SCREENING FOR ALKALINE RESISTANT SPORE FORMING BACTERIA AS CONCRETE HEALING AGENTS

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

December 2020

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 her guidance, patience, and encouragement. She not only helped me to finish my thesis but assisted me to go through depressions and difficulties. I am so blessed to have Prof. Ting as my thesis advisor.

I would also like to thank my thesis committee: Prof. Chien-Chung Chen who helped me prepared experimental materials and taught me background knowledges of civil engineering and Prof. Scott Bates who provided me various ideas in terms of my experimental design and thesis writing. Without all these helps, this study could not be successfully finished.

I would also like to thank Jessica Orr and Hsin-Wen Liang who helped me with my research. They gave me many suggestions and supports.

Finally, I must express my very profound gratitude to my parents, Hung-Ju Chiao and Ling-Yen Huang, for their love and supports. They mean more to me than they will ever know.

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ABSTRACT

In order to find suitable bacteria as concrete healing agents, we examined a total of 50 bacterial isolates from an alkaline soil sample. These isolates were subsequently tested for sporulation rates, ability to induce calcium carbonate precipitation, tolerance to alkaline conditions, as well as their capacity to heal cracks in mortar samples. Of the 50, two bacterial isolates showed promising results across all these test categories. These isolates were identified as *Bacillus horneckiae* and *B. kochii*. Both were able to grow on LB agar at a pH of 10, within 5 days had sporulation rates over 90% on the AR2A agar plates, and precipitated calcium carbonate on B4 agar plates.

Both *B. horneckiae* and *B. kochii* had preferences for high alkaline environments. The OD 540nm readings of these two bacteria in pH 9 and 10 LB broths were significantly higher than the readings of their counterparts in pH 8 LB broth after 48 h of incubation. The growth of *B. horneckiae* and *B. kochii* in different concentrations of YE broths were tested. These two bacteria both had worse growth in 0.5 and 1% YE broths than in 2% YE broth. The spores of *B. horneckiae* and *B. kochii* were also tested for germinations in the same test environments. Results showed that either high pH or low nutrient levels did not have many impacts on spore germinations of these two bacteria.

Calcium carbonate precipitation from these two bacteria were quantified. *Bacillus horneckiae* and *B. kochii* reduced approximately 980 and 650 ppm of free Ca^{2+} ion respectively from a 1/10 LB broth containing 2500ppm of Ca^{2+} within 7 d and precipitated $CaCO_3$.

The mean viable counts of *B. horneckiae* and *B. kochii* decreased 1.2 and 1.5 orders of magnitude respectively in the first 24 h, dropped additional 0.6 and 0.4 orders of magnitude between day 1 and 14, and then, remained constant between day 14 and 28 after being mixed in mortar samples. Healing abilities were tested by incorporating bacterial spores in mortar samples. Cracks up to 0.25 mm were healed in mortar samples containing *B. horneckiae* or *B. kochii* spores. All the results suggested that both the bacterial isolates, *B. horneckiae* and *B. kochii*, may be used as bacterial healing agents in self-healing concretes.

CHAPTER 1. INTRODUCTION

1.1 Self-healing Concrete

Concrete is one of the most widely used construction material in the world due to its low cost and versatility. However, the durability of the concretes is always a concern due to crack formation. Small cracks tend to form in concrete naturally due to physical and chemical factors. Physically, erosion, freezing, and thawing may cause concrete damages. Chemically, salt and carbon dioxide in the air may also lead to concrete corrosion. Additionally, human factors such as overloading the concrete structures may also result in crack formation (Şahmaran et al., 2010). Hairline cracks typically have no effects on the strength of the concrete. Nevertheless, water and harmful chemicals that infiltrate through the cracks may lead to the degradation of the concrete matrix and the corrosion of the steel bars within the concrete. As a result, the service life of the concrete structure is shortened.

To enhance the service life of concrete, surface coatings, which form a polymer film over the concrete may be used to physically block harmful substances from penetrating into the concrete. Multiple substances have been used for coatings, which each have pros and cons. For example, epoxy resin is a traditional coating material. It has excellent chemical resistance and adhesive strength but has low thermal stability and poor hydrophobicity (Pan et al., 2017). Concrete structure may also be maintained either passively or actively. The passive approach is a series of procedures that include monitoring, damage detection, and repair (Wang et al., 2012). When concrete cracks are detected, sealants, such as epoxy, siloxane, acrylics, and polyurethanes, are commonly used to fill the cracks and protect the concrete structure from further deterioration (Pacheco-Torgal et al., 2013). Active approaches, on the other hand, involve adding self-healing ingredients into the concrete that facilitate autogenously crack healing ability. Self-healing concretes are made by incorporating alkaline resistant bacterial spores, a calcium source, and organic nutrients for bacterial growth into the concrete mix, and self-healing concretes have been researched over the course of the past 20 years (Jonkers, et al., 2007). As Figure 1 shows, once the concrete cracks, water can penetrate into the concrete structure and dissolve the organic nutrients. The water allows the dormant bacterial spores to germinate.

Germinated bacteria serve as nuclei to aggregate calcium carbonate on their cell walls and then repair the cracks (Figure 2; Gollapudi et al., 1995; Muynck et al., 2010).

Passive treatment, which requires constant inspection and repair, is very costly. For instance, the USA government spends around 7 trillion dollars a year on concrete maintenance. (Delatte et al., 2009). Moreover, Organic polymers, such as epoxy and polyurethanes, tend to have some degree of toxicity (Pacheco-Torgal et al., 2018). In contrast, self-healing concrete does not require maintenance and does not pollute the environment. Concrete self-healing mechanisms repair both interior and exterior cracks (Pan et al., 2016). They also block further penetration of harmful substances and restores concrete compressive strength (Jonker et al., 2007). As a result, self-healing concretes are more cost-efficient and eco-friendlier overall (Pacheco-Torgal et al., 2013; Wang et al., 2012).

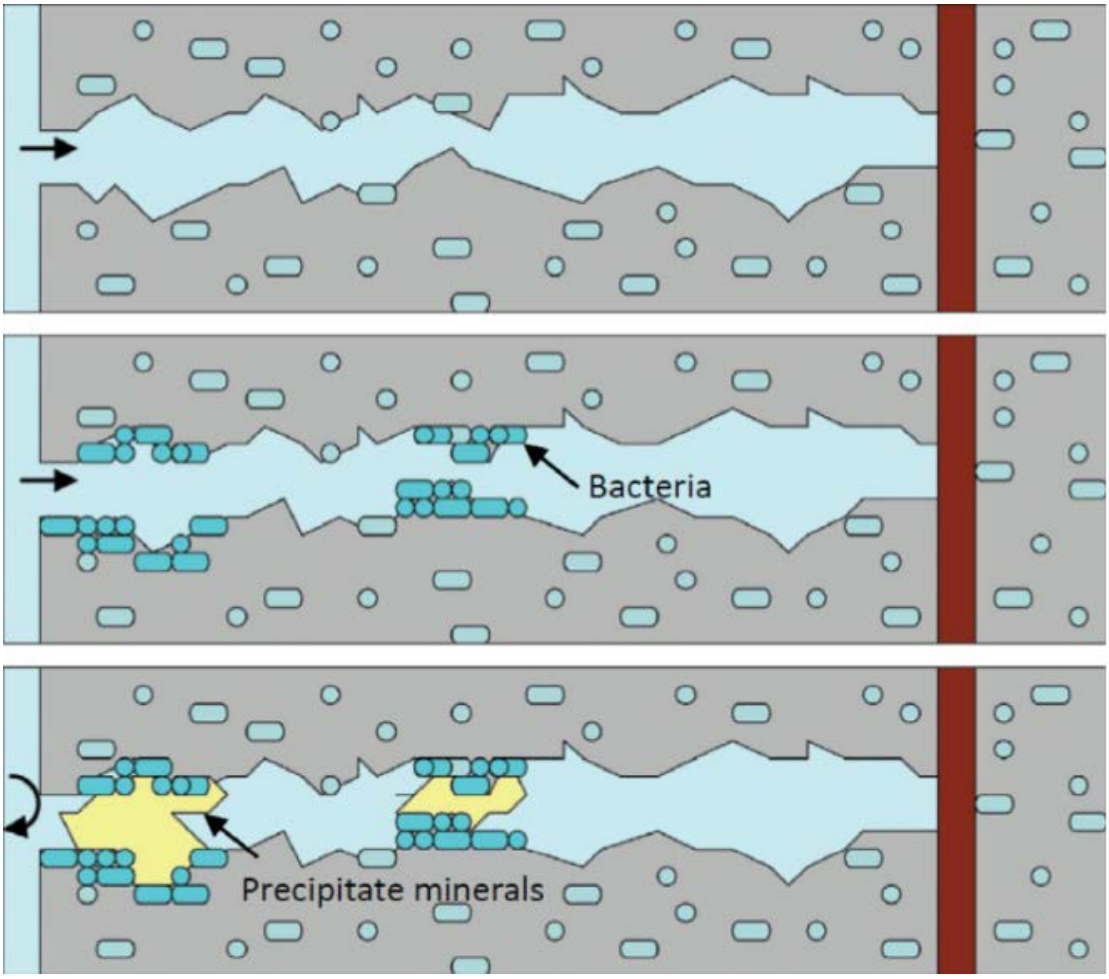


Figure 1. Bacteria revives by the water, precipitate calcite, and fill cracks (Jonkers et al., 2007).

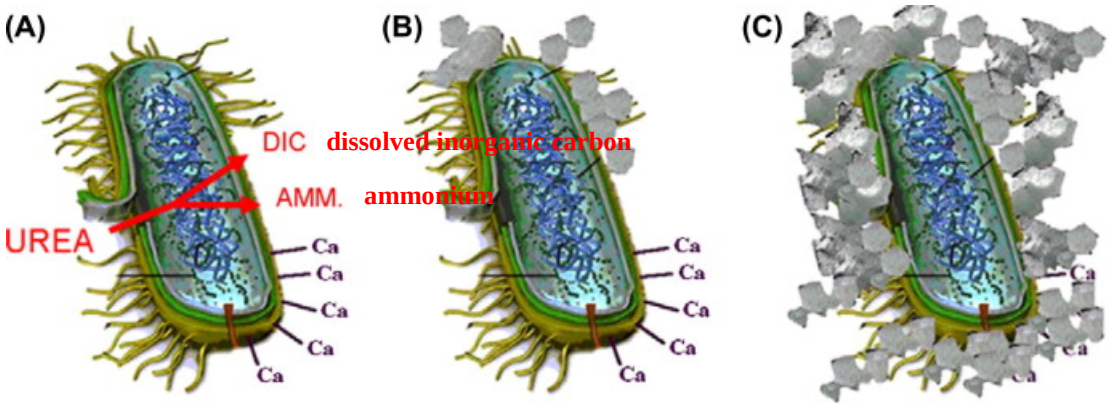


Figure 2. Process of calcium carbonate precipitation on the bacterial cell wall (Pacheco-Torgal et al., 2013).

1.2 Microbially Induced Calcium Carbonate Precipitation Mechanism

Microbially induced calcium carbonate precipitation (MICP) is a common phenomenon found in nature, including marine water, freshwater, and soil environments (Hammes et al., 2002). A previous study isolated 210 soil bacteria that can precipitate calcium carbonate ($CaCO_3$) on the B4 plate (Boquet et al., 1973).

Bacterial mineralization of $CaCO_3$ requires the presence of Ca^{2+} and carbonate ion (CO_3^{2-}) generated from bacterial metabolism (Boquet et al.,1973). With the presence of both Ca^{2+} and CO_3^{2-}, Ca^{2+} will attach to the negatively charged cell wall of bacteria, and positive Ca^{2+} will react with available CO_3^{2-}. After the reaction, $CaCO_3$ will have precipitated on the bacterial cell wall, which serves as a nucleation site. Many bacteria, including both autotrophic and heterotrophic bacteria, have been shown to have MICP activity. There are three autotrophic pathways, non-methylotrophic methanogenesis, oxygenic photosynthesis, and anoxygenic photosynthesis, can perform MICP (Castanier et al., 1999), and each requires specific environmental parameters. Non-methylotrophic methanogenesis pathway requires and anaerobic environment, while the oxygenic photosynthesis and anoxygenic photosynthesis pathways require light to trigger the reactions (Seifan et al., 2016).

The environmental conditions of heterotrophic pathways, on the other hand, are diverse and occur under various environmental conditions, including aerobiosis, anaerobiosis, and microaerophily (Castanier et al., 1999), and they only require organic nutrients to facilitate the reactions. Thus, heterotrophic bacteria were more commonly used as self-healing agents.

For heterotrophs, both ureolytic and non-ureolytic bacteria can mediate the formation of $CaCO_3$. Ureolytic bacteria can produce urease, which degrades urea $CO(NH_2)_2$ into ammonium (NH_4^+) and carbonic acid (CO_3^{2-}). Carbonic acid can subsequently react with Ca^{2+} to form $CaCO_3$. (DeJong et al., 2010; Vijay et al., 2017). Many pathways from non-ureolytic bacteria were found to precipitate calcite, bacteria which precipitate calcium carbonate through organic carbon oxidation is now widely discussed. Heterotrophic bacteria can use different organic nutrients as their energy source and form CO_3^{2-} as the end product to precipitate calcium carbonate. For example, organic salts (calcium acetate and calcium lactate) can serve as both the calcium source and the organic nutrients supporting bacterial growth in the self-healing concrete (Seifan et al., 2016).

1.3 Ureolytic or Non-ureolytic Bacteria

Both ureolytic and non-ureolytic bacteria are widely used as concrete healing agents (Huynh et al., 2017; Khaliq et al., 2016; Wang et al., 2014; Wiktor et al., 2011; Xu et al., 2018) (Table. 1). However, ureolytic bacteria were not considered in this study. Ureolytic bacteria had high calcium carbonate productivity through urea degradation (Wang et al., 2014), however, many researches indicated that using ureolytic bacteria could have some disadvantages. Urea degradation can generate ammonium ions which have negative effects on the environment and human health (Zhang, 2017). In addition, the oxidation of urea produces nitric acids (HNO_3) that cause corrosions of concretes (Sharma et al., 2017).

1.4 Nutrient Composition in Mortar/Concrete Specimen

There is no standard nutrient concentration when making self-healing concrete samples. Therefore, nutrient compositions in self-healing concrete/mortar are different among different research groups (Table 1). However, a previous research showed that changing the growth media could alter MICP ability of concrete healing bacteria (Alazhari et al., 2017). It showed that *B pseudofirmus* DSM 8715 converted a greater amount of calcium carbonate precipitation from a media with additional supplemented chemicals and a higher concentration of yeast extract.

Table 1. The nutrient composition used by different research groups.

Bacteria	Nutrient composition	Reference
Ureolytic		
Bacillus subtilis HU58	0.44% urea, 0.22% $CaCl_2$. H_2O and 0.11% nutrient broth (w/w) of mortar sample	Huynh et al., 2017
Bacillus sphaericus	18 g urea, 36 g $Ca(NO_3)_2 4H_2O$, and 3.84 g yeast extract, in 1750g cement	Wang et al., 2014
Sporosarcina pasteurii	1.5 g calcium nitrate, 2.5 g urea in 1 kg cement sample	Xu et al., 2018
Non-Ureolytic		
Bacillus alkalinitrilicus	17.52g calcium lactate and 0.22g yeast extract in 1800g mortar	Wiktor et al., 2011
Bacillus subtilis	18 kg calcium lactate in $1m^3$ of cement sample	Khaliq et al., 2016

1.5 Characteristics of Bacteria in Self-healing Concrete

Ideally bacteria used in self-healing concretes should have high sporulation rates within a short time in growth media, tolerate high pH, survive long term in the concrete environment, and precipitate calcium carbonate effectively to repair cracks. Figure 3 shows the process of screening bacterial isolates with these characteristics from alkaline soil samples.

Bacterial spores (e.g., endospores) allow for bacterial dormancy, and these spores can remain dormant in an extreme environment for many years and germinate when water and nutrients are available. In contrast, vegetative cells require water and nutrients for growth, and they will quickly die when water and nutrients are no longer available. Therefore, bacterial spores are more typically being used as self-healing concrete agents, rather than vegetative cells.

Bacterial metabolism is influenced by pH values in the environment. For example, pH may cause spore germination time to be prolonged, and the growth rate of vegetative cells may be decreased in an environment with extreme pH values. Therefore, a successful bacterial healing agent should tolerate high alkaline pH since the pH values of concrete range from 10 to 13 (Wang et al., 2017).

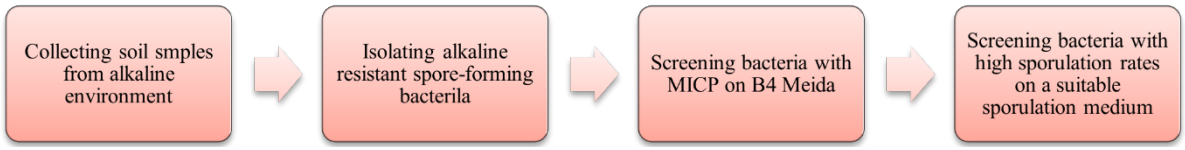


Figure 3. Process of screening new bacterial isolates as concrete healing agents.

1.6 Isolation of Alkaline Resistant Bacteria

1.6.1 Choosing Proper Sampling Sites

Aerobic spore-forming bacteria are the most commonly used concrete healing agents (Huynh et al., 2017; Jonkers et al., 2007; Khaliq et al., 2015; Kim et al., 2015; Sharma et al., 2017). Although they are widely distributed in nature, they are mostly found in soil (Logan et al., 2011; Table 2).

Soils with alkaline pH are an ideal source for the isolation of alkaline resistant spore-forming bacteria. For instance, a *Lynsinibacillus* strain was a newly isolated concrete healing agent from alluvial soil in India (Vashisht et al., 2018). This kind of soil is typically alkaline since it is rich in iron oxide and lime. This *Lynsinibacillus* strain was found to survive under alkaline environments and increased the compressive strength of cracked concrete samples (Vashisht et al., 2018). In this study, alkaline metal polluted soils (pH 11.4) were chosen for the screening of new bacterial isolates.

1.6.2 Screening for Bacteria with High Sporulation Rates

To isolate spore formers, samples are typically heated at 70 °C for 30 min to kill bacterial vegetative cells. Only dormant endospores will survive for isolation (Zhang et al., 2016; Lee et al., 2017).

Nutrient depletion in the environment was found to be the primary factor that triggers the sporulation of bacteria (Ryu et al., 2005). Therefore, sporulation media should provide this kind of environment. In addition, incubation temperature and chemicals, like magnesium, may also affect bacterial sporulation (Ryu et al.,2005). Table 3 shows various sporulation media used by other research groups and sporulation rates of concrete healing bacterial strains in their studies. An alkaline mineral medium has been used for enhancing the sporulation of *B. pseudofirmus* and *B. cohnii* (Jonkers et al., 2010), whereas minimal basal salts (MBSs) medium

has been used for cultivating bacteria spores of *Bacillus sphaericus* (Wang et al., 2015). The sporulation rates of *B. sphaericus* can reach 90% in 14 to 28 d. Sporulation of *Lysinibacillus* strain YS11 were observed on B4 medium after a 10 d incubation at 30 °C (Lee et al., 2017). Both *B. cohnii* and *B. pseudofirmus* can reach a 90% sporulation rate after 3 d on the alkaline R2A (AR2A) plate at 30 °C (Chiu, 2019). 2×Schaeffer's glucose (2× SG) agar plate was used for the sporulation of a *bacillus* strain (H4 strain) and spores can be found in 1 d at 37 °C (Zhang et al., 2016). Some bacterial strains only sporulate on certain media; therefore, finding an excellent sporulation media for a newly isolated bacterial strain is important.

Table 2. Isolation of bacterial concrete-healing agents.

Sampling site	Isolation medium	pH	Isolated bacteria	Reference
Concrete	LNC (lactate-nitrate-carbonate) agar plate	11	*Bacillus* strains *Sporosarcina* strain	Kim et al., 2015
Rhizosphere	LB agar plate Mineral salt agar plate	9	*Lysinibacillus* strain	Lee et al., 2017
Alluvial soil	Urea agar plate	9.4	*Lysinibacillus* strain	Vashisht et al., 2018
Alkaline soil	Mineral medium	10	*Bacillus alkalinitrilicus*	Sorokin et al., 2008
Loamy soil	B4 medium	8	*Bacillus licheniformis*	Vahabi et al., 2015
Rhizosphere	LB broth	11.2	*Bacillus* strain	Jung et al., 2020
Livestock soil	Nutrient broth	7	*Bacillus* strains	Khadhim et al., 2019

Table 3. Sporulation media used in previous self-healing concrete studies.

Media	Ingredients	pH	Bacteria strain	Sporulation	Reference
Minimal basal salts (MBSs) medium	0.3 g $MgSO_4 \cdot 7H_2O$, 0.02 g $MnSO_4$, 0.02g $Fe_2(SO_4)_3$, 0.02g $ZnSO_4 \cdot 7H_2O$, 0.2g $CaCl_2$, 10 g tryptose, 2g yeast extract (/L)	7.4	*Bacillus sphaericus*	90% sporulation rates in 14 to 28 d.	Wang et al., 2015
Alkaline Mineral medium	0.2 g NH_4Cl, 0.02 g KH_2PO_4, 0.225 g $CaCl_2$, 0.2 g KCl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.01 g $MnSO_4 \cdot 2H_2O$, 1 ml trace element solution SL12B, 0.1 g yeast extract, 5.16 g citric acid trisodium salt, 4.2 g $NaHCO_3$ and 5.3 g Na_2CO_3 (/L)	10	*Bacillus. pseudofirmus Bacillus. coh*nii	Not recorded	Jonkers et al., 2010
B4 medium	yeast extract 4 g, glucose 5 g, calcium acetate 25 g (/L).	8	*Lysinibacillus* strain YS11	Spore formed within 5 d	Lee et al., 2017
Alkaline R2A medium	Proteose peptone 0.05%, Casamino acids 0.05%, Dextrose 0.05%, Soluble starch 0.05%, Dipotassium phosphate,0.03%, Magnesium sulfate 0.005%, Sodium pyruvate 0.03% Agar 1.5%	9.5	*Bacillus. pseudofirmus Bacillus. coh*nii	Both reached 90% sporulation rates within 3d	Chiu, 2019
2×Schaeffer's glucose (2× SG) medium	yeast extract 1g, casamino acid 0.8g, sodium citrate 0.3g, $MnSO_4 \cdot 7H_2O$ 2.0g, KCl 0.2g, $NaCl_2$ 5g, agar 2g (/100ml).	7.3	H4 strain (Close to *B. pseudofirmus*)	Spore formed in 1 d	Zhang et al., 2016

1.6.3 Screening Bacteria with MICP

Finding bacteria that can carry out calcium carbonate precipitations is the last part of the screening process, B4 medium which is made by 0.25g calcium acetate, 0.4g yeast extract, and 1.0g glucose (pH adjusted to 8.0 by NaOH) was designed and widely used for this purpose. If a bacterial cell can precipitate calcium carbonate, after being streaked on the B4 agar plate and allowed to grow for 18 d, calcium carbonate crystals will deposit on the bacterial cell surface and the calcium carbonate deposit can then be observed microscopically on the plate (Boquet et al., 1973).

1.7 Using Protective Materials to Enhance Bacterial Survival

Even though bacterial spores could theoretically survive under environmental stresses, a previous research shows that the viability of bacteria spores decreases dramatically (a three-fold logs drop) in concrete samples within 22 days (Jonkers et al., 2010). Therefore, protective materials were studied here to increase the viability of bacteria in the concrete. Such materials protect bacteria spores by immobilizing bacteria endospores into the porous structure of the protective materials. Because microcapsules can resist the mechanical forces that occur during concrete preparation, bacteria can be protected from these stresses (Seifan et al., 2016). Biocompatible hydrogel silica gel is one such material that has been used to immobilize bacteria endospores. In a previous study, *B. sphaericus* was embedded into the gel and mixed with nutrients and bacterial survival was demonstrated through the detecting of ureolytic activity by *B. sphaericus* (Wang et al., 2014) The ureolytic activity remained stable for 24 weeks after being incorporated into the concrete, which suggested that the viability of bacteria spore was stable (Wang et al., 2014; Bundur et al., 2017). Lightweight aggregates (LWA) and graphite Nanoplatelets (GNP) have also been tested for their protective ability. *Bacillus subtilis* endospores were incorporated into LWA and GNP, and the results showed that these two materials could successfully increase the viability of the embedded bacterial spores (Khaliq et al., 2016). Expanded clay (EC) was also tested to protect *Bacillus alkalinitriculus* endospores. Spores and organic nutrients were vacuumed into EC, and the result showed that concrete cracks

were healed, while the long-term durability of bacteria spores was also demonstrated (Wiktor et al., 2011).

However, the effect of concrete strength needs to be considered when incorporating material into the concrete mix. For example, a previous studie (e.g., Wang et al., 2017) has shown that mixing silica gel into the concrete will decrease the flexural strength of the concrete, which is undesired. Other study (e.g., Erşan et al., 2015) has compared the influences of mixing different commercially available protective materials (diatomaceous earth, metakaolin, expanded clay, granular activated carbon, zeolite, and air entrainment) into the mortar sample. In this study, setting times and the compressive strength of samples with different protective materials were compared, and only zeolite was found to significantly decreased the compressive strength of the mortar samples.

1.8 Identification of Calcium Carbonate Precipitation

Several methods have been used for calcium carbonate identification. Scanning electron microscope (SEM) uses a laser beam to scan the surface of the sample and creates a three-dimensional image. It is not only used for revealing the size and structure of the specimen but also for observing crystal formation on the surface of the concrete and the healing of microcracks (Leng et al., 2013; Sharma et al., 2017). X-ray diffraction is a method for characterizing nanostructure. The emission of an X-ray beam will diffract by the specimen. After measuring the angle of the diffraction, the diffraction pattern can be calculated, and each specimen has a unique pattern. The morphology and the crystal structure can be determined by comparing the results to known diffraction patterns (Lee et al., 2012; Vahabi et al.,2015). X-ray absorption spectroscopy (EDX) is also used to analyze the elemental composition of the specimen. It measures the X-ray emission from the specimen by a spectrometer after its electrons are excited by absorbing an X-ray beam. The different elements will discharge different energy of X-ray. By comparing the emission energy to a database, the chemical composition of the specimen can be determined and analyzed (Groot et al., 2006; Sharma et al., 2017).

1.9 Quantification of Calcium Carbonate Precipitation

The quantification of calcium carbonate can be directly determined by its dry weight, and previous research has used a filtration method for this process (Vahabi et al., 2015). Bacteria strains were inoculated into B4 broth (Ingredients showed in Table 2) and incubated at 32°C for 1 week. In brief, this method uses the following protocols: the precipitants are filtered onto a Whatman No. 1 filter paper; the filter paper with calcium carbonate precipitation is dried at 37 °C for 2 d; and finally, the dry weight (g/L) is measured (Vahabi et al., 2015). Alternatively, calcium carbonate in a broth culture may be collected by centrifugation. For example, in another study, one percent of bacteria culture was added into urea broth with $CaCl_2$ supplement., After incubation at 37 °C in a shaker incubator (130 rpm) for 3–5 d, the tubes were centrifuged at 8000*g* for 15 min. The pellets were dried at 80 °C for 24 h and weighed as the quantity of calcium carbonate precipitation (Vashisht et al., 2018). Calcium carbonate precipitation can also be measured by dissolving the pellet in acid and measuring calcium ion concentration in the solution. For instance, Sharma et al. (2017) collected calcium carbonate precipitate from an 8 d1/10th strength LB acetate culture by centrifugation, and then the pellet was washed and dissolved in 0.6 M HCl into free Ca^{2+} which was then measured using atomic absorption spectroscopy.

The quantity of calcium carbonate precipitation can also be indirectly determined by measuring the decrease of calcium ions in the culture medium. Zhang et al., (2016) inoculated 10 µl spores at a concentration of 1×10^9 spores/ml into 100 µl of calcium precipitation medium in a well of a 96-well plate. The spores were incubated in a CO_2-free vacuum desiccator at 30 °C for 7 d. At the end of the incubation, 3.75 µl of supernatant was collected by centrifugation and transferred to another 96 well plate to determine Ca^{2+} concentration using an O-Cresolphthalein Complexone (OCPC) method. Complexone is a chromophore that attaches to Ca^{2+} and turns purple. After adding OCPC to the well, Ca^{2+} concentration in the well can be measured by determining absorbance (575 nm) by a 96-well plate spectrometer (Zhang et al., 2016; Zhang et al., 2017).

Calcium ion concentration in a medium can also be measured using a calcium-ion selective electrode (ISE). The electrode directly detects free calcium ions in the liquid medium by converting calcium activity into electrical potential.

1.10 Objectives

Numerous bacteria isolates were used as bacterial healing agents by other research groups. It is unknown whether these bacterial agents have all the desirable traits to produce an ideal concrete healing agent. This research aimed to screen new bacterial isolates that possess the traits of alkaline tolerance, high sporulation rates, long survival times inside of mortar samples, while being able to effectively heal concrete cracks.

CHAPTER 2. MATERIALS AND METHODS

2.1 Bacteria Isolation

Alkaline soil samples (pH 11.4), which were collected at Arcelor Mittal, Indiana, in both spring and summer of 2018, were used for isolating alkaliphilic bacteria. One gram of soil sample was suspended with 1ml diH_2O in a sterile tube and heated at 70 °C for 30 min using a heating block to kill all the vegetative cells. Then, 100-µl of each heated soil suspension was streaked on Alkaline Luria Bertani agar (ALBA) plates for isolation (Table 4) and incubated at 30 °C for 24 h. Bacterial colonies with different colonial morphology were isolated.

2.2 Bacterial Cultures.

New isolates were preserved on ALBA slants at 4 °C. Working cultures were prepared by transferring stock cultures into ALB broths (pH 10) at 30 °C for 24 h. Positive control bacteria, *B. pseudofirmus* (ATCC 11859), were obtained from American Type Culture Collection (Manassas, VA, USA). It was preserved on alkaline nutrient agar (ANA) slants (Table 4) which were recommended by ATCC. The working cultures of these two bacteria were cultivated in alkaline nutrient broth (ANB; Table 4) at 30 °C for 24 h.

2.3 Screening for Bacteria with Calcium Carbonate Precipitation Ability

B4 agar plates (B4A; Table 4) were used for screening bacterial isolates with MICP activity. Each bacterial isolate was streaked on B4 plates and incubated at 30 °C for 7 d. Colonies with crystal formation were considered as bacteria with MICP activity.

2.4 Identification of Bacterial Isolates by 16S RNA Sequencing

Two bacterial isolates that grew in ALB broths had over 87% sporulation rates within 5 d and showed MICP abilities, were further identified by 16S RNA sequencing. Each bacterial isolate was cultivated in ALB for 24 h at 30 °C and centrifuged at 13,000 rpm for 3 min at room temperature. The DNA of each isolate was extracted from the pallet following the protocol of the QIAprep Spin Miniprep Kit (Qiagen, Germany). The mix of the polymerase chain reaction

(PCR) includes 22 µl of diH_2O, 1 µl of extracted DNA sample, 1 µl of 10 µm fd1 primer (5'-AGAGTTTGATCCTGGCTCAG-3'), and 1 µl of 10 µm rp1 primer (5'-AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA-3`) in a PCR tube. The reaction cycle of PCR is 95 °C for 2 min, followed by 35 cycles at 95 °C for 20 sec, 55 °C for 30 sec, and 72 °C for 2 min, with then a final extension step at 72 °C for 8 min. Amplified DNA fragments were purified following the protocol of the QIAquick Gel Extraction Kit (Qiagen, Germany). After 16S RNA sequencing, 16S RNA data of the two isolates were analyzed by using Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/).

2.5 Bacterial Sporulation Rates Determination

2.5.1 Sporulation Media Preparation

Sporulation rates of each bacterium were determined on four media, alkaline R2A agar (AR2A), alkaline nutrient agar, alkaline sporulation agar (ASA), and modified B4 agar (mB4A). The chemical composition of each medium is shown in Table 4.

2.5.2 Sporulation Rates Determination

Working cultures of bacteria isolates were streaked separately on the four types of agar plates: AR2A, ASA, ANA and mB4A (Table 4), and incubated at 30 °C. Sporulation rates of each were determined after 3, 5, and 7 d incubation. After spore staining using the Bartholomew and Mittwer's method (Bartholomew, J.W. et al., 1950), bacterial vegetative cells stain red and bacterial spores stain green. A compound microscope was used for observing staining results. Sporulation rates on each medium were determined using the following equation.

$$\text{Sporulation rate (\%)} = \frac{\text{Number of spores}}{\text{Total number of cells and spores}} \times 100\%$$

Table 4. Media used in this study.

Media	Ingredients	pH adjustment	Usage in this study
ALBA	Peptone (10 g) Yeast Extract, (5 g) Sodium Chloride (5 g), and agar (12 g) in 1 L of distilled water.	adjusted to pH 10 with 5 N NaOH before autoclaving.	Culture media Preservation
AR2A	Proteose peptone #3 (0.5 g), magnesium sulfate heptahydrate, (0.1g), sodium pyruvate (0.3g), casein acid hydrolysate (0.5g), dipotassium phosphate (0.3g), yeast Extract (0.5g), soluble Starch (0.5g), and glucose (0.5g) in 900 ml of distilled water and 100 ml of alkaline solution.	Addimg alkaline solution after autoclaving	Sporulation
ASA	Peptone (5.0 g), meat extract (3.0 g), $MnSO_4H_2O$ (10.0 mg) and agar (12 g) in 1 L of distilled water.	adjusted to pH 8 with 5 N NaOH before autoclaving.	Sporulation
ANA	Glucose (6.0 g), peptone (15.0 g), sodium chloride (6.0 g), yeast extract (3.0 g), and agar (12 g) in 900 ml of distilled water and 100 ml of alkaline solution.	Addimg alkaline solution after autoclaving	Culture media Preservation
ANB	Glucose (6.0 g), peptone (15.0 g), sodium chloride (6.0 g), and yeast extract (3.0 g), and agar (12g) in 900 ml of distilled water and 100 ml of alkaline solution.	Addimg alkaline solution after autoclaving	Culture media
B4A	Glucose (0.5 g), yeast extract (0.4 g), calcium acetate (0.25 g), agar (12 g) in 1 L of distilled water.	adjusted to pH 8 with 5 N NaOH before autoclaving.	Testing MICP
mB4A	Glucose (0.5 g), yeast extract (0.4 g), $MnSO_4H_2O$ (10.0 mg) and calcium acetate (0.25 g) in 1 L of distilled water.	adjusted to pH 8 with 5 N NaOH before autoclaving.	Sporulation
1/10 LB	Peptone (1 g) Yeast Extract, (0.5 g) Sodium Chloride (0.5 g), and calcium acetate (10g) in 1 L of distilled water.	adjusted to pH 8 with 5 N NaOH before autoclaving.	Quantify MICP

*Alkaline solution: filter-sterilized Na-sesquicarbonate solution (4.2g $NaHCO_3$ and 5.3g Na_2CO_3/100 ml H_2O)
*Autoclaving: 121 °C for 15 min

2.6 Effects of pH and Nutrient Composition on Bacterial Growth

Bacterial growths were measured by using a plate reader (Molecular Devices, Sunnyvale, CA). Working cultures of *B. horneckiae, B. kochii, and B. pseudofirmus* were prepared and transferred individually into different empty sterilized tubes with PBS buffer. All bacterial suspensions were adjusted to 0.8 $OD_{540nm,}$ which contained approximately 10^8 CFU/ml bacterial cells, before each test. After adding 200 µl of test medium and 5 µl of bacterial suspension in each well of a 96-well plate, the plate was incubated at 30 °C for 48 h in the plate reader. The OD_{540nm} reading of each well was measured every 6 h.

To test the effect of pH on bacterial growth, LB broth was adjusted to pH 8, 9, or 10 using 5N NaOH before autoclaving. Different concentrations of pH 9 (pH was adjusted by adding 5N NaOH before autoclaving) yeast extract (YE) broth (0.5, 1, and 2 % yeast extract in diH_2O) were used for analyzing the influence of nutrient concentrations on bacterial growth.

2.7 Effect of pH and Nutrient Concentration on Bacterial Spore Germination

Spores of each bacterial culture were produced on AR2A plates after 7 d incubation. Spores of each bacteria were washed and scraped down by a sterilized loop with sterilized water from the agar plates. Then, spore solutions were transferred into sterile tubes. Bacterial spore suspensions of *B. horneckiae, B. kochii, and B. pseudofirmus* were adjusted to OD_{540nm} of 0.8 to test spore germination. For testing the influence of pH on spore germination, spores of all bacterial isolates were added separately in pH 8 or pH 10 LB broth. The impact of nutrient concentration on spore germination was analyzed by cultivating different bacterial spores individually in 2% or 0.5% YE broth. The 96-well plate was prepared by pipetting 200 µl of testing medium and 5 µl of spore suspension in each well. The plate was incubated in a plate reader at 30 °C for 24 h. The OD_{540} of each well was measured every hour.

2.8 Calcium Precipitation Efficiency

One ml of *B. horneckiae* or *B. kochii* working culture was added into 100 ml of 1/10 LB broth (Table 4) and incubated at 30 °C on a shaker (200 rpm; Lab line instruments, Illinois). Free calcium ion concentrations in the broth were measured on day 1, 2, 3, 5, 7, and 12 using a calcium ion-selective electrode (ISE, Thermo Fisher, Massachusetts).

2.9 Bacterial Spore Survival in Mortar Samples

2.9.1 Immobilization of Bacterial Spores in Expanded Shale

The working cultures of *B. horneckiae*, *B. kochii*, and *B. pseudofirmus* were spread separately on AR2A agar plates using sterile cotton swabs. After incubation at 30 °C for 7 d, each plate was washed by 5 ml of sterile diH_2O before collecting the bacterial spore suspensions. Each spore suspension was heated at 70 °C for 30 min to inactivate vegetative cells. Then, the viable count of each bacterial spore suspension was determined using the plate count method.

Expanded shale (ES) was used as the bacterial spore carrier in this study. The ES was sterilized by heating at 180 °C for 24 h in a hot air oven. Bacterial spores were immobilized in the ES by soaking 500 g of sterile ES in 250 ml of bacterial spore suspension (2×10^6 CFU/ml) in a sterile beaker and exposed to -0.8 bar for 20 min and +0.6 bar for 10 min (Figure 4). After immobilization. ES samples containing immobilized bacterial spores were dried at 55 °C for 48 h and stored in sterile bottles at room temperature.

After the 48 h drying period, viable counts of *B. horneckiae*, *B. kochii, and B. pseudofirmus* spores embedded in ES were verified. To determine viable counts, 6 g ES sample was mixed with 30 ml of sterile dH_2O in a 50 ml sterile centrifuge tube for 1 min using a vortex. The wash water of ES containing *B. horneckiae* or *B. kochii* was spread plated on ALB agar plates while the wash water of ES containing *B. pseudofirmus* was spread plated on ANA plates to determine the viable counts

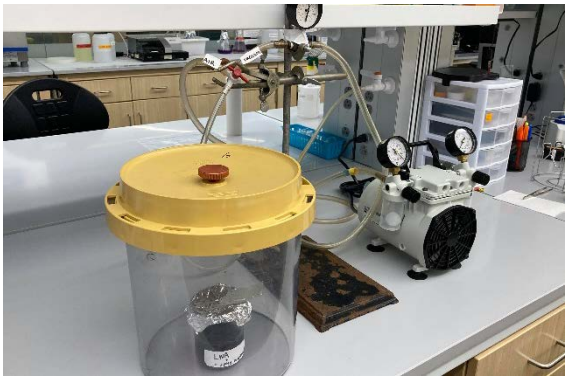


Figure 4. Immobilize bacterial spores into expanded shale.

2.9.2 Preparation of Mortar Sample

River sand and type I Portland cement were sterilized in a hot air oven at 180 °C for 24 h before making the mortar samples. Each mortar sample was made by mixing 9.13 g ES with immobilized spores, 0.18g yeast extract, 0.64g calcium acetate, 16.85 g sand, 16.51 g cement, and 9.17 ml of sterile diH2O. Mortar samples were cured at 20 °C for 1 week (Figure 5 shows the cured mortar sample). Mortar samples with sterilized ES were served as control.

Viable counts of mortar samples were recorded on 1, 7, 14, and 28 d after curing. For viable counts, the mortar sample were crushed into powder. Next, 30 µl of sterile diH_2O was mixed with 6 g crushed mortar sample in a clean test tube and vortexed for 1 min to wash out embedded bacterial spores. Then, the wash water was serial diluted and spread plated on ALB agar plate for the plate counts.

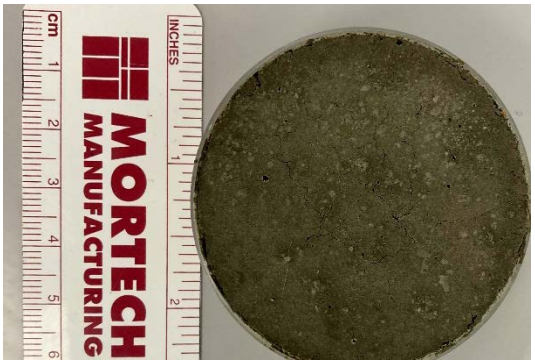


Figure 5. Cured mortar sample

2.10 Mortar Samples Crack Healing Test

Mortar samples were cured at room temperature for 7 d and then cracked by applying pressure on two opposite ends of the samples using a clamp (Figure 6). Crack width was measured. Then, each cracked mortar sample was immersed 1 cm under the surface of tap water and incubated at 30°C until the cracks were healed. Crack healing was observed under a digital microscope (Celestron, California) and measured using Celestron Micro CapturePro software.

Figure 6. Clamp for cracking mortar samples.

CHAPTER 3. RESULTS

3.1 Isolation of Spore forming Alkaline Resistant Bacteria from Alkaline Soil Samples

A total of 50 colonies with different colonial morphology were isolated from alkaline soil samples on ALBA plates (Figure 7). They are further screened for MICP activity on B4A plates.

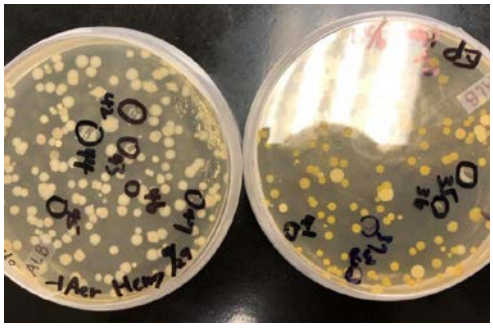


Figure 7. Bacterial isolation on alkaline LB plates

3.2 Screening Bacterial Isolates with MICP Activity

After incubated for 7 d at 30 °C, bacterial colonies with crystal formation on B4A plates were considered positive MICP activity. Among the 50 isolates, only isolates #5, 12, 43, 44, and 47 showed crystal formation on B4 agar plates (Figure 8).

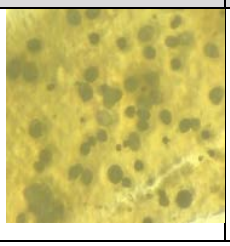
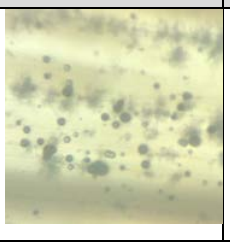
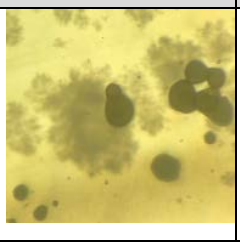
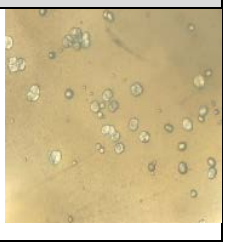
Isolate	# 5	# 12	# 43	# 44	# 47
Calcium carbonate formation					

Figure 8. Crystal formations by five isolates (#5, 12, 43, 44, and 47) after incubation on B4 agar plates for 7 d at 30 °C. (Viewed under 40×magnification).

3.3 Screening Bacterial Isolates with High Sporulation Rates on Four Sporulation Media

Sporulation rates of isolates #5, 12, 43, 44, and 47 on AR2A, ASA, ANA, and MB4A were examined after incubation at 30 °C for 7 d. Some of the isolates did not grow on all the sporulation media types. The letter X in Figure 9 means no growth was observed on the media. Isolates #5 had no growth on ASA or ANA plates. It only produced spores on MB4A plates with 16 % sporulation rate. Isolates #43 (*B. horneckiae*) and #44 (*B. kochii*) had over 90% sporulation rates recorded on AR2A plates. Isolate #47 had 65% sporulation rates on MB4A. Isolate # 12 only grew on AR2A and MB4A, but it did not produce any spores on the two media.

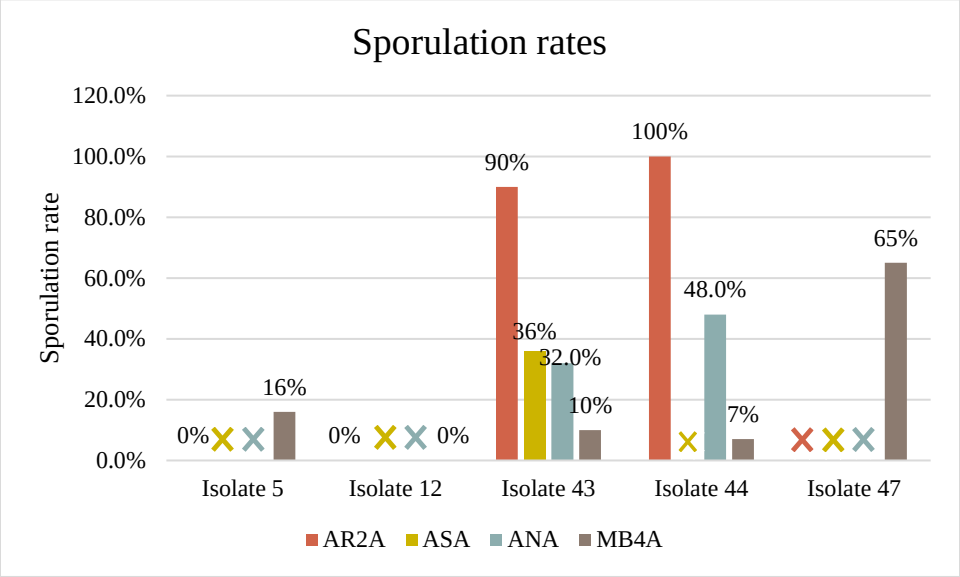


Figure 9. Comparison of sporulation rates of isolates #5, #12, #43 (*B. horneckiae*), #44 (*B. kochii*) and #47, after 7 d incubation at 30 °C on alkaline R2A (AR2A), alkaline sporulation agar (ASA), alkaline nutrient agar (ANA) and modified B4 agar (MB4A). (n=1)
*X in the figure means no growth was observed on the agar plates.

Initial screening showed isolates #43 (*B. horneckiae*) and #44 (*B. kochii*) had over 90% sporulation rates on AR2A plates. Figure 10 shows spore stains of *B. horneckiae* and *B. kochii* on AR2A plates after 7 d incubation at 30 °C.

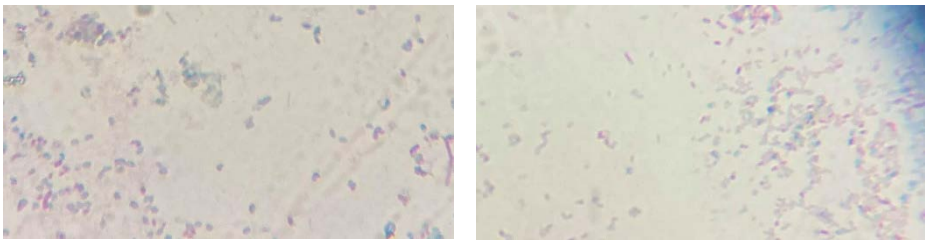
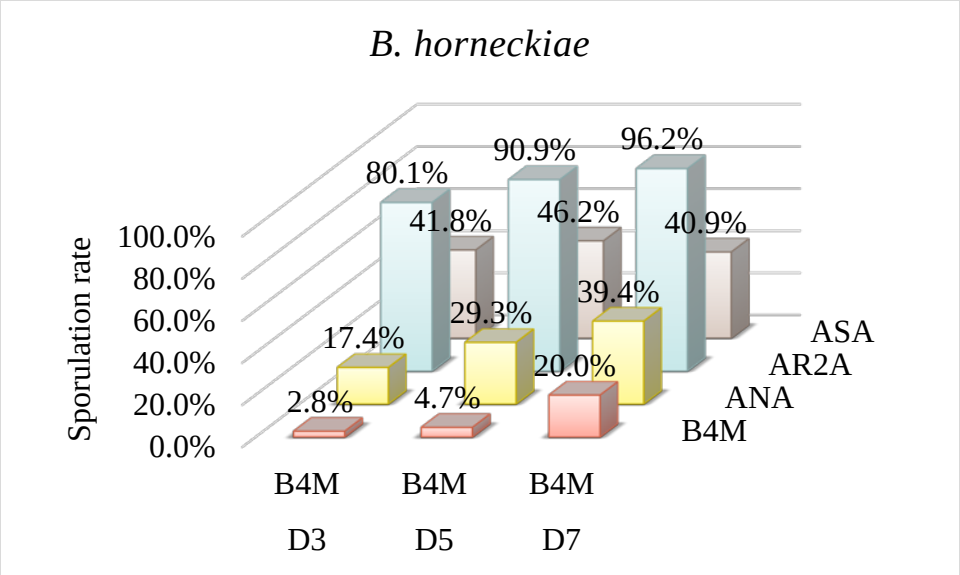


Figure 10. Spore stains of *B. horneckiae* (left) and *B. kochii* (right) on AR2A plates after 7 d incubation at 30 °C (1000X).

The sporulation rates of these two bacteria after incubation at 30 °C for 3, 5, and 7 d were further evaluated. As shown in Figure 11, AR2A was the best sporulation medium for *B. horneckiae*. The mean sporulation rates of *B. horneckiae* on AR2A plates were 80.7, 90.9, and 96.2 % on day 3, 5, and 7 respectively. However, there was no significant difference among the mean sporulation rates obtained from the three incubation periods. The mean sporulation rates of *B. horneckiae* on AR2A plates were significantly higher than those on the other three media on day 3, 5, and 7 ($P < 0.05$).



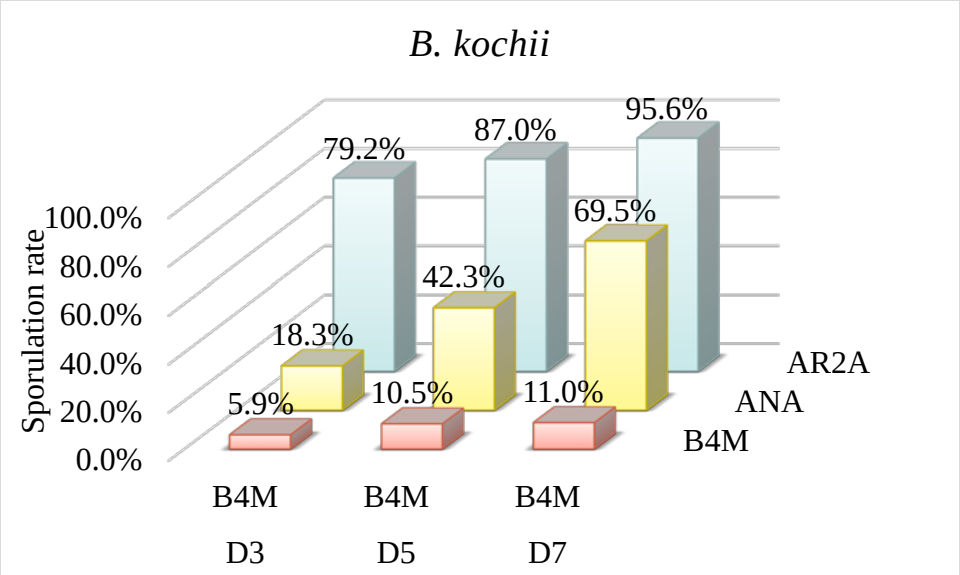
	Day 3	Day 5	Day 7
MB4A	2.8±0.0%^{AW}	4.7±0.0%^{AZ}	20.0±0.3%^{AX}
ANA	17.4±0.0%^{AX}	29.3±0.2%^{ABXZ}	39.4±0.3%^{BX}
AR2A	80.1±0.2%^{AZ}	90.9±0.1%^{AY}	96.2±0.3%^{AY}
ASA	41.8±0.1%^{AY}	46.2±0.0%^{AX}	40.9±0.3%^{AX}

Figure 11. Mean sporulation rates of *B. horneckiae* on modified B4 agar (MB4A), alkaline nutrient agar (ANA), alkaline R2A (AR2A), and alkaline sporulation agar (ASA) plates after 3, 5, and 7 d incubation at 30 °C (n=3).

[1] Values within columns with different letters (W, X, Y, Z) are significantly different ($P < 0.05$)

[2] Values within rows with different letters (A, B, C) are significantly different ($P < 0.05$)

Since *B. kochii* did not grow on ASA plates, only MB4A, ANA, and AR2A were used in this study. As shown in Figure 12, the mean sporulation rates of *B. kochii* on AR2A were 79.2, 87.0, and 95.6% on day 3, 5, and 7 respectively, which were significantly higher than the sporulation rates on MB4A, ANA or ASA at the same incubation periods ($P < 0.05$).-In addition, there were no significant differences among the mean sporulation rates obtained from the three incubation periods. This suggested that AR2A was the best sporulation medium for *B. horneckiae*.



	Day 3	Day 5	Day 7
MB4A	5.9±0.0%^{AX}	10.5±0.05%^{AX}	11.0±0.1%^{AX}
ANA	18.3±0.2%^{AY}	42.3±0.1%^{ABY}	69.5±0.1%^{BY}
AR2A	79.2±0.1%^{AZ}	87.0±0.1%^{AZ}	95.6±0.1%^{AZ}

Figure 12. Mean sporulation rates of *B. horneckiae* on modified B4 agar (MB4A), alkaline nutrient agar (ANA), alkaline R2A (AR2A), and alkaline sporulation agar (ASA) plates after 3, 5, and 7 d incubation at 30 °C (n=3).

[1] Values within columns with different letters (X, Y, Z) are significantly different ($P < 0.05$).

[2] Values within rows with different letters (A, B, C) are significantly different ($P < 0.05$).

3.4 Identification of Bacterial Isolates Using 16S RNA Gene Sequencing

Bacterial isolate #43 and #44 were identified by 16SRNA gene sequencing. Based on the result of BLAST analysis on the NCBI website, bacterial isolate#43 shares 99.37% similarity of 16S RNA gene sequence with *Bacillus horneckiae* strain 1P01SC. Bacterial isolate #44 shares 99.33% similarity of 16S RNA gene sequence with *Bacillus kochii* strain WCC 4582.

3.5 Morphology of *B. horneckiae* and *B. kochii*

Figure 13 shows the colonial morphology of *B. horneckiae* and *B. kochii* on ALB plates after 24 h of incubation at 30 °C. Based on this figure, *B. horneckiae* colonies are irregular,

raised and translucent. The margins of them are curled. The colonies of *B. kochii* are irregular, raised and translucent with undulate margins.

Figure 13. Colonial morphology of *B. horneckiae* (left) and *B. kochii* (right) on ALB plates after 24 h of incubation at 30 °C.

Figure 14 shows the gram stains results of *B. horneckiae* and *B. kochii* after incubated on ALB plates after 24 h of incubation at 30 °C. Both *B. horneckiae* and *B. kochii* are gram positive rods bacteria.

Figure 14. Gram stains of *B. horneckiae* (left) and *B. kochii* (right) after incubated on ALB plates for 24 h of at 30 °C.

3.6 Comparison of Calcium Precipitation Efficiency of *B. horneckiae* and *B. kochii*

Figure 15 shows the reduction of free Ca^{2+} in 1/10 LB broth (pH 8) over a 12-day incubation period at 30 °C due to MICP activity. The rates of MICP by *B. horneckiae* and *B. kochii* were quite similar during the first five days. Both of them reduced approximately 600 ppm from 2500 ppm of free Ca^{2+} in the broth. *Bacilllus horneckiae* reduced an additional 400 ppm of free Ca^{2+} in the broth by MICP from day 5 to 7, whereas. *B. kochii* did not further reduce free Ca^{2+} concentration after day 5.

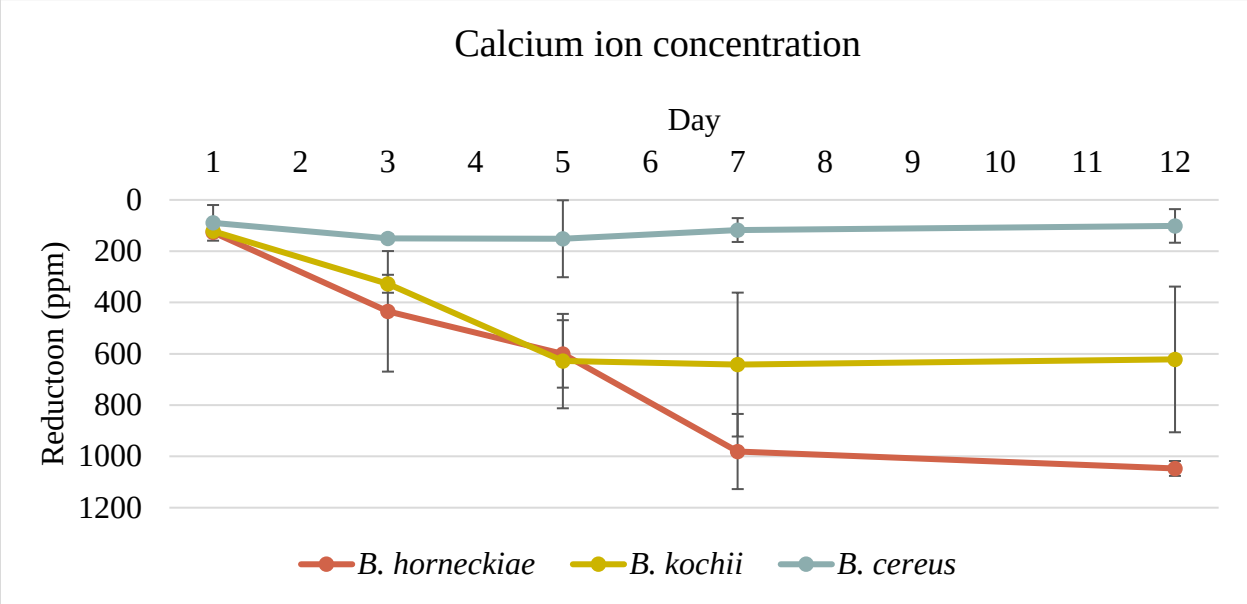


Figure 15. Free Ca^{2+} reduction by *B. horneckiae*, *B. kochii*, and *B. cereus* (negative control) in 1/10 LB broth (pH8) with 10 g/L of calcium acetate at 30 °C for 12 d (n=3).

3.7 X-Ray Diffraction Analysis of Precipitate in 1/10 LB Broth

Precipitate collected in the 1/10 LB broth culture of *B. horneckiae* was identified by X-ray diffraction (XRD) analysis. According to the XRD result showed in Figure 16, the peak obtained at 2θ angle with values around 30° and 50° indicates calcium magnesium carbonate 37.1% (w/w) and vaterite 62.1% (w/w) structure respectively in the precipitate. Detecting vaterite, a polymorph of calcium carbonate in the precipitate, indicated the MICP ability of *B. horneckiae*.

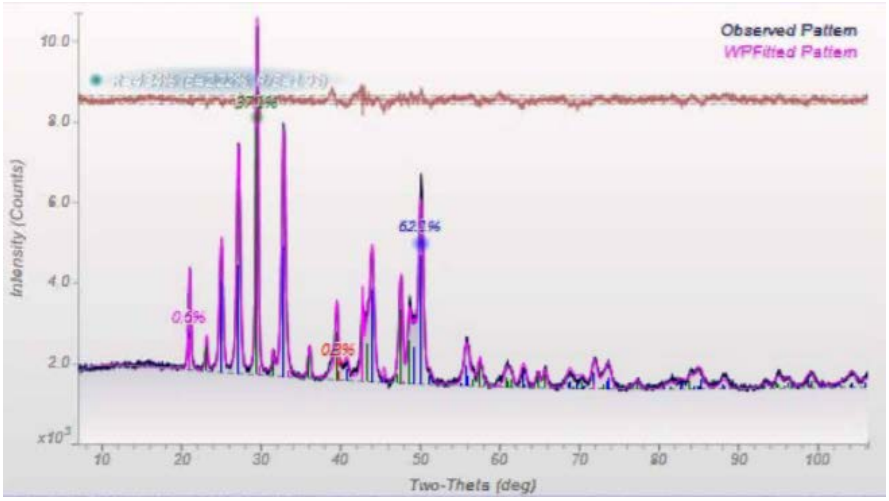


Figure 16. X-ray diffraction (XRD) spectrum of precipitate collected in 1/10 LB broth (pH 8) culture of *B. horneckiae* after incubation at 30 °C for 7 d.

3.8 Effect of Different Environmental Conditions on Bacterial Growth and Spore Germination

3.8.1 Growth of *B. horneckiae, B. kochii,* and *B. pseudofirmus* in LB broth with Different pH Values

The three bacteria's growth curves in LB broths with different pH values (pH 8, 9, and 10) at 30 °C are shown in Figure 17. The mean OD_{540nm} readings of *B. horneckiae* and *B. kochii* in pH 9, and 10 LB broths were significantly higher ($p<0.05$) than the mean readings of their counterparts in pH 8 LB broth after 12 h incubation (Figure 18). On the other hand, there was no significant difference among the OD_{540nm} readings of *B. pseudofirmus* in three pH values of LB broth.

The growth of *B horneckiae* and *B. kochii* was compared with the growth of *B. pseudofirmus* in LB at pH 9 as well as in LB pH 10 after 24 and 48 h incubation. As shown in Figure 18, the mean OD_{540nm} readings of the former two bacteria were lower than the OD_{540nm} reading of B. pseudofirmus. However, after 48 h incubation, there were no significant differences among the three mean readings in either LB pH 9 or pH 10. This indicated that although these three bacteria could have similar growth in both pH 9 and 10 LB broths, *B. pseudofirmus* grow more rapidly

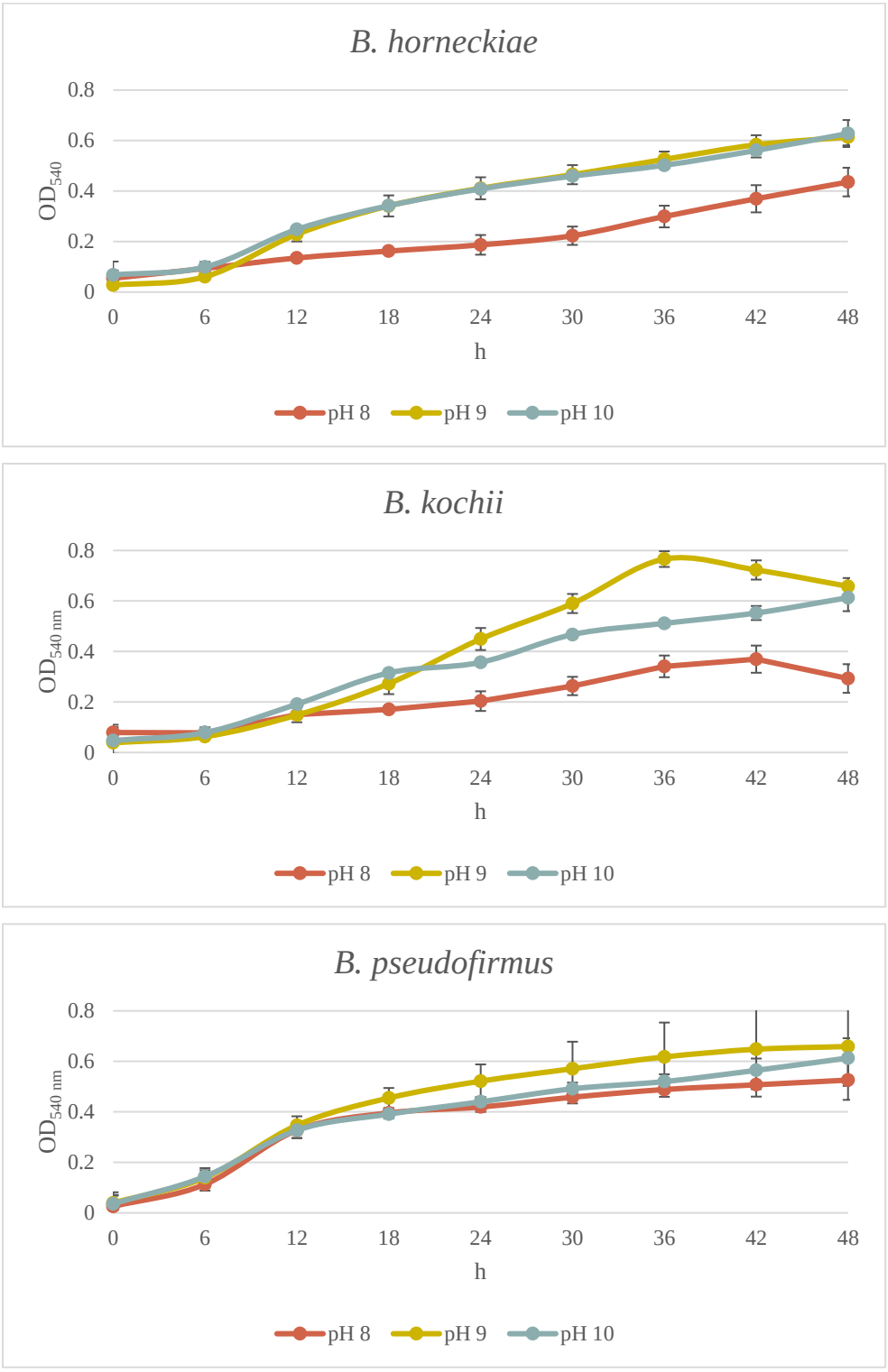
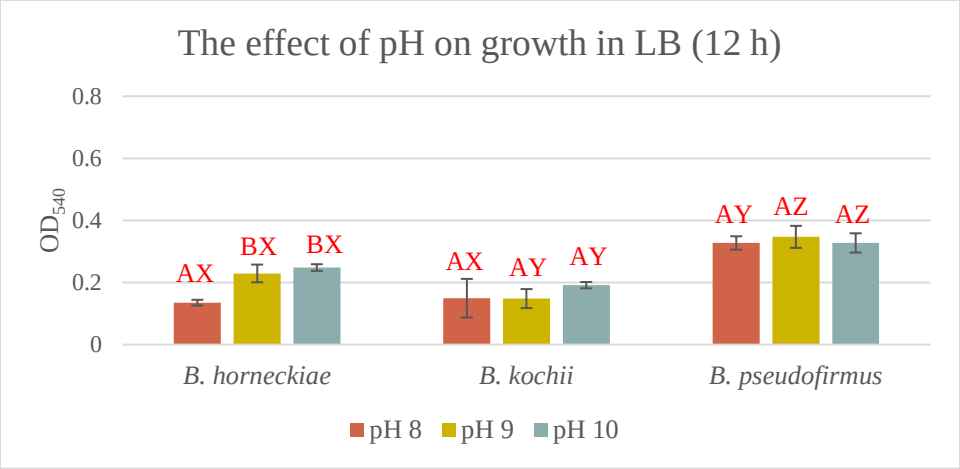
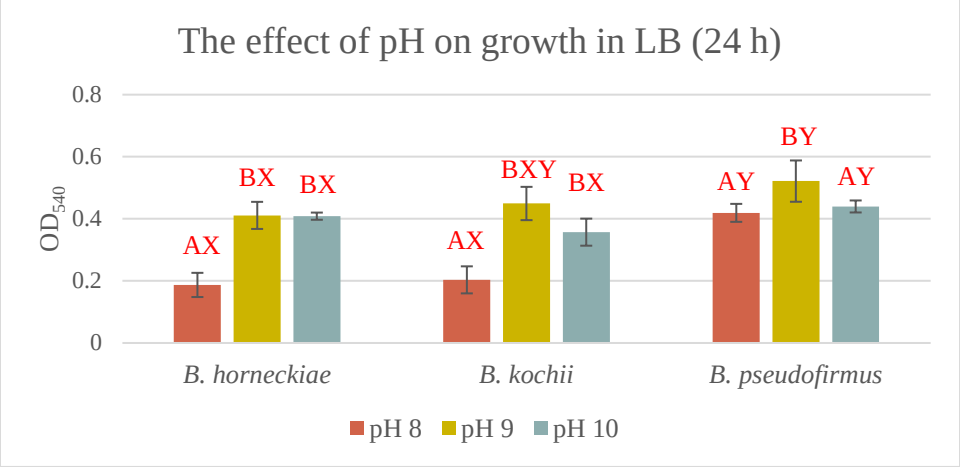


Figure 17. Mean OD_{540nm} readings of *B. horneckiae, B, kochii, and B pseudofirmus* in LB broths (pH 8, 9, and 10) for 2 d at 30°C (n=3).





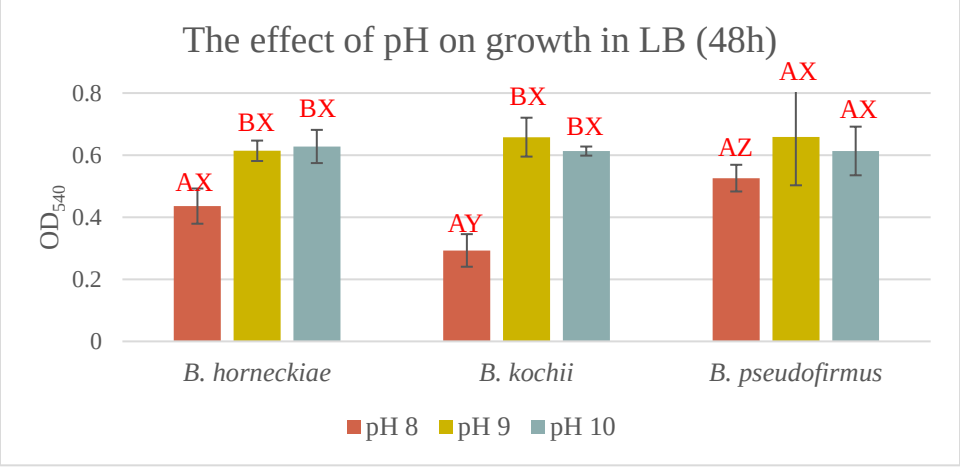


Figure 18. Mean (n = 3) OD_{540} readings of *B. horneckiae, B, kochii, and B pseudofirmus* in LB broth (pH 8, 9, and 10) for 2 d at 30°C.

[1] Different letters (A, B, and C) mean significant difference ($P<0.05$) of a test culture in different test media.

[2] Different letters (X, Y, and Z) mean significant difference ($P<0.05$) among different cultures in the same test medium.

3.8.2 Growths of *B. horneckiae, B. kochii,* and *B. pseudofirmus* Spores in LB Broth with Different pH Values

Figure 19 shows the growths of *B. horneckiae*, *B. kochii*, and *B. pseudofirmus* from spore inocula in pH 10 LB broth. Both the lag phase of *B. horneckiae* and *B. pseudofirmus* ended within 4 h. A more extended lag phase was reported for *B. kochii* spores in pH 10 LB broth (4-5 h). The results suggested that all three bacteria spores can germinate in pH 10 LB broth.

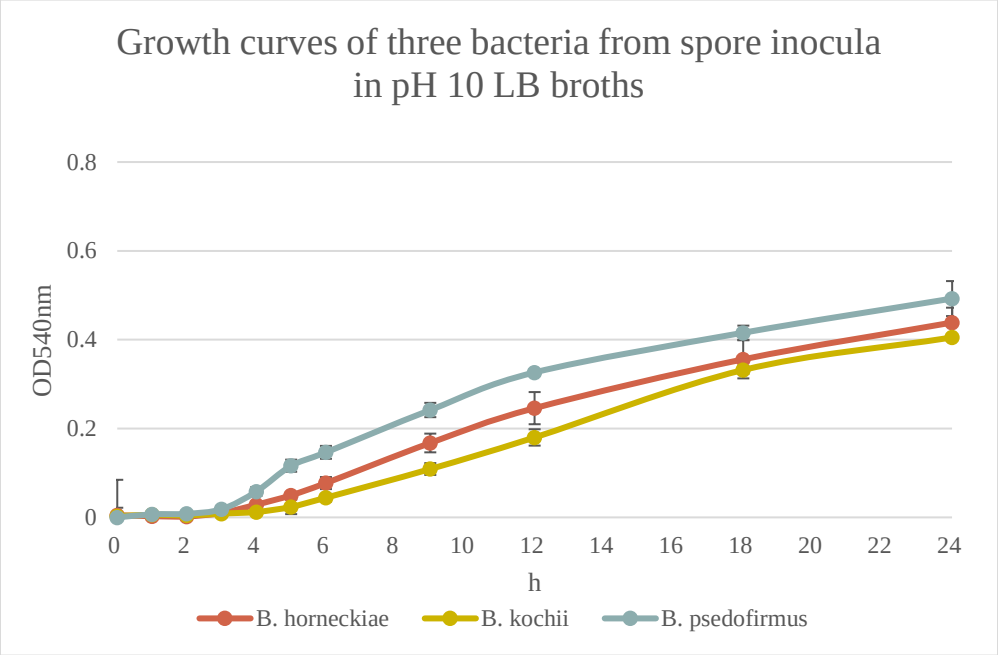


Figure 19. Mean OD_{540nm} readings of *B. horneckiae, B. kochii, and B. pseudofirmus* spores in 10 LB broth for 2 d at 30°C (n=3).

3.8.3 Growth of *B. horneckiae, B. kochii,* and *B. pseudofirmus* in Different Concentrations of Yeast Extract Broth at pH 9

As shown in Figure 20, both *B. horneckiae* and *B. kochii* grew most rapidly in 2% YE broth, and they were sensitive to different nutrient conditions. The OD_{540nm} readings of them in 2% YE broths were both constantly higher ($p<0.05$) than their counterparts in 0.5% and 1% YE broths after 12 h of cultivations. *Bacillus pseudofirmus* also had most rapid growth recorded in 2% YE broth. The OD_{540nm} readings of *B. pseudofirmus* obtained from 2% YE broth were significantly higher than the readings getting from 0.5 and 1 % YE broths at 24 h (Figure 20).

However, after another 24 h of incubation, the OD_{540nm} readings of *B. pseudofirmus* culture in 0.5 and 1 % YE broths reached the same levels of the 2% YE broth cultures.

By comparing the growth of these three bacteria form Figure 21, *B. pseudofirmus* grew more rapidly than *B. horneckiae* and *B. kochii* in all three concentrations of YE broths. The OD_{540nm} readings of it were significantly higher ($p<0.05$) than the other two bacteria in 0.5 and 1% YE broths from 12 to 48 h. *Bacillus kochii* showed the slowest growth in all three concentrations of YE broths.

This experiment showed that *B. pseudofirmus* had better growth in low concentrations of YE broths than the other two bacteria. It also indicated that yeast extract was not a good nutrient source for supporting the growth of *B. kochii* since the OD_{540nm} readings of *B. kochii* was under 0.3 even in the 2% YE broths. If compare it to the other two bacteria, the OD_{540nm} readings of the other two bacteria in 2% YE could reach 0.4 within 7 d.

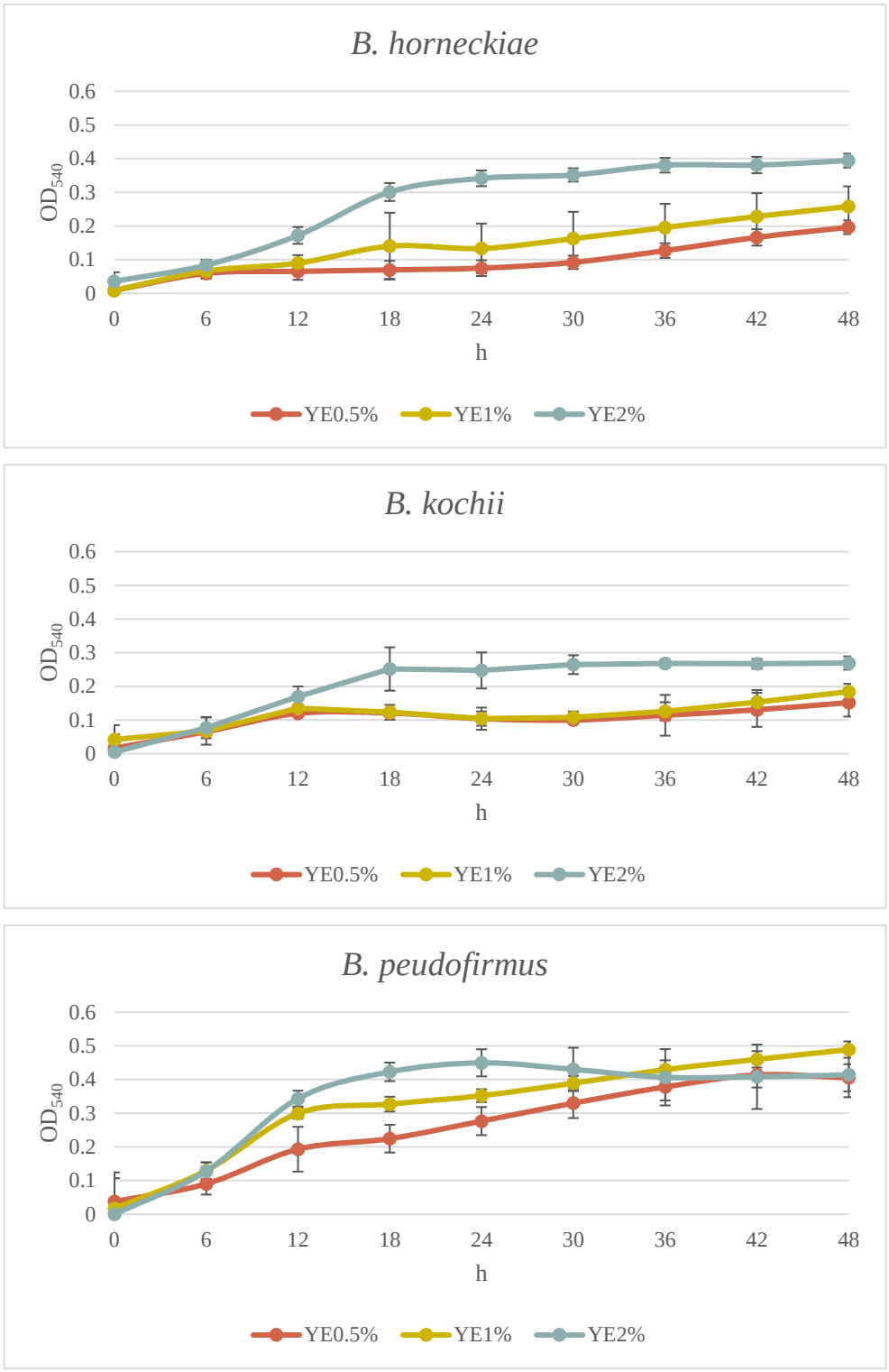
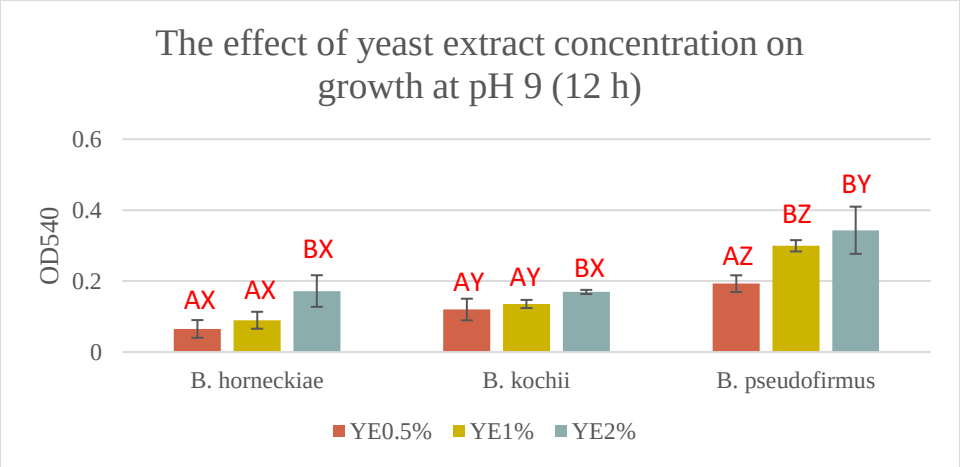
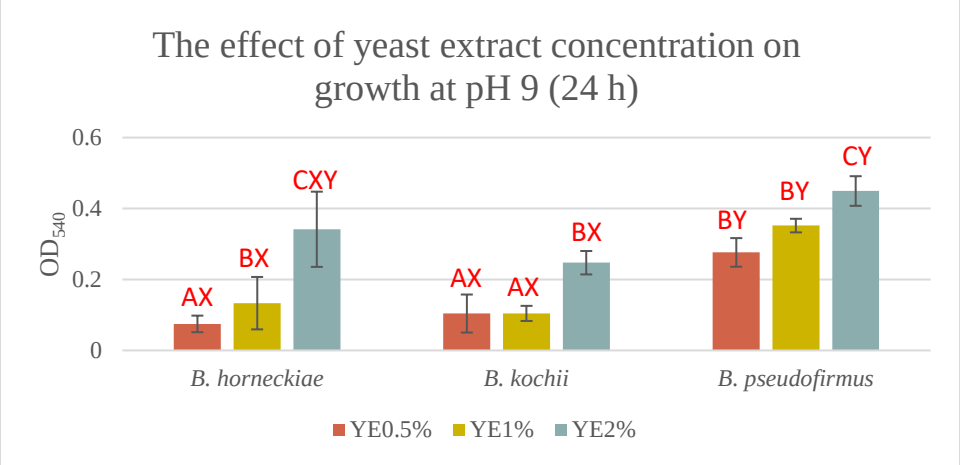


Figure 20. Mean OD_{540} readings of *B. horneckiae, B. kochii, and B. pseudofirmus* in pH 9 YE broth (0.5, 1 and 2%) for 2 d at 30°C (n=3).





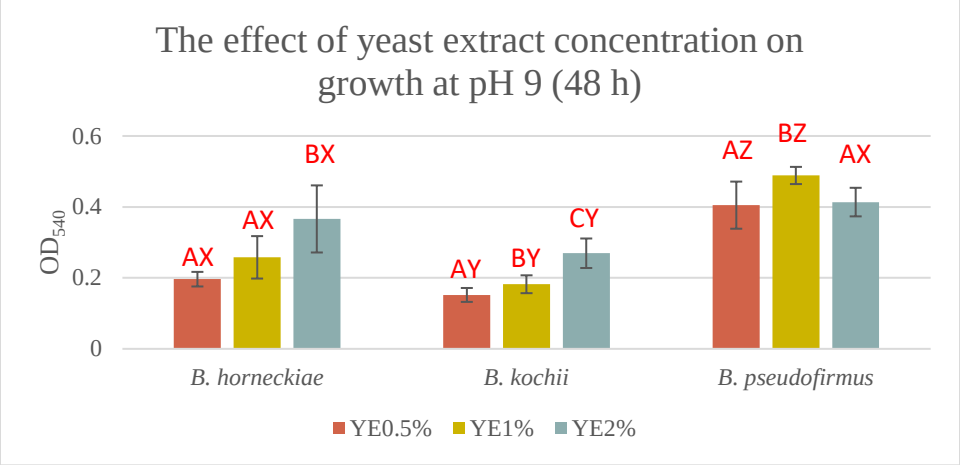


Figure 21. Mean OD_{540} reading of *B. horneckiae, B. kochii, and B. pseudofirmus* in YE broth (0.5, 1 and 2%) for 2 d at 30°C (n=3).

[1] Different letters (A, B, and C) mean significant difference (P<0.05) of a test culture in different test media.

[2] Different letters (X, Y, and Z) mean significant difference (P<0.05) among different cultures in the same test medium.

3.8.4 Growth of *B. horneckiae, B. kochii,* and *B. pseudofirmus* Spores in Different Concentrations of Yeast Extract Broth at pH 9.

The growths of *B. horneckiae, B. kochii,* and *B. pseudofirmus* from spore inocula in 0.5, 1, and 2% YE broths were shown in Figure 22. Lag phases of both *B. horneckiae* and *B. kochii* spores in all three concentrations ended within 4 h. The length of the lag phase of *B. pseudofirmus* was similar to the other two bacteria in 0.5 and 2% YE broth. However, no growth was recorded in 1 % YE broth. This result suggested that *B. horneckiae* and *B. kochii* spores can germinate and grow in lower concentrations (0.5 and 1%) YE broths.

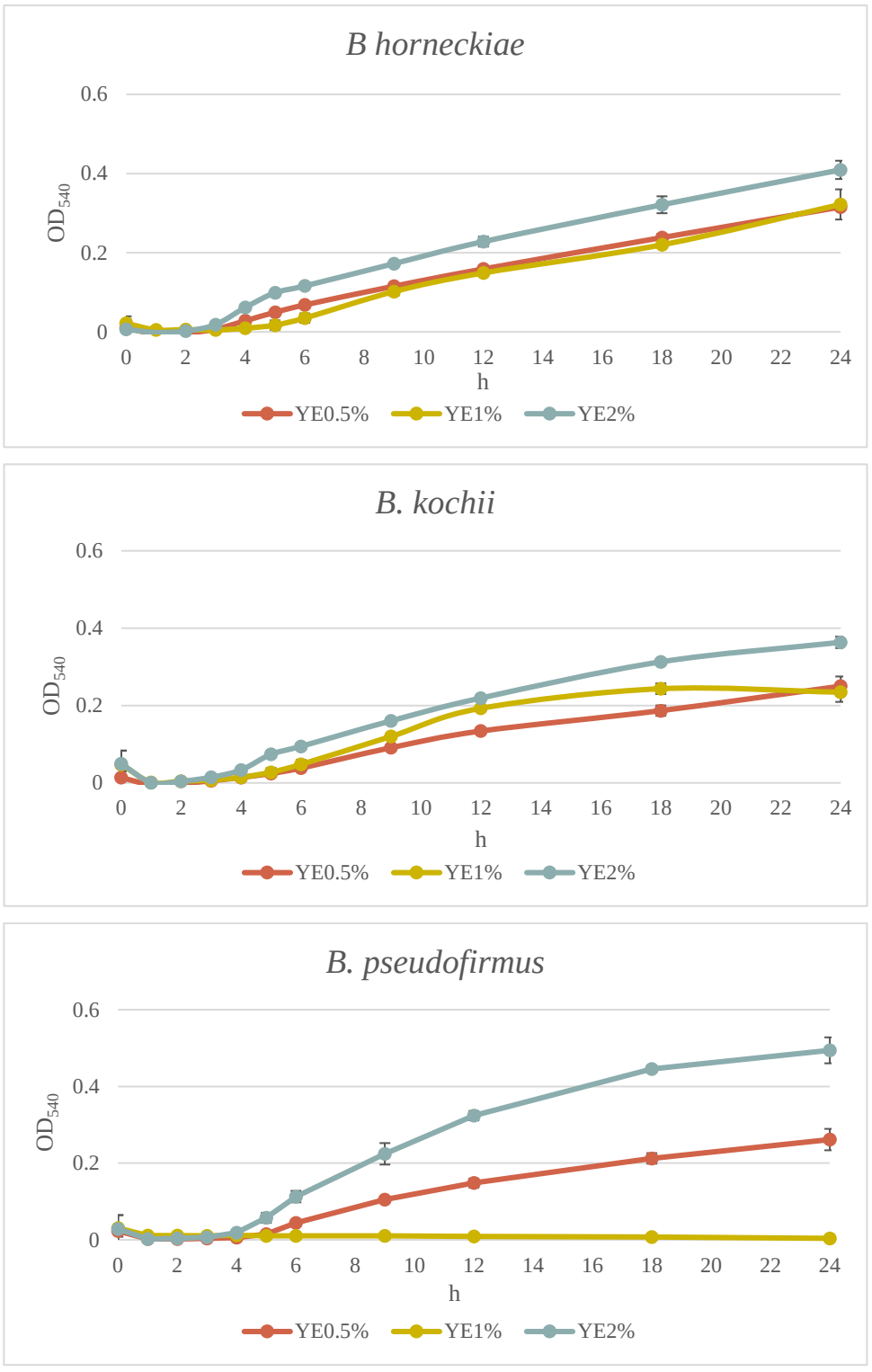


Figure 22. Mean OD_{540} reading of *B. horneckiae, B kochii, and B pseudofirmus* spores in pH 9 YE (2% and 1%) broth.

3.9 Survival of Bacteria Spore in Mortar Samples

As shown in Figure 23, the mean viable counts of *B. horneckiae*, *B. kochii,* and *B. pseudofirmus* dropped 1.7, 1.7, and 2.4 orders of magnitude CFU/g, after being incorporated into mortar samples for 7 d. Then the mean viable counts of these three bacteria stayed stable. By comparing the mean viable counts among each bacterium, there is no significant difference ($P<0.05$) in the mean viable counts of *B. horneckiae* and *B. kochii* from day 7 to 28. In addition, both *B. horneckiae* and *B. kochii* constantly had higher mean viable bacteria counts than *B. pseudofirmus* did on day 7 (Table 5).

Table 5. Mean viable bacteria counts of immobilized *B. Horneckiae, B kochii,* and *B. pseudofirmus* in mortar samples at days 1, 7, 14, and 28 at 20°C (n =3).

Time (Day)	*B. horneckiae*	*B. kochii*	*B. pseudofirmus*
0	6.7 ± 0.2^{AX}	6.8 ± 0.1^{AX}	6.6 ± 0.2^{AX}
1	5.5 ± 0.1^{BX}	5.3 ± 0.2^{BX}	4.9 ± 0.3^{BX}
7	5.0 ± 0.2^{CDX}	5.1 ± 0.3^{BX}	4.2 ± 0.2^{BY}
14	4.9 ± 0.3^{CXY}	5.0 ± 0.2^{BX}	4.3 ± 0.2^{BY}
28	5.1 ± 0.2^{BDX}	4.8 ± 0.6^{BX}	4.1 ± 0.3^{BY}

[1]Values within columns with different letters (A, B, C) are significantly different ($P < 0.05$).
[2]Values within rows with different letters (X, Y, Z) are significantly different ($P < 0.05$).

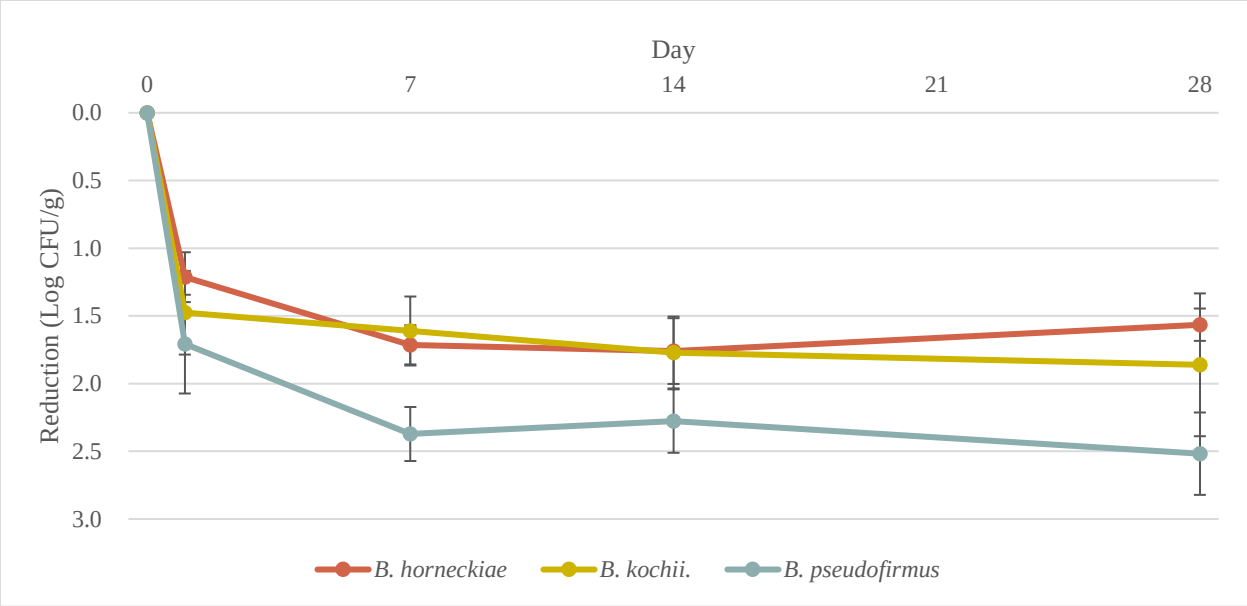


Figure 23. Log reduction of mean viable bacteria count of *B. Horneckiae*, *B kochii* and *B. pseudofirmus* in mortar samples over 28 d at 20°C (n=3).

3.10 Mortar Sample Healing

Figure 24 shows the dissecting microscopic images of crack-healing of mortar samples by *B. horneckiae*, *B kochii*, and *B. pseudofirmus* after 40 d of immersion in tap water at 20°C. All three bacteria were capable of healing cracks less than 0.25 mm. Control samples that did not contain bacteria also showed some degree of healing. However, the crack cannot be healed entirely without the presence of the bacterium.

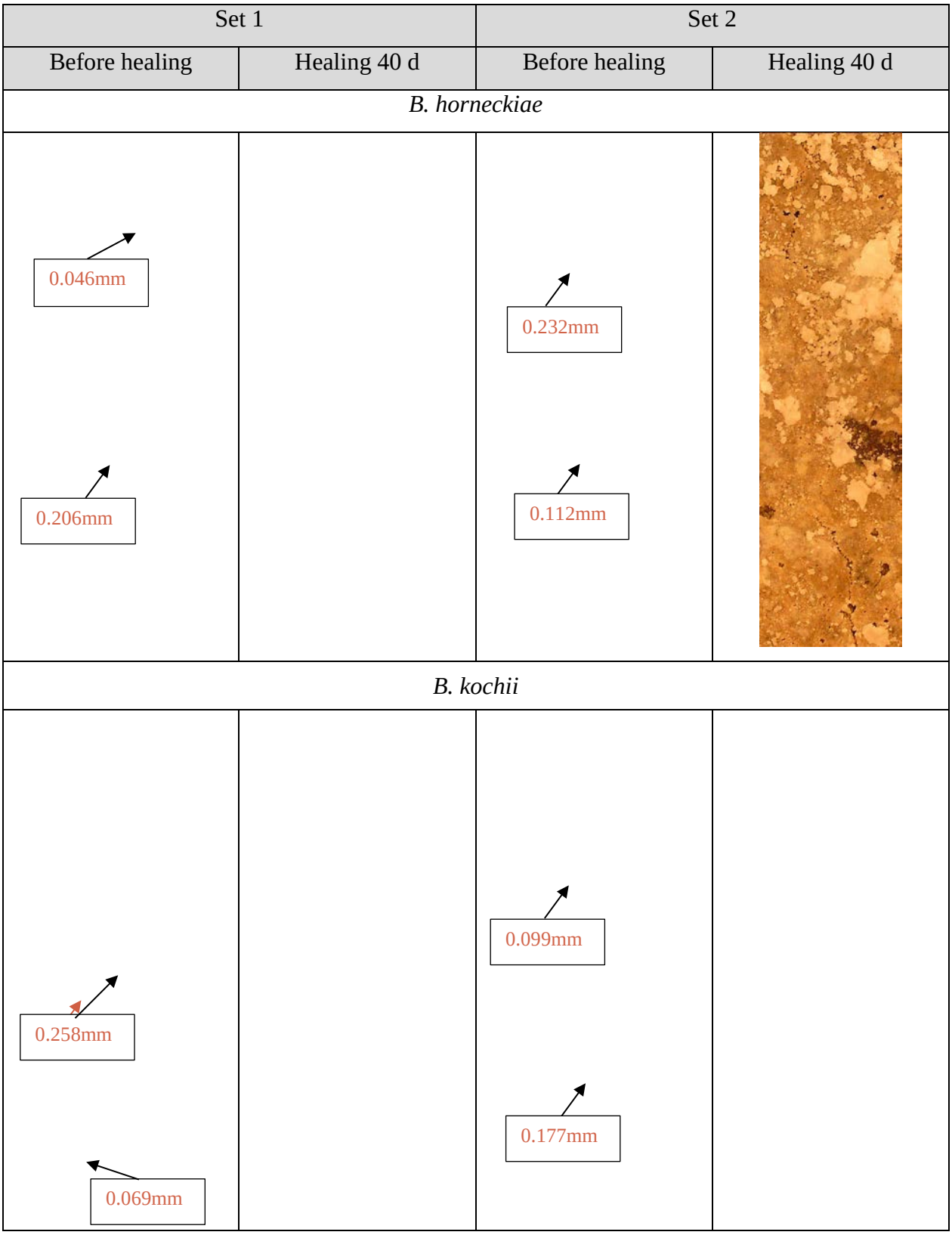
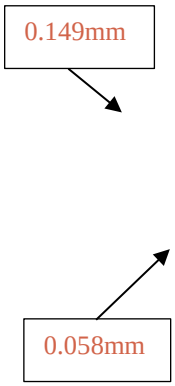
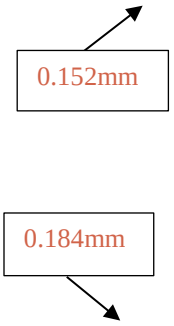


Figure 24. Dissecting microscopic images of negative control and crack-healing by *B. horneckiae*, *B kochii*, and *B. pseudofirmus* in mortar samples at 30°C for 40 d

Figure 24. continued

Set 1		Set 2	
Before healing	Healing 40 d	Before healing	Healing 40 d
B. pseudofirmus			
			
Negative Control			
			

CHAPTER 4. DISCUSSION

4.1 Bacterial Isolation

Bacillus horneckiae and *B. kochii* isolated in this study are gram-positive, spore-forming, aerobic rods. Crystal formation by these two bacteria on B4A plates (Figure 8) showed bacterial MICP ability.

These two bacteria were not widely found and studied in the past. *Bacillus horneckiae* was once isolated from an assembling room in a space center, and it appeared to be a novel species of the genus *Bacillus* (Vaishampayan et al., 2010). The spores can form biofilms which help the bacteria spores resist desiccation and UV radiation up to 1000 J m^{-2} (Vaishampayan et al., 2010). *Bacillus kochii* was isolated from dairy products and a clean room of pharmaceutical manufacturing site. This bacterium was reported to tolerate 10% NaCl and pH 10.5 (Seiler et al., 2012).

Based on previous studies, these two bacteria are spore formers with high pH and salt tolerance (Vaishampayan et al., 2010; Seiler et al., 2012). Those findings match the results obtained from this study. Both bacteria showed heavy growth on pH 10 LBA, which indicated that *B. horneckiae* and *B. kochii* could survive under alkaline environments.

4.2 Sporulation Rates

To reduce the cost and time of preparing a large number of bacterial spores for making self-healing concrete, it is essential to use a bacterial culture with high sporulation rates and sporulation efficiency. Numerous sporulation experiments of concrete healing bacteria have been carried out in the past. Some reaching more than 90% sporulation rates within 5 d. For example, *Bacillus* sp. JH7 and *Sporosrcina* sp. HYO08 fully sporulated on tryptone yeast extract broth within five days (Kim et al., 2015); *B. pseudofirmus* LMG 17944 and *B. cohnii* ATCC 51227 had over 90% rates on AR2A plates within 3d; *S. pasteurii* ATCC 11859 had their best sporulation rates of 94 % respectively on tryptic soy agar with 2% urea at day five (Chiu, 2019). However, not all the concrete healing bacteria had such high sporulation rates. For instance, *L. sphaericus* LMG 22257 required more than two weeks to reach 90% sporulation rate on liquid minimal basal salts medium (Wang et al., 2017).

In comparison, *B. horneckiae* and *B. kochii* had mean sporulation rates of 87% and 91% respectively on AR2A plates within 3 days, and they are considered as having reasonable sporulation rates. The best medium for the sporulation of *B. horneckiae* and *B. kochii* was AR2A. The base medium of R2A, the R2A medium, which contains a variety of nutrients in very low concentrations, is commonly used for enumerating heterotrophic bacteria in water (Table 2; Reasoner et al., 1985). Gray et al. (2019) found *Bacillus* and *Clostridia* sporulated when they encounter nutrient depletion. This finding may explain why AR2A media favored bacterial sporulation in this research.

4.3 MICP Identification and Quantification

Based on the results showed in Figure 15, *B. horneckiae* is better than *B. kochii* in terms of MICP ability. *Bacillus horneckiae* transformed around 1000 ppm of free Ca^{2+} into calcium carbonate, whereas *B. kochii* only precipitated 600 ppm Ca^{2+}. The maximum amount of free Ca^{2+} that *B. horneckiae* can precipitate matches the results from a previous MICP study of *Lysinibacillus* sp. YS11 which consumed 1000 ppm free Ca^{2+} in pH 8 B4 medium (Lee et al., 2017).

Both *B. horneckiae* and *B. kochii* utilized around 400 ppm of free Ca^{2+} in 72 hours, *Bacillus* sp. AK13 was also found to utilize free Ca^{2+} after 72 hours of incubation at pH of 11.2 B4 medium and consumed 400 ppm of free Ca^{2+} in the next 12 hours (Jung et al., 2020). *Lysinibacillus* sp. YS11 has shown a higher Ca^{2+} consumption speed. It only took 48 hours to consume 1000 ppm of free Ca^{2+} with the pH of 8 in B4 medium (Lee et al., 2017). After all, *B. horneckiae* and *B. kochii* showed promising results in a medium with a lower nutrient level (1/10 LB). However, it is difficult to compare free Ca^{2+} consumption rates among different bacteria grown in different media and culture conditions. Further experiments are required to answer this question.

Readings from the Ca^{2+} selective probe were not very consistent, as indicated by the vast error bars in Figure 15. The organic chemicals inside the media likely caused the inconsistent Ca2+ readings. Therefore, results obtained from a Ca^{2+} selective probe may not be very accurate. However, this method may be useful for screening bacteria with MICP ability especially as it has a shorter testing time. Based on this study's results, MICP of bacterial isolates could be detected

by the Ca^{2+} selective probe within three days. In contrast, commonly used B4A plates required a seven days incubation period to test for MICP.

4.4 Bacterial Growth and Spore Germination

This study tested the alkaline tolerance of isolated bacteria in pH 8, 9, and 10. The growth in pH higher than 10 was not tested because both the YE and LB broth started to precipitate when their pH reached 11. The results showed that *B. horneckiae* and *B. kochii* did not grow well in pH 8 broth. They had significantly higher OD_{540nm} readings in pH 9 and 10 LB broths. Previous work tested the pH tolerance of concrete healing bacteria. For example, *B. sphaericus* successfully grew in pH 12 urea yeast extract broth (UYE); however, not only the lag phase of the bacteria was prolonged when the broth reached a pH of 10, with the growth of the bacteria also being reduced (Wang et al., 2017). Unlike *B. sphaericus*, the growth of these two newly isolated bacteria did not slow down or reduce in LB at pH 10. Instead, the growth of these two bacteria benefited from the high pH.

To figure out the abilities of *B. horneckiae* and *B. kochii* to reproduce in different levels of nutrients, the growth of these two bacteria in different concentrations of pH 9 YE broths were compared. The results showed that the growths of both *B. horneckiae* and *B. kochii* significantly decreased in 0.5 % and 1 % YE broths. This suggested that an adequate amount of nutrients can support these two bacteria to grow more rapidly. However, adding more organic nutrients might decrease the compressive strength or increase the concrete setting time (Erşan et al., 2015; Jonkers et al., 2010; Wang et al., 2018). Therefore, more experiments are required to find out if nutrient concentrations that support high growth of *B. horneckiae* and *B. kochii* and do not have negative effects on concrete strength.

Figure 19 and Figure 22 show that the lag phases of both *B. horneckiae* and *B. kochii* ended within four hours on LB broths at different pH values (pH 8, 9, and 10) and in different concentrations (0.5, 1, 2%) for the YE broths. This means that spores of *B. horneckiae* and *B. kochii* can germinate in less than four hours in the tested growth environments. Germination within four hours is reasonable fast when compared to other concrete healing bacteria. *Bacillus sphaericus* spores required 12 hours and 24 hours of growth to geminate on UYE broth, with a pH of 9 and 10 respectively (Wang et al., 2017). Germination may be induced by adding chemical agents. *Bacillus pseudofirmus* spores can germinate within two hors when incubated

with the proper germination triggers (0.1 mol^{-1} NaCl, 10 μmol^{-1} of L-alanine and inosine, at pH 9.5; Sharma et al., 2017) Therefore, these chemicals may be considered to be added into the self-healing concrete.

Measuring the optical density of spores inocula can identify whether spores are germinated, however, germination rates of spores inocula were still unknown. To improve this study, observing the germination under a compound microscope may be required. Measuring dipicolinic acid (DPA) can also be considered in the future. Dipicolinic acid is a chemical substance that releases during the germination of *Bacillus* sp. (Vepachedu, 2004). Using this method would allow for better quantify the spore germination of *B. horneckiae* and *B. kochii*.

Another problem of this study was that not all the growth curves from spore inocula match growth curves from vegetative cell inocula. This was possibly because they had different inocula. In this study, both spores and vegetative cells samples were adjusted to 0.8 (OD_{540nm}) before testing. Since the sizes of spores and vegetative cells were different, the amounts of bacterial cells differed when they had the same OD_{540nm} readings.

4.5 Bacteria Survival in Mortar Samples

To guarantee long-term healing, spores of bacterial healing agents inside the concrete need to remain viable for a long period of time. Survivals of many concrete healing bacteria have been tested in the past. Viable counts of *Bacillus megaterium* embedded in fly ash amended mortar samples dropped three orders of magnitude after 28 days (Achal et al., 2011). Jonkers et al. (2010) also reported that the viable counts of *B. cohnii* spores decreased three orders of magnitude in concrete samples within 22 days (Jonkers et al., 2010). Spores of *B. pseudofirmus, B. cohnii,* and *B. halodurans* were also reported to have had a two to three orders of magnitude decrease after 7 days in cement samples (Sharma et al., 2017). These suggested that bacterial spores could have a significant decrease in concrete/mortar samples.

In this research, the control bacteria, *B. pseudofirmus,* had two and a half orders of magnitude drop in its mean viable count after seven days inside the mortar samples. This level of reductions matches the survival results mentioned previously. However, *B. horneckiae* and *B. kochii* only had 1.6 orders of magnitude and 1.9 orders of magnitude drops, respectively, in mean viable counts after being mixed into mortar samples seven days and remained stable. This

suggested that the two new isolates can better tolerate the environment inside the mortar samples and provide long-term healing capability in self-healing concrete.

4.6 Mortar Crack Healing

Concrete healings of *B. horneckiae, B. kochii,* and *B. pseudofirmus* are shown in Figure 24. *Bacillus horneckiae* and *B. kochii* successfully healed cracks up to 0.25 mm on mortar samples. It suggested that, inside the mortar samples, spores of these two new bacterial isolates can remain viable and germinate when nutrients and water are available. And the vegetative cells of them heal concrete cracks through MICP.

The widest crack that was made and healed in this research was around 0.25 mm. It is unknow whether the two bacteria could heal cracks wider than 0.25 mm. Previous studies have shown that self-healing concrete could heal cracks wider than 0.25 mm. For example, *Sporosarcina pasteurii*, which encapsulated in sulphoaluminate cement, was found to heal concrete cracks up to 0.42 mm after 28 days (Xu et al. 2018). Expanded perlite immobilized *B. cohnii* successfully healed 0.79 mm wide concrete cracks in 28 days (Zhang et al., 2017). Spores powder of *Bacillus sp.* AK13 can heal cracks of 1 mm width after being mixed into mortar samples for 14 days (Jung et al., 2020). These studies were carried out in different the experimental conditions with different incorporated materials. Both bacterial healing agent and self-healing concrete formula may influence the crack healing performance. Therefore, exploring the concrete healing ability of *B. horneckiae* and *B. kochii* in different growth conditions is necessary to optimize the self-healing concrete formula and test the maximum crack width that can be healed by *B. horneckiae* and *B. kochii.*

CHAPTER 5. CONCLUSION

The goal of this study was to screen new bacterial concrete healing agents. According to the results, the following conclusions can be drawn.

- Among the 50 bacteria isolates from alkaline soil, only *B. horneckiae* and *B. kochii* met the criteria of good concrete self-healing agents. Both bacteria reached around 80% and over 90% sporulation rates within three days and five days incubation respectively on AR2A plates, showed MICP activities on B4A plates, and grew heavily in alkaline medium (pH 10 LB plates). There were other three bacterial isolates showed positive results of alkaline pH tolerance and crystal formation. However, optimum sporulation media for each of these three bacteria were not found in this study.
- Precipitates collected from the 1/10 LB broth (pH 8) culture of *B. horneckiae* were confirmed by XRD analysis to be vaterite, a polymorph of calcium carbonate.
- The results showed that the mean viable counts of *B. horneckiae* and *B. kochii* dropped 1.2 and 1.5 orders of magnitude respectively during the first 24 hours, dropped another 0.5 and 0.2 orders of magnitude respectively between day one and day seven, and then remained stable after day seven in the mortar samples. Also, the mean viable counts of the two bacteria in mortal samples were constantly higher than the mean viable counts of *B. pseudofirmus*, a commonly used concrete healing bacteria. Meanwhile, a one-trial test showed that the mean viable counts of the two bacteria remained stable in mortar samples after six months at room temperature. More tests can be done in the future to figure out how long can these two bacteria last in the mortar samples. Overall, the high survival rates were the best advantages that *B. horneckiae* and *B. kochii* possess.
- The growth study indicated that both *B. horneckiae* and *B. kochii* grew better at pH 9 and 10 than at pH 8. Therefore, the alkaline environment inside the concretes may not be a challenge for these two bacteria. However, when *B. horneckiae* and *B. kochii* were cultivated in different YE broths concentrations, the results showed that their growth significantly declined at lower concentrations of YE broths. Therefore, finding other organic nutrients that support better growth of these two bacterial isolates can be a future goal.

- A growth study using spores as the inocula showed that both *B. horneckiae* and *B. kochii* successfully germinated and entered log phases within 2-4 hours in B broths at pH 8, 9, or 10 and in different concentrations of YE broths (0.5, 1, and 2%).
- Both *B. horneckiae* and *B. kochii* could heal mortar cracks up to 0.25 mm within 40 days. More experiments are required in the future to test their ability to heal cracks wider than 0.25 mm. However, successful concrete crack healings confirmed that spores of both *B. horneckiae* and *B. kochii* could germinate, reproduce, and precipitate calcium carbonates inside mortar samples.

This research demonstrated a protocol that successfully screening concrete healing agents from alkaline soil samples.

REFERENCES

Achal, V., Mukherjee, A., Kumari, D., Zhang, Q., 2015. Biomineralization for sustainable construction - A review of processes and applications. Earth-Science Rev. https://doi.org/10.1016/j.earscirev.2015.05.008

Achal, V., Pan, X., Özyurt, N., 2011. Improved strength and durability of fly ash-amended concrete by microbial calcite precipitation. Ecol. Eng. 37, 554–559. https://doi.org/10.1016/j.ecoleng.2010.11.009

Alazhari, M., Sharma, T., Heath, A., Cooper, R., Paine, K., 2018. Application of expanded perlite encapsulated bacteria and growth media for self-healing concrete. Constr. Build. Mater. https://doi.org/10.1016/j.conbuildmat.2017.11.086

Bartholomew, J.W., Mittwer, T., 1950. A simplified bacterial spore stain. Biotech. Histochem. 25, 153–156. https://doi.org/10.3109/10520295009110979

Boquet, E., Boronat, A., Ramos-Cormenzana, A., 1973. Production of calcite (Calcium carbonate) crystals by soil bacteria is a general phenomenon. Nature 246, 527–529. https://doi.org/10.1038/246527a0

Bundur, Z.B., Amiri, A., Ersan, Y.C., Boon, N., Belie, N.De, 2017. Impact of air entraining admixtures on biogenic calcium carbonate precipitation and bacterial viability. Cem. Concr. Res. 98, 44–49. https://doi.org/10.1016/j.cemconres.2017.04.005

Castanier, S., LeMétayer-Levrel, G., Perthuisot, J.P., 1999. Ca-carbonates precipitation and limestone genesis - the microbiogeologist point of view. Sediment. Geol. https://doi.org/10.1016/S0037-0738(99)00028-7

Chiu, C.H., 2019. Screening of Microorganisms, Calcium Sources, and Protective Materials for Self-healing Concrete. Purdue Univ. Grad. Sch.

Civantos, E., Thuiller, W., Maiorano, L., Guisan, A., Araújo, M.B., 2012. Potential Impacts of Climate Change on Ecosystem Services in Europe : The Case of Pest Control by Vertebrates. Bioscience 62, 658–666

DeJong, J.T., Mortensen, B.M., Martinez, B.C., Nelson, D.C., 2010. Bio-mediated soil improvement. Ecol. Eng. 36, 197–210. https://doi.org/10.1016/j.ecoleng.2008.12.029

Delatte, N., 2009. Failure, Distress and Repair of Concrete Structures, Failure, Distress and Repair of Concrete Structures. https://doi.org/10.1533/9781845697037

Douglas, S., Beveridge, T.J., 1998. Mineral formation by bacteria in natural microbial communities. FEMS Microbiol. Ecol. 26, 79–88. https://doi.org/10.1016/S0168-6496(98)00027-0

Erşan, Y.Ç., DaSilva, F.B., Boon, N., Verstraete, W., DeBelie, N., 2015. Screening of bacteria and concrete compatible protection materials. Constr. Build. Mater. 88, 196–203. https://doi.org/10.1016/j.conbuildmat.2015.04.027

Gollapudi, U.K., Knutson, C.L., Bang, S.S., Islam, M.R., 1995. A new method for controlling leaching through permeable channels. Chemosphere 30, 695–705. https://doi.org/10.1016/0045-6535(94)00435-W

Gray, D.A., Dugar, G., Gamba, P., Strahl, H., Jonker, M.J., Hamoen, L.W., 2019. Extreme slow growth as alternative strategy to survive deep starvation in bacteria. Nat. Commun. 10, 1–12. https://doi.org/10.1038/s41467-019-08719-8

Groot, F.DE, Vogel, J., 2006. Fundamentals of X-ray Absorption and Dichroism M : the Multiplet Approach. Inorg. Chem. 3–66.

Hammes, F., Verstraete, W., 2002. Key roles of pH and calcium metabolism in microbial carbonate precipitation. Rev. Environ. Sci. Biotechnol. https://doi.org/10.1023/A:1015135629155

Heede, R., 2014. Tracing anthropogenic carbon dioxide and methane emissions to fossil fuel and cement producers, 1854-2010. Clim. Change 122, 229–241. https://doi.org/10.1007/s10584-013-0986-y

Huynh, N.N.T., Phuong, N.M., Toan, N.P.A., Son, N.K., 2017. Bacillus Subtilis HU58 Immobilized in Micropores of Diatomite for Using in Self-healing Concrete. Procedia Eng. 171, 598–605. https://doi.org/10.1016/j.proeng.2017.01.385

Jonkers, H.M., 2007. Self Healing Concrete: A Biological Approach, in: Springer Series in Materials Science. https://doi.org/10.1007/978-1-4020-6250-6_9

Jonkers, H.M., Thijssen, A., Muyzer, G., Copuroglu, O., Schlangen, E., 2010. Application of bacteria as self-healing agent for the development of sustainable concrete. Ecol. Eng. 36, 230–235. https://doi.org/10.1016/j.ecoleng.2008.12.036

Jonkers, H.M., Schlangen, E., 2007. Crack Repair By Concrete-Immobilized Bacteria. Civ. Eng. 1–7.

Jung, Y., Kim, Wonjae, Kim, Wook, Park, W., 2020. Complete genome and calcium carbonate precipitation of alkaliphilic Bacillus sp. AK13 for self-healing concrete. J. Microbiol. Biotechnol. 30, 404–416. https://doi.org/10.4014/jmb.1908.08044

Khadhim, H.J., Ebrahim, S.E., Ammar, S.H., 2019. Isolation and Identification of Ureolytic Bacteria Isolated from Livestock Soil to Improve the Strength of Cement Mortar. Eng. Technol. J. 37, 24–28. https://doi.org/10.30684/etj.37.3C.3

Khaliq, W., Ehsan, M.B., 2016. Crack healing in concrete using various bio influenced self-healing techniques. Constr. Build. Mater. 102, 349–357. https://doi.org/10.1016/j.conbuildmat.2015.11.006

Kim, H.K., Park, S.J., Han, J.I., Lee, H.K., 2013. Microbially mediated calcium carbonate precipitation on normal and lightweight concrete. Constr. Build. Mater. 38, 1073–1082. https://doi.org/10.1016/j.conbuildmat.2012.07.040

Kim, H.J., Eom, H.J., Park, C., Jung, J., Shin, B., Kim, W., Chung, N., Choi, I.G., Park, W., 2015. Calcium carbonate precipitation by Bacillus and sporosarcina strains isolated from concrete and analysis of the bacterial community of concrete. J. Microbiol. Biotechnol. 26, 540–548. https://doi.org/10.4014/jmb.1511.11008

Lee, C.F., Chang, C.L., Yang, J.C., Lai, H.Y., Chen, C.H., 2012. Morphological determination of face-centered-cubic metallic nanoparticles by X-ray diffraction. J. Colloid Interface Sci. 369, 129–133. https://doi.org/10.1016/j.jcis.2011.12.053

Lee, Y.S., Kim, H.J., Park, W., 2017. Non-ureolytic calcium carbonate precipitation by Lysinibacillus sp. YS11 isolated from the rhizosphere of Miscanthus sacchariflorus. J. Microbiol. 55, 440–447. https://doi.org/10.1007/s12275-017-7086-z

Leng, Y., 2013. Materials characterization: Introduction to microscopic and spectroscopic methods: Second edition, Materials Characterization: Introduction to Microscopic and Spectroscopic Methods :Second Edition. wiley. https://doi.org/10.1002/9783527670772

Logan, N.A., DeVos, P., 2011. Endospore-forming Soil Bacteria. Springer 27, 1–5. https://doi.org/10.1007/978-3-642-19577-8

Muynck, W.De, Belie, N.De, Verstraete, W., 2010. Microbial carbonate precipitation in construction materials: A review. Ecol. Eng. 36, 118–136. https://doi.org/10.1016/j.ecoleng.2009.02.006

Pacheco-Torgal, F., Labrincha, J.A., 2013. Biotech cementitious materials: Some aspects of an innovative approach for concrete with enhanced durability. Constr. Build. Mater. 40, 1136–1141. https://doi.org/10.1016/j.conbuildmat.2012.09.080

Pacheco-Torgal, F., Melchers, R.E., Shi, X., DeBelie, N., VanTittelboom, K., Sáez, A., 2018. Eco-efficient Repair and Rehabilitation of Concrete Infrastructures, Eco-efficient Repair and Rehabilitation of Concrete Infrastructures. https://doi.org/10.1016/c2016-0-04100-1

Pan, X., Shi, Z., Shi, C., Ling, T.C., Li, N., 2017. A review on concrete surface treatment Part I: Types and mechanisms. Constr. Build. Mater. 132, 578–590. https://doi.org/10.1016/j.conbuildmat.2016.12.025

Reasoner, D.J., Geldreich, E.E., 1985. A new medium for the enumeration and subculture of bacteria from potable water. Appl. Environ. Microbiol. 49, 1–7. https://doi.org/10.1128/aem.49.1.1-7.1985

Ryu, J.H., Kim, H., Beuchat, L.R., 2005. Spore formation by Bacillus cereus in broth as affected by temperature, nutrient availability, and manganese. J. Food Prot. 68, 1734–1738. https://doi.org/10.4315/0362-028X-68.8.1734

Şahmaran, M., Li, V.C., 2010. Engineered cementitious composites: Can composites be accepted as crack-free concrete? Transp. Res. Rec. 1–8. https://doi.org/10.3141/2164-01

Schreiberová, H., Bílý, P., Fládr, J., Šeps, K., Chylík, R., Trtík, T., 2019. Impact of the self-healing agent composition on material characteristics of bio-based self-healing concrete. Case Stud. Constr. Mater. 11. https://doi.org/10.1016/j.cscm.2019.e00250

Seifan, M., Samani, A.K., Berenjian, A., 2016. Bioconcrete: next generation of self-healing concrete. Appl. Microbiol. Biotechnol. 100, 2591–2602. https://doi.org/10.1007/s00253-016-7316-z

Seiler, H., Schmidt, V., Wenning, M., Scherer, S., 2012. Bacillus kochii sp. nov., isolated from foods and a pharmaceuticals manufacturing site. Int. J. Syst. Evol. Microbiol. 62, 1092–1097. https://doi.org/10.1099/ijs.0.027771-0

Sharma, T.K., Alazhari, M., Heath, A., Paine, K., Cooper, R.M., 2017. Alkaliphilic Bacillus species show potential application in concrete crack repair by virtue of rapid spore production and germination then extracellular calcite formation. J. Appl. Microbiol. 122, 1233–1244. https://doi.org/10.1111/jam.13421

Sorokin, D.Y., VanPelt, S., Tourova, T.P., 2008. Utilization of aliphatic nitriles under haloalkaline conditions by Bacillus alkalinitrilicus sp. nov. isolated from soda solonchak soil. FEMS Microbiol. Lett. 288, 235–240. https://doi.org/10.1111/j.1574-6968.2008.01353.x

Vahabi, A., Ramezanianpour, A.A., Sharafi, H., Zahiri, H.S., Vali, H., Noghabi, K.A., 2015. Calcium carbonate precipitation by strain Bacillus licheniformis AK01, newly isolated from loamy soil: A promising alternative for sealing cement-based materials. J. Basic Microbiol. 55, 105–111. https://doi.org/10.1002/jobm.201300560

Vaishampayan, P., Probst, A., Krishnamurthi, S., Ghosh, S., Osman, S., McDowall, A., Ruckmani, A., Mayilraj, S., Venkateswaran, K., 2010. Bacillus horneckiae sp. nov., isolated from a spacecraft-assembly clean room. Int. J. Syst. Evol. Microbiol. 60, 1031–1037. https://doi.org/10.1099/ijs.0.008979-0

Vashisht, R., Attri, S., Sharma, D., Shukla, A., Goel, G., 2018. Monitoring biocalcification potential of Lysinibacillus sp. isolated from alluvial soils for improved compressive strength of concrete. Microbiol. Res. 207, 226–231. https://doi.org/10.1016/j.micres.2017.12.010

Vepachedu, V.R., Setlow, P., 2004. Analysis of the germination of spores of Bacillus subtilis with temperature sensitive spo mutations in the spoVA operon. FEMS Microbiol. Lett. https://doi.org/10.1016/j.femsle.2004.08.022

Vijay, K., Murmu, M., Deo, S.V., 2017. Bacteria based self healing concrete – A review. Constr. Build. Mater. 152, 1008–1014. https://doi.org/10.1016/j.conbuildmat.2017.07.040

Wang, J.Y., DeBelie, N., Verstraete, W., 2012. Diatomaceous earth as a protective vehicle for bacteria applied for self-healing concrete. J. Ind. Microbiol. Biotechnol. 39, 567–577. https://doi.org/10.1007/s10295-011-1037-1

Wang, J.Y., Snoeck, D., VanVlierberghe, S., Verstraete, W., DeBelie, N., 2014. Application of hydrogel encapsulated carbonate precipitating bacteria for approaching a realistic self-healing in concrete. Constr. Build. Mater. 68, 110–119. https://doi.org/10.1016/j.conbuildmat.2014.06.018

Wang, J., Jonkers, H.M., Boon, N., DeBelie, N., 2017. Bacillus sphaericus LMG 22257 is physiologically suitable for self-healing concrete. Appl. Microbiol. Biotechnol. 101, 5101–5114. https://doi.org/10.1007/s00253-017-8260-2

Wang, J., Mignon, A., Snoeck, D., Wiktor, V., VanVliergerghe, S., Boon, N., DeBelie, N., 2015. Application of modified-alginate encapsulated carbonate producing bacteria in concrete: A promising strategy for crack self-healing. Front. Microbiol. 6, 1–14. https://doi.org/10.3389/fmicb.2015.01088

Wang, X., Stewart, M.G., Nguyen, M., 2012. Impact of climate change on corrosion and damage to concrete infrastructure in Australia. Clim. Change 110, 941–957. https://doi.org/10.1007/s10584-011-0124-7

Wiktor, V., Jonkers, H.M., 2011. Quantification of crack-healing in novel bacteria-based self-healing concrete. Cem. Concr. Compos. 33, 763–770. https://doi.org/10.1016/j.cemconcomp.2011.03.012

Willem De Muynck, Nele De Belie, Willy Verstraete, 2017. Bacteria based self healing concrete – A review. Constr. Build. Mater. 36, 1–14. https://doi.org/10.1007/s10584-013-0986-y

Worrell, E., Price, L., Martin, N., Hendriks, C., Meida, L.O., 2001. Missions from ∗ the. Carbon N. Y. 26, 303–329. https://doi.org/10.1146/annurev.energy.26.1.303

Xu, J., Wang, X., 2018. Self-healing of concrete cracks by use of bacteria-containing low alkali cementitious material. Constr. Build. Mater. 167, 1–14. https://doi.org/10.1016/j.conbuildmat.2018.02.020

Zhang, J.L., Wu, R.S., Li, Y.M., Zhong, J.Y., Deng, X., Liu, B., Han, N.X., Xing, F., 2016. Screening of bacteria for self-healing of concrete cracks and optimization of the microbial calcium precipitation process. Appl. Microbiol. Biotechnol. 100, 6661–6670. https://doi.org/10.1007/s00253-016-7382-2

Zhang, J., Liu, Y., Feng, T., Zhou, M., Zhao, L., Zhou, A., Li, Z., 2017. Immobilizing bacteria in expanded perlite for the crack self-healing in concrete. Constr. Build. Mater. 148, 610–617. https://doi.org/10.1016/j.conbuildmat.2017.05.021

Zhang, J., Mai, B., Cai, T., Luo, J., Wu, W., Liu, B., Han, N., Xing, F., Deng, X., 2017. Optimization of a binary concrete crack self-healing system containing bacteria and oxygen. Materials (Basel). 10. https://doi.org/10.3390/ma10020116