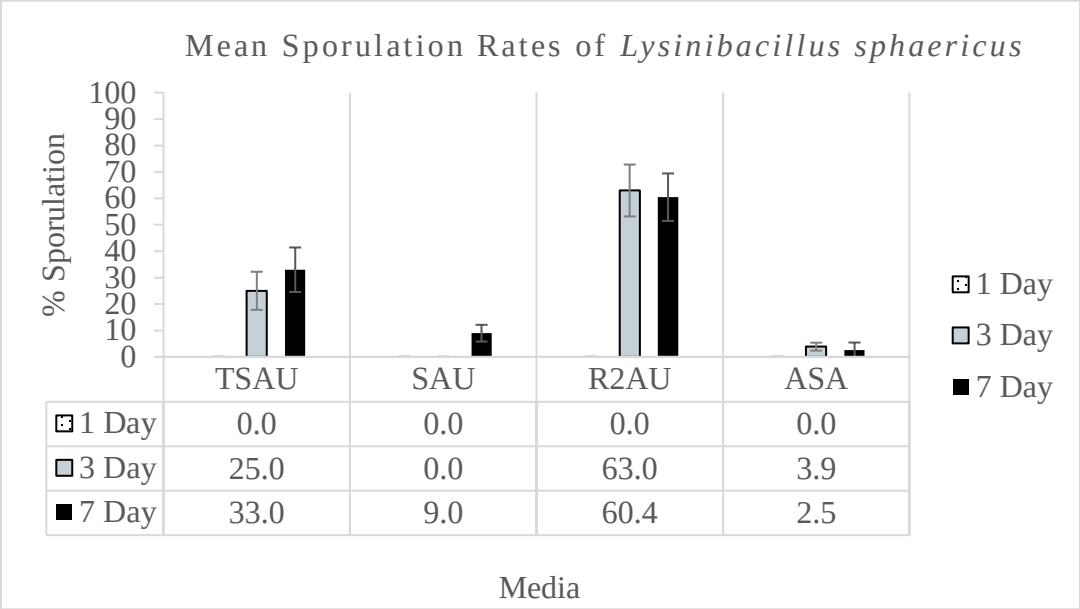
All four bacteria grew heavily on TSAU (Table 8). The two ureolytic bacteria, *L. sphaericus* and *S. pasteurii*, showed growth on the three media supplemented with urea (TSAU, SAU, and R2AU) and on ASA. The two non-urolytic bacteria, *B. cohnii* and *B. pseudofirmus*, grew on all tested media, and heavy growth was observed on TSAU, ANA, and AR2A.

Table 8 Growth and mean (n=3) sporulation rates of *L. sphaericus*, *S. pasteruii*, *B. cohnii*, and *B. pseudofirmus* on tryptic soy agar with 2% urea (TSAU), sporulation agar with 2% urea (SAU), R2A with 2% urea (R2AU), alkaline sporulation agar (ASA), alkaline nutrient agar (ANB), and alkaline R2A (AR2A) plates at 30°C after three days.

	L. sphaericus		*S. pasteruii*		*B. cohnii*		*B. pseudofirmus*	
	Growth	Spores (%)	Growth	Spores (%)	Growth	Spores (%)	Growth	Spores (%)
TSAU	+++[1]	25	+++	89	+++	0	+++	0
SAU	++	0	++	28	+	0	+	0
R2AU	++	63	++	45	+	0	+	0
ASA	++	4	++	61	+	0	+	0
ANA	-	ND[b]	-	ND	+++	0	+++	0
AR2A	-	ND	-	ND	+++	98	+++	93

[1] +++ heavy growth, ++ moderate growth, + poor growth, - no growth

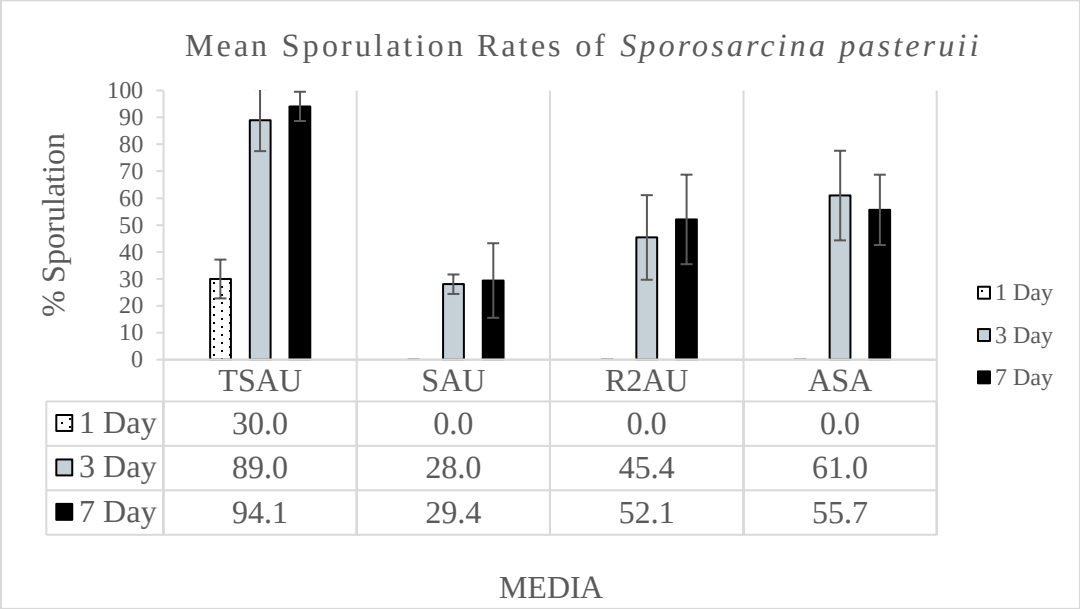
Sporulation rates of the four bacterial on media that supported bacterial growth were compared. The highest mean sporulation rate (63%) of *L. sphaericus* was found on R2AU agar plates after 3 days of incubation, followed by 25% and 4% mean sporulation rates on TSAU and ASA, respectively (Figure 6). After 7 days of incubation, the mean sporulation rates for this bacterium increased from 25 to 33% on TSAU and from 0 to 9% on SAU, while the mean sporulation rates remained similar on R2AU and ASA (Figure 6). There was no significant difference ($P > 0.05$) in sporulation rates after 3-day and 7-day incubation on TSAU or R2AU.



	TSAU	SAU	R2AU	ASA
1 Day	0.0	0.0	0.0	0.0
3 Day	25.0	0.0	63.0	3.9
7 Day	33.0	9.0	60.4	2.5

Figure 6 Mean (n =3) sporulation rates of *L. sphaericus* on tryptic soy agar with 2% urea (TSAU), sporulation agar with 2% urea (SAU), R2A with 2% urea (R2AU), and alkaline sporulation agar (ASA) after 1, 3, and 7 days of incubation at 30°C.

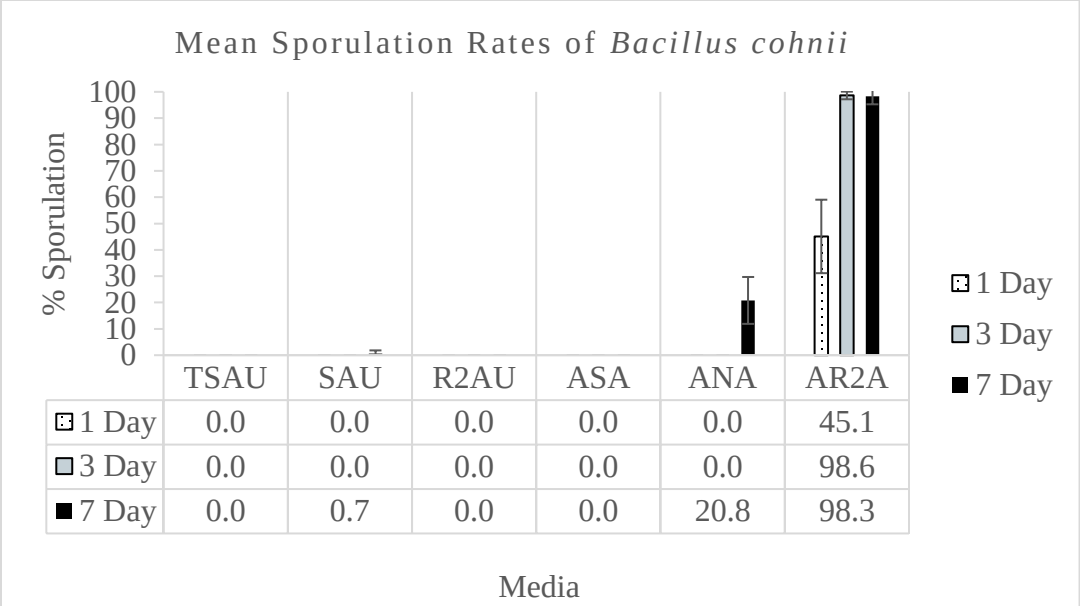
Sporosarcina pasteurii produced spores vigorously on TSAU with 30% mean sporulation rate after incubation for 1 day. However, all media showed the production of spores after 3 days. The highest mean sporulation rate was 89% on TSAU, followed by 61% on ASA, 45% on R2AU and 28% on SAU. There was no significant difference ($P > 0.05$) in sporulation rates after 3-day and 7-day incubation on these media (Figure 7).



	TSAU	SAU	R2AU	ASA
1 Day	30.0	0.0	0.0	0.0
3 Day	89.0	28.0	45.4	61.0
7 Day	94.1	29.4	52.1	55.7

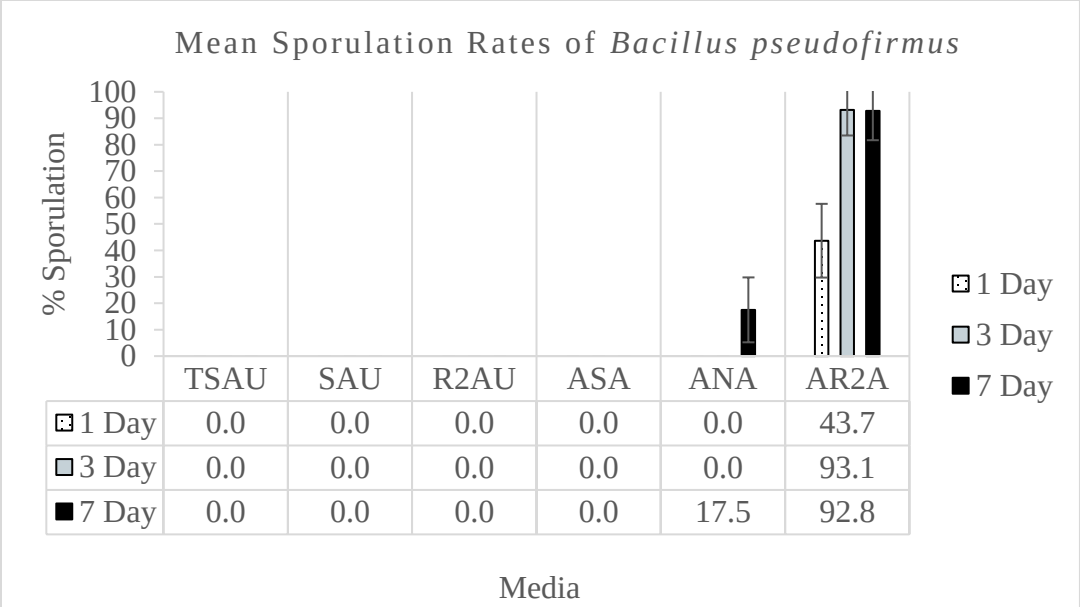
Figure 7 Mean (n =3) sporulation rates of *S. pasteruii* on tryptic soy agar with 2% urea (TSAU), sporulation agar with 2% urea (SAU), R2A with 2% urea (R2AU), and alkaline sporulation agar (ASA) after 1, 3, and 7 days of incubation at 30°C.

Although *B. cohnii* and *B. pseudofirmus* grew on all six tested media, both microorganisms produced spores only on ANA and AR2A. Between these two media, AR2A supported faster growth and more abundant sporulation of the two bacteria. On AR2A, approximately 90% of sporulation rates of both bacteria could be reached after three days. On the other hand, ANA was an inefficient sporulation medium providing 21% and 18% sporulation rates of *B. cohnii* and *B. pseudofirmus*, respectively after 7 days of incubation at 30°C (Figure 8 and Figure 9).



	TSAU	SAU	R2AU	ASA	ANA	AR2A
1 Day	0.0	0.0	0.0	0.0	0.0	45.1
3 Day	0.0	0.0	0.0	0.0	0.0	98.6
7 Day	0.0	0.7	0.0	0.0	20.8	98.3

Figure 8 Mean (n =3) sporulation rates of *B. cohnii* on tryptic soy agar with 2% urea (TSAU), sporulation agar with 2 % urea (SAU), R2A with 2% urea (R2AU), alkaline sporulation agar (ASA), alkaline nutrient agar (ANA), and alkaline R2A (AR2A) after 1, 3, and 7 days incubation at 30°C.



	TSAU	SAU	R2AU	ASA	ANA	AR2A
1 Day	0.0	0.0	0.0	0.0	0.0	43.7
3 Day	0.0	0.0	0.0	0.0	0.0	93.1
7 Day	0.0	0.0	0.0	0.0	17.5	92.8

Figure 9 Mean (n =3) sporulation rates of *B. pseudofirmus* on tryptic soy agar with 2 % urea (TSAU), sporulation agar with 2% urea (SAU), R2A with 2% urea (R2AU), alkaline sporulation agar (ASA), alkaline nutrient agar (ANA), and alkaline R2A (AR2A) after 1, 3, and 7 days incubation at 30°C.

3.2 Growth Media Study

The four bacteria were inoculated into TSBU, AYEU, AYE, YEU, YE and ANB and incubated at 30°C for 48 h and were then measured in a plate reader. The maximum OD_{540nm} readings of each bacterium in the six broths were compared.

As shown in Figure 10, *B. pseudofirmus* grew best in TSBU after a 6 h lag phase, and the maximum OD_{540nm} readings of TSBU (0.5) were detected after 48 h incubation respectively. In AYE, YE, and ANB, growth occurred after a 6 h lag phase and the maximum OD_{540nm} readings of AYE (0.2), YE (0.2), and ANB (0.1) were detected after 48 h incubation respectively. The bacteria grew poor in YEU and AYEU throughout 48 h incubation, and the maximum OD_{540nm} readings of these two media were lower than 0.1.

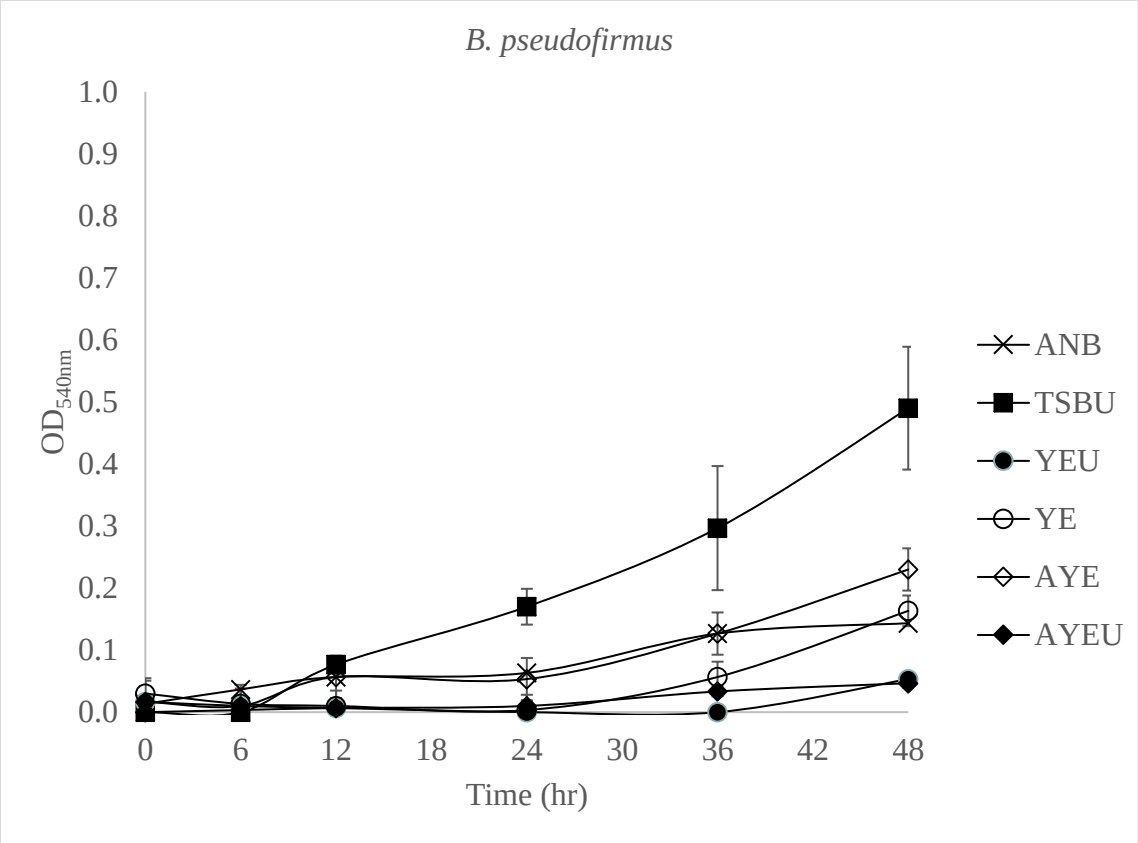


Figure 10 Growth patterns of *Bacillus pseudofirmus* in alkaline nutrient broth (ANB), tryptic soy broth with 2% urea (TSBU), 2% yeast extract (YE), 2% yeast extract supplemented with 2% urea (YEU), 2% alkaline yeast extract (AYE), and AYE with 2% urea (AYEU) after 48 h incubation at 30°C

Comparing with *B. pseudofirmus*, *B. cohnii* grew more rapidly in all media except ANB. *Bacillus cohnii* grew best in TSBU followed by AYEU, AYE, YEU, YE and ANB (Figure 11). In TSBU, AYEU, AYE, and ANB, growth occurred after a 6 h lag phase and the maximum OD_{540nm} readings of TSBU (0.7), AYEU (0.5), AYE (0.4), and ANB (0.2) cultures were detected after 48 h, respectively. In YEU and YE, the growth occurred after a 12 h lag phase, and the OD_{540nm} readings of YEU (0.2) and YE (0.2) were recorded after 24 h incubation. The OD_{540nm} readings in YEU and YE decreased from 0.2 to 0.1 after 24 h incubation, and there was no spore detected these two media.

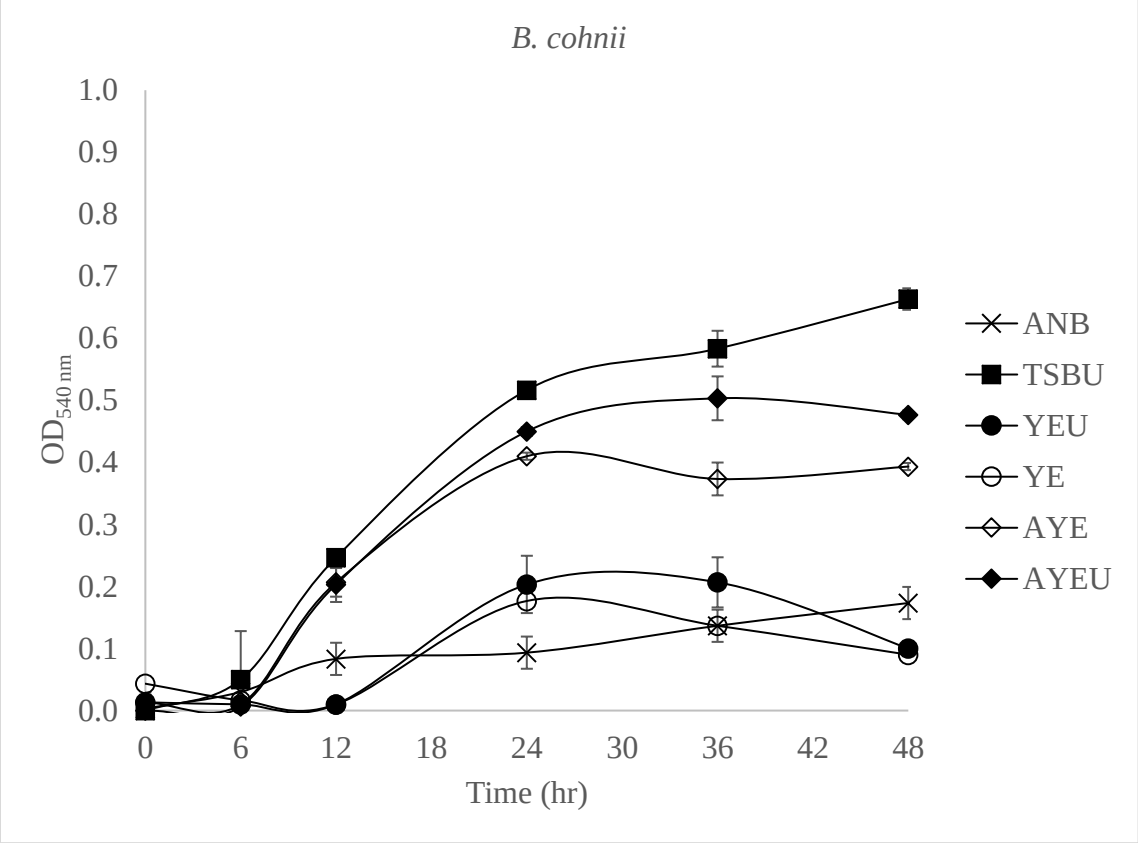


Figure 11 Growth patterns of *Bacillus cohnii* in alkaline nutrient broth (ANB), tryptic soy broth with 2% urea (TSBU), 2% yeast extract (YE), 2% yeast extract supplemented with 2% urea (YEU), 2% alkaline yeast extract (AYE), and AYE with 2% urea (AYEU) after 48 h incubation at 30°C

Sporosarcina pasteurii grew best in TSBU followed by AYE, AYEU, YEU, YE and ANB (Figure 12). In TSBU and YEU, growth occurred immediately and the maximum OD_{540nm} readings of TSBU (0.7) and YEU (0.4) were reached after 24 h incubation respectively. The OD_{540nm} reading in YEU decreased from 0.4 to 0.2 after 24 h incubation. Spores were found in the YEU culture. Spores are smaller than their vegetative counterparts. It is speculated that spore formation contributed to the decrease in OD_{540nm} readings in the YEU culture. In AYE, AYEU, and ANB, growth occurred after a 6 h lag phase and the maximum OD_{540nm} readings of AYE (0.7), AYEU (0.5) and ANB (0.2) were detected after 48 h, 48 h and 24 h incubation respectively. In YE, growth started after a 12 h lag phase and the maximum OD_{540nm} (0.2) was detected after 24 h incubation.

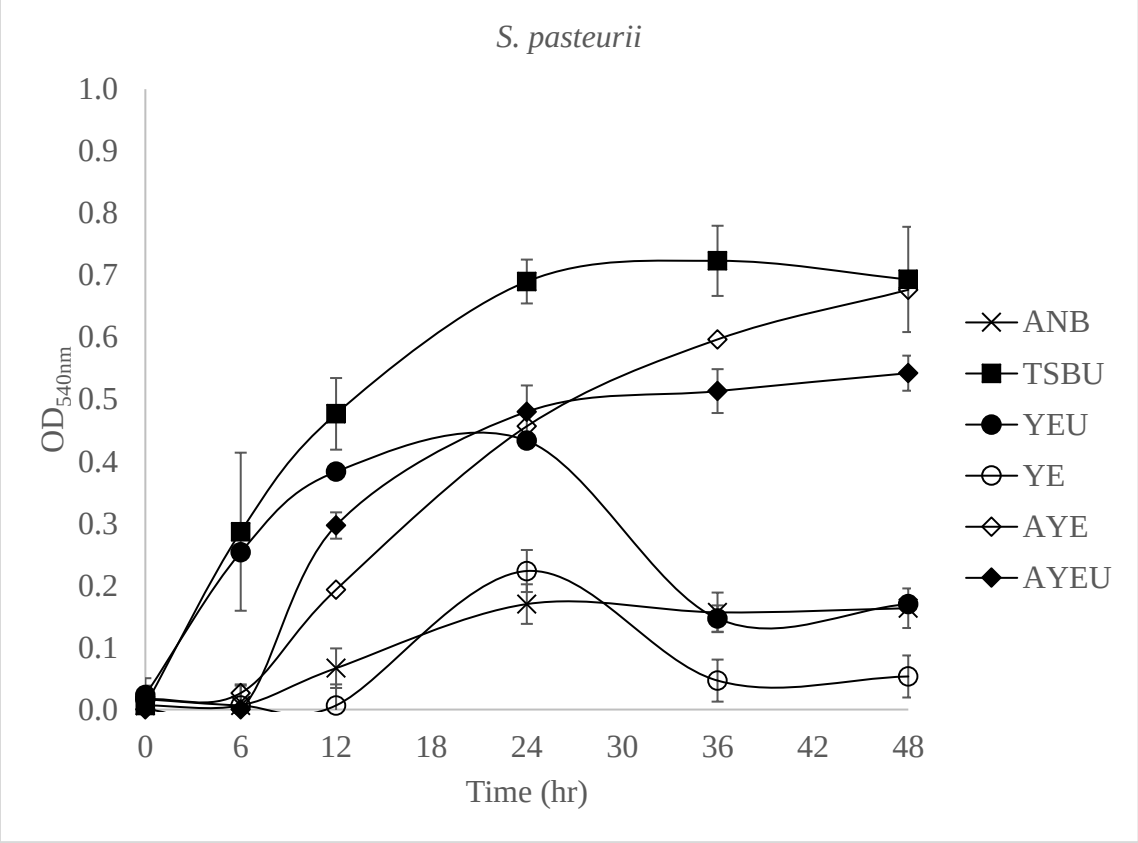


Figure 12 Growth patterns of *Sporosarcina pasteruii* in alkaline nutrient broth (ANB), tryptic soy broth with 2% urea (TSBU), 2% yeast extract (YE), 2% yeast extract supplemented with 2% urea (YEU), 2% alkaline yeast extract (AYE), and AYE with 2% urea (AYEU) after 48 h incubation at 30°C. Sporulation was detected in YEU.

Lysinibacillus sphaericus grew best in YEU, followed by TSBU, AYEU, YE, AYE, and ANB (Figure 13). In TSBU and YEU, growth occurred immediately and the maximum OD_{540nm} readings of TSBU (0.8) and YEU (0.9) were reached after 48 h incubation respectively. In AYEU, growth occurred after a 6 h lag phase and the maximum OD_{540nm} readings of AYEU (0.6) after 36 h incubation respectively. In YE, AYE, and ANB growth started after a 12 h lag phase and the maximum OD_{540nm} readings of YE (0.6), AYE (0.3), and ANB (0.1) were detected after 48 h incubation.

Overall, all four bacteria grew well in TSBU with maximum OD_{540nm} readings ranging from 0.5 to 0.8 and grew poorly in ANB with OD_{540nm} readings ranging from 0.1 to 0.2. The two ureolytic bacteria grew faster and better than did the two non-ureolytic bacteria (Figure 10–13).

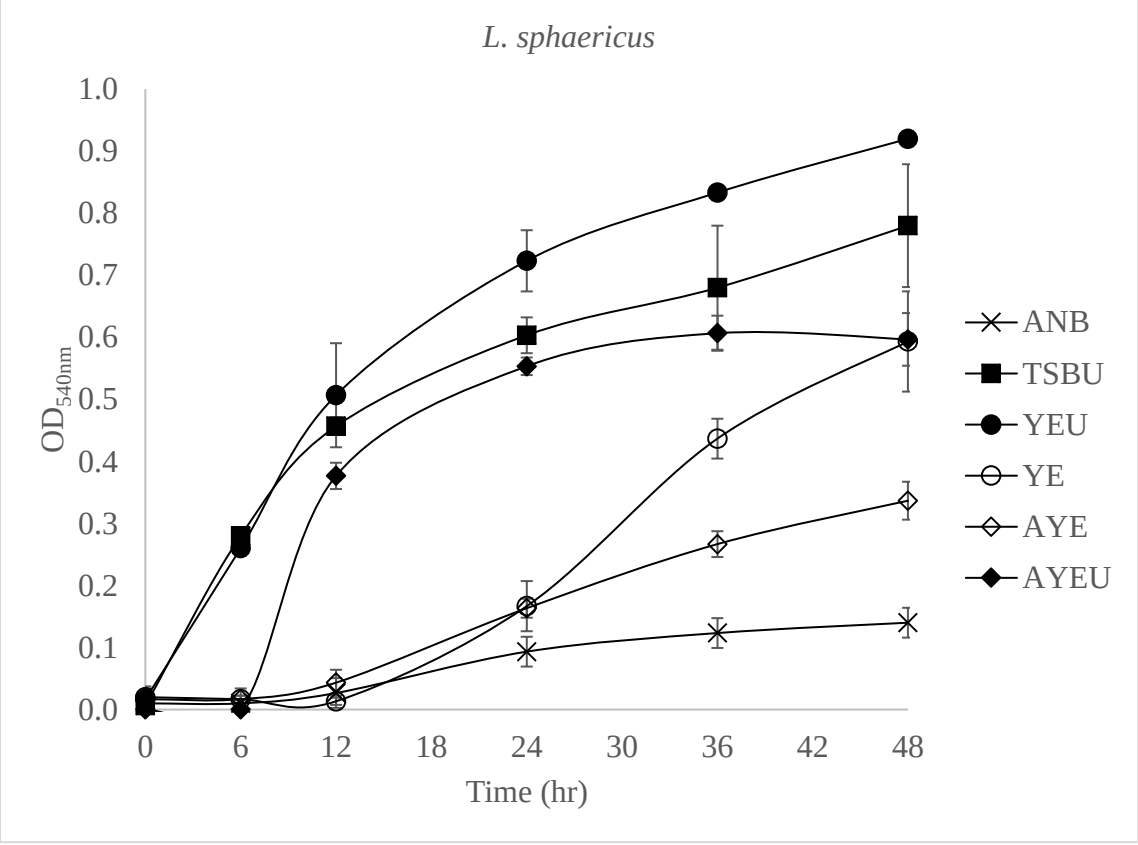


Figure 13 Growth patterns of *Lysinibacillus sphaericus* in alkaline nutrient broth (ANB), tryptic soy broth with 2% urea (TSBU), 2% yeast extract (YE), 2% yeast extract supplemented with 2% urea (YEU), 2% alkaline yeast extract (AYE), and AYE with 2% urea (AYEU) after 48 h incubation at 30°C.

3.3 Self-Healing Concrete Microbe Survival Study

The survival of the four LWA-immobilized bacterial spores in mortar samples with or without nutrient immobilization were compared, and the viable counts of each microorganism were shown in Table 9. After mixing into mortar samples, the viable counts of each bacterium decreased significantly during the first day ($P < 0.05$). Figure 14 shows the reduction of mean viable counts of the four immobilized bacterial spores in mortar samples without nutrient (1 g/L yeast extract and 80 g/L calcium lactate) immobilization. After 1 day, the mean viable count of *B. cohnii, B. pseudofirmus, S. pasteurii*, and *L. sphaericus* dropped 3.1, 3.3, 2.4, and 3.3 Log CFU/g. The viable counts of each bacterium remained stable during the next 27 day testing period in mortar samples. There was no significant difference ($P > 0.05$) in viable counts of each bacterium on day 7, 14, and 28. (Table 9).

Figure 15 shows the reduction of viable counts of the four different bacterial spores in mortar samples made with nutrients (1 g/L yeast extract and 80 g/L calcium lactate) and spore immobilized ES. After 1 day, the viable counts of *B. cohnii, B. pseudofirmus, S. pasteurii*, and *L. sphaericus* decreased 2.5, 2.6, 1.8, and 2.6 Log CFU/g, respectively. The viable counts of each bacterium also remained stable during the next 27 day testing period in mortar samples. There was no significant difference ($P > 0.05$) in viable counts of each bacterium on day 7, 14, and 28 (Table 9). As shown in Figure 14 and 15, the four bacteria survived better in mortar samples when nutrients were also immobilized in LWA. However, the difference was not significant ($P > 0.05$). Moreover, *S. pasteurii* survived better than the other three bacteria in mortar samples but the difference was not significant ($P > 0.05$).

Table 9 Mean viable counts (CFU/mL) of *B. cohnii* (Bc), *B. pseudofirmus* (Bp), *S. pasteurii* (Sp), and *L. sphaericus* (Ls) spores immobilized in small size expanded shale (ES) with and without nutrient immobilization [1] over 28 days in mortar samples at room temperature. (n =3)

	Without nutrient				With nutrient			
	Bc	Bp	Sp	Ls	Bc	Bp	Sp	Ls
Day 0	$6.5 \pm 0.3^{A\,2\,a\,3}$	6.8 ± 0.3^{Aa}	6.7 ± 0.2^{Aa}	6.8 ± 0.3^{Aa}	6.0 ± 0.1^{Aa}	5.9 ± 0.3^{Aa}	6.2 ± 0.2^{Aa}	6.1 ± 0.2^{Aa}
Day 1	3.4 ± 0.8^{Ba}	3.5 ± 0.5^{Ba}	4.3 ± 0.2^{Ba}	3.5 ± 0.5^{Ba}	3.5 ± 0.4^{Ba}	3.3 ± 0.3^{Ba}	4.4 ± 0.1^{Ba}	3.5 ± 0.6^{Ba}
Day 7	3.1 ± 0.8^{Ba}	2.8 ± 0.7^{Ba}	3.0 ± 0.5^{Ba}	3.0 ± 0.3^{Ba}	3.5 ± 0.2^{Ba}	3.2 ± 0.2^{Ba}	3.5 ± 0.4^{Ba}	3.5 ± 0.3^{Ba}
Day 14	3.2 ± 0.4^{Ba}	3.1 ± 0.4^{Ba}	3.2 ± 0.2^{Ba}	3.6 ± 0.3^{Ba}	3.2 ± 0.3^{Ba}	3.2 ± 0.3^{Ba}	3.5 ± 0.1^{Ba}	3.3 ± 0.2^{Ba}
Day 28	3.1 ± 0.6^{Ba}	3.1 ± 0.3^{Ba}	2.9 ± 0.2^{Ba}	3.2 ± 0.3^{Ba}	3.2 ± 0.3^{Ba}	2.8 ± 0.4^{Ba}	3.4 ± 0.4^{Ba}	2.9 ± 0.2^{Ba}

[1] Contained 1 g/L yeast extract and 80 g/L calcium lactate

[2] Values within columns with different letters are significantly different ($P < 0.05$)

[3] Values within rows with different letters are significantly different ($P < 0.05$)

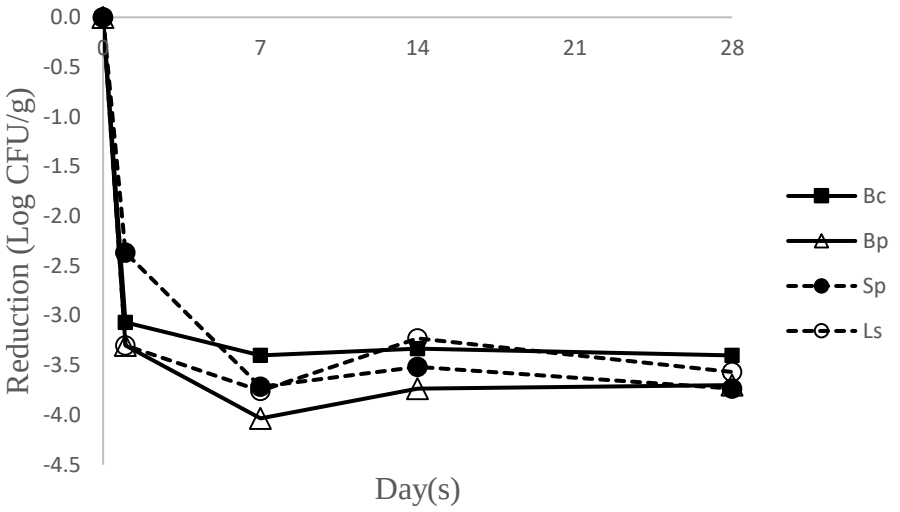


Figure 14 Log reduction in viable counts of *B. cohnii* (Bc), *B. pseudofirmus* (Bp), *S. pasteurii* (Sp), and *L. sphaericus* (Ls) spores immobilized in small size expanded shale (ES) over 28 days in mortar samples at room temperature. (n =3)

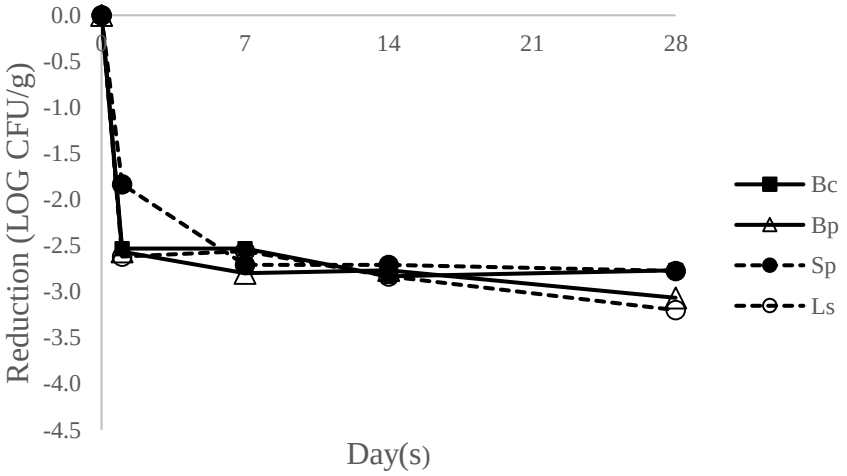


Figure 15 Log reduction in viable counts of *B. cohnii* (Bc), *B. pseudofirmus* (Bp), *S. pasteurii* (Sp), and *L. sphaericus* (Ls) spores immobilized in small size expanded shale (ES) containing 1 g/L yeast extract and 80 g/L calcium lactate over 28 days in mortar samples at room temperature. (n =3)

3.4 Microbial Mediated Calcium Carbonate Production Study

The two ureolytic bacteria, *S. pasteurii* and *L. sphaericus*, had respectively 98.4% and 101.8% mean recovery rates of $CaCO_3$ from YEUCL cultures (Figure 16). However, the mean $CaCO_3$ recovery rates of the two bacteria from YEUCN were 81.7% and 89.7% respectively (Table 10). Although *L. sphaericus* showed higher $CaCO_3$ recovery rates from the two calcium sources than did *S. pasteurii*, the differences were not significant ($P > 0.05$).

Compared to ureolytic bacteria, *B. pseudofirmus* and *B. cohnii* presented low efficiencies in converting both calcium lactate and calcium nitrate to $CaCO_3$. *Bacillus pseudofirmus* showed 10.4 and 11.0% mean $CaCO_3$ recovery rates from YEUCL and YEUCN respectively, and *B. cohnii* showed approximately 2.9% mean $CaCO_3$ recovery rates from AYECL. No precipitation (-12.5% mean $CaCO_3$ recovery rate) was found in AYECN inoculated with from *B. cohnii* (Table 10).

Figure 16 Precipitation collected from YEU supplemented with calcium lactate medium inoculated with *L. sphaericus*.

Table 10 Calcium carbonate production (g) by *S. pasteurii, L. sphaericus, B. pseudofirmus*, and *B. cohnii* using calcium lactate and calcium nitrate (Ca source 183.39 mM) in 2% yeast extract with 2% urea (YEU) and 2% alkaline yeast extract (AYE) after 24 h incubation at 30°C with 200 rpm.

Bacteria	Media	Mean Net yield ± SD (g/100 mL) (n=3)	Mean % Recovery± SD (n=3)
S. pasteruii	YEUCL	$1.805 \pm 0.121^{CD*}$	98.4 ± 6.6^{C}
	YEUCN	1.499 ± 0.109^{DE}	81.7 ± 5.9^{DE}
L. shaericus	YEUCL	1.868 ± 0.045^{CE}	101.8 ± 2.5^{CE}
	YEUCN	1.644 ± 0.102^{G}	89.7 ± 5.5^{DG}
B. pseudofirmus	AYECL	0.154 ± 0.031^{AB}	10.4 ± 2.1^{ABH}
	AYECN	0.171 ± 0.020^{F}	11.0 ± 1.3^{H}
B. cohnii	AYECL	0.043 ± 0.024^{A}	2.9 ± 1.6^{A}
	AYECN	-0.195 ± 0.043^{BF}	-12.5 ± 2.8^{BF}

* Yields within columns with different letters are significantly different ($P < 0.05$)

3.5 Microbial Mediated Mortar Crack Healing Study

Small cracks (~0.58 mm) were created on each mortar samples after curing at room temperature for 3 days. Mortar samples prepared with microbial self-healing agents exhibited crack healing results after curing in water at room temperature for 10 days (Figure 17). Control mortar samples that did not contain self-healing agents showed slight crack healing effects after curing, while mortar samples that contained self-healing agents (bacterial spores and nutrients) showed complete healing for small cracks on the surface (Figure 17).

	Control	*S. pasteurii #1*	*S. pasteurii #2*
Day 0			
Day 10			

Figure 17 Healing effects of cracked mortar samples made with *S. pasteruii* and nutrients after 10 days in dH_2O

3.6 Summary Performances of the Four Bacteria

Overall performance of the four bacterial strains is summarized in Table 11. The highest three-day-sporulation rates of *B. pseudofirmus, B. cohnii, S. pasteurii*, and *L. sphaericus* were 93% on AR2A, 99% on AR2A, 89% on TSAU, and 63% on R2AU respectively. There was no significant difference in the three-day-sporulation rates of *B. cohnii, B. pseudofirmus,* and *S. pasteurii* ($P > 0.05$). However, the mean three-day-sporulation rate of *L. sphaericus* was significantly lower than the rates of the other three bacteria ($P < 0.05$).

The maximum OD_{540nm} readings of *B. pseudofirmus*, *B. cohnii*, *S. pasteurii*, and *L. sphaericus* after 48 h at 30°C were 0.5, 0.7, and 0.7 in TSBU, and 0.9 in YEU respectively. The maximum OD_{540nm} readings in AYE, which represented strong alkaline condition (pH = 8.9), was also compared. *Sporosarcina pasteurii* showed the best growth, followed by *B. cohnii, L. sphaericus,* and *B. pseudofirmus* with the maximum OD_{540nm} readings 0.7, 0.4, 0.3, 0.2, respectively in AYE after 48 h incubation.

The highest $CaCO_3$ recovery rate (101.8%) was found in YEUCL inoculated with *L. sphaericus*, followed by 98.4% mean recovery rate from YEUCL inoculated with *S. pasteurii*. However, there was no significant difference between the two ($P > 0.05$). The highest mean $CaCO_3$ recovery rates of *B. pseudofirmus* and *B. cohnii* were 11% in AYECN, and 2.9% in AYECL respectively. There was significant difference between these two values ($P < 0.05$). The mean recovery rates of the $CaCO_3$ from the two ureolytic bacteria were significantly higher than those from the two non-ureolytic bacteria ($P < 0.05$). Overall, *S. pasteurii, B. cohnii,* and *L. sphaericus* produced significantly more $CaCO_3$ ($P < 0.05$) from calcium lactate than from calcium nitrate, whereas, *B. pseudofirmus* had similar recovery rates of $CaCO_3$ from calcium lactate and calcium nitrate.

The four bacterial strains showed no significant differences ($P > 0.05$) in their survival rates in mortar samples. However, *S. pasteurii* showed a slightly better survival over *B. cohnii, L. sphaericus,* and *B. pseudofirmus* after one day in mortar samples.

Table 11 Summary performances (n=3) of *B. pseudofirmus*, *B. cohnii*, *S. pasteurii*, and *L. sphaericus* from sporulation study, growth study, $CaCO_3$ production study, and survival test in mortar samples.

	Sporulation medium / Mean sporulation rates [1]	Growth medium / Mean maximum OD_{540nm}[2] ± SD	Calcium source / Mean $CaCO_3$ recover rates [3] (%) ± SD	Mean Log CFU/g reductions in mortar samples [4] after 1 day / 28 days
B. pseudofirmus	AR2A / 93 % ± 9.2 ^{A} [5]	TSBU / 0.52 ± 0.3^{A} AYE / 0.2 ± 0.0^{B}	Calcium lactate / 10.4 % ± 2.1^{ABH} Calcium nitrate / 11.0 % ± 1.3^{H}	2.6 ± 0.5 ^{A}/ 3.1 ± 0.5 ^{A}
B. cohnii	AR2A / 99 % ± 1.5 ^{A}	TSBU / 0.72 ± 0.1^{AE} AYE / 0.4 ± 0.0^{A}	Calcium lactate / 2.9 % ± 1.6^{A} Calcium nitrate /-12.5 % ± 2.8^{BF}	2.5 ± 0.4 ^{A}/ 2.8 ± 0.3 ^{A}
S. pasteurii	TSAU / 89 % ± 7.6 ^{A}	TSBU / 0.72 ± 0.2^{AD} AYE / 0.7 ± 0.0 ^{AC}	Calcium lactate / 98.4 % ± 6.6^{C} Calcium nitrate / 81.7 % ± 5.9^{DE}	1.8 ± 0.2 ^{A}/ 2.8 ± 0.6 ^{A}
L. sphaericus	R2AU / 63 % ± 9.4 ^{B}	YEU / 0.9 ± 0.3 ^{CDE} AYE / 0.3 ± 0.0 ^{A}	Calcium lactate /101.8 % ± 2.5^{CE} Calcium nitrate / 89.7 % ± 5.5^{DG}	2.6 ± 0.7 ^{A}/ 3.2 ± 0.2 ^{A}

[1] The sporulation rates collected after three day incubation

[2] After 48 h incubation at 30°C

[3] Yeast extract with 2% urea are the nutrient source for *S. pasteurii* and *L. sphaericus*. Alkaline yeast extract is the nutrient source for *B. pseudofirmus* and *B. cohnii*.

[4] With nutrient immobilization (calcium lactate, 80 g/L and yeast extract, 1 g/L)

[5] Values within columns with different letters are significantly different ($P < 0.05$)

3.7 Comparison between ES and EC

3.7.1 Survival of Spores in LWA

Viable counts of *B. cohnii* and *B. pseudofirmus* immobilized in both EC and ES were shown in Figure 18. Both *B. cohnii* and *B. pseudofirmus* spores remained stable over 150 days of incubation. There was no significant difference ($P > 0.05$) in viable counts recovered from three tested sizes of LWA, but slightly higher viable counts were found in small size LWA. The viable counts of spores recovered from EC were similar to those from ES.

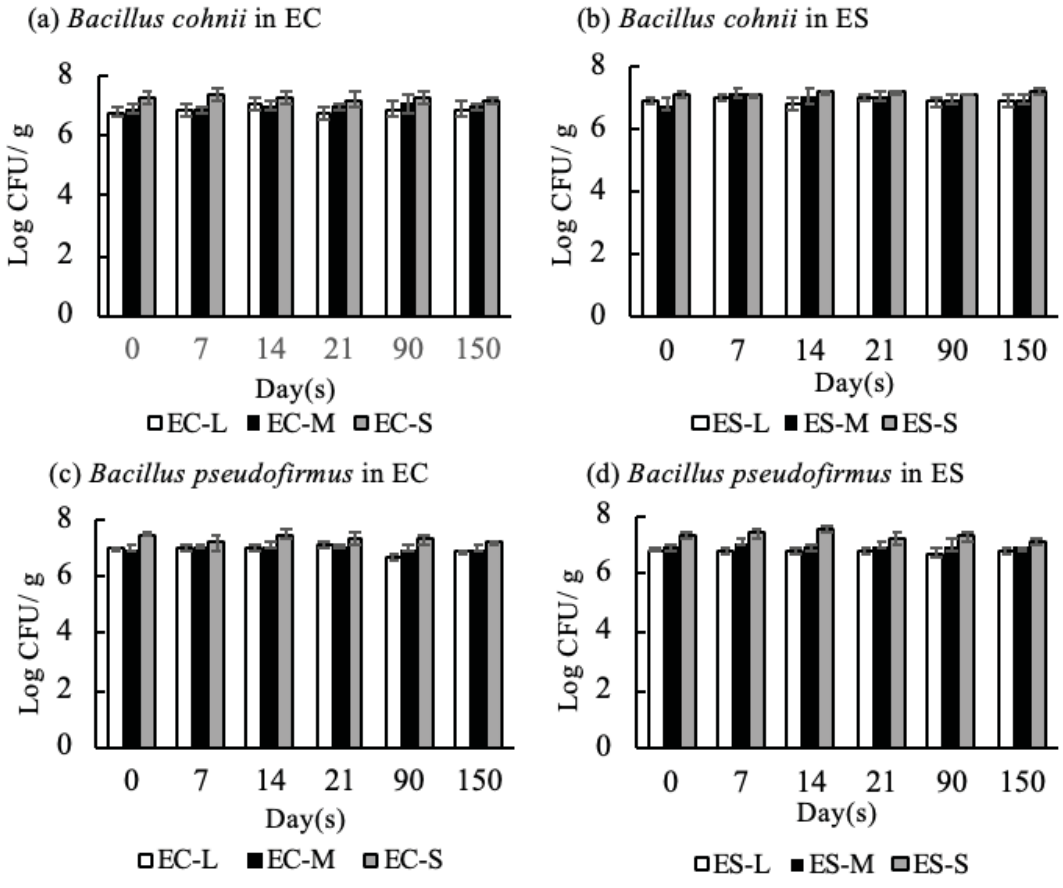


Figure 18 Mean (n=3) viable counts (Log CFU/ g LWA) of *B. cohnii* and *B. pseudofirmus* recovered from Large (2.00– 4.75 mm), Medium (0.85– 2.00 mm), and Small (<0.85 mm) expanded clay (EC) and expanded shale (ES), ($P > 0.05$)

3.7.2 Viable Counts of bacterial spores in mortar samples

Figure 19 shows the survival of spores immobilized in small LWA, spores immobilized in small LWA with nutrients, and spores without immobilization in mortar samples. Mean viable counts of spores immobilized in small LWA dropped 1.9– 2.2 logs during the first day in the mortar samples and decreased additional 1.3– 1.7 logs during the following 27-day incubation. Embedding nutrients in LWA slightly improved mean viable counts of immobilized spores in mortar samples, but the difference was not significant ($P > 0.05$). In contrast, the mean viable counts of spores without immobilization decreased 3.3– 3.4 logs during the first day and remained relatively the same for the following 27 days in control mortar samples.

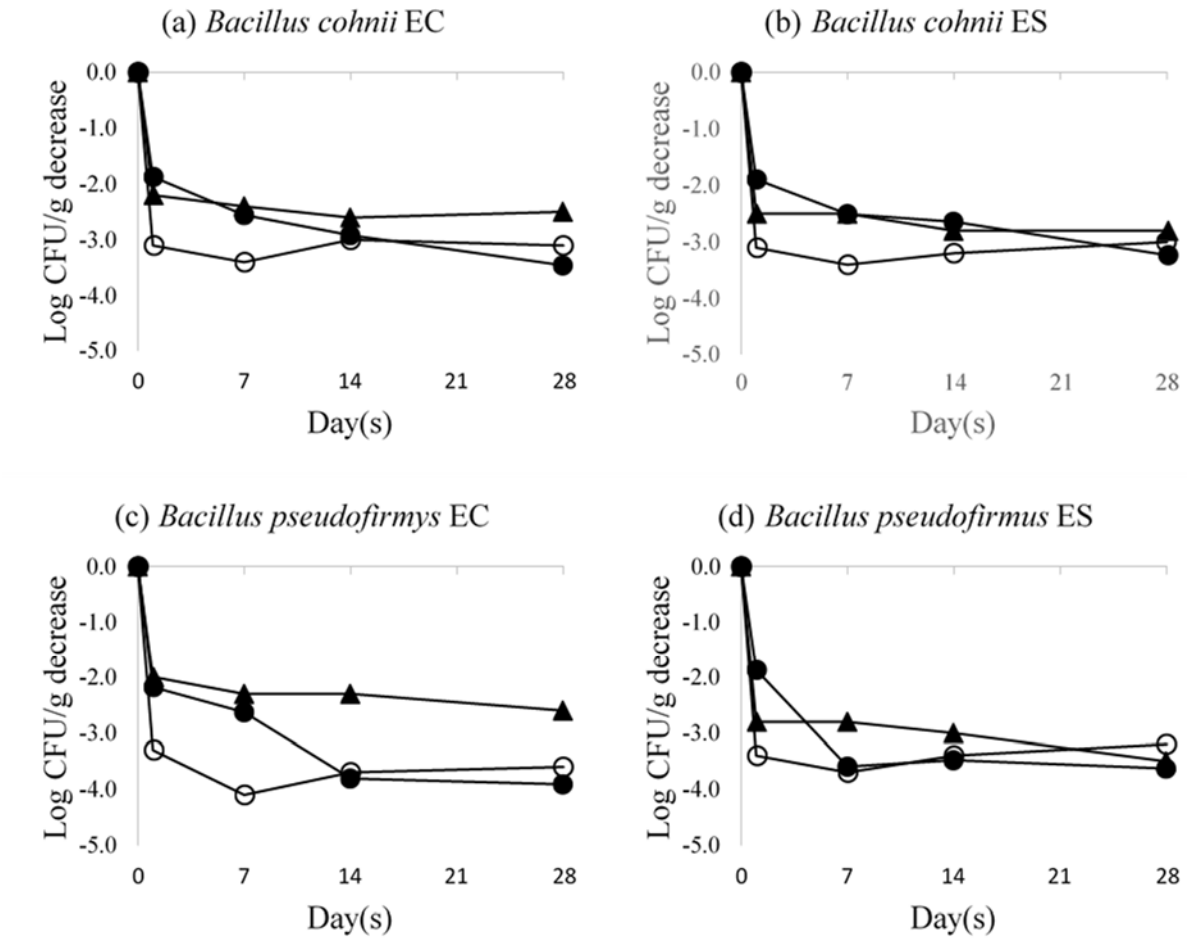


Figure 19 Log reduction in viable counts of *B. cohnii* (Bc) and *B. pseudofirmus* (Bp) spores in mortar samples prepared with small size LWA (expanded clay (EC) or expanded shale (ES)) with different immobilization treatments over 28 days. (n=3)
(● – immobilization without nutrients, ▲-immobilization with nutrients, ○-no immobilization)

CHAPTER 4. DISCUSSION

4.1 Sporulation

The four bacteria showed distinct growth and sporulation requirements. Results of this study showed that bacteria can grow rapidly in a medium but produce spores inefficiently. Tryptic soy agar supplemented with 2% urea supported heavy growth for all the four bacteria, but only *S. pasteurii* sporulated vigorously on this medium. Although *B. cohnii* and *B. pseudofirmus* grew on all six media, they produced spores only on two media, ANA and AR2A. In a previous study, 75 and 25% sporulation rates of *B. cohnii* and *B. pseudofirmus*, respectively, were obtained from a liquid minimal basal salts medium [23]. In the same study, it was reported that *S. pasteurii* did not produce any spores in the same medium. This study showed that AR2A promoted the highest spore formation of *B. cohnii* and *B. pseudofirmus* with over 90% sporulation rates, and TSAU promoted the highest spore formation of *S. pasteurii* after 3 days of incubation at 30°C (Table 11). The results of this study also showed that R2AU supported maximum sporulation rates of *L. sphaericus*. R2A is considered a nutrient-poor medium which is intended for isolation of bacteria in environmental samples such as soil and potable water [49]. This medium was selected for this study because it stimulates sporulation of many soil bacteria and it is commercially available. Additionally it can be easily modified to R2AU for sporulation of *L. sphaericus*, and AR2A for sporulation of *B. cohnii* and *B. pseudofirmus*.

Among the four tested bacteria, *L. sphaericus* was the poorest spore-forming bacteria. The highest mean sporulation rate of *L. sphaericus* was 60% on R2AU agar after 3 days incubation at 30°C. In previous studies. a minimal basal salt medium was used for *L. sphaericus* sporulation [15, 21, 22]. It was reported that 14 to 28 days of incubation time was required for *L. sphaericus* to reach 90% or more sporulation rate in this medium [22]. Based on the results of this study, R2AU is not only a medium that can be easily prepared than the minimal basal salt medium, but it also yielded 60% sporulation rates within 3 days. Future study can be focus on finding alternative sporulation media that will yield higher sporulation rate of *L. sphaericus* within 3 days of incubation.

4.2 Growth Media

In this study, growth of *S. pasteurii*, *L. sphaericus*, *B. pseudofirmus*, and *B. cohnii* in TSBU, ANB, and four YE-based broths (YE, YEU, AYE, and AYEU) was tested. Tryptic soy broth (TSB) and nutrient broth (NB) are nutrient-rich media that have been used to grow a wide variety of bacteria. It is suggested by the culture providers to cultivate the two ureolytic bacteria with TSB supplemented with either 2 % urea (TSBU) and cultivate the two non-ureolytic bacteria with NB supplement with an alkaline solution (ANB). Therefore, TSBU and ANB were included in this growth study as positive controls. However, it was found that the two non-urolytic bacteria, *B. pseudofirmus* and *B. cohnii* showed better growth in TSBU than in ANB. It was hypothesized that higher nutrient composition of TSB promoted microbial growth (Table 2).

Yeast extract has been used in self-healing concrete as a nutrient to support growth of concrete healing bacteria such as *S. pasteurii*, *L. sphaericus, B. pseudofirmus,* and *B. cohnii* [13, 14, 18, 22]. In this study, YEU (YE supplementated with 2 % urea) was used to grow ureolytic bacteria. The AYE (pH8.9), modification of YE with alkaline solution containing $NaHCO_3$ and Na_2CO_3, was to mimic the alkaline condition of the embedded YE in a mortar sample. Based on the results of this study, the supplementation of 2 % urea enhanced the growth of the two ureolytic bacteria in YE (Figure 12 and 13). The AYE medium supported the maximum growth of the two non-ureolytic bacteria after 48 h incubation. *Bacillus cohnii* reached stationary phase in AYE and AYEU faster than did *B. pseudofirmus* (Figure 10 and 11). Therefore, this result suggested that *B. cohnii* could possibly show better and faster growth than *B. pseudofirmus* in a self-healing concrete containing YE.

Reductions in OD_{540nm} readings were observed in YE and YEU inculated with *B. cohnii* or *S. pasteurii* during 24–36 h incubation at 30°C. In order to explain these results, samples were taken after 48 h incubation and inspected for the presence of bacterial spores. Spores of *B. cohnii* were found in YE but not in YEU after 48 h incubation. It was speculated that the supplementation of urea in the medium did not induce the sporulation. The bacterium remained vegetative form and died during the incubation. Similar sporulation results of *B. cohnii* were observed in the sporulation test, in which *B. cohnii* did not produce spores on sporulation media supplemented with 2 % urea (Figure 8). Spores of *S. pasteurii*, however, were found in YEU but not in YE. It was hypothesized that YEU supported a rapid growth of *S. pasteurii*, and the sporulation was induced by the wastes and nutrient depletion in the medium. According to Table

2, YE provided fewer nutrient sources than did TSB. Therefore, nutrients in YEU will be used up faster than did in TSBU if the bacterium showed similar growth in both media. Figure 12 shows that the growth of *S. pasteurii* in TSBU and in YEU during the first 18 h incubation was similar. Therefore, it was suggested that *S. pasteurii* produced spores in YEU due to the fast growth and nutrient depletion

Overall, TSBU supported the rapid growth of all the four microorganisms in the five tested media. However, a previous study showed that embedding TSB in mortar samples negatively affected the compressive strength of the mortar samples; whereas, embedding YE did not show impact on compressive strength of the mortar samples [48]. The growth of the two ureolytic bacteria in four YE-based media was overall higher than non-ureolytic bacteria, and *B. pseudofirmus* showed the poorest growth among four bacteria. Moreover, *S. pasteurii* showed the highest growth (OD_{540nm}= 0.6) in YE media with an alkaline pH (AYE) after 48 h incubation (Figure 10-13). Therefore, *S. pasteurii* is expected to have the best growth over other three microorganisms in mortar samples.

4.3 Survival of Spores in Mortar Samples

The viable count results of immobilized *S. pasteurii*, *L. sphaericus*, *B. pseudofirmus* and *B. cohnii* spores from mortar samples decreased dramatically in 1 day (Figure 14). The mean viable counts of *B. cohnii*, *B. pseudofirmus*, and *L. sphaericus* dropped 3.1, 3.3, and 3.3 logs respectively, whereas, the mean viable counts of *S. pasteurii* only reduced 2.4 logs. Such results were similar to a previous study [12], in which *B. cohnii*, *B. halodurans*, and *B. pseudofirmus* spores were embedded into mortar samples, and the viable counts after 10 days showed approximately 2.0 log reductions. A similar reduction was also observed in another study, in which the number of *B. cohnii* spores decreased about 3.0 logs after 22 days in cement stone and dropped below detection level ($<5 \times 10^2/ cm^3$) after 135 days [24]. In a recent studies [25], freeze-dried spores of *B. cohnii*, *B. halodurans*, and *B. pseudofirmus* were incorporated into cement mix, and it was reported that approximately 2.2– 3.0 log reductions of spores were observed after 7 days.

It was found that immobilizing organic nutrients in LWA with bacterial spores could enhance the survival rates of bacterial spores (Figure 15). Decreased reductions in viable counts were observed from all four bacterial spores in mortar samples with nutrient immobilization. It is hypothesized that organic compounds are rehydrated and provided protection to microorganisms

when water is present during mortar sample preparation. The viable counts obtained from all four microorganisms after 1 day in mortar samples reached constant. Such results were similar to a previous study [24], in which the viable counts of *B. cohnii*, *B. halodurans*, and *B. pseudofirmus* reduced < 1.0 log between 7 and 28 days and then remained relative constant between 42 and 93 days.

According to the results of this study, viable counts of *S. pasteruii* spores were higher than viable counts of *L. sphaericus*, *B. pseudofirmus* and *B. cohnii* after 1 day in mortar samples. However, the differences among the four viable counts after 1 day were not significantly different ($P > 0.05$) (Figure 14 and Figure 15).

4.4 Calcium Carbonate Precipitation from Two Calcium Sources

An alkaline solution containing $NaHCO_3$ and Na_2CO_3 was used to prepare AYECL and AYECN. Both $NaHCO_3$ and Na_2CO_3 provided CO_3^{2-} in the alkaline media. Therefore, it was hypothesized that the $CaCO_3$ precipitation occurred in the media was a result of reaction between CO_3^{2-} and Ca^{2+} from the calcium source. The $CaCO_3$ precipitation in AYECL and AYECN media might contribute to the low $CaCO_3$ recovery rates of the two non-ureolytic bacteria in these two media (Table 10). It is necessary to study a different calcium precipitation media for the two non-ureolytic bacteria in the future.

Both ureolytic bacteria, *S. pasteurii* and *L. sphaericus*, produced large amounts of $CaCO_3$ within 24 h in YEUCN and YEUCL at 30°C. *Sporosarcina pasteurii* showed approximately 98 and 82% rates in converting calcium lactate and calcium nitrate respectively into $CaCO_3$. The recovery rates of $CaCO_3$ from these two calcium sources were significantly different ($P < 0.05$). *Lysinibacillus sphaericus* showed >100% and approximately 90% rates in converting calcium lactate and calcium nitrate respectively into $CaCO_3$ and the $CaCO_3$.recovery rates from calcium lactate was significant higher than the recovery rates from calcium nitrate ($P < 0.05$). A previous study also reported that *S. pasteurii* produced more $CaCO_3$ from calcium lactate than form $Ca(NO_3)_2$ [28].

Calcium lactate is an organic compound that provided extra carbon sources for microbial growth, while calcium nitrate is not. In the survival test, it was found that calcium lactate could enhance bacterial viability in mortar samples (Figure 19). Therefore, it was concluded that calcium

lactate could potentially be an ideal calcium source for bacterial healing agents because it not only increases the survival of bacteria in mortar samples but also contributes to $CaCO_3$ production.

The $CaCO_3$ precipitation test was completed after 24 h of incubation. Compared to results from microbial growth (Figure 10– 13), *S. pasteurii* and *L. sphaericus* were in the log phase ($OD_{540\ nm}$= 0.4 and 0.7, respectively) after 24 h of incubation in YEU. Microorganisms showed the highest metabolic activities during the log phase. In this case, high urease activity led to higher production of CO_3^{2-} to react with Ca^{2+} in the medium. Therefore, $CaCO_3$ formation by these two ureolytic bacteria was higher. A recent study showed that the $CaCO_3$ recovery rate of *S. pasteurii* from a medium containing NB, urea, and calcium lactate was only 24% [31]. The difference in recovery rates might be due to the different composition in the media used. Yeast extract might provide more rapid growth for *S. pasteurii* than did NB.

Although *B. cohnii* grew well in AYE with 0.4 OD_{540nm} readings after 24 h of incubation, $CaCO_3$ production was very low. The mean $CaCO_3$.recovery rates were 10.4 and 11.0% in AYECL and AYECN respectively.

Bacillus pseudofirmus grew poorly in AYE and the $OD_{540\ nm}$ reading was <0.1 (after 24 h of incubation (Figure 10). These low $CaCO_3$.recovery rates may be due to the poor growth in AYECL and AYECN.

Another speculated reason for the low $CaCO_3$ performances of the two non-ureolytic bacteria was the susceptibility of these two bacteria to calcium concentrations. Previous studies, showed that both *B. pseudofirmus* and *B. cohnii* produced $CaCO_3$ in 0.02 M calcium acetate *in vitro* [24] and in 0.1 M calcium lactate in mortar samples [25]. However, 0.183 M calcium lactate and calcium nitrate were used in this study. The two bacteria might be inhibited by the higher Ca^{2+} concentration in tested media, and resulted in trace or no $CaCO_3$ production in this study.

Although *B. pseudofirmus* and *B. cohnii* could produce spores easily within 3 days, they showed poor performances in growth and $CaCO_3$ production in YE-based media. In contrast, *L. sphaericus* showed efficient $CaCO_3$ production and grew rapidly in YE-based media with urea, it did not produce spores efficiently. Overall, *S. pasteurii* was the most ideal concrete-healing bacteria among the four because it grew rapidly in YE-based media with urea, had slightly better survival rates in mortar samples, and showed efficient performances in sporulation and $CaCO_3$ production.

4.5 Protective Carrier

Long term success of bacterial based self-healing concrete is contingent upon long term survival of the bacterial spores used in the concrete mixture. In this study, two LWA, EC and ES, were compared. Both materials are porous which can potentially protect bacterial spores in concrete. Expanded shale, is readily available in the area where this research was taken place, and it has not been studied by other researchers. Spores of *B. cohnii* and *B. pseudofirmus* were immobilized in EC and ES separately and then viable counts of the two types of spores were determined periodically. The results of this study indicated that spores of both microorganisms survived well in dried EC and ES of all sizes for the 150 day testing period (Figure 18). Among the three sizes, higher viable counts were observed from small-sized particles. It was hypothesized that small-sized LWA was able to retain more liquid than did medium or large size LWA due to its larger surface area s. Therefore, more spores were recovered from small size LWA. Using EC as a protective material and a carrier for microorganism has been studied in previous research [21]. Results of this study showed that ES could be an alternative carrier for bacterial spores in self-healing concrete.

Viable counts of bacterial spores with or without immobilization in LWA (EC and ES) and in mortar samples were determined. Without immobilization, the mean viable counts of *B. cohnii* and *B. pseudofirmus* dropped 3.4 and 3.7– 4.1 logs respectively after seven days in mortar samples. With immobilization in EC or ES, the mean viable counts of *B. cohnii* and *B. pseudofirmus* dropped 2.5– 2.6 and 2.6– 3.6 logs respectively after 7 days in mortar samples (Figure 19). This result suggested that immobilization of spores in EC or ES provided some protection to the spores during the first seven days in the mortar samples. However, at the end of the 28-day testing period, viable counts of immobilized spores and non-immobilized spores were about the same. It was noted that when mortar samples aged, it became more difficult to crush into fine powder. Some viable spores inside LWA may not be fully released into wash water for enumeration. Consequently, the viable counts of immobilized spores appeared to be lower than those of non-immobilized spores in the mortar samples. Our results also showed that incorporating nutrients such as calcium lactate and yeast extract into EC and ES before immobilizing the spores may improve survival rates of immobilized spores in mortar samples (Figure 19). Organic nutrients possibly protected bacterial spores in adverse environmental conditions.

Overall, the two types of LWA, EC and ES, are very similar in ability to protect microbes in self-healing concrete. Both are porous and can be used to carry bacterial spores. However, results of this study revealed that neither EC nor ES effectively prevented the death of the two bacterial healing agents in the mortar samples after 28 days. Although previous studies suggested that LWA protected bacterial spores from compression in concrete, LWA may not shield the bacterial spores from other stresses in the concrete environment, *e.g.*, harmful chemicals released from concrete may inhibit microbial activities. However, immobilizing healing agents into LWA may benefit the contractor preparing materials for making bacteria-based self-healing concrete. Bacterial healing agents, nutrients, and calcium precursors can be embedded into LWA, and bacterial healing agents can remain stable in these materials based on the results of this study. In such way, these materials serve as carriers to deliver bacterial-healing agents into cement mix, and the concerns for contractors to handle biohazards materials can be avoided.

Future research should focus on protecting bacterial healing agents against both chemical and physical stresses in concrete.

CHAPTER 5. CONCLUSION

The goal of this study was to compare different healing agents, including microorganisms, calcium sources, and protective materials for concrete cracks repair.

The following conclusions can be drawn concerning the comparison of the four microorganisms, *Sporosarcina pasteurii*, *Lysinibacillus sphaericus*, *Bacillus cohnii*, and *Bacillus pseudofirmus*:

- Spores of each bacterial strain could be induced by different sporulation agar plates within 3 days. *Sporosarcina pasteurii* produced spores efficiently on TSAU with an 89% of sporulation rate. *Bacillus cohnii* and *B. pseudofirmus* showed 93 and 99 % sporulation rates respectively on AR2A. The highest sporulation rate (60%) of *L. sphaericus* was on R2AU. Further studies are required to improve the sporulation rates of *L. sphaericus*.
- Ureolytic bacteria, *S. pasteurii* and *L. sphaericus*, showed rapid growth in AYE and YEU respectively. In contrast, *B. cohnii*, and *B. pseudofirmus* grew poorly in all media except in the TSBU.
- Approximately 1.8– 3.3 log reductions in viable counts of the four bacterial strains were observed within 1 day in mortar samples, and *S. pasteurii* showed the smallest reduction in viable counts (1.8– 2.4 logs). However, all four bacterial strains showed similar viable counts after 1 days in mortar sample with 2.5– 4.0 log reductions. Further studies are required to improve survival rates of bacterial healing agents in self-healing concrete.
- Results from this study showed that the two ureolytic bacteria *S. pasteurii* and *L. sphaericus,* precipitated $CaCO_3$ more efficiently than did the two non-urolytic bacteria *B. cohnii,* and *B. pseudofirmus. Sporosarcina pasteurii* and *L. sphaericus* converted two calcium sources: calcium lactate or calcium nitrate in YEU into $CaCO_3$ efficiently within 24 h at 30°C, and the recovery rates were above 80%. However, *B. cohnii* and *B. pseudofirmus* showed low $CaCO_3$ recovery rates (< 10%) from AYE containing calcium lactate or calcium nitrate. Further studies are required to improve the $CaCO_3$ production from *B. cohnii* and *B. pseudofirmus*.
- Among four tested bacterial strains, *S. pasteurii* showed the best overall performance as a microbial self-healing agent as it: (1) had good growth rates in the nutrient supplements

(AYE and AYEU), (2) had high sporulation rates, (3) showed high $CaCO_3$ production, and (4) showed a slightly better capacity to survive in mortar samples through time.

- Calcium lactate, overall, induced a significantly higher ($P < 0.05$) microbial $CaCO_3$ production over calcium nitrate by three microorganisms except *B. pseudofirmus*.

The following conclusions can be drawn from the study of comparison of two light weight aggregates (LWA), expanded shale (ES) and expanded clay (EC), as a protective material:

- Spores of *B. cohnii* and *B. pseudofirmus* remained viable in both EC and ES throughout the 150-day testing period. Therefore, both EC and ES can be used as bacterial carriers in concrete.
- Smaller sizes of LWA yielded slightly higher viable counts than did large and medium sizes.
- In mortar samples, viable counts of *B. cohnii* and *B. pseudofirmus* immobilized in EC or ES decreased 3.0– 3.9 logs after 28 days. Viable counts of immobilized spores were slightly higher than their non-immobilized counterparts in mortar samples during the first 7 days. This result suggested that both EC and ES provided some protection for the spores. However, on day 28, the viable counts of spores with or without immobilization were similar.
- Embedding nutrients into LWA before immobilizing spores may provide some protection to spores in mortar samples.

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