The self-healing mechanisms of bacterial cementitious materials via novel isolated ureolytic bacterium species

A Thesis Submitted for the Degree of Doctor of Philosophy

By

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Abstract

The self-healing approach used in this study is based on the urease hydrolysing bacterium, namely, Bacillus Sphaericus. The scope of the work is diverse as it combines and brings together both aspects of the bioengineering and civil engineering in order to provide better understanding of the self-healing mechanisms that lie behind the yielding of the calcium carbonate precipitation. The initial stage of this study, comprised of collecting different soil samples from different alkaline sources to extract and isolate urease bacterium species. In addition, a collection of more than 100 different bacterial strains belong to bacillus Sphaericus were screened for the presence of urease enzyme and ability to produce copious amount of calcium carbonate under extreme alkaline conditions.

In-vitro calcium carbonate precipitation experiments were performed to stress the selected bacterial strains prior to the application for the self-healing mortar. Parameters such as temperature, pH, shaking conditions, ability to form endospores and the production of copious amount of calcium carbonate were placed under scrutiny. The biochemical properties of the selected bacterial strain were studied and investigated further by monitoring the evolution in pH, the production of ammonium, insoluble and soluble calcium and the colony forming unit. Following the characterisation, three strains were promoted forward for the use of self-healing mortar.

In-vitro calcium carbonate perception in broth state showed that the yielding of $CaCO_3$ was maximum in the case of strain 89 and strain 67 at 0.5932g/100ml and 0.8398 g/100ml, respectively.

The need for an encapsulating material that provides suitable environment for the bacteria to endure the mechanical and physical forces in addition to the high alkaline environment of the cement matrix, is indeed a key component towards the self-healing applications. As a result, the autoclaved aerated recycled concrete (AAC) aggregates were selected for this study, due to the high porous structure which implies high absorption properties. Three healing systems were proposed for the application of the self-healing mortar. The first approach comprises of bacterial spores impregnated under vacuum into the AARC aggregates along with a suitable nutrient designed specifically for maximum yielding

of calcium carbonate precipitation. The second approach comprised of a three-component healing system with the introduction of a mixed culture to enhance the healing capacity in addition to providing reinforcement for the cement matrix. The third approach comprised of direct incorporation of different bacterial cells into cement mortar to test for the capacity of the strain to heal cracks under high alkaline environment.

Mortar specimens were cracked at 28 days of curing, ranging from 0.127 to 0.875mm, the introduction of the three-healing system provided promising results in regard to healing cracks of 0.875mm over the period of 289 days of curing. Direct incorporation of bacterial strains at different concentrations 10^7cells/ml and 10^8cells/ml showed the tendency to heal cracks of 0.127 and 0.253mm, respectively.

Furthermore, direct incorporation of bacterial cells into the cement matrix, supported the analysis that urea hydrolysing bacteria is indeed an enzymatic activity. Two key enzymes were defined and strongly linked to the calcium carbonate precipitation process that is the urease enzyme and the carbonic anhydrase enzyme, where the latter is a zinc enzyme that catalyses the conversion of calcium dioxide into carbonate acid and ultimately promotes further insoluble calcium carbonate precipitation.

The introduction of a three-component healing system showed enhanced healing capacity, Direct Incorporation of 10^7cells/ml with 30% impregnated aggregates showed the capacity to partially heal cracks of 0.791mm by 28.3% over 14 days and 100% over 28 days healing period. Increasing the number of cells showed expected higher healing efficiency, specimens in set 22.1B were able to completely seal cracks of 0.875mm by 99.085 % over the period of 28 days.

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List of abbreviations

MICP	Microbial induced calcium carbonate
MIB	Mineralisation induced biologically
MICCP	Microbially induced calcium carbonate precipitation
JCI	Japan Concrete Institute
ESEM	Environmental Scanning Electron Microscopy
FTIR	Fourier-transform infrared spectroscopy
XRD	X-ray powder diffraction
AARC	Autoclaved aerated recycled concrete
ρ d	Apparent density
i	Water uptake
SC	Sorption Coefficient
t	Time
CFU	Colony forming unit
$CaCO_3$	Calcium carbonate precipitation

Chapter 1

1.1 Introduction

Inspired by biological systems, in which the damage triggers a healing process, the self-healing is the process of partial/total recovery of at least one of the material properties, such as mechanical strength and durability. The phenomenon of self-healing in concrete structures has been known and extensively investigated in recent years. Concrete is a composite construction material made primarily of cement, aggregates and water. In construction sector, Portland cement concrete is considered as an essential building material around the world and the second most consumed building material, twice as much as steel, plastics and aluminium combined. One drawback however is its massive production which exerts negative effects on the environment. Recent studies showed that cement production contributes 7% CO2 to the global anthropogenic CO_2 emissions, which is particularly due to the sintering of limestone and clay at a temperature of 1500°C. During this process the calcium carbonate ($CaCO_3$) is converted to calcium oxide (CaO), while releasing CO_2 (Worrall et al., 2001).

Cracks in concrete are almost inevitable, a phenomenon that hampers the structural integrity and durability of a material. Cracks can occur in concrete structures due to several reasons such as drying shrinkage, freeze thaw reactions, mechanical compressive and tensile forces. When cracks present in concrete, aggressive compounds such as chloride ions or carbon dioxide (CO_2)can penetrate through the concrete matrix and ultimately get to the reinforcement, causing corrosion and reduction in the strength and durability, which may lead to irreparable damages in the structure. Large costs are incurred all over the globe to repair cracks in concrete and a variety of techniques are available, however, majority of traditional repair systems are expensive and chemical based, and lead to environmental and health hazards.

Up till now, applying some compounds either to fill the cracks, such as epoxy resins, or to prevent the formation of these cracks, such as plastic polymers applied on the surface of the concrete (repairing and curing compounds), are the common ways to improve or extend the life of the concrete structure. However, both processes require human intervention which leads to a labour cost. Statistics revealed that large amounts of money spent reconstruction due to the lack of quality and durability in concrete structures. It is estimated the cost of construction in the USA has been estimated between $20 billion and $200 billion. In the United Kingdom, the repair and maintenance accounts for almost 45% of the UK's activities in the construction and building industry.

As a result of these facts, self-healing of cracked concrete has been studied for many years. Infact concrete had itself some self-healing ability; the traditional concrete mixtures are made with w/c ratio of 0.4 to 0.55. Theoretically speaking, a water to cement ratio of 0.35 is enough to complete all the reactions in cement. In practice, a water to cement ratio of 0.35 results in only 70% of the cement to react and the remaining 30% is left unreacted in the cement paste. When a crack occurs, unreacted cement cores may become exposed to moisture that penetrates through the cracks. In autogenously self-healing concrete, four processes that can block the crack, the formation of $CaCO_3$ or calcium hydroxide to block the crack, impurities in the presence of water, further blocked by hydration in the unreacted cement and by the expansion of hydrated cementitious matrix in the crack flanks (swelling of calcium silicate gel)

Free calcium ions released, as a result of cement hydration and dissipation through concrete as well as cracking surfaces, will reacts with CO_2 and HCO3. Consequently, $\mathrm{CaCO_3}$ crystals are formed and the reaction can happen at PH above 8 or between 7.5 and 8. The crystals grow at the surface of the cracks and finally fills the gap.

Due to the fact, autogenous self-healing can be useful for cracks with width of only 0.1 to 0.2 mm, another mechanism which is currently under investigation and developed in several laboratories, i.e. a technique based on the application of mineral producing bacteria. An efficient sealing of surface cracks by mineral precipitation was observed when bacteria were applied onto damaged surfaces or manually inserted into cracks. Due to the fact that bacteria were manually applied and externally applied to existing structures, this type of repair cannot be categorised as truly self-healing. As result, the prospect of using viable bacteria as a sustainable self-healing agent was investigated. Li et al. (2007) observed the formation of a white crystallising material on cracked concrete old structures and suggested that self-healing concrete using chemical products is viable.

So far, several healing approaches have been studied (Huang et al 2014), including autogenous, adhesive based, mineral admixture based and bio-based healing methods. Microbial self-healing concrete has gained strong interest in recent years, the system is based on mineral precipitating bacteria that is non-pathogenic and environmentally friendly (Wiktor et al 2011; Khaliq et al 2016; Jonkers et al 2007; Wang et al 2012; Jonkers et al 2010; Gollapudi et al 1995; Ramachandran et al 2001; Bang et al 2001; Dick et al 2006; De Muynck 2008; Van Tittelboom; Wiktor et al 2015; Wang et al 2014). Self-healing concrete using ureolytic bacteria was first introduced by Gollapudi et al, through the enzymatic hydrolysis of urea to carbon dioxide and ammonia.

The viability of using non ureolytic bacteria (Bacillus cohnii, Bacillus alkalinitrilicus and Bacillus pseudofirmus) for self-healing concrete was first introduced by Jonkers et al, 2007, 2010, 2011. Upon germination, bacteria spores catalyse the conversion of organic compounds to produce/precipitate insoluble calcium carbonate.

The self-healing of materials using microencapsulated healing agents has yielded high healing capacity (Brown et al. 2002, 2004, 2005b; Jones et al. 2006, 2007; Mauldin et al. 2007; Rule et al. 2005; White et al. 2001). White et al., 2001 developed a structural polymeric material that possesses the capacity to automatically seal cracks. The proposed system is achieved by embedding micro-encapsulated healing agent and a chemical catalyst within the materials matrix. Upon rupture, the healing agent is released via capillary action and becomes in contact with the embedded catalyst, resulting into crack closure/bridging through the process of polymerisation.

However, the self-healing using chemical agents requires complex set of requirements in regard to storage, transportation and rupture. Moreover, the completion of healing process is highly dependent on several parameters without affecting the inherent properties of the material, such parameters are mechanical triggering, stable storage of healing agent, chemical triggering, release of healing agent and polymerisation.

In one exception the spores of specific alkali resistant bacteria related to genus bacillus could be added to the concrete mixture as a self-healing agent, these spores germinate after activation by crack ingress water and start to produce copious amounts of crack filling calcium carbonate-based minerals through the conversion of precursor organic compounds, which were also added to the concrete mixture. Interesting feature of these bacteria is that they are able to form spores, which are specialised spherical thick-wall cells that has the same structure as to plant seeds, these spores are viable but dormant cells, can withstand mechanical and chemical stresses, and remain in dry state for periods of over than 50years.

It's worth mentioning that the matrix of fresh concrete is highly alkaline mainly due to the formation of calcium hydroxide, which forms after calcium silica hydrate that is considered the most important product of ordinary Portland cement. Matrix capillary water of fresh concrete is characterised by PH values between 11 and 13, therefore bacteria should withstand or resist the mechanical stresses and survive in high alkalinity environments for a prolonged period of time.

As mentioned above, the most promising bacteria to be incorporated in the concrete matrix appear to be alkali- resistant spore forming bacteria. Bacteria also need to be oxygen tolerant due to the

ingress oxygen causes the concrete matrix to be toxic. Due to the harsh environment in concrete (pH~ 13, small pore sizes <0.1µ and dry conditions) protective material or encapsulation techniques have been tested, such as silica gel, polyurethane, ceramic capsules, hydrogel.

Several protection materials have been examined by Ersan et al., 2015, where he investigate the six different protection materials approaches, such as diatomaceous earth, metakaolin, expanded clay particles (EC), granular activated carbon (GAC), zeolite and air entrainment, denitrifying pack (DP) which consisted of diaphorobacter nitroreducens +=ca(NO3)2 + ca(HCOO)2. Furthermore, R+GAC resulted in a 13% increase in compressive strength compared to the reference. Recent studies by Wang et al., 2012 showed that bacterial vegetative cells imbedded in silica gel and polyurethane were able to heal cracks of up to 0.4mm, in another study Wang et al., 2014 showed that hydrogel allowed bacteria to heal crack between 0.2 and 0.5mm.

Dong et al., 2014 used microcapsule that consisted of sodium monofluorophosphate and microcrystalline cellulose into polysorbate 80. In their study they used $Ca(OH)_2$ as a solution with different PH levels to stimulate a cementitious environment.

As test passes, PH levels will trigger the microcapsules where the sodium monofluorophosphate, ionising PO3F and Na as the reaction and PO3f proceeds to react with the hydration product of cementitious materials in light of the equilibrium. Such programs produce an increase in OH ion activity in the medium as well as the PH 12.4 of the calcium hydroxide suspension increases to 13.5 after the release of aqueous MFP in microcapsule.

Urease enzyme activity in microorganisms has also been tested for calcium carbonate precipitation (bekheet and syrett, 1977:Mc.Connaughy, 2011). Calcium carbonate precipitation is governed by several factors, such as PH, calcium ion concentration, concentration of dissolved inorganic carbon in addition to the presence of nucleation sites. The cell wall of the bacteria acts as a nucleation site while the factors are provided by the metabolism of the bacteria.

1.2 Problem statement

Cracks in concrete are almost inevitable, a phenomenon that hampers the structural integrity and durability of materials. The treatment of concrete using bacteria have indicated promising results towards civil engineering applications and two major pathways have been extensively explored in regard to the self-healing concrete using bacteria. The first path is through the metabolic conversion calcium lactate by bacteria species belonging to bacillus pseudofirmus, whereas a more efficient and widely used approach is through urease hydrolysing bacterium species. Considering the latter approach, the reported literature portrayed faster metabolism in comparison to the former in terms of the production or yielding of calcium carbonate precipitation.

The direct incorporation of bacteria into the cement matrix along with different types of nutrients to produce calcium carbonate portrayed inconsistent results. The potential in applying nutrients in real life civil engineering applications is very minimal due to the high cost and incompatibility with the cement matrix. The introduction of bacteria to heal cracks via suitable carrier to act as a smart material that activates upon exposure to oxygen and moisture have been explored. Different materials have been proposed, however, the majority are either expensive, not compatible with concrete structure or showed poor performance when exposed to high cracks. Utilisation of the hydration products as a source of nutrients for the metabolic production of calcium carbonate is anticipated to be an efficient and cost-effective strategy in addressing the above issues, given its structural affinity to concrete.

1.3 Research objectives

The aim of this study is to develop a new-generation and smart self-healing cementitious material with maximised healing efficiency via hydration product-aided bacterial approach. The importance of exploring the biological side of the self-healing mortar is to understand the real factors that contribute towards the overall healing mechanisms in concrete structure. The study also aims to isolate alkaline and screen bacterial species with a positive urease enzymatic activity and with the capacity to produce copious amounts of insoluble calcium carbonate.

Stressing of the bacteria in-vitro to produce calcium carbonate is a vital component in this study, using different bacterial strains to bioengineer a healing solution that is feasible for the civil engineering industry. Understanding of the biochemical properties of the calcite producing bacterium is also a major part of this study, this is done through simultaneous monitoring of the change in pH, ammonium production, conversion of calcium in solution into calcium carbonate, and the number of cells that are viable at each certain point during the precipitation process.

The study is to address the major limitations and setbacks in the technique used in the introduction of bacteria in self-healing mortar. Upscaling is a key factor for the success and viability of self-healing mortar in real life applications. The study further investigates and focuses on designing a biological cementitious material that utilises the micronutrients available inside the spore along with the micro incorporated nutrients to contribute towards restoring the overall mechanical properties. The study investigates the use of several techniques in the introduction of bacteria into cementitious material, and the potential of using autoclaved aerated recycled concrete (AARC) aggregates as carrier using a two-component healing system and an optimised three component healing system.

1.4 Outline of thesis

The study consists of six major chapters, comprising of introduction, literature review bridging bio-engineering and civil aspects towards the application and introduction of the self-heling cementitious material, the third chapter being the methodology, followed by testing the effect of bacterial on the mechanical properties of cement mortar and understanding the healing mechanisms on the self-healing mortar being the fifth chapter. The major conclusions drawn and future works are presented in chapter 6. The brief overview of each chapter is outlines below:

Chapter 1 Introduction

Overview of the importance and issues related to the concrete structures and the available solutions is briefly explained. The aim and objectives of this study in addition to the problem statement are stated.

Chapter 2 Literature review

This part of the study provides overview of the major problems associated with concrete production and structures in terms of carbon foot print, cost and the short and long terms performance of the available technique in carrying maintenance to concrete structures. Overview of the capacity for the concrete to heal due to several parameters, such as autogenous healing, has also been produced to provide better understanding of the behaviour of the material. The methodologies and approaches of self-healing concrete are deeply scrutinised in this part of the chapter to provide strong and deep understanding of factors that are related to the enhancing and optimising the healing efficiency. The introduction of bacteria into the cement matrix and the tests followed in assessing the efficiency of the healing and mechanical properties of bio-based cementitious materialshave also been studied in this chapter. A tabulated summary of a key experiments and results are also portrayed.

Chapter 3 Methodology

This chapter thoroughly describes the materials and methods that have been used to achieve self-healing mortar via ureolytic bacterium species. The chapter consists of two major parts of bioengineering and civil engineering, where the former includes detailed explanation of the protocols used in isolating, purifying and characterising different bacterial species. The types of media used and the make of each composition are tabulated. The design and make of the in-vitro calcium carbonate experiments in broth state are deeply explained. The procedures used in the analysis of the precipitates and in assessing the biochemical properties of different isolates are presented.

The casting and fabrication of cement mortar in addition to detailed encapsulation processing techniques are presented. The methods used in inducing cracks, curing and monitoring of the healing efficiency of pre-cracked mortar specimens are explained in details.

Chapter 4 Characterisation of various bacillus Sphaericus strains for the use of self-healing mortar

A detailed analysis of the yielding capacity of different bacterial strains using In-vitro calcium precipitating media was performed in this study. Bacterial strains were assessed for their capacity to endure alkaline condition, produce spores, and produce copious amount of calcium carbonate. Several important factors were carefully considered in assessing the applicability of the selected strains to be used in cement mortar, such as temperature, pH, shaking conditions and types of nutrients including calcium source. The effect of each parameter on the final calcium carbonate yielding was also considered.

The chapter also presents monitoring change in pH, ammonium production, calcium and the number of cells present during the calcifying process. Stat graphic experiment was also conducted in order to examine the effect of urea calcium acetate, yeast extract in addition to other nutrients on the capacity of the bacteria to produce calcium carbonate.

Chapter 5 Understanding the mechanisms of the self-healing mortar using AARC impregnated aggregates

The introduction of fresh bacteria and thier role in enhancing the mechanical and impermeable properties of cement mortar are discussed. The effect of various nutrients on the mechanical strength of cement mortar was studied, such as calcium lactate and calcium acetate.

The ability of the promoted bacterial strains to use micronutrients and the hydration products in cement towards the calcifying process was explored. The study further examined the effect of different curing conditions, i.e. water, wet-dry cycle, dry and media curing on the mechanical properties of hardened cement mortar.

The impregnation technique used and the efficiency of the drying methods on reducing the spore germination for maximum viability are also discussed. Following the impregnation, the change in density after each impregnation cycle and after applying the coating material was noted to aid in calculating the amount calcium, urea and nutrient broth present per gram of the aggregate.

The introduction of the impregnated aggregates was done as part of the sand used, i.e. 20%, 30%, 40%(v/v), which were used as a carrier for the two-component healing system comprising of bacterial spores and nutrient. Non-impregnated aggregates were used as control. Different cracks were induced at 28 days of curing using flexural testing in order to assess the healing capacity of such approach.

An optimised three component healing system is proposed with fresh bacterial cells acting as a reinforcement in the cement matrix to provide better healing and enhance material restoration. The approach was also conducted with a mixed culture that doesn’t have the capacity to produce spores but is able to grow in alkaline condition and produce copious amount of calcium carbonate.

Assessing of the healing material was done by microscopic monitoring over 3, 7, 14, and 28 days of healing. Furthermore, permeability via water uptake on the healed/unhealed specimens was conducted to provide an indication of how far the precipitation was able to penetrate through the crack. Analysis of the produced precipitates was performed by means of Field emission scanning electron microscope (FESEM), FTIR and X-ray powder diffracted.

Chapter 6 Final appraisal and future work

This chapter provides concise summary and conclusive statements established within the thesis, in addition to future prospective and recommendations discussed.

Analysis of the produced precipitates was performed by means of Field emission scanning electron microscope (FESEM), FTIR and XRD.

Chapter 2.0 Literature review

2.1 Application of bacteria as self-healing agent for the development of sustainable concrete

Jonkers, H et al 2008 investigated the applicability or the use of alternative metabolic minerals producing pathways by incorporating bacteria into the concrete matrix prior to casting, where the bacteria embedded in the matrix should remain viable for a prolonged period of time and be able to produce copious amounts of sufficient minerals in order to plug or seal the crack.

Bang et al, 2001: Ramachandran et al 2001; De Muynck et al 208a, b and Ramakrishan et al 2007, are all previous studies on self-healing concrete where they used ureolytic bacteria, mainly from the genus bacillus, as an agent for biological production of calcium carbonate-based minerals. In the aforementioned studies, CaCo3 formation is based on enzymatic hydrolysis of urea to ammonia and co2 where the pH of the reaction increases from neutral to alkaline condition (as a result of Pk-value of the equilibrium constant is close to 9.2) as a consequence, bicarbonate and carbonate ions are formed and precipitate with presence of calcium ions from CaCo3 minerals.

One major problem that has been reported with this reaction is nitrogen loading which occurs due to the fact that for each carbonate ions, two ammonium ions are produced at the same time. The matrix of concrete is highly alkaline especially due to the formation of Portland (CaOH) which is after calcium silicate hydrate that is considered the most important hydration product of ordinary Portland cement (OPC).

As a result, alkaliphilic spore forming Bactria, specifically genus Bacillus, appear to be the most promising bacterial agents. Incorporated bacteria also need to be oxygen tolerant as the concrete matrix is very oxic.

Jonker's (2008) proposal represents two component healing agents (bacteria + calcium lactate), where he used calcium lactate as a nutrient or mineral precursor for the bacteria. Due to the fact that the addition of mineral precursors may impact other concrete properties such as initial and final setting times, the quantity of mineral precursors added to the concrete is limited. As a result, a crack of <1 mm diameter could be healed but not larger cracks.

$CaC6H10O6 + 6O2 \rightarrow CaCo3 + 5Co2 + 5H2o$

CaCo3 precipitation will increase more when CO2 produced react with Ca(OH)2 minerals 5CO2

$+Ca(OH)2 \rightarrow 5CaCO3 + 5H2O$

The above process represents an alternative approach to urea's-based system. One major advantage of metabolic conversion of calcium lactate is that it does not cause a massive production in of ammonia i.e. preventing the risk of degradation and reinforcement corrosion specially when further oxidised by bacteria to yield nitric acid (Dierck et al 1991)

The effect of adding magnesium to the growth medium was also examined, where light microscopic analysis of the growing culture revealed that the addition of Mg has significantly stimulated the formation of bacterial spores where spores were produced in the vegetative cells.

One major problem that has been recorded is the loss of viability of bacterial spores which is found to be related to the continuous decrease in pore diameter resulting in crushing or killing of bacteria. The decrease in pore diameter was apparent from the MIP analysis where large pore size was evident in young concrete but disappeared in favour of aged concrete. However, this problem could be solved by microencapsulation.

In another study Jonkers et al 2011 used two components healing agent (bacteria + calcium lactate) to the examine the potential of self-healing concrete, where he investigated the viability of incorporating bacteria along with organic bio-mineral precursor compound (calcium lactate) into porous expanded clay particles (as a protection material). Such proposal should protect the bacteria from activating too early yet remain viable for a prolonged period of time when integrated into the concrete matrix.

The amount of light weight aggregate used in this study compromise of 50% of the total aggregate volume. Control specimens which incorporated with expanded clay particles (without bacteria) and specimen loaded with expanded clay particles (with bacteria) were prepared. In the clay particles specimens loaded with bacteria, part of the aggregate material (2-4 mm size class) was replaced by similarly sized expanded clay particles loaded with chemical self-healing agent (bacterial spores 1.7* 10^5 expanded clay particles, corresponding to 5* 10^7 spores concrete, plus 5% w/w fraction calcium lactate,). Based on the compressive test results, 50% decrease in compressive strength was observed after 28 days curing for the expanded clay particles specimens, however expanded clay particles loaded with bacteria showed a substantial healing capacity.

Considering the water permeability results, considerable difference was observed between control and bacterial specimens, where all bacterial specimens, which were completely healed, recorded no measurable permeability which is in contrast to the control specimens which featured a permeability between 0 and 2ml/h. SEM results revealed that both control and bacterial specimens had the capacity to precipitate calcium carbonate but in different amounts, however for the control specimens $\mathrm{CaCO_3}$ occurred adjacent to the crack rim (partially healed) while the $\mathrm{CaCO_3}$ precipitation in the bacterial specimens appeared within the crack itself.

The rationale behind the $\mathrm{CaCO_3}$precipitation in the control specimens is related to the concept of secondary hydration, where un-hydrated cement particles present near the crack undergoes secondary hydration when exposed to water. As a result of the presence of carbon dioxide produced from water, carbon dioxide will react with calcium hydroxide Ca $(\mathrm{OH})_2$ produced from C2S and C3S hydration reactions to finally precipitate calcium carbonate. The large production of $\mathrm{CaCO_3}$near the crack rim might be attributed to the high concentration of calcium hydroxide and carbon dioxide as a result of the opposing diffusion gradients of the respective reactants i.e. carbon dioxide spreads out from bulk water in the direction of the crack interior while calcium hydroxide diffuses away from the crack interior toward the overlying bulk water. This can be seen in the equation below:

CO_2+ Ca $(OH)_2$ → $CaCO_3$+ H_2O

Figure 1 Light microscopic images of pre-cracked concrete in control and bacterial concrete, Jonker et al 2010.

Considering the self-healing using the bacterial approach, the process results in substantial amounts of calcium carbonate, produced within the crack interior due to the active metabolic conversion of organic bio cement precursor compound by the present bacteria. In addition to the latter source of calcium carbonate, carbon dioxide formed from the metabolic process at the crack's surface will react with portlandite particles that are present inside the crack; as a result, the portlandite directly reacts with carbon dioxide that is produced by the bacteria, resulting in additional amounts of calcium carbonate being produced. Therefore, the bacterial calcium lactate conversion results in a total of calcium carbonate equivalents to be produced, hence, more efficient in regard to sealing or plugging of the crack. The bacterial metabolic conversion of calcium lactate can be seen in the following equation:

Ca $(C_3H_5O_2)2$ + $7O_2$ → $\mathrm{CaCO_3}$+ $\mathrm{5CO_2}$ + $\mathrm{5H_2O}$

It's worth pinpointing that the effect on crack healing efficiency by using calcium lactate solely was not investigated in this experiment, as such investigation requires the experiment to be performed sterile conditions which is considered technically difficult due to the introduced effects needed for heat sterilisation or chemicals on specimen characteristics. To summarise the experiment was performed under non-sterile conditions (realistic to the real-world applications) which made it difficult to exclude any contaminates that might be present from tape water which may have contributed in further calcium carbonate to be precipitated. Measurements of oxygen consumption was performed (on specimens containing bacterial spores and on specimens containing calcium lactate plus bacteria) in order to provide an indication of the amount of calcium carbonate produced, it was observed that specimens containing bacterial spores plus calcium lactate consumed large amounts of oxygen (after submersed in water) compared to calcium lactate specimens and bacterial spores specimens, where the former had large delays in oxygen consumption and the latter with no oxygen consumption at all.

The above analysis suggests that the use of bacterial spores as a two component self-healing biochemical agent is not essential as the bacteria present in the crack ingress water is able to metabolically convert calcium lactate into calcium carbonate, however the former option still remain as the favourable option as it provides sufficient amounts of calcium carbonate i.e. ensuring or providing a more efficient crack healing capacity. To conclude, the result of this experiment revealed a 100% crack healing in clay particle specimens contacting bacteria and calcium lactate compared to control specimens where they had a crack healing efficiency of 33%. However, before it becomes available for real world application, further studies to be on the amount of healing agent to be used in order to reduce the adverse effects on the compressive strength in addition to becoming economically competitive.

2.2 Crack repair in concrete using bio-deposition

Belie N et al 2009, investigated the potential of crack healing by using different bio deposition treatments in an attempt to reduce water permeability and increase the resistance against damage processes such as chloride ingress and carbonation. In this study, Bacillus sphaericus was used and protected in silica sol (by suspending the sample in SiO_2) then immersed in different treatment in order to examine the effect of each treatment on the healing efficiency.

Microbiologically induced carbonate precipitation involves ureolytic micro-organisms that can induce or activate extracellular precipitation of CaCo3 by decomposing urea into ammonia and carbon dioxide, this cause an increase in PH level at the cell surface and promote the microbial deposition of $\mathrm{CO_2}$as $\mathrm{CaCO_3}$.

The use of $\mathrm{CaCl_2}$as a calcium source was investigated in many studies (Bang et al.2001; Adolphe et al.190; Ferris&Stehmeier 1992). Due to the fat that chloride ions are detrimental to the concrete's reinforcement, Belie N et al. 2009, investigated the use of calcium nitrate Ca $(NO_3).4\mathrm{H_2O}$ as an alternative calcium source. The following treatments were carried out:

Medium

this involves immersing the samples for 3 day in equimolar solution of ureum (20g/l) and

$\mathrm{CaCl_2.H_2O}$ (49 g/l) or Ca (NO_3).$4\mathrm{H_2O}$ (78 g/l)

BS + $\mathrm{CaCl_2}$

Samples were immersed in a Bacillus sphaericus culture grown overnight, growth medium consisted of 20g/l yeast extract and 20 g/l ureum.

BS + sol-gel + $\mathrm{CaCl_2}$or BS + sol-gel + Ca $(\mathrm{NO_3})_2$

This involves centrifuging 50 ml of culture grown overnight at 4C and 700Rpm, then suspending the resulting pellet in demineralised water (10ml) and sol-gel (40ml) .1.2g of NaCl is then to the solution then vortexed for 30s.It's worth mentioning that the suspension was added to the crack by using a syringe. Following the gel formation samples were placed in the equimolar solution for 3 days.

Sol-gel

Samples were immersed into silica gel for 20 min then dried for 24h at room temperature.

Sol-gel + BS + $CaCl_2$

Samples were treated in sol-gel and immersed in BS+$CaCl_2$solution.

Based on visual examination and ultra sound test, all treatment resulted or were able to bridge cracks except treatment with 'medium' and 'BS + $CaCl_2$as they had limited $CaCO_3$deposition. Furthermore, it should be noted that complete sealing was only achieved in artificial crack of 0.3mm wide and 10mm deep.

Considering the permeability test (test was conducted in accordance with Wang et al.1997), all treatments resulted in a decrease in water permeability in comparison to untreated cracks. However, grout and BS + $CaCl_2$were least efficient. In contrast, treatments with BS + sol-gel + $CaCl_2$or BS + sol-gel + Ca $(NO_3)_2$and epoxy were the most efficient in regard to decreasing water permeability.

2.3 Use of bacteria to repair cracks

In this study bacillus sphaericus was used to heal cracks, such strain was selected based on its high urease activity, continuous formation of dense calcium carbonate crystals and its very negative zeta potential. The concept of inducing calcium carbonate precipitation has been used by Bang et al, Ramakrishnan et al, day et al, Dick et al and Santhosh et al. in the aforementioned studies $CaCO_3$have been used to heal cracks in granite, consolidation of sand columns and as a surface treatment of limestone. Due to the high alkalinity of concrete environment, which limits the growth of the bacteria, it is important to incorporate bacterial cells in order to protect them from the high PH in the concrete.

Polyurethane (PU) possess strong mechanical properties and due to it biochemical inert characteristics, PU has been commonly used as a bacterial carrier. In his study Bang et al (2001) investigated the use of cylindrical shaped polyurethane foam immobilised bacteria (10mm diameter and 50mm length) in remediating cracks (3.18mm width), where the PU foam along with the bacteria were applied directly into the crack. However, since this treatment is time consuming and it limits the urease activity when the bacteria is incorporated, silica gel was used as an alternative protection material where the bacteria along with silica gel were applied into the crack using a syringe. Therefore, silica gel was used as a filling material prior to the $CaCO_3$trigger in addition to as a protection vessel for the bacteria.

De muynck 2008, conducted a research examining the effect of calcium chloride as a calcium source on the precipitation of calcium carbonate. To summarise, chloride ions have been reported to cause deterioration to the concrete matrix, as a result, calcium nitrate and calcium acetate have been used as an alternative calcium source. The efficiency in healing cracks was evaluated by performing and conducting a series of tests such as low-pressure permeability test, ultrasonic measurements and by visual examination of the degree of crack filling.

Two traditional repair techniques have been examined and compared with the microbiologically induced $CaCO_3$technology namely; 2-component epoxy resin (sikadur 52) and 2-component cement bound mortar (sika to 111). Considering the bacterial technology, Bacillus sphaericus strains have been chosen, where six unique strains have been isolated and selected on the basis of their potential in precipitating calcium carbonate. Following the treatments specimens were suspended in a urea-calcium solution for three days, the urea calcium solution consisted of urea(20g/l) and $CaCl_2.H_2O$ (49 g/l) or $Ca(NO_3)_3.4H_2O$ (79/L) or $Ca(CH_3COO)_2.H_2O$ (59 g/L). the treatment carried out in this study were the followings:

- BS + sol-gel + $CaCl_2$or BS + sol-gel + Ca $(NO_3)_2$or Bs in sol-gel+$Ca(CH_3COO)_2$
- Sol-gel
- Bs+CaCl2
- Sol-gel + Bs +$CaCl_2$
- Autoclaved BS + sol-gel +$CaCl_2$ or autoclaved BS + sol-gel + Ca $(NO_3)_2$or autoclaved Bs in sol- gel + $Ca(CH_3COO)_2$

The treatment with autoclaved bacteria is analogous to the first treatment except that specimens for water permeability test were treated with active bacteria and autoclave bacteria to be certain that the reduction of water flow was not only due to crack filling of silica gel-sol and biomass. Table 1 shows the crack techniques that have been performed in this study.

A) Traditional							
Type	Description						
Epoxy	Injection of epoxy resin with needle						
Grout	Application of mortar with spatula						
B) Non-traditional							
	Autoclaving bacteria	Injection Levasil	Injection Levasil and BS	Immersion in BS culture	Immersion in $CaCl_2$ and urea solution	Immersion in $Ca(NO_3)_2$ and urea solution	Immersion in $Ca(CH_3COO)_2$ and urea solution
BS + $CaCl_2$				x	x		
Sol-gel		x					
Sol-gel + BS + $CaCl_2$		x		x	x		
BS in sol-gel + $CaCl_2$			x		x		
BS in sol-gel + $Ca(NO_3)_2$			x			x	
BS in sol-gel + $Ca(CH_3COO)_2$			x				x
Autoclaved BS in sol-gel + $CaCl_2$	x		x		x		
Autoclaved BS in sol-gel + $Ca(CH_3COO)_2$	x		x				x

Table 1 Crack repair techniques, De muynck et al 2008.

Crack healing efficiency of different repair techniques have been evaluated by conducting the water permeability test, which was in accordance to Wang et al (1997) and Aldea et al (2000). The water permeability coefficient (k) was determined by producing waster pressure at the top of the concrete specimen via water column and by following the drop of the water column in time. Specimens with realistic cracks were selected for this test with a crack width ranges from 0.01mm to 0.9mm. Subsequent to crack treatment, specimens were vacuum saturated in a demineralised water in accordance to NBN B24-213(1976). The fall of the water level in the pipette, as a result of the water flowing through the crack, was recorded at regular intervals (contingent to the rate of water flowing through the specimens). Water was then restored to its original level.

Water permeability coefficient was calculated by using Darcey's law: K = $\frac{aT}{AT\ln{(h_0\ /h_f)}}$

Where:

- a: is the cross-sectional area of the pipette (m^2)

- A: is the cross-sectional area of the specimen(m^2)
- T: is the specimen thickness (m)
- t: is the time (s)
- h_0 , h_f are the initial and final water heads(cm)

Since ultrasonic waves travel quicker in hardened concrete (4000-5000 m/s) than in air (350m/s) or water (1480 m/s), ultrasonic measurement test was conducted for each crack repairing technique, in order to examine the effectiveness of such repair on the propagation of the waves through the concrete specimen. In other words, ultrasonic waves will travel through open fissures slowly leading to an increase in transmission time, in contrast to the sealed cracks where the waves travel through the sealant faster leading to a decrease in transmission time. Concrete specimens with standardised cracks of 0.3mm width were prepared for this test.

Visual examination of crack repair was conducted on a 1cm slice which was sawn from the specimens with standardised cracks. The purpose of this test is to evaluate the cross section of the treated cracks under optical microscope (Moritex micro scope man MS-500B). The same test was conducted on the surface of the specimens selected for water permeability test to examine the repaired cracks.

Thermo-gravimetric analysis (TGA) was carried out in to order to test or examine the material characteristics. Samples treated with autoclaved Bs in sol-gel $+CaCl_2$ or autoclaved Bs in sol-gel $+Ca(CH_3COO)_2$ were selected for this test, where the specimens were left to dry at room temperature followed by taking a sample of the repaired material using a needle. TGA analysis was conducted on every crack repair technique where ± 30mg of the repair material was brought in a sample cup and placed in the apparatus then subject to heat ranging from 20°C to 900°C at a rate of 10°C /min.

Through this process, the amount of calcium carbonate precipitated can be determined by the difference in weight loss. To clarify, since the autoclaved bacteria are not expected to precipitate CaCO3 crystals, a difference in weight loss may be observed for treatment with autoclaved or active bacteria. Upon heating of $CaCO_3$, crystals in the repair material where $CaCO_3$ will decompose and bound CO_2 will be released from the material, leaving only calcium oxide, this can be seen in the following equation:

CaCO2 → Ca O + CO

Therefore, when $CaCO_3$ is subjected to heat typically in the range of 650-750°C (oniyama Eetal 1995), a decrease of weight loss will be observed (as a result of CO_2 release) in the samples treated with non-autoclaved or active bacteria.

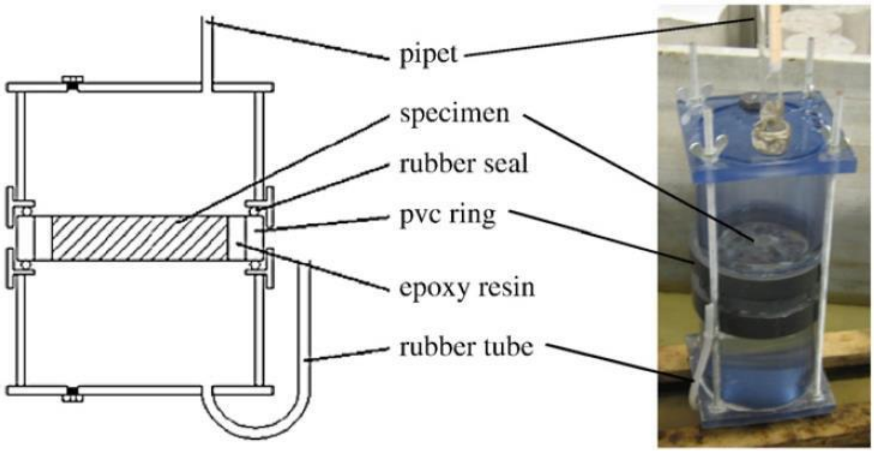

Figure 3 Water permeability test, De muynck et al 2008.

Considering the water permeability test (Figure 3). The water permeability gradually decreased until it reached stable, a possible explanation for this phenomenon is due to the incomplete saturation of the concrete specimens along with the presence of air bubbles. Previous research by Nanayakkara et al (2003), justified the loss in water permeability to be linked to the autogenic healing of cracks as a result of further hydration of un-hydrated cement particles, carbonation of Ca $(OH)_2$ and the deposition of soluble hydrates in the direction of the flow.

Contrast to the above, Hearn et al (1998) reported that the decrease in water permeability due to secondary hydration of un-hydrated cement particles, would have negligible effect under the condition that specimens were no tested at an early age. The effect of secondary hydration on carbonation have been neglected in this experiment as the test specimens used in this experiment were cured for more than a year (at a temperature of 20 °C and RH of 90%). Furthermore, carbonation of Ca $(OH)_2$ was also disregarded due to the fact that specimens were always covered up with water in the upper section of the water permeability test.

Considering all the points above, only air bubbles due to incomplete saturation could be responsible for the initial reduction in water permeability. Figure 4shows water permeability results for different repairing techniques. Figure 4, 5, and 6 represent permeability coefficient (k) against crack width for traditional rapier technique, biological treatment and finally a comparison of samples treated with active and autoclaved bacteria, respectively.

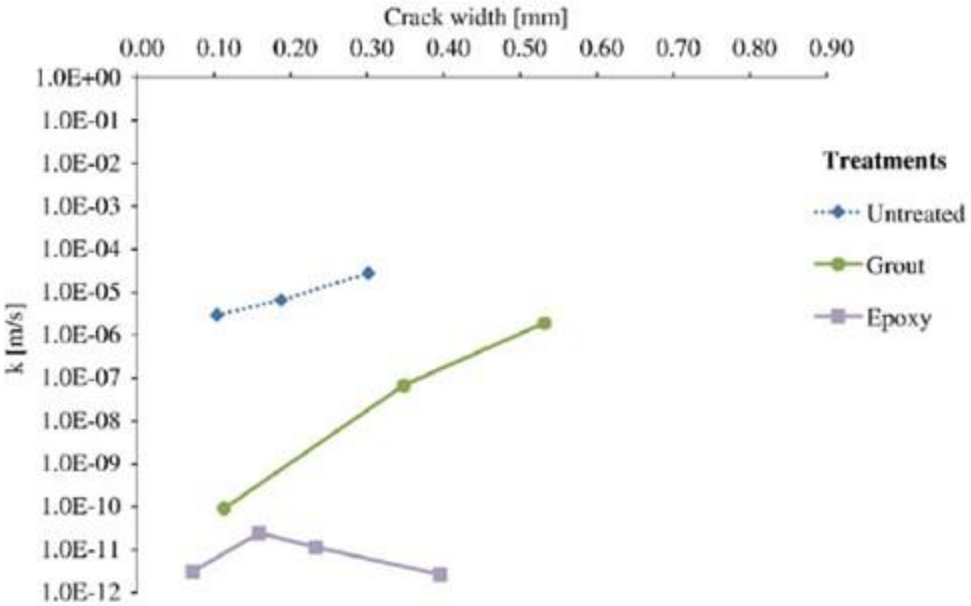

Figure 4 Water permeability of treated and untreated samples (traditional techniques), De muynck et al 2008.

Based on figure 5, all treatment (excluding Bs+$CaCl_2$, as the bacteria were unable to survive the high PH in the concrete i.e. no crystals were observed under the SEM analysis) showed a decrease in

Water permeability when compared to untreated cracks. Cracked specimens treated with grout, sol-gel showed a slightly better performance in regard to water permeability, however grout treatment had a lower efficiency due to the grain size of the paste which made it difficult to seal the crack with paste. On the other hand, Bs in sol-gel +$CaCl_2$ or Ca $(NO_3)_2$ or $Ca(CH_3COO)_2$ treatments had a better efficiency i.e. low water permeability (analogous to epoxy treatment).

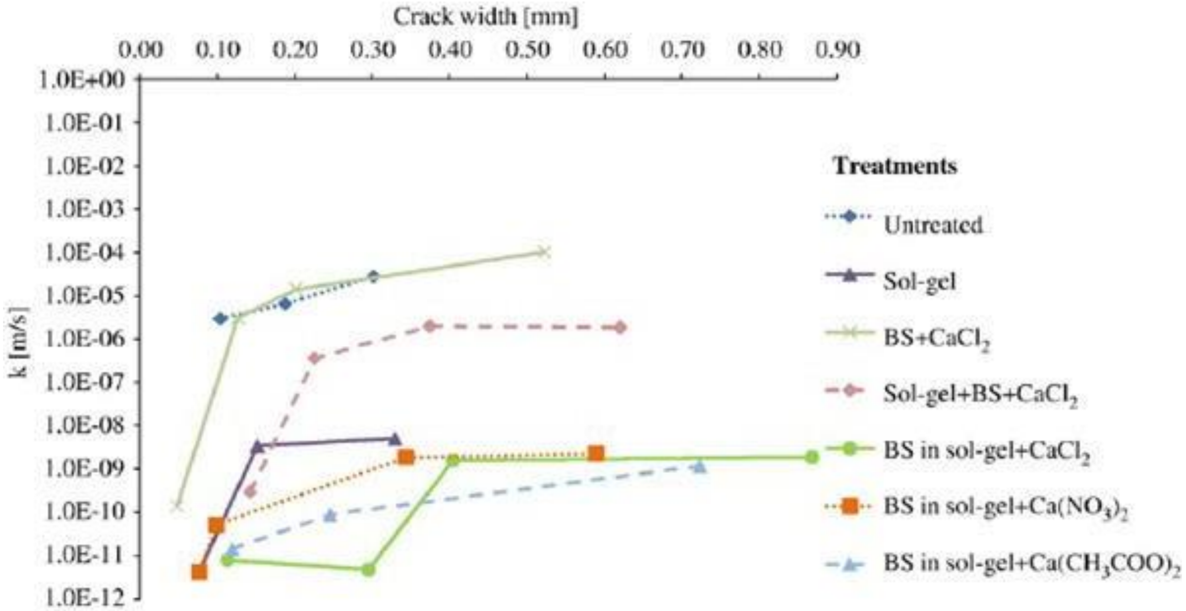

Figure 5 Water permeability of treated and untreated specimens (biological repair), De muynck et al 2008.

In regard to the calcium source, since the use of chloride have been reported to cause deterioration to the concrete reinforcement and no difference in efficiency between Bs in sol-gel+ $CaCl_2$or Ca $(NO_3)_2$ or $Ca(CH_3COO)_2$, it can be concluded that the use of calcium nitrate and acetate as an alternative calcium source would disregard the adverse effect of chloride ions.

Previous research by De Muynck et al (2008) found that the type of the bacterial culture in addition to the composition medium had significant influence on the morphology of calcium carbonate crystals, using SEM analysis it was evident that the presence of acetate ions resulted in the formation spherical crystals while calcium ions resulted in a rhombohedral crystal.

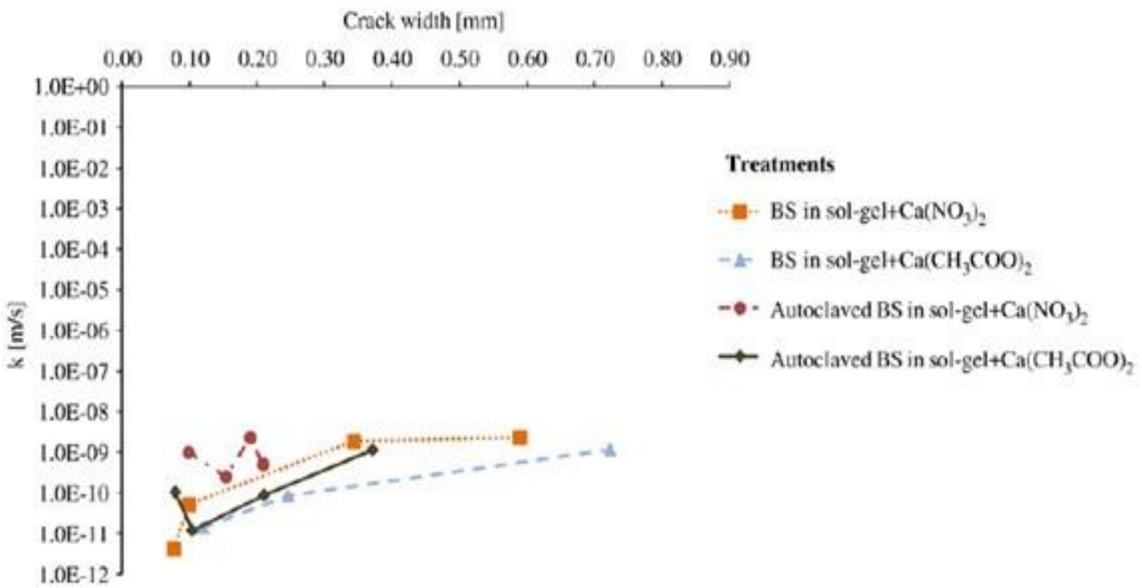

Figure 6 Comparison specimens treated with active and autoclaved B. Sphaericus, De muynck et al 2008.

Figure 6 shows a comparison in terms of water permeability between specimens treated with active and autoclaved B. sphaericus. It can be seen that specimens treated with sol-gel and autoclaved B. sphaericus had a lager decrease in water permeability, the decrease however is attributed to the fact that silica so-gel had filled the crack i.e. limiting the flow of the water. Treatment using active bacteria and sol-gel had slightly lager decrease in water permeability compared to autoclaved due to the presence of the CaCO3 crystals which resulted in a better sealing efficiency.

Ultrasonic measurements were taken before and after treatment. Treatment with epoxy or Bs in sol-gel +$CaCl_2$resulted in the largest decrease in transmission time for crack of 10 mm deep. Specimens treated with sol-gel +Bs +$CaCl_2$ and Bs + $CaCl_2$had a bigger decrease than what initially was expected, this could be due to the fact that all treated samples were suspended in urea-calcium solution which resulted in filling of submerged pores I the concrete matrix. Sol-gel treatment resulted in a minimal decrease in transmission time due to the shrinkage of the sol-gel. Untreated samples on the other hand had an increase in transmission time.

Considering the 20mm cracks, treatment with Bs in sol-gel + +$CaCl_2$or Ca $(NO_3)_2$or $Ca(CH_3COO)_2$

showed a lower efficiency compared to the results obtained from the 10mm cracks due to the incomplete fillings of the cracks. Sol-gel and grout resulted in the lowest decrease in transmission time. It's worth mentioning that grout treatment was only applied on the surface of the sample i.e. no filling occurred, however ultrasonic results showed a slight decrease, indicating that the waves still travelled around the crack. In the case of untreated cracks, increase in transmission was observed i.e no healing or filling of the cracks occurred.

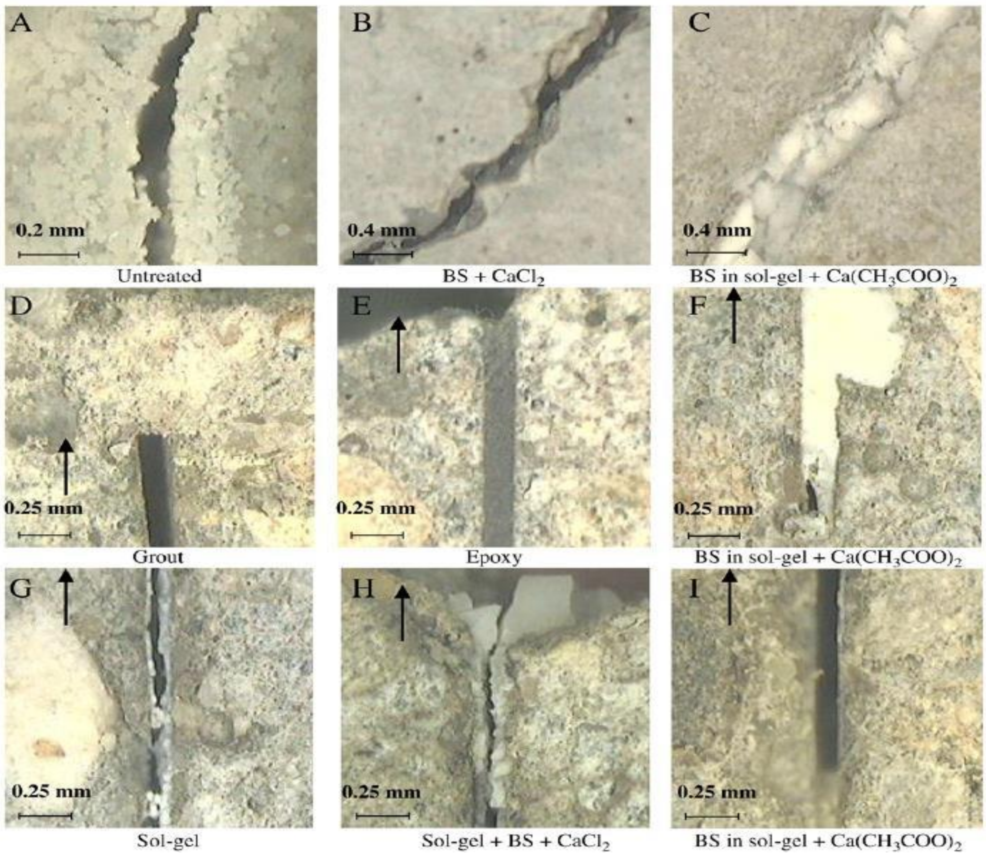

Figure 7 Specimens with different treatments, De muynck et al 2008.

Figure 7, illustrates a top view of specimens with different treatments. Figure 8A shows a concrete specimen with untreated crack, however, crystal deposition at the crack face was observed i.e. indicating that the specimen had undergone autogenous healing during the water permeability test. For cracks treated with Bs +$CaCl_2$, no calcium carbonate crystals were observed as the bacteria were unable to survive the high PH I the concrete. In contrast, when the bacteria were incorporated in sol-gel plus calcium acetate (Figure 7C), cracks were completely healed or sealed.

For the traditional repair techniques i.e. epoxy and grout. It can be seen from figure 8D that grout treatment was unable to fill the crack due to the large grains of the grout paste, figure 8E however shows that specimens treated with epoxy were able to completely fill cracks of 10mm and 20mm deep. As stated earlier specimens treated with Bs in sol-gel +$CaCl_2$ or Ca $(NO_3)_2$or $Ca(CH_3COO)_2$ were suspended in a urea-calcium solution in order to induce the calcium carbonate precipitation. After suspension CaCO3 precipitation occurred and complete filing of crack size of 10mm was observed (figure 8F). however, when the crack size increased to about 20mm deep, incomplete filling was observed as the CaCO3 precipitation was not sufficient in sealing of crack of 2mm deep (figure 7I).

Therefore, Bs in sol-gel +$CaCl_2$or Ca $(NO_3)_2$or $Ca(CH_3COO)_2$treatments were as efficient as epoxy treatment in sealing cracks of 10mm deep but not cracks of 20mm deep whereas epoxy treatment was able to completely fill cracks of both 10mm and 20mm deep.

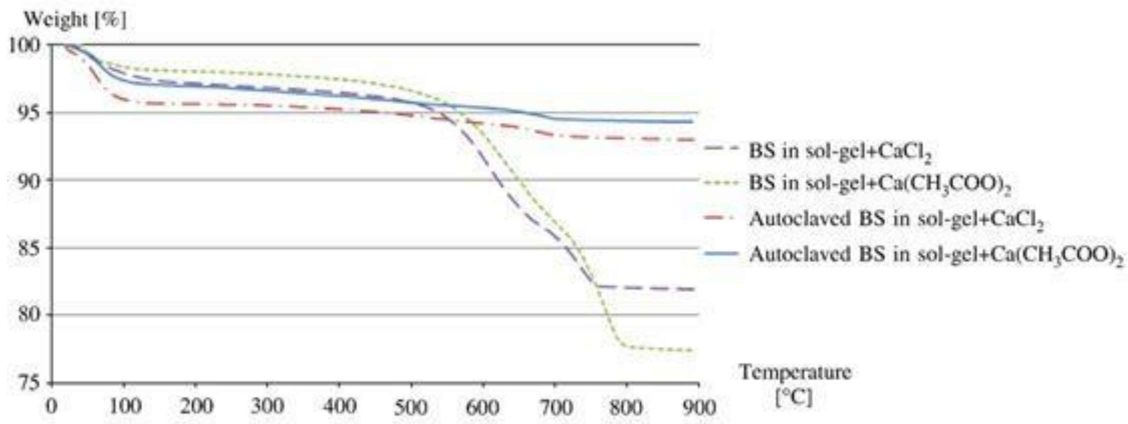

Figure 8 TGA results for different repair techniques, decrease in weight vs temperature, De muynck et al 2008.

From the TGA analysis (Figure 8), it is evident that water started to evaporate when the temperature increased to about 100c i.e. resulting in a weight loss due to the decomposition of $CaCO_3$. Furthermore, it can also be seen that there is an apparent difference in terms of the weight loss between treatment with active and autoclaved bacteria. Specimens treated with autoclaved bacteria had a small decrease in weight loss (1.69% and 1.34 for $CaCl_2$and $Ca(CH_3COO)_2$, respectively) compared to active bacteria where the weight loss was quite large (136.6% and 18.85% respectively) i.e. indicating that calcium precipitation has occurred in the case of active bacteria.

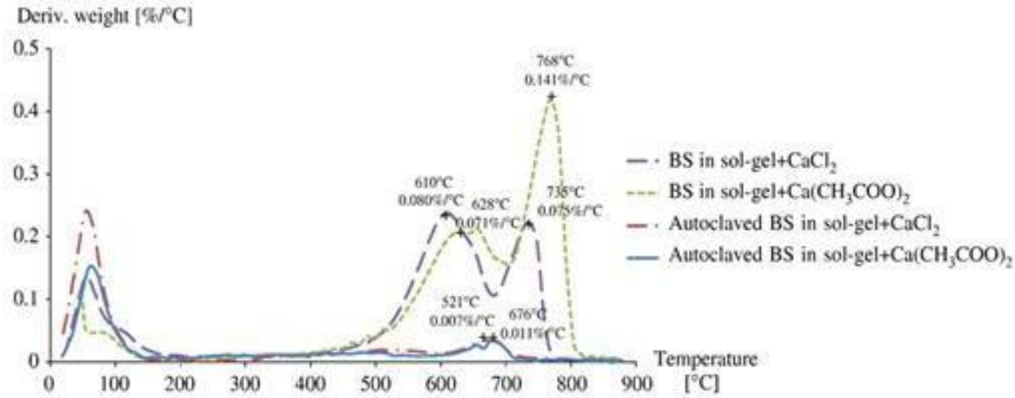

Figure 9 TGA results, derivative of weight loss versus the increase in temperature, *De muynck et al 2008.*

Figure 9,represents the derivative weight loss versus the increase in temperature, in other words, it provides an indication of the point where the weight loss is quite dominant. For the specimens treated with autoclaved Bs in sol-gel +$CaCl_2$and autoclaved Bs in sol-gel +$Ca(CH_3COO)_2$, minimal amount of CaCO3 was detected at the temperature of 521c and 676c, respectively.

In the case of treatment with active bacteria, CaCO3 decomposition was more obvious as two peaks were observed for each material. Specimens treated with Bs in sol-gel +$CaCl_2$ and Bs in sol-gel +$Ca(CH_3COO)_2$had an initial and final peak of 610°C and 735°C, 628°C and 768 °C, respectively. Therefore, this support the hypothesis of ONYAMA et al (1995) where he reported that when $CaCO_3$is heated at a rate of 10°C /min, $CO_2$is expected to be released at a temperature ranging from 690-760°C. Furthermore, it can be concluded from the above findings that the first peak represents $CaCO_3$decomposition while the second peak corresponds to the evaporation of another substance produced by the bacteria.

XRD test revealed that the crystalline material consisted mainly of calcite; vaterite and aragonite, with the calcite being the most stable form i.e. when calcium chloride and acetate were used as a calcium source, calcite and vaterite crystals were observed, respectively. Previous studies by Galwey et al 1999, stated that aragonite and vaterite can be converted to calcite when subjected to a temperature of 455°C and between 350°C and 400°C, respectively i.e. above 600°C, $CaCO_3$ would be present in the form of calcite and will only correspond to the second peak in the TGA analysis.

Taking everything into account, it can be observed that the first peak was caused by the bacteria themselves as the peak was not detected when the autoclaved bacteria was used. Considering the water permeability results, a decrease in water permeability was observed when sol-gel with active or autoclaved bacteria was used. in contrast, TGA analysis showed that only active bacteria with sol- gel was efficient in sealing cracks due to its ability in precipitating calcium carbonate crystals which ultimately results in sealing or plugging the cracks.

2.4 The use of bacterial cell walls to improve the mechanical performance of concrete

Bacterial cell walls which comprise the cell surface are known to be central to MICP. Bacterial cell walls are negatively charged under alkaline environment which attracts calcium ions in the extracellular environment to react with carbonate ions and forms $CaCO_3$ minerals on the cell surface i.e. cell wall will act as a nucleation site.

Pei, R et al 2013, showed that cell walls are more effective than live bacteria in binding calcium ions and forming $CaCO_3$ crystals since live bacteria actively pump H ions across the cell membrane to compete with ca ions for binding to the negatively charged cell walls. In his study, Peri, R et al 2015 reported the effect of bacterial cell walls on the mechanical properties of cement mortar and quantification of the amount of $CaCO_3$ formed in cement mortar, Results showed that cell walls at a concentration of 3.3 mg/l(10x) and 0.33 mg/ml resulted in a 15.6% and 14.8% increase in compressive strength. Addition of dead cells had no effect at a concentration of 3.3 mg/ml but decreased at 3.3

mg/ml(10x) (for 28 days but not at the 7 days), live cells added at a concentration of 0.33mg/ml(10x) or 33.3mg/ml resulted in a slight decrease in terms of compressive strength.

In addition to cell walls, the effect of dead cells and live cells in accelerating cacor3 formation in Ca (OH)2 and Cacl2 solution was also examined. It was reported that CaCo3 formation was accelerated when Cacl2 was used as a solution in the presence of cell walls or dead cells of B. subtilis (Cacl2 reached max. at day 6), however, when $Ca(OH)_2$was used as a solution, only the cell walls were present in accelerating the formation of $CaCO_3$. The rational explanation for the difference in the carbonation rate relate to the fact that $Ca(OH)_2$ provide an alkaline environment for better dissolution and dissociation of carbonate ions.

The effect of other bacterial cell walls and dead cells along with peptidoglycan were also examined, results showed that cell walls of both gram positive (M.luteus) and negative bacteria (E.coli) accelerated the $CaCO_3$formation in Ca $(OH)_2$solution, whereas dead cells and peptidoglycan had no effect on the $CaCO_3$formation.

As mentioned above, dead cells of bacillus subtilis had no effect on the compressive strength, cell walls however increased compressive strength, the reason for this difference is that bacterial cells such protein and polysaccharides may carry negative charge under pH in $Ca(OH)_2$ solution which might interfere with the effect of negatively charged cell walls as nucleation site for further Caco3 formation.

Previous research showed contradictory results in regard to the effect of bacteria and nutrients on the mechanical strength. Gosh et al, showed that the addition of media had no effect on the compressive strength which contradicts with Jonkers et al finding's, where he showed that the addition of calcium lactate, peptone or yeast extract (components of media) caused a slight decrease in compressive results. As a result, the effect of $CaCl_2$, sodium bicarbonate, ammonium chloride, nutrient broth and urea were investigated, where the observed results were indistinguishable from the use of water. It's worth mentioning that the negative effect of $CaCl_2$could be eliminated by using a dosage between 0.5-2 % (a dosage of 25.2 mM ~0.28%)

Considering the effect on porosity, cell walls have shown a significant decrease in porosity by 2.47 and 4.64 % at 7 and 28 days respectively. The increase in compressive strength and the decrease in porosity can be explained in the following points:

- By converting the Ca $(OH)_2$ which is a vital source of calcium into a solid Caco3 with strong structural role i.e. increases compressive strength
- $CaCO_3$can then fill the voids present in concrete which decreases porosity by improving particle packing efficiency i.e. making the concrete denser.
- Sato, T (2008) and Lothenbach, B et al 2010, showed that direct addition of CaCo3 into the cement paste becomes nucleation centre for C-SH formation during cement hydration a i.e. increases cement hydration
- Another possible explanation is that cell walls carry negative charge which may act as a nucleation site for concrete hydration.

For explanation reasons, gram positive bacteria consist of a thick cell wall and inner cell membrane. Thickness that typically fall in the range of 20-80nm with a 50% peptidoglycan with a covalently linked polymers that includes teichoic acids and proteins. Gram negative on the other hand contain thin cell wall, typically 10nm, that is sandwiched between an inner and outer cell membrane.

It must be noted that cell wall was prepared in accordance to the literature provided by Mastromei G et al 2008. One issue associated with the experiment is the size distribution of the cell wall which was not addressed in this study.

2.5 Industrial application of biological self-healing concrete: challenges and economical feasibility

The phenomenon of self-healing concrete has been already under investigation since 1970, Malinskii Y 1970 examined the process of self-healing of cracks in polymers where he illustrated that the self-healing concrete occurs at the tip of a crack in an unloaded specimen which leads to an increase in strength during relaxation time (Malinskii, 1970). White at el 2001, developed a structural polymeric material that possesses the ability to automatically seal cracks along with recovering its structural strength, in his model the material was integrated into a microencapsulated healing agent that becomes activated upon crack formation. Polymerisation of the healing agent is then activated by contact with the incorporated catalyst, leading to the bonding of the crack face.

Species of the bacillus group are able to precipitate calcite on their cell constituents and their micro environment by conversion of urea into ammonia and carbon dioxide (Castanier et al. 1999;Hammes et al. 2003). As a consequence of urea degradation, the PH of the microorganism environment will increase and stimulates the deposition of carbon dioxide as a calcium carbonate in an environment that is rich in calcium.

A novel approach to re-establish corroded limestone is the bio-mineralisation of calcium carbonate. Bio-mineralisation can be defined as a biologically induced precipitation where the bacteria forms a local microenvironment that has ideal condition that permits the optimal extracellular chemical precipitation of mineral phases.

The term bio mineralisation can be defined as a biologically induced mineralisation where the organism morphs its local micro environment in order to create conditions that are suitable for the chemical precipitation of mineral phases extracellularly. Bio mineralisation often occurs from oxidation or reduction carried out by some microbial species with the formation of a recognised bio mineralised product. These reactions are of vital importance to the engineering industry, as microbial mining and microbially influenced corrosion, and play an important role in the microbial physiology and ecology. (Hamilton, 2003)

Microbial induced carbonate precipitation (MICP) has been considered as a promising approach for healing cracks by several authors. The concept of the MICP uses the hydrolysis of urea to place a resorting and protective layer of calcium carbonate on degraded lime stone.

The basic reaction of the calcoc carbonic system is:

CO_3 + Ca $\leftrightarrow$ $CaCO_3$ (Eq1)

(Kso = 3.8*10^9), where Kso is the solubility product

The super saturation level S, defined by the ratio of the ionic product, is considered as the driving force for CaCO3 precipitation

S = (Ca)*(CO3)/Kso (Eq2)

In the process of microbial urease activity, 1 mol of urea is hydrolysed intracellularly to 1 mol of ammonia and 1 mole of carbamate (Eq.3) which in turn disintegrates to produce additional 1 mole of ammonia and carbonic acid (Eq4) (Burne and Chen 2000)

CO $(NH_2)_2$+ H_2O → NH_2COOH +NH_3 (Eq3)

NH_2COOH +H_2O → NH_3+H_2CO_3 (Eq4)

As result of the above reactions or ureolytic metabolism, the carbonic acid and the two molecules of ammonia equilibrate in water and the ammonia becomes protonated to yield ammonium and hydroxide ions. This can be seen in Eq5 and Eq6 (Burne and Chen 2000)

H_2CO_3↔HCO_3+ H (Pk = 6.37) (Eq5)

2 NH_3 + 2H_2O ↔ 2NH_4 +2OH (Eq6)

Equations 4 and 5 cause a pH increase in the local micro environment (around the bacterial cell) and propagates in the bulk solution of the bacterial cell suspension. As a result of the PH increase, this will shift the bicarbonate equilibrium (Eq7)

$HCO3 + H + 2NH4 + 2OH \leftrightarrow CO3 + 2NH4 + 2H2O$ (Eq7)

Therefore, CaCO3 precipitation will occur with the presence of soluble calcium ions, as a result of the rise in the carbonate concentration and the increase in S (Eq1). in other words, CaCO3 precipitation becomes complete when calcium ions are existent and the reaction between carbonate ions and calcium ions, resulting in the deposition of white precipitate.

According to Hammes et al 2002, crystal nucleation sites are essential for an efficient deposition of calcium carbonate, this is due to the fact that bacterial cell walls are negatively charged and calcium ions can be bound to it. Furthermore, this fact is associated with the release of carbonate ions from the hydrolysis of urea which results in the deposition of calcium carbonate crystals on the cell wall , figure 10

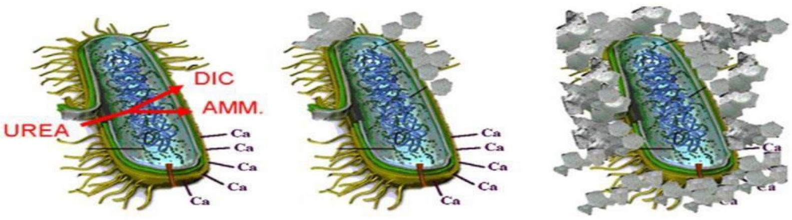

Figure 10 overview of the ureolytic carbonate precipitation at the microbial cell wall. DIC: dissolved inorganic Carbon; AMM: ammonia. (Muynck et al 2008)

2.6 Screening of bacteria and concrete compatible protection materials

Ersan et al 2015, evaluated the effect of various promising protection materials for the so-called self-healing in order to examine the influence of bacterial agents, strain specific nutrients and protection materials on the mortar setting and strength properties.

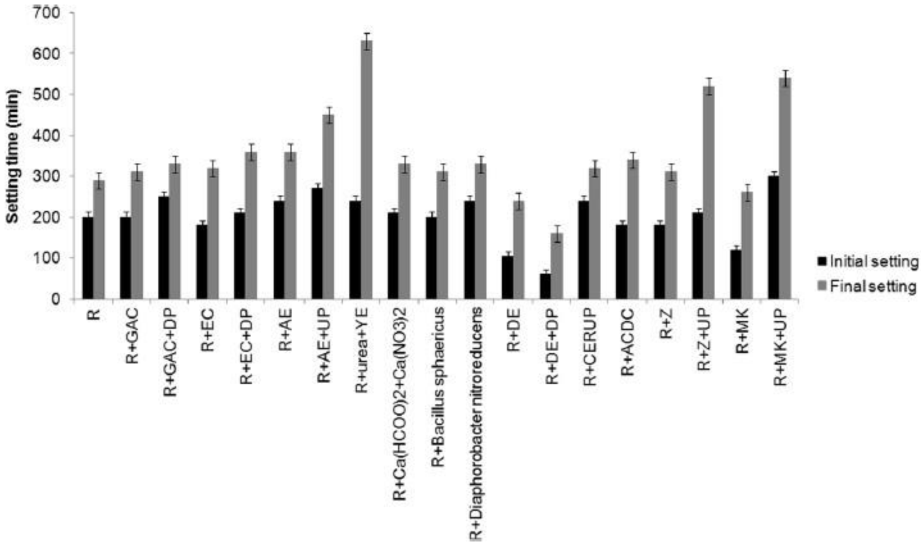

*Figure 11 I*nitial and final setting times of different mixtures, *Ersan et al 2015.*

Figure 11 initial and final setting times of different mixtures

In the same study, R+GAC+DP and R+EC+DP showed great improvement in terms of compressive strength at 7 and 28 days compared to reference, Table 2. Also, the addition of granular activated carbon (R+GAC) resulted in a 13% increase in compressive strength. On the other hand, the addition of zeolite as a protection material showed a negative effect on the compressive strength. Furthermore, the study recommended the use of Pozzolan particles that are fine enough to provide an enhanced superficial reactive area for the solid-state chemical reaction.

Mixture	7 days	28 days	result
R	50.3±1.6	58.6 ±2.0	Not recorded
R+ B. sphaericus	18.5±1.4	23.9±1.0	60% decrease
R+Diaphorobacter nitroreducens	43.4±0.7	52.3±1.5	Not recorded
R+urea+yeast extract	38.0±0.5	49.3±0.4	No major effect
R+Ca(NO3)2 + Ca)hcoo)2	52.0±2.1	62.6±1.8	Slight increase

R+DE	46.2±2.0	56.5±0.3	No major difference on 7 and 28 days
R+EC	55.0±2.3	57.4±9.3	50% decrease
R+GAC	54.9±1.7	66.4±1.3	13%increase after 28 days
R+MK	55.2±0.9	63.1±0.4	Not recorded
R+Z	39.2±9.5	46.4±4.3	Negative effect
R+AE	32.8±0.8	36.0±1.7	36% decrease
R+DE+DP	49.1±1.7	59.9±1.4	No effect
R+EC+DP	57.8±0.4	67.8±1.8	Very good effect
R+GAC+DP	61.1±1.4	71.0±2.3	Very good effect
R+MK+UP	16.1±0.5	19.6±1.1	65% decrease
R+Z+UP	37.9±0.5	49.0±1.4	25% and 16 decrease
R+AE+UP	17.3±3.4	20.3±3.4	65% decrease
R+ACDC	48.2±2.8	63.1±1.5	Not recorded
R+CERUP	48.2±4.4	57.6±3.3	Not recorded
5% lsd	4.7	4.7	Not recorded

Table 2 The effect of different encapsulating material on the compressive strength of hardened concrete at 7 and 28 days, Wang et al 2011.

It's worth mentioning that in this study, the use of expanded clay particles was only conducted by replacing the aggregate content by expanded clay particles (by 50%).

Wang et al 2011, investigated the possibility to use silica gel or polyurethane as a carrier for protecting the bacteria. Results revealed that bacteria incorporated in silica gel demonstrated a higher activity when compared to polyurethane incorporation, in other words, more $CaCO_3$precipitated in silica gel (25% by ass) than polyurethane (11% by mass) based on the thermo-gravimetric analysis

Self-healing concrete can be achieved by different approaches, such as Secondary hydration of un-hydrated cement, addition of fibre and encapsulation of polymers. An alternative approach is the bio mineralisation of calcium carbonate which is more compatible with the concrete matrix and more sustainable option when compared to healing agents like expanded additives and polymers. Wang et al 2011 reported three mechanisms associated with bio carbonate precipitation:

- The distillatory sulfate reduction carried out by sulfate reducing bacteria under anoxic conditions.
- Degradation of organic acid
- Nitrogen cycle, in particular the degradation of urea by ureolytic bacteria which is easier to operate and control comared to the above approaches.

Healing efficiency was evaluated by measuring the strength regain and decrease in water permeability, also, the amount of urea decomposed was indicated by the amount of NH_4 produced. In his study, Wang et al 2011 used more than one component healing agent (figure 12), where bacteria along with nutrients and other agents in tube glass then embedded into the specimens during casting. When cracking occurs, glass tubes in the crack zone will break and the healing agent will seep out into the crack. Silica gel then forms as ca ions (from the concrete matrix and ad rom the healing agent that has been already incorporated in the specimen) and silica sol come together.

The word or term of silica sol is emanated from silicic acid sol and it represents a colloidal dispersion of silicic acid in water. Due to the fact that sol gel has suitable matrix porosity for transmitting molecules and ions in addition to good mechanical, thermal a, photochemical stability, biological inertness, it has been widely used as a carrier for micro-organisms such as bacterial cells, algae and yeast.

In previous studies by Tittelboom et al 2009 investigated the use of silica gel as a carrier for bacteria in order to manually heal cracks, where the mixture consisted of silica sol and bacterial suspension (bacterial cells and NaCl) which was applied into the cracks by using a syringe. As a result of high concentration of Na and Cl, gel formation started to take place; it's worth noting that the injection was repeatedly carried until the crack was completely filled. When silica sol turned into gel, test specimens were suspended in a medium that consisted of urea and calcium ions (Ca) and as consequence $CaCO_3$precipitation occurred. In regard to water permeability, specimens showed a decrease of about 3 magnitudes after the treatment.

Polyurethane (PU) which is a waterproof material was also used in this study. In contrast to previous studies, where PU foam and bacteria where applied externally. In this study however, PU foam along with the bacteria were applied internally for the purpose of automatic healing of cracks. In previous studies, Bang et al 2001 was first to incorporate bacteria in a PU foam to be externally applied for manual repairing purposes where the specimens containing PU and bacteria were cut into equal sized and placed directly into the crack, followed by incubation of specimens in a medium that consisted of urea and $CaCl_2$. Compressive test results revealed that specimens containing PU foam and bacteria had a 13% increase compared to specimens with PU foam only.

The survival rate of the bacteria was also tested in this study, the amount of urea hydrolysed by the bacteria was determined on the basis of the total ammonium nitrogen (TAN) measured in the deposition medium. Also, since 1 mole of urea produces 2 moles of NH4 (as shown in the chemical reactions), thus the amount of NH_4provide an indication of hydrolysed urea and hence $CaCO_3$.

The bacterial activity after incubation was also calculated on the basis of bacterial ureolytic activity which is the ability to decompose urea and on the basis of carbonate-genesis activity which is the ability to for $CaCO_3$precipitation. Bacterial ureolytic activity was defined as the amount of urea decomposed by bacteria in the urea solution, which was calculated by measuring the urea conductivity of the solution. Since 1 mole of urea provide (Dunn, n.d.) 2 moles of NH_4 and 1 mole CO_3, the higher the urea hydrolysed the higher the conductivity of the urea solution, as shown in the equation below:

Urea decomposed (Mm) = conductivity (mscm^-1) *9.6

Bacterial carbonate-genesis was obtained by determining the amount of urea decomposed in the deposition medium. Therefore, the amount of urea decomposed in deposition medium represents the amount of $CaCO_3$that is formed.

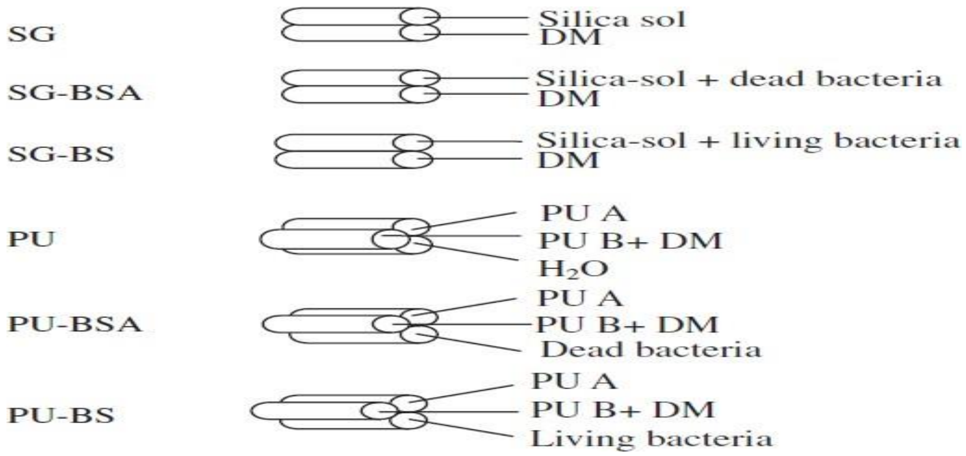

Figure 12 Glass tubes with different healing agents used by Wang et al 2011.

In regard to bacteria incorporated in silica gel and polyurethane foam, test results showed that dead bacteria did not increase the conductivity of urea' s solution. Figure 12 shows the specimens prepared with different agents. The addition of silica gel and dead bacteria to the urea solution resulted in an increase in conductivity for the first 24h due to the release of Na and Cl ions which were used to transform silica into gel. The conductivity in the urea solution with the addition of free bacterial cells resulted in a rapid increase due to the volatilization of NH_3formed from NH_4, this increase however decreased after 24h before becoming stable.

The conductivity in the urea solution with the addition of silica gel incorporated bacteria increased at a slower rate compared to free bacteria cells, the increase in conductivity is due to the diffusion of $NH_4$and CO_3through silica gel and into the solution (NH4 and $CO_3$are produced when bacterial cells inside the silica becomes in contact with urea and decomposes it). Furthermore, the slower decomposition is related to the fact that bacterial activity became limited or decreased after incorporated into silica gel, in other words it took more time to hydrolyse the same amount of urea than non-incorporated. Another reason that might attribute to the decrease in conductivity might be the retarding effect of gel on the diffusion of urea.

Urea solution with SG + BS had slightly higher conductivity than free cells due to trapped ammonia inside the silica gel and couldn’t escape anymore. Urea solution with polyurethane or polyurethane and dead bacteria had a limited increase in conductivity i.e. no urea decomposed. In contrast, polyurethane with live bacteria had a recognizable increase at a slower rate than non-immobilised bacteria; the slower increase was due to the influence of the polyurethane foam. Furthermore, no urea decomposed in the deposition medium with silica or PU alone or silica gel with dead bacteria or PU foam with dead bacteria.

Considering the strength regain which was performed in this study, despite the fact that silica gel enables more $CaCO_3$precipitation than PU foam, silica gel has a limited strength to contribute to the overall strength regain. PU foam on the other hand, is an organic polymer and contributes to the strength regain by making the bond between the polymer and the crack wall stronger. Therefore, higher strength regain can be obtained in PU foam.

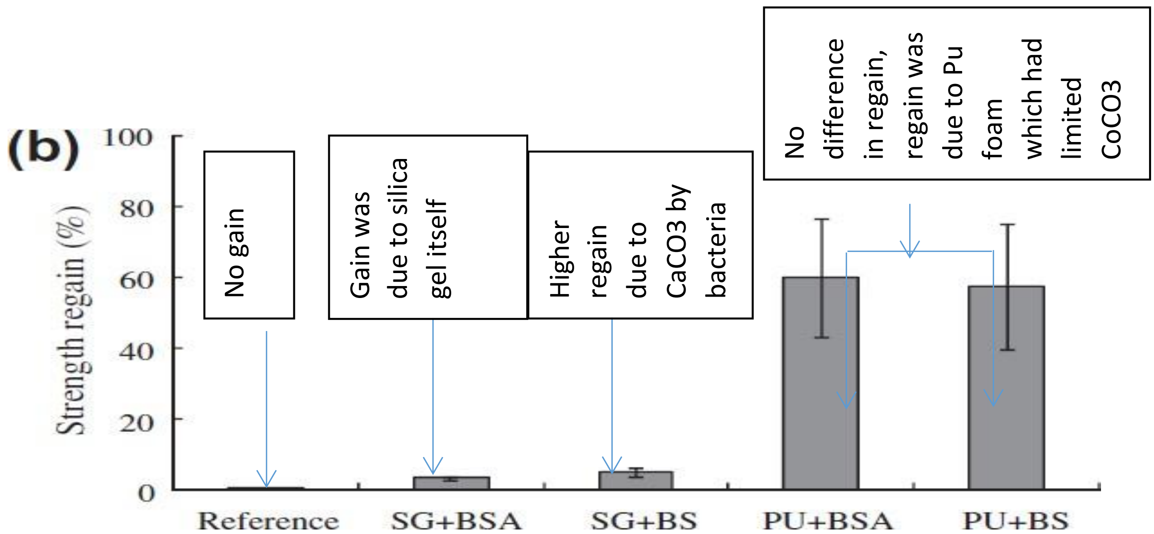

Figure 13 Strength regain of cracked specimens*, Wang et al 2011.*

Considering the water permeability test, test results (Figure 14) for specimens with only PU were similar to that with reference i.e. cracks healed with silica gel had a limited capacity to decrease water permeability. Silica gel and bacterial suspension (living bacteria resulted in a decrease in water permeability of about two orders in comparison to the reference, living bacteria with silica gel also decreased water permeability, the decrease in permeability is attributed to the $CaCO_3$precipitation which plugged the crack.

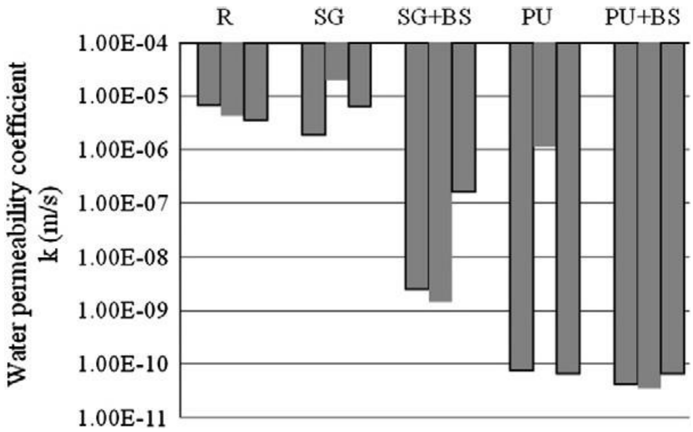

Figure 14 Water permeability of specimens after being healed, *Wang et al 2011.*

Specimens incorporated with polyurethane (with or without bacteria) illustrated a lower permeability compared to specimens with silica. Polyurethane and live bacteria had a lower permeability than those specimens of PU without living bacteria, which is believed to be due $CaCO_3$which filled the pores of some PU foam.

All in all, higher ureolytic activity were obtained when using silica gel as a carrier i.e. more calcium carbonate precipitation, hence the difference between silica gel and silica gel with incorporated bacteria. Since PU foam is waterproof material there should be a large decrease in water permeability when used alone, however its effect was not as obvious as silica gel, because bacteria incorporated in polyurethane showed less $CaCO_3$yielding when compared to silica gel. From the SEM analysis it was noticed that particles were more uniformly distributed in silica gel as PU foam were more viscous.

Therefore, more healing efficiency in terms of strength re-gain and water permeability was obtained when using polyurethane and bacteria

2.7 Calcite precipitation induced by polyurethane immobilised bacillus pasteurii.

There are several properties that can aid in sustaining environmental changes in the concrete matrix, such properties are thought to offer reinforcement against these conditions and must be considered in practical use for remediating cracks, such as:

- High retention of filling materials containing micro-organisms
- The reduction of permeability
- High bonding strength between crack filling and concrete

In an attempt to seek alternative encapsulation materials, Bang et al 2000 investigated the viability of microbial cells encapsulated in polymers where such proposal is expected to help the cells or bacteria in retaining high metabolic activities and protect them from adverse environmental changes. Previous studies by and Wang et al, 1993 used polyurethane as a carrier for enzymes due to its mechanically strength and its biochemically inert characteristics. As a result of condensation of polycyanates and polyols this enables polyurethane to make open cells foam, when small molecules join together to make long molecules i.e. when polymerisation occurs, carbon dioxide diffuses away from the matrix leaving behind pore spaces, this results in a porous matrix that increases the surface area and minimises the diffusion limitation which is a common disadvantage of polymers such as acrylamide, alginate and carrageenan. Studies by Fukushima et al 1978 and Oreilly et al, 1989 introduced a variety of hydrophilic PU polymers that help in overcoming difficulties related to diffusion limitations.

Therefore, this study examines the amount of $CaCO_3$ and ammonia produced when using polyurethane as a bacterial carrier. The effect on the tensile, elasticity and compressive strength were recorded in order to identify the effectiveness of microbial precipitation on crack remediation.

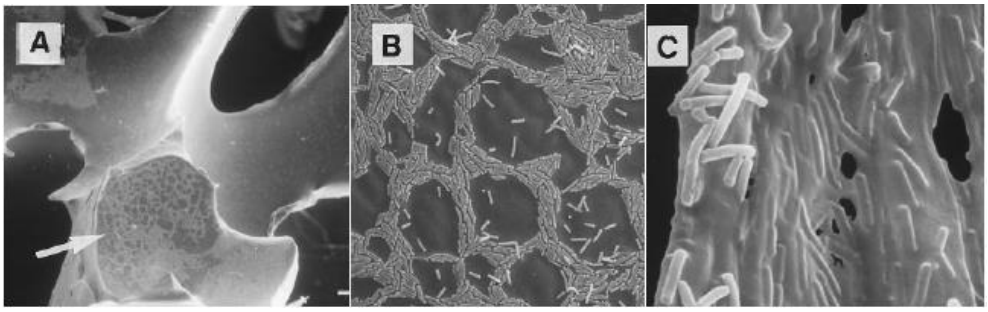

Figure 15 Scanning electron micrograph of bacillus pasteurii encapsulated in polyurethane, (A) distribution of PU with bacteria, (B) distributioin of bacteria on the PU surface, (C) bacteria embedded in PU matrix, Bang et al 2000

SEM analysis revealed that no apparent morphological damage has been done to the cell by using polyurethane, as shown in Figure 15A-C bacteria were entrapped throughout the polymer matrix where the cells were embedded in the matrix or adhered to the surface area with some clumping. It is worth mentioning that the physical properties of polyurethane such as tensile, elastic modulus and elongation were examined with the polymer incubated in saline (NaCl). Incubation of polyurethane immobilised bacteria in saline and citrate buffer for more than 2 hours resulted in cell leakage, however this leakage is considered negligible as less than 5 cells leaked out from the entire polyurethane matrix encapsulated with 5*10^7 cell/ml.

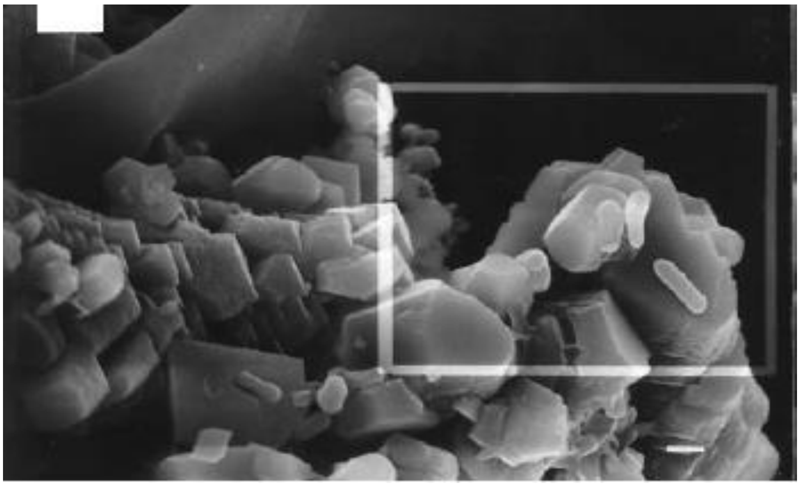

Figure 16 *Scanning electron micrographs of calcite precipitated by polyurethane loaded with bacteria, Bang et al 2000.*

Figure 16 shows scanning electron micrograph of polyurethane (with bacteria) incubated in a urea-$CaCl_2$medium for 12 hours. It is evident that bacteria were embedded in calcite crystals that grew throughout the polymer matrix i.e. displaying higher density in pores.

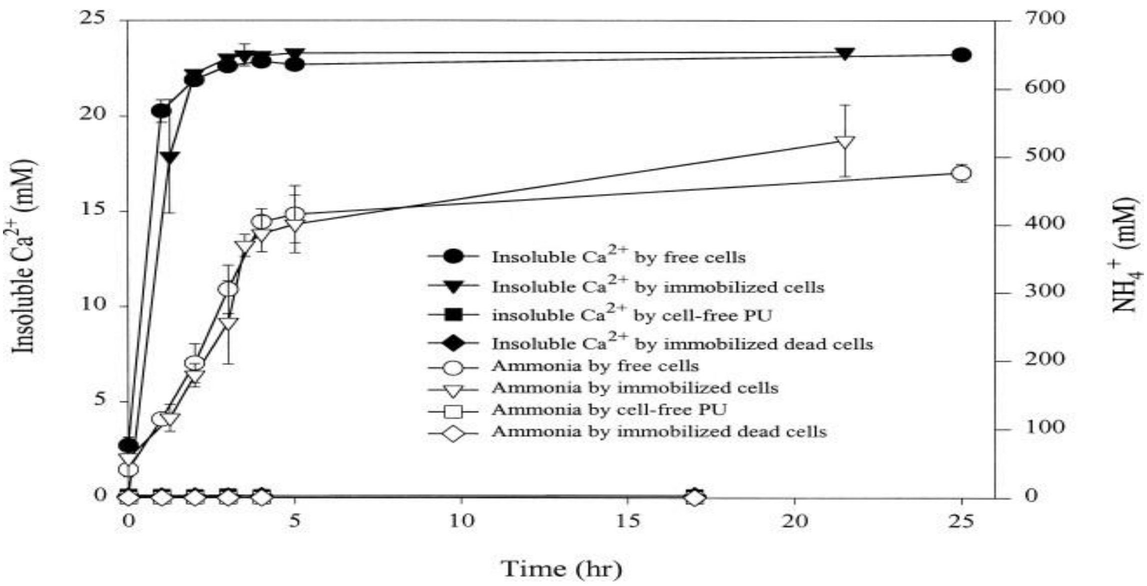

Figure 17 Calcite precipitation and ammonia production by free and immobilised bacteria, Bang et al 2000.

Figure 17shows calcite precipitation and ammonia production patterns by free and immobilised cells with both incubated with 5*10^7 cells/ml. it can be seen that both free and immobilised cells had calcite precipitation that was completed in 4 hours, where 98% of soluble calcium ions became insoluble. Considering polyurethane loaded with no cells or dead bacterial, neither calcite precipitation nor ammonium production was observed.

The effect of calcite precipitation on tensile strength elastic modulus of polyurethane was also investigated (as shown in figure 18). PU immobilised bacteria had an initial increase of 42% in tensile strength (21.18psi) compared to PU without bacteria (14.94 psi), however the tensile strength decreased as the incubation period in medium increased. The decrease in tensile strength was predominant in the first 12hours before reaching a final decrease of 21% at the end of day 7.

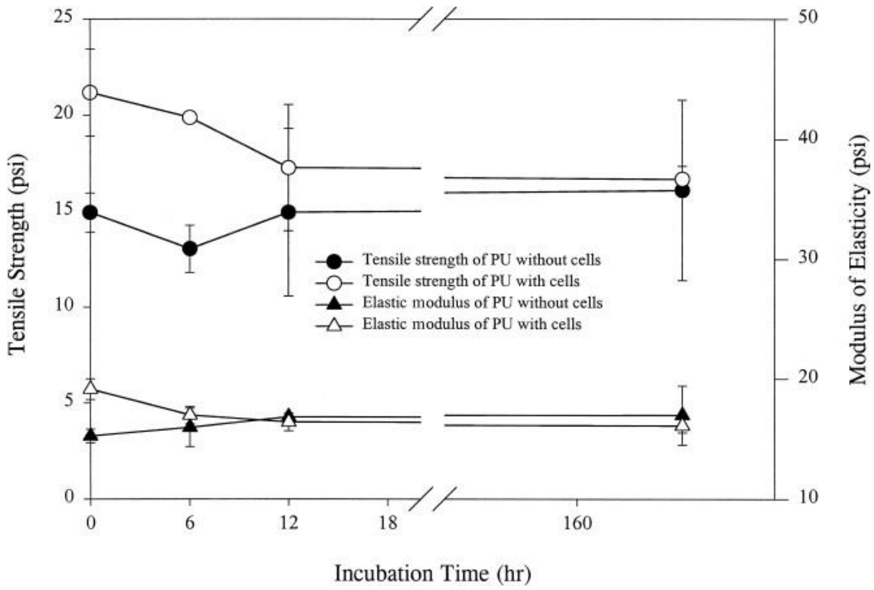

Figure 18 Effect of calcite precipitation on tensile strength and elastic modulus of polyurethane, Bang et al 2000.

In regard to the effect on the modulus of elasticity, polyurethane with bacteria had a 26% increase in stiffness compared to PU with no cells, similar to the tensile test results, the variation in stiffness was evident for the first 12hours until it reached stable at the end of day7, where the stiffness of PU with cells was slightly lower than PU without cells, Figure 18.

Incubation time	Compressive strength (psi)			
	Cell concentration (cells crack^{-1})			
	0	5×10^7	5×10^8	5×10^9
7 days	4261 (±172)	4172 (±179)	4599 (±210)	4772 (±94)
28 days	4191 (±56)	4198 (±309)	4266 (±2)	4303 (±139)

Table 3 *Compressive test result of specimens treated with PU immobilised B. pasteurii, Bang et al 2000.*

Considering the compressive test results of concrete specimens treated with different cell concentrations (Table 3), the highest compressive strength was observed in specimens treated with a concentration of 5*10^9 incorporated cell cracks for 7 days incubation, longer incubation period of 28 days resulted in a slight increase in compressive strength in comparison to 7 days incubation.

The fact that PU immobilisation is accompanied with CO_2 due to foam formation, this raises some concern in regard to the its effect on the ureas activity of the bacteria, however the cell free from showed little $CaCO_3$precipitation i.e. CO_2generated from polymerisation has no effect on the calcite precipitation in the concrete matrix, instead porous polyurethane polymer served as an additional nucleation site for calcite crystals.

Calcite precipitation resulted from polyurethane immobilised bacteria did not generate an increase in tensile strength, a rational explanation for this, is that the increase in tensile strength and modulus of elasticity is dependent upon the chemical bonding between the polyurethane foam and the precipitated calcium carbonate. The initial increase in tensile strength and modulus of elasticity indicates that there is a possible interaction between the foam and the precipitated calcite, however, this interaction appeared to be weakened when immersed in medium i.e. resulting in a decrease in both tensile and stiffness.

Previous studies by Ramakrishnan et al 1999 and 1998, showed that the use of sand and bacteria in remediating cracks have increased the compressive strength, however, SEM analysis revealed that most of the calcite precipitation adjacent to the crack surface area. Bang et al, 2000 however, calcite precipitation occurred thorough out the concrete matrix i.e. resulting in a better healing efficiency. The main advantage of using polyurethane is that it provides a shelter for the bacteria against the extreme high alkalinity in the concrete environment in addition to serving as a nucleation site for calcite crystals.

Therefore, polyurethane has proved to be an effective tool to be used in microbiologically induced calcite precipitation in concrete cracks, on the other hand it was noticed that ureases activity was reduced as a result of using polyurethane and that in turn reduced the calcium precipitation rate, therefore it is of vital importance to understand the biochemical behaviour of urease as it plays a vital role in enhancing the calcite precipitation.

2.8 Role for calcium sources in the strength and microstructure of microbial concrete

This study investigates the uniaxial compressive strength, splitting tensile strength in addition to water absorption of microbial mortars treated with different calcium sources such as $CaCl_2$, $Ca(CH_3COO)_2$ and Ca $(NO_3)_2$. In most studies calcium chloride as a calcium source for microbially induced precipitation have been used, due to the fact that calcium chloride can be very detrimental for concrete structure when it penetrates through the crack it will cause loss of protection or de-passivation and electrochemical corrosion to the steel bars, other calcium sources such as calcium nitrate, calcium chloride and calcium acetate have been investigated in this study in order to reduce the adverse effects of calcium chloride, Zhang et al 2014.

Previous studies have been done on the aforementioned calcium sources which were applied as a surface treatment. Van Tittelboom et al 2009 compared the crack healing potential of bacteria and traditional repair techniques by means of water permeability, ultrasound measurements and visual examination. In his study it was observed that all three calcium sources performed equally in terms of water permeability. De muynck et al 2006, reported the effects of bacterial CaCO3 precipitation on parameters affecting the durability of concrete mortars, in his study it was reported that the type of bacterial culture and nutrients composition or deposition medium had a significant impact on the crystal morphology of$CaCO_3$, where specimens treated with CaCl2 had a rohmbohedral $CaCO_3$crystals and spheroidal when $Ca(CH_3COO)_2$ was used. In contrast, li and qu used calcium chloride and calcium acetate as a calcium source for preparing microbial specimens for repairing cracks, in their findings, it was reported that calcium sources had no impact on real impact on the compressive strength however the repair have resulted in an increase of 15% in regard to the compressive strength.

It's worth mentioning that the bio grouting and preparation of the test samples were in accordance to Yang and Cheng 2013. Table 3 shows detailed parameters of the test samples which were prepared in this study.

Group		A			B			C			D		
Pumped Batches[a]		1			2			3			3		
Urease activity (mS/cm/min)		1.20			1.00 ± 0.08			1.10 ± 0.09			2.35 ± 0.15		
Biomass (OD_{600})		2.96			3.08 ± 0.25			3.24 ± 0.23			1.81 ± 0.14		
Environmental temperature (°C)		19			21 ± 1			21 ± 1			29		
Calcium source		Cl^-	NO_3^-	CH_3COO^-	Cl^-	NO_3^-	CH_3COO^-	Cl^-	NO_3^-	CH_3COO^-	Cl^-	NO_3^-	CH_3COO^-
Number of samples	Dry density [b]/water absorption[b]	7	5	11	9	7	12	11	13	6	5	6	5
	UCS [b]	0[c]	2	4	8	6	10	8	5	8	5	6	5
	BTS[d]		/		4	5	9		/			/	
	MIP		/			/			/		1	1	2
	SEM	1	1	1	1	1	1	1	1	1	3	3	3
	XRD	1	1	1		/			/		3	3	3

Table 4 Parameters of the samples used, Zhang et al 2014.

In this study it was observed that the average dry density of specimens treated with $Ca(CH_3COO)_2$ was the same as the sample treated with $CaCl_2$in group D and larger than group B and less than the sample in group C. in regard to the water absorption which was needed to be tested in order to assess the material properties, the effect of calcium sources on water absorption was uncertain.

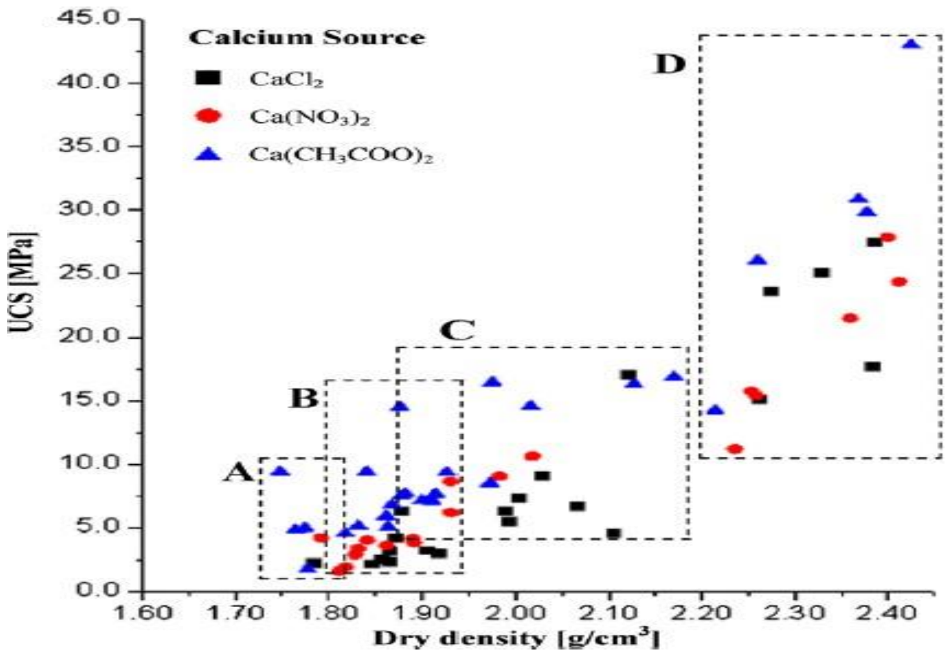

Figure 19 C*ompressive strength against dry density of samples treated with different calcium sources, Zhang et al 2014.*

Considering the compressive strength results, Figure 19shows that the compressive strength increases as the dry density increases where the compressive strength is linked to the amount of $CaCO_3$ precipitated, it can be seen that samples treated with calcium acetate illustrated a higher compressive strength than those samples treated with calcium nitrate and calcium chloride. Figure 20 shows the effect of different calcium sources on the dry density, it can be seen that the compressive strength was greatly enhanced when treated with calcium acetate, the compressive strength of the acetate samples was 2.45, 1.58 and 1.32 times that of those samples treated with calcium chloride in groups B, C and D respectively.

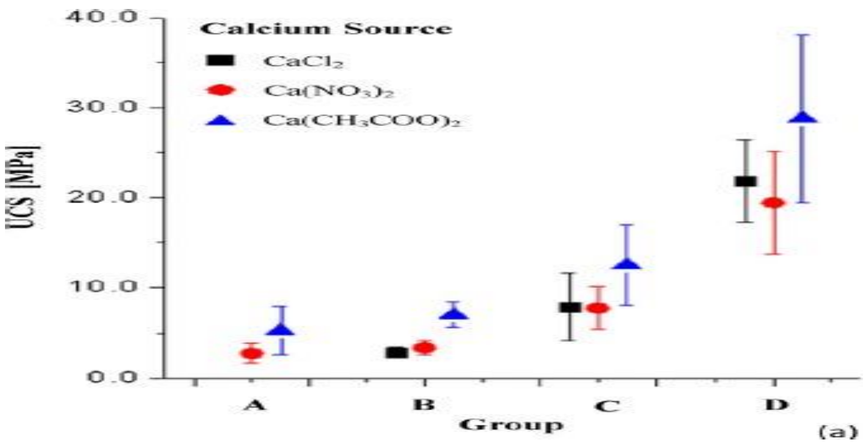

*Figure 20 T*he average and standard deviation values of compressive strength of each group, Zhang et al 2014.

Figure 20 the average and standard deviation values of compressive strength of each group

A mercury instruction porosimeter was applied on three samples from group D in order to assess the effect on the pore size distribution. The bio-grouting process resulted in a decrease in pore size of the originally loose sand and the porosity decreased as the dry density increased. Considering the Nitrate-1, chloride-1 and acetate-1 where they had a similar compressive strength, the pore size distribution of chloride and nitrate samples were similar with the same mode pore diameter of 14µm, while the acetate-1 sample appeared to be larger with a mode pore diameter of 23µm. in regard to the acetate samples, it can be seen that the mode pore size diameter of the acetate-1 was reduced by 50% in acetate 2, from 23 to 11 respectively, therefore it can be concluded that the higher the strength the lower the median pore diameter and the more uniform the pore size distribution (Table 5).

Table 4 mercury intrusion test results

Sample	Calcium source	Dry density	UCS	Water absorption	Porosity	Mode pore diameter	Median pore diameter
Nitrate-1	Nitrate	2.4	27..9	2.71	12.4	14	13.1
Chloride-1	Chloride	2.39	27.6	2.98	12.1	14	12.2
Acetate-1	Acetate	2.26	26.0	4.2	14.7	23	7.3
Acetate-2	acetate	2.43	43.0	2.5	10.6	11	3.4
Loose sand	-	-	0	-	45.4	146	144

Table 5 Mercury intrusion test results, Zhang et al 2014.

From Figure 21it can be seen that the median pore diameter of the acetate sample is 7.3 which is lower than the chloride and nitrate samples where they had 12.2 and 13.1 respectively. However, the pore structure of samples treated with calcium acetate illustrated a more uniform distribution over a wide range (from 0.002 to 20) when compared to the chloride and the nitrate samples.

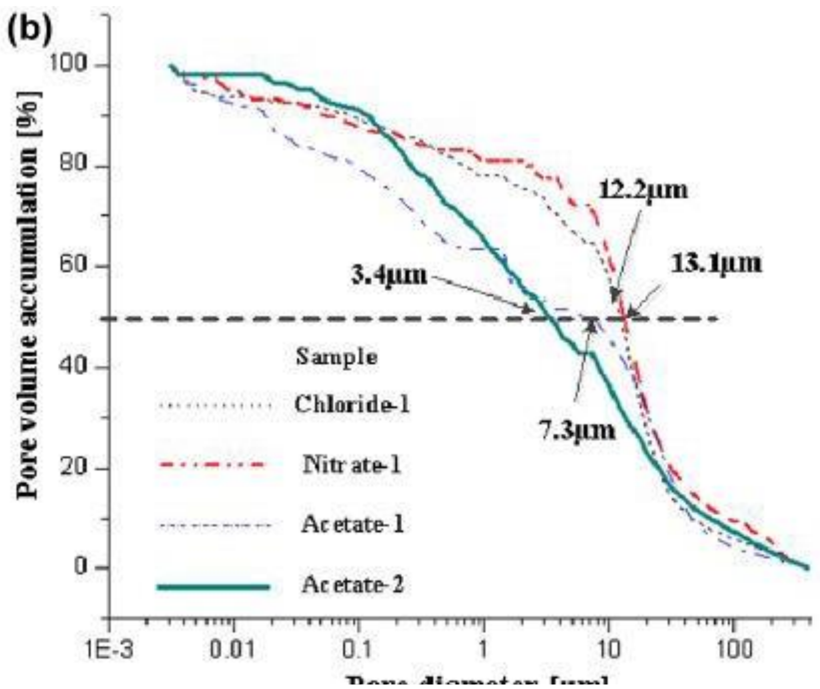

Figure 21 Pore size distribution of the microbial mortars, Zhang et al 2014.

SEM and XRD analysis were also performed in order to understand the external morphology, chemical composition, the crystalline structure and orientation of the microbiologically induced calcite precipitation samples, SEM analysis revealed a hexahedral structure (most likely calcite) for the calcium chloride and nitrate samples in groups A, B and C but the crystal surface of the nitrate samples was rough. Samples in group A treated with calcium acetate had both hexahedral and acicular crystals (most likely aragonite) with 5-10μmin length. The acicular crystals in the acetate samples in groups B and C were more than spherical and lettuce likes crystals. Considering the samples in group D, a large amount of calcium crystals was observed in the samples treated with chloride, the crystal surface was smooth and hexahedral structure. In the nitrate samples, hexahedral structure with a rough surface was observed which consisted of many small prismatic crystals of about 10 in length, while the acetate samples had acicular crystals of about 30 in length.

The XRD test revealed that samples treated with chloride and nitrate in group B had a calcite crystal while the acetate samples within the same group composed of 88% aragonite and 12% calcite. On the other hand, XRD pattern for group A showed that samples treated with acetate had calcium carbonate that composed of 90% calcite, 6% aragonite and 4% vaterite. Previous studies showed that calcium carbonate has crystals that is composed mainly of calcite, vaterite and aragonite, with calcite being the most common amongst the three, however due to some organic molecules' aragonite and vaterite precipitation may be induced therefore aragonite crystal structure was observed in the samples treated with calcium acetate.

To conclude the calcium source is of vital importance as it will change the pore structure of the sample and this will indeed affect the compressive strength of the sample, this was demonstrated in the acetate samples as they distributed more uniformly over a wide area when compared to the other two calcium sources, therefore calcium acetate is believed to be a better calcium source when compared to nitrate and chloride. (group D showed a superior urea's activity and a higher compressive strength)

Jonkers et al 2006, attempted to exploit the potential to apply calcite precipitating bacteria as a crack healing agent in concrete, in his study, he investigated the potential of four strains to precipitate calcite, produce spores, survive concrete production and finally heal cracks.

This study proved that alkaliphilic endospore forming bacteria embedded in the concrete matrix can actively precipitate calcium carbonate minerals, when water seeps in through the freshly formed cracks, endospores will be activated. for mineral precipitation to occur, active cells require organic substrate that can metabolically be converted to inorganic carbon which then reacts with the present free calcium to precipitate calcium carbonate. However, in this experiment, organic carbon was added as part of the incubation medium while it should have been embedded in the concrete matrix, where only water is needed to activate the concrete immobilised bacteria which can then convert organic carbon into calcium carbonate and by doing so seal freshly formed cracks.

All in all, Jonkers et al 2006 proved that incorporation of bacteria in high numbers at a concentration of 10^9cm^3 can still remain viable without affecting the strength of the concrete matrix, where these bacteria can precipitate more calcium carbonate to improve the efficiency in healing or sealing freshly formed cracks.

Spore suspension and tap water (control):	Number cm^{-3}:
B.cohnii	5.73 E7 (1.76-18.58)
B.halodurans	5.63 E6 (1.74-18.17)
B.pseudofirmus	7.98 E6 (2.63-24.24)
Tap water (control)	< 500
Cement stone samples:	
B.cohnii	1.15 E6 (3.80-34.80) [2.0]
B.halodurans	1.07 E5 (0.36-3.20) [1.9]
B.pseudofirmus	5.62 E5 (1.74-18.14) [7.0]
Control	< 500

Table 6 Estimated number of viable bacterial spores, *Jonkers et al 2006*

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The estimated number of viable spores retrieved from young cement stone, i.e. after ten days curing was between 1.9 and 7.0% of the number of viable spores present in the original spore suspension used for the preparation of cement stone samples, Table 6. According to Jonkers these number are still high even if the obtained numbers reflect truly viable spores, only one viable spore cell is theoretically enough for the microbial growth and calcite precipitation to occur.

Previous studies where bacteria were externally applied for concrete and monument crack repair (bang et al.2001; Ramachandran et al.2001; Dick et al.2006; Rodriguez-navarro et al.2003), CO_2what can locally reach high concentrations due to rapid metabolic conversion of organic compounds, will chemically react with Ca $(OH)_2$produced from C2S and C3S hydration reactions. The Ca $(OH)_2$that leaks out of the concrete's pore system reacts with $CO_2$and precipitates as calcite or any other calcium carbonate-based mineral.

In another study, five bacterial strains were isolated from alkaline soil samples and tested for their potential to form endospores, urease activity and their ability to precipitate calcium carbonate, based on the latter test results three stains were selected and identified by the 16s rRNA gene sequencing, namely, bacillus megatrium BSKAU, Bacillus licheniformis BSKNAU and Bacillus flexus BSKNAU. In an attempt to reduce the cost incurred from the substrate used for cultivating bacterial cultures, Krishnapriya et al 2015 used wheat bran as an alternative substrate.

Endospores are dormant, tough and non-reproductive structures that are capable of surviving and resisting very hostile environments when formed within the cell, they are also capable of opposing or withstanding heat, desiccation, chemicals and radiation. Upon exposure to hostile conditions, bacteria are able to form endospores within 6-8hours. Spores are metabolically inactive unless or otherwise exposed to their favourable conditions, then they can germinate into vegetative cell within 90mins of exposure (Geeta et al 2009). The formation of endospores is quite vital as they aid the bacteria in withstanding the harsh environment during the mixing of concrete and remain viable for a period of up to 200 years, stresses such as large mechanical stress and chemically induced stress can greatly affect the viability of the bacteria. Therefore, when air and water leak through cracks and fissures, endospores become activated by germinating into vegetative cells within the concrete. This however, initiates calcium precipitation and ultimately closes or seals off the crack.

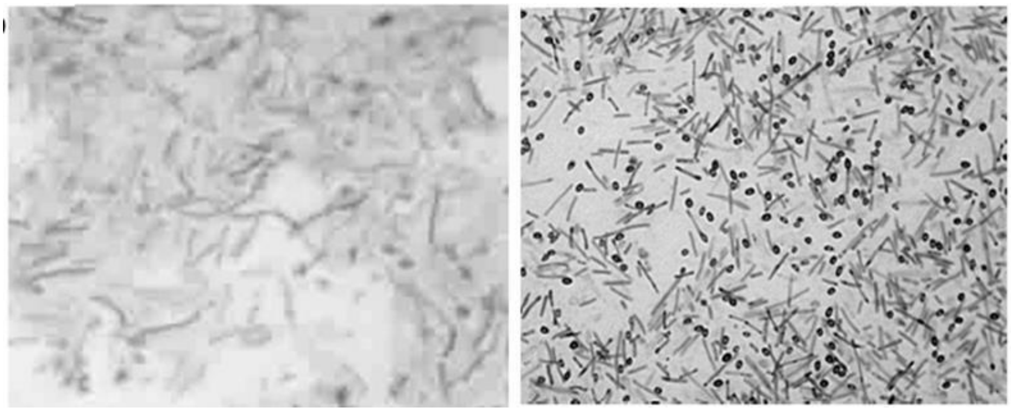


Figure 22 Endospore staining picture for Megatrium and B.flexus, Jonker et al 2010.

All bacteria are capable of forming spores, Figure 22shows two species forming different amounts of spores; vegetative cells are visible as light-coloured rods. The left hand picture represents B.flexus which had limited amount of spores and the right hand picture represents B. megatrium which had greater amounts of spores. As a result B. megatrium were able to completely seal of cracks after 81 days in addition to better compressive strength whereas B.flexus was able to partially heal the crack.

2.9 Microbial participation in the formation of calcium silicate hydrated (CSH) from bacillus subtilis

In attempt to use bacteria as alternative agent to form C-S-H gel, Afifudin et al 2011 investigated the potential of using micro-organisms to induce silica precipitation, in his study, untreated bacillus and chemically modified bacillus subtilis (CMBS) were prepared then incorporated in an aqueous solution of calcium hydroxide to examine the amount of silica precipitated by both untreated and CBMS to form C-S-H. Furthermore, the effect of incorporating such bacteria on the compressive strength was also reported in this study.

In regard to chemically modifying bacillus subtilis, the cell wall of the bacillus subtilis was chemically reacted with ethylene diamine solution (which was stimulated by reacting with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride solution) for the purpose of modifying so that it becomes electropositive; such modification is expected to enhance the binding of silicate from the silica acid during the incubation process. Therefore, bacillus subtilis was mixed with 0.5M ethylene diamine and 0.2 M 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide hydrochloride in order to become aqueous solution then stirred for 6 hours at room temperature. The mix was then centrifuged for 3 minutes at 10000 rpm then washed four times before re-centrifuged again; the resultant cells were finally and chemically modified.

Following the modification process, both untreated and chemically modified bacillus subtilis (CMBS) were separately incubated in a solution that contains H_4SiO_4 at room temperature then shaken for 10 days at 150rpm. After a 10 days period, the incubated cell which is expected to have absorbed silica was centrifuged for 3 minutes at 10000 rpm. It should be noted that that PH level of the silica solution was kept constant during the incubation process. For the purpose of forming C-S-H gel, the centrifuged cell resulted from the silica incubation in both untreated and CMBS, were vacuumed until it turned into powder then reacted with calcium hydroxide solution before vacuuming again for 2 days to remove excessive water from the reaction. The final produced was ready in the form of a powder and ready for the XRD analysis to examine the amount of silica absorbed by both cells.

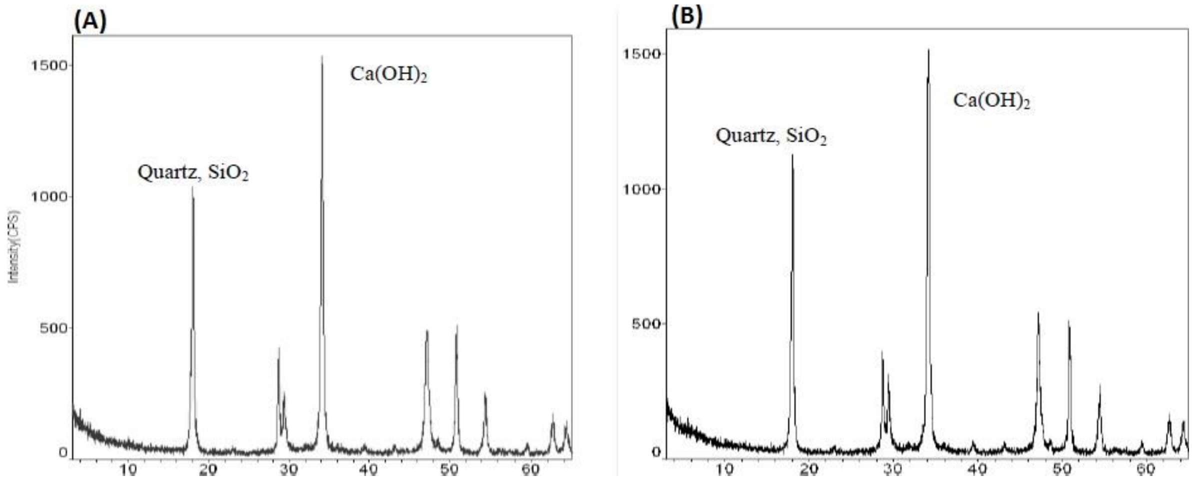


Figure 23 *XRD peaks (A) untreated bacillus subtilis (B) CMBS,* Afifudin et al 2011.

The amount of silica absorbed was detected by the presence of silica oxide, Figure 23shows the amount silica oxide absorbed by both untreated and chemically modified bacillus subtilis (CMBS) after 10 days incubation in silica solution at a PH of 5.5. it can be seen from the figure above that there is no significant difference in the amount of silica absorbed by both untreated and chemically modified bacillus subtilis, which is in contrast to Mera and Beveridge 2013 findings, where CMBS absorbed significant amounts of silica in comparison to the untreated bacillus subtilis.

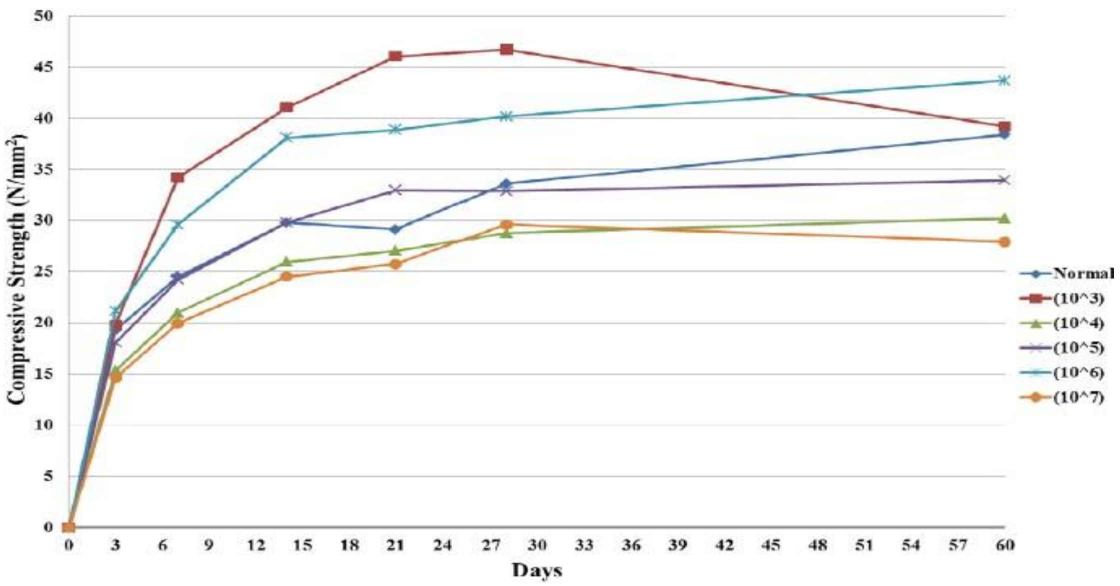


Figure 24 Compressive strength results for concrete specimens at different cell concentrations, *Afifudin et al 2011.*

Considering the compressive strength test, six specimens were prepared with or without bacillus subtilis to examine the effect on the compressive strength. As shown in Figure 24, It can be seen that concrete specimen at a cell concentration of 10^6 cell/ml showed the greatest compressive strength compared to the control specimen, such improvement in compressive strength is related to the silica precipitation that has occurred from bacillus subtilis which was deposited on the cell surface and within the concrete matrix and resulted in plugging off the small pores and finally sealing off the crack.

Concrete specimen prepared at a call concentration of 10^5 had no improvement in compressive strength, however concrete specimen prepared at a cell concentration of 10^3 cell/ml had a higher compressive strength for the first 28 days in comparison to control specimen, but a drop in compressive strength was observed at day 60 where the drop in compressive strength was reported as an unknown phenomenon.

To conclude, the inclusion of bacillus subtilis had a positive effect on the compressive strength results, the optimum cell concentration for bacillus subtilis was found to be 10^6 ell/ml where the maximum compressive strength was obtained (28% increase compared to control).

2.10 Effect of curing conditions

Self-healing systems comprising of microcapsules embedded with healing agents have been extensively studied in recent years mainly polymers and composites. Such approach for the use of self-healing concrete, without the need for human intervention, has been a hot topic in recent years. The concept of using microcapsules as a carrier for self-healing applications has been studied by Yang et al, where the addition of microencapsulated Mthylethacrylate based healing agent into carbon microfiber-reinforced mortar, proved to be efficient in improving crack resistance and toughness when subjected to fatigue loading. Calcium precipitation via bacterial metabolism is considered as an environmentally friendly and economical approach due to its promising potential and wide range of applications. MICP has been used in several engineering applications such as, repair of defects and flaws, consolidation of loose particles (especially in soil and sands) and protection of concrete and stone surfaces. The basic concept behind self-healing using microbial approach is that bacteria (spore/ cells) are added into the concrete matrix directly during mixing as part of the makeup water. Bacterial spores will remain dormant until the presence of cracks and fissures, where moisture and oxygen seep in through and result in activation of the bacterial spores. Under suitable conditions (such as temperature, PH etc.) bacterial spores activate initially with the help of micronutrients available in the spore, upon activation live cells will feed on the nutrients available and, finally precipitate $CaCO_3$ as a by-product.

Wang et al investigated the use of hydrogel as an encapsulating material for the self-healing applications. Bacillus Sphaericus LLMG 2257 was used in this study, 1ml bacterial spores at a concentration of 10^9 cells/ml were encapsulated into one hydrogel sheets during the initial preparation or synthesis of the pure hydrogel i.e. during the cross-linking stage, followed by freeze drying process to obtain micro encapsulated hydrogel powder.

Crack healing of efficiency of specimens with/without hydrogel encapsulated bacteria incorporated, was monitored via light microscopy and 3D X-ray CT for full quantification of the amount of precipitate. Specimens were subjected to different curing conditions, namely, 60% relative humidity (RH), 95% relative humidity (RH) and wet dry cycle, where specimens immersed in water for 1 hour and left to breath in air for 12hours.

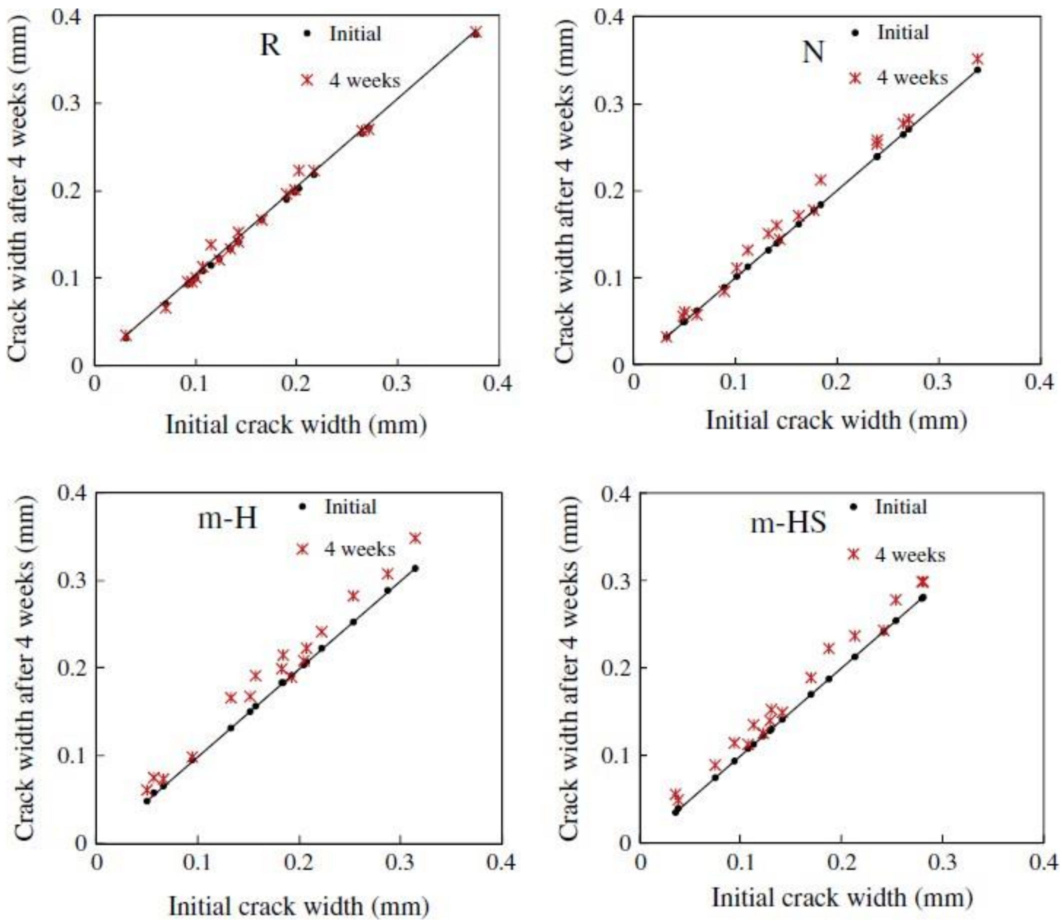


Figure 25 Change in crack width for specimens stored at 60% RH, *Wang et al.*

In this study, experimental work was divided into groups, where group R, N, m-H and m-HS represent control, control with nutrients, mortar with hydrogel and mortar with encapsulated bacterial hydrogel, respectively. Figure 25shows 28 days age cracked mortar specimens subjected to 60% RH. No major/significant healing observed including specimens with/without encapsulated bacterial hydrogel.

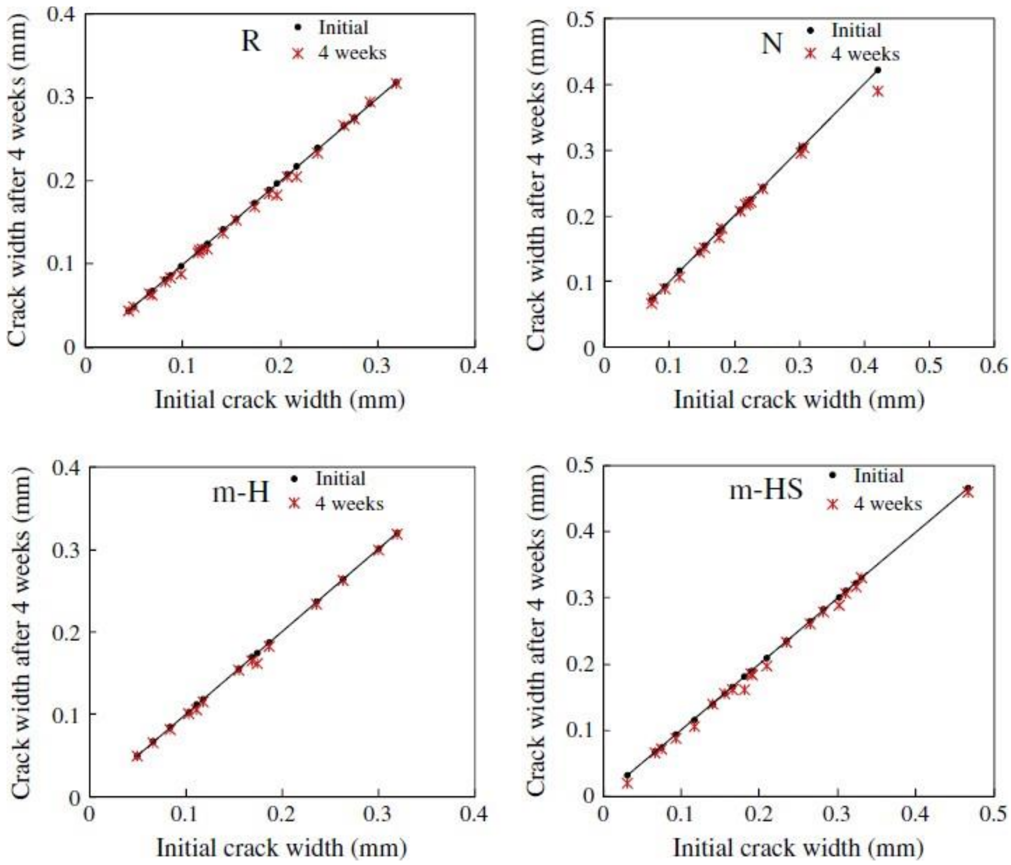


Figure 26 Change in crack width for specimens stored at 95% RH, *Wang et al.*

Similar observation was noted for specimens cured at 95%RH, where the crack's width at 28 days remained unhealed for all specimens, figure 26.

On the contrary, cracked specimens subjected to wet dry cycle showed significant healing, compared to specimens subjected to the 60% RH and 95% RH. Healing efficiency for all specimens was greatly affected by crack size, where the healing efficiency for control and nutrient specimens reduced to zero when the cracks were ranging from 0.25-0.7mm.

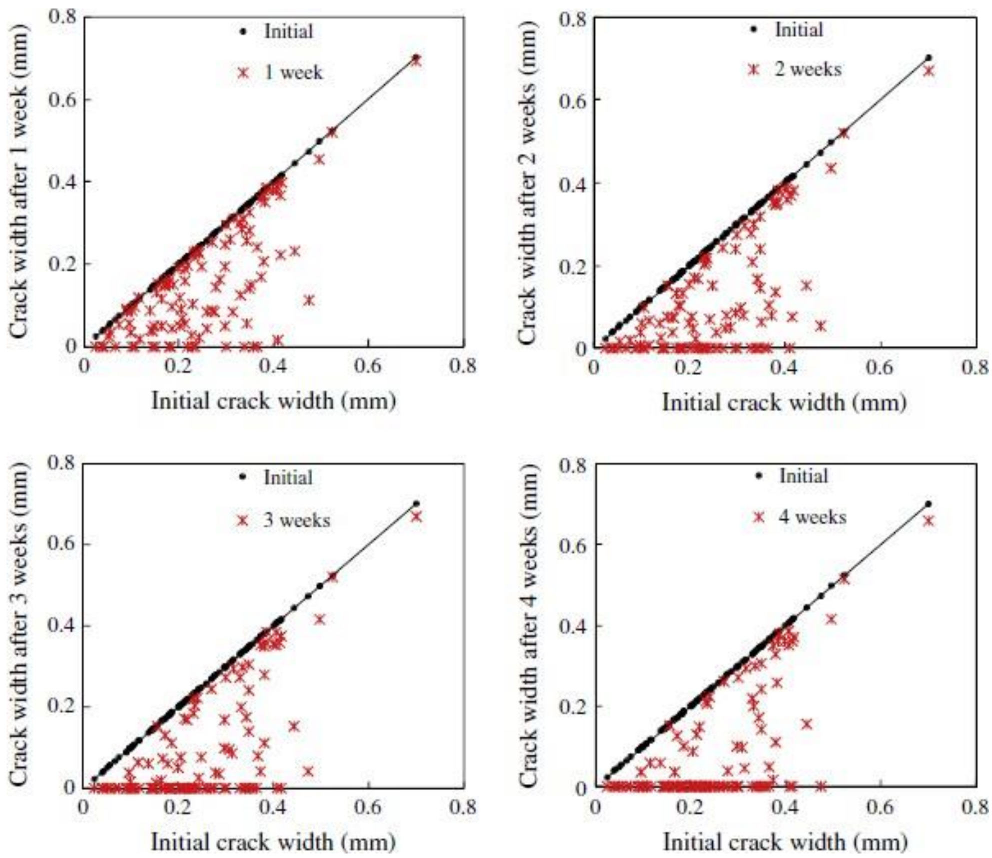


Figure 27 *Crack healing evolution in the specimens of m-HS under wet-dry cycles,* Wang et al.

Furthermore, Specimens with hydrogel only had healing ratio of 10-75% when cracks ranged from 0-0.25mm but was greatly reduced to less than 5% when cracks were larger than 0.25mm. The evolution of crack healing, under wet-dry cycle, in specimens with bio hydrogel (m-HS) is shown in Figure 27. It can be seen that higher healing capacity has been observed in comparison to the other groups. Complete healing was observed for cracks less than 0.05mm, the maximum crack healed over a period of 4 weeks was about 0.5mm, Figure 28.

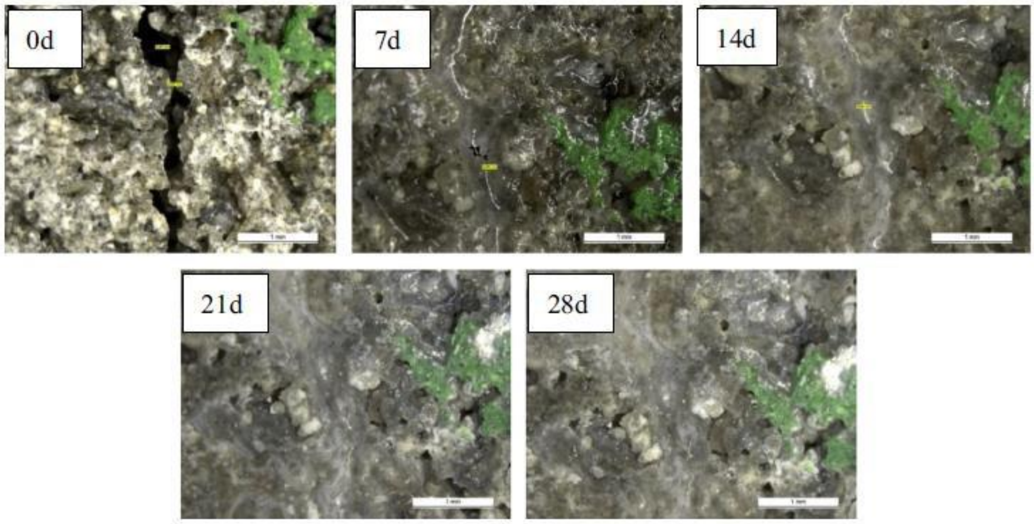


Figure 28 *Crack healing evolution in bio hydrogel specimen m-HS under wet-dry cycle,* Wang et al.

Figure 28 Crack healing evolution in bio hydrogel specimen m-HS under wet-dry cycle

The efficiency of crack healing ratio is highly dependent on the size of crack i.e. the healing ratio decrease as the crack size increase. As shown in Figure 29, specimens in group R and N healing ratios were greatly affected by crack size, where the healing ratio reduced to zero when the cracks were ranging from 0.25-0.7mm. The healing ratio for cracked specimens incorporated with hydrogel only (m-H), was in the range of 10-75% for crack of 0-0.25mm.

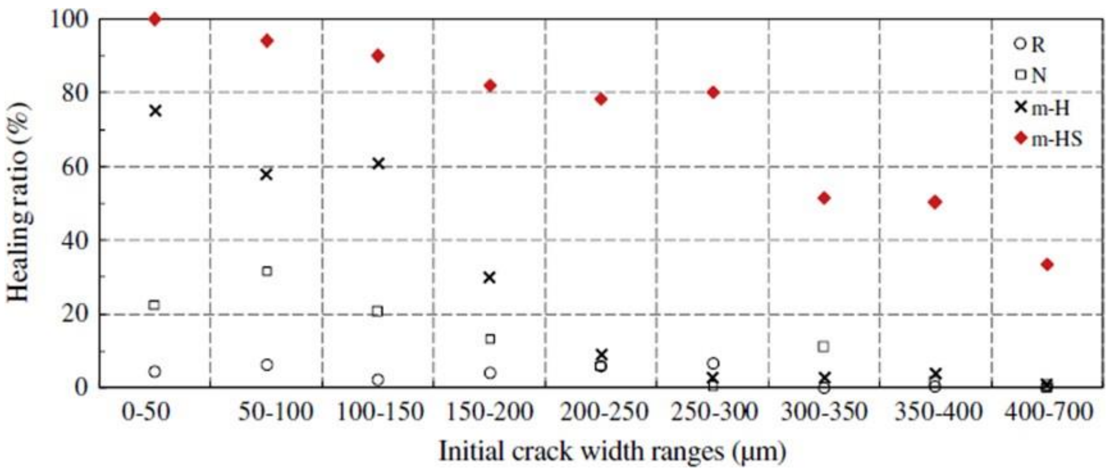


Figure 29 Average crack healing ratio for specimens with different cracks under wet dry cycle, *Wang et al.*

Higher healing ratios were observed for specimens loaded with bio hydrogel (m-HS). Crack of less than 0.05mm were completely healed, however, healing ratios of 80-95% was observed, when cracks where in the range of 0.05-03mm. Healing ratio was reduced to 30-50% for cracks ranging from 0.3-0.7mm. To summarise, bacterial specimens had a healing ratio ranging from 70-100% for cracks smaller than 0.3mm, which 50% higher than that in non-bacterial specimens.

The application of hydrogel as an encapsulating material has proved to be feasible in this study. Both autogenic and bacterial based self-healing system is water dependant. Up till now, water is provided to cracked specimens via full immersion. However, this approach is feasible or limited to certain application such as underground and underwater structure, but not for realistic environments such as dry conditions or wet from rain or snow. Therefore, for self-healing to be feasible to be realistic, it should occur without human intervention. In this study, hydrogel was able to supply sufficient water for self-healing to occur. Furthermore, bacterial specimens had 70-100% healing ratio for cracks smaller than 0.3mm. Maximum crack closure or bridging was 0.5mm at 28 days, in specimens with bio-hydrogel, while 0.18mm for specimens with hydrogel only.

Healing efficiency was greatly enhanced when mortar specimens subjected to wet dry cycle. Hydrogel has high water absorption and retention, in other words it promotes or facilitates autogenous healing. As a result, healing efficiency was enhanced in hydrogel embedded specimens (with or without bacterial spores). The healing ratio of mortar hydrogel embedded specimens was 70-100% for cracks of 0.05-0.3mm i.e. 50% higher than that of nonbacterial specimens. The enhancement in crack healing in bacterial specimens was due to calcium carbonate precipitation formed at the crack mouth. Where the impregnated bacterial spores germinate and activate up on suitable environmental condition such as availability of nutrient, water, oxygen, PH and moisture. Upon germination, spores will turn into live cells, due to urease enzyme and carbonic anhydrase, decomposes urea into carbonate ions to finally forming $CaCO_3$precipitation.

To conclude, this study portrayed the importance of incubation conditions for ordinary and bacterial concrete. Water is a crucial component in self-healing mechanisms for both bacterial healing and autogenous healing. Where the later requires water to promote the occurrence of the secondary hydration of un-hydrated cement, precipitation of $CaCO_3$ and swelling of hydration products. In regard to bacterial based mortars, water is absolutely crucial as spores require water for the precipitation to occur. Therefore, healing efficiency would be negligible without the presence of water. Wet dry cycle specimens with/ without hydrogel encapsulated spores had better healing efficiency due to the availability of more free water which facilitates both autogenous and bacterial healing. Wang et al, reported higher healing efficiency when subjected in water for 16h and 8 h in air, which again shows the importance of water. Furthermore, specimens embedded with hydrogel subjected to wet dry cycle, had higher healing ratio than the specimens without hydrogel in the same group. This is due to the fact that hydrogel possess the capacity to absorb and retain water. It is worth pinpointing that the healing efficiency can be limited as a result of the water evaporation when specimens exposed to air during the dry cycle.

In addition to autogenous healing, several studies have reported the use various healing agents for enhancing the healing capacity of concrete as a self-healing material (Schlangen et al, Dry et al, Joseph et al, Li et al). Such approach autonomously repairs cracks in concrete when embedded into the concrete matrix. Extensive research on self-healing materials has been done over the past years. However, the majority of the available techniques available are expensive, chemical based and require the human intervention.

A wide variety of self-healing techniques are available. However, microbial induced calcium carbonate (MICP) via urea hydrolysis has proved to be the most efficient and promising pathway towards a wide range of engineering applications. Bacterial cell/ spores along with the mineral precursors are directly embedded into the concrete matrix during the mixing process. Upon the presence of cracks, bacterial cells/ spores will activate as a result of moisture and oxygen travelling through the cracks and fissures, leading to a by-product of $CaCO_3$ due to bacterial metabolic process, and plugging of the cracks (Achal et al).

The potential of using calcite precipitating bacteria via urea hydrolysis for crack remediation or durability improvement has been investigated (De Muynck et al, Ramachandran et al, Bang et al, Dick et al). However, ureolytic enzymes were externally applied for mortar specimens or concrete structures. As a result, Jonker et al 2010 used a two-component healing agent comprising of bacterial spores and organic compound, added directly into the concrete matrix. Upon germination, bacterial spores′ catalysis the conversion of organic compounds into insoluble calcium carbonate, according to the following reaction:

$$CaC_6H_{10}O_6 + 6O_2 \rightarrow CaCO_3 + 5CO_2 + 5H_2O$$

However, the study reported that bacterial precipitation/activity was limited to young concrete specimens (1-7 days old), when the self-healing agent (bacterial spores and precursor) added directly during mixing. Such findings were linked to several reasons such as loss of viability of bacterial spores during mixing, inactivation due to high alkalinity.

Therefore, Jonker et al 2011 immobilized bacterial spores and organic precursor into expanded clay particles prior to adding into concrete matrix. In this manner, expanded clay particles acts as an internal structural element that provides protection for impregnated self-healing agent. Such approach, decreases the loss in viability of spores and increases the functionality of bacteria based self-healing systems. Expanded clay particles (1-4mm) were impregnated twice under vacuum, with the former being bacterial spores and the latter being organic precursor which comprises of calcium lactate (80g/l) and yeast extract (1g/l). The mixing proportions used by Jonker is shown in table 6.

In addition to monitoring the healing efficiency of cracked specimens via light microscopy, optical oxygen consumption measurements were taken as an indication to monitor the metabolic activity of aerobic bacteria during the conversion of calcium lactate into calcium carbonate.

Table 7 mixing proportions used in Jonker's study

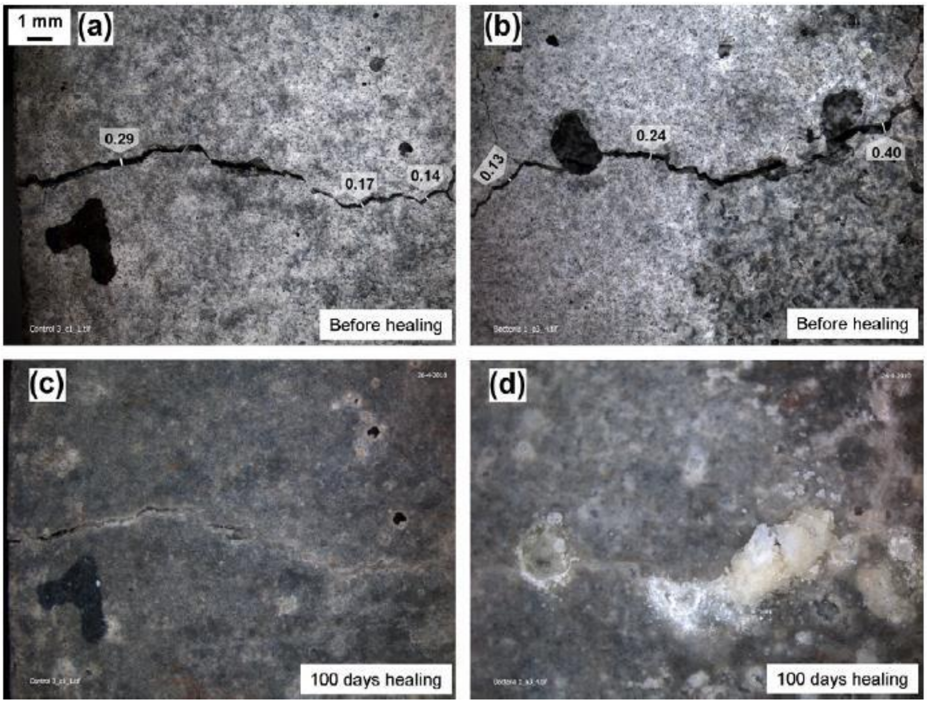


Figure 30 Crack healing process in control before (a) and after 100 days healing, bio-based healing before (b) and after 100 days healing (d), Jonker et al 2011.

Figure 30 shows the healing efficiency of cracked control and bacteria-based specimens before/ after 100 days of immersing in water. In regard to bacteria based cracked specimens, Abundance calcium precipitation has been observed which resulted in crack closure of 0.46mm compared to control specimens. In addition to the formation of $CaCO_3$due to the bacterial metabolic conversion of calcium lactate. Previous studies by Jonkers et al 2010 noted that the yielding of calcium precipitation is increased when the produced CO_2 molecules react with Ca $(OH)_2$ minerals (carbonation), which are important hydration products.

As a result of autogenous healing, the healing of control specimens was limited to 0.18mm, which is in a good agreement with the reported literature where autogenous healing has been reported to be between 0.1 and 0.3mm (Jacobsen et al 1996; Reinhardt et al 2003; Li et al 2007; Neville et al 1995; Ter et al 2005; Qian et al 2010). Schlangen et al 2009 and Edvardsen et al 1999, suggested that abiotic autogenous healing is due several factors such as swelling and hydration of cement pastes, blockage due to water impurities, calcium carbonate precipitation or movement of concrete fragments that separate during the occurrence of cracking process. Therefore, the formation of $CaCO_3$ crystals in control specimens might be attributed to the natural carbonation of cement paste. Despite the fact that no direct permeability measurement was conducted in this study, monitoring the evolution of cracks over time, indeed provides a good indication in regard to the enhancement and recovery of material's permeability properties.

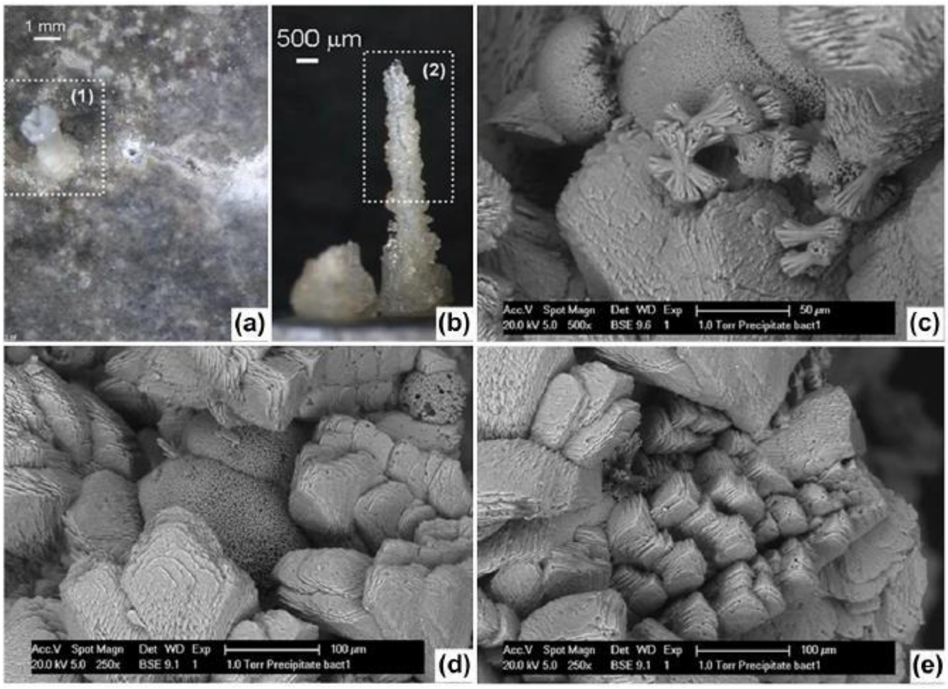


Figure 31 ESEM images of the collected crystals at the crack mouth of healed specimens, Jonker et al 2011.

ESEM of the collected precipitate (Figure 31), showed deformed lamellar rhombohedra and needle-like clusters assembled in dumbbell shapes. Several studies reported that the type of calcium and bacteria affect the morphology of the produced calcium carbonate crystals (Rodriguez-Navarro et al 2003, Warren et al 2001). Lian et al 2006, observed noticeable difference in shape and texture of the formed calcite crystals in soils as a result of bacterial activity. In another study, Rodriguez-Navarro et al 2003, studied the viability of calcium carbonate producing bacteria in porous ornamental limestone. Vaterite which is the less stable phase/polymorph of calcium carbonate precipitation, was observed. Crystals were reported to be rhombohedral exhibiting crystallographic orientation. Furthermore, Vaterite has been reported to chemically occur in highly calcium carbonate supersaturate solutions and transform into calcite and aragonite in aqueous solutions, at room temperature.

The above findings promote two mechanisms in which bacteria enhance the production of calcium carbonate precipitation i.e. through metabolically promoting calcium carbonate supersaturated conditions and the availability of nucleation sites to seed $CaCO_3$precipitation.

Mian et al 2015 studied bacteria-based self-healing concrete using spore forming alkali resistant bacterium. A 24h bacterial culture (corresponding to 10^9cells/ml) along with nutrients were added directly into the concrete matrix during mixing. Specimens with/without healing agents were cracked at 21 days and cured in water with constant supply of oxygen to promote bacterial metabolism for the conversion of calcium carbonate. The effect of curing on self-healing specimens was also examined. Cracked specimens with/without bacteria were left to cure in three incubation conditions: (1) 25°C, immersed in water; (2) 25°C, 90%RH; (3) 25°C, wet-dry cycle (during one cycle, specimens immersed in water for 12h and left in air to breath for 12h.

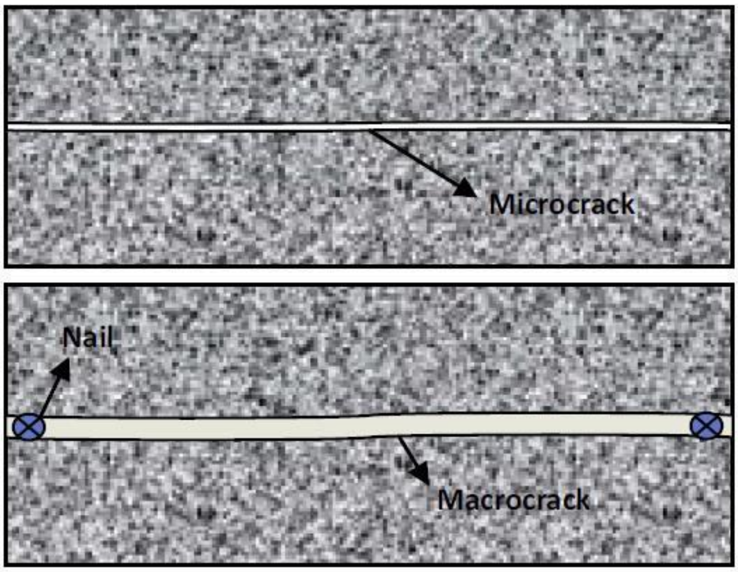


Figure 32 *Crack creation method used by Mian et al 2015.*

Crack creation method is shown in Figure 32. Specimens first were coated with adhesive tape on all sides except the crack mouth, then subjected to compression until crack appearance. Different diameter nails were then applied on both ends of the crack to achieve various crack width ranging from 0.1mm to 1mm.

Surface images of the healed specimens showed abundance white precipitation formed at the crack mouth (Figure 33). Cracks of 0.3mm were almost fully healed after 20 days of curing water, while cracks of 0.8mm were partially healed, figure 33.

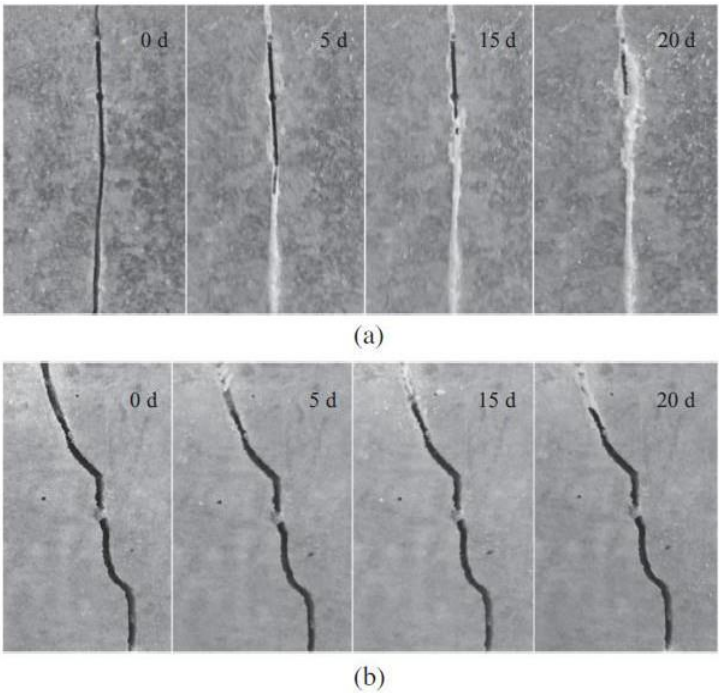


Figure 33 Crack healing in bacteria-based specimens over time: (a) specimen with crack width of 0.3mm; (b)) specimen with crack width of 0.8mm, Mian et al 2015.

ESEM analysis revealed the morphology of formed crystals at the crack mouth, where crystals appeared lamellar close packing orientation (Figure 34, left). Further analysis of crystals showed that calcite was the dominant phase in terms of crystallinity (Figure 34, right).

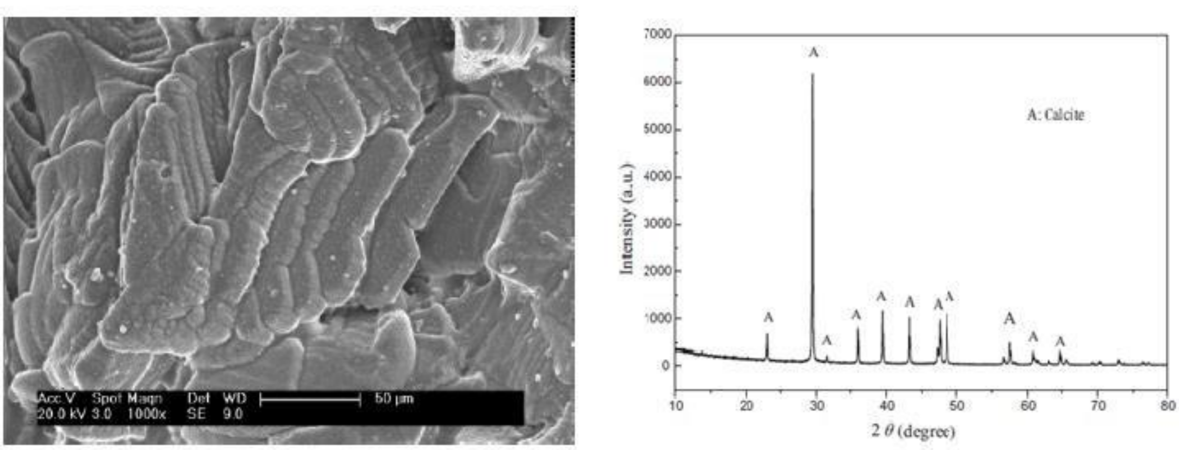


Figure 34 34 ESEM analysis (left) and XRD (RIGHT) of the formed crystals at the crack mouth.

Crack healing ratio was conducted to provide an indication of the effect of curing under different conditions on the healing mechanisms, Figure 35). Wet curing proved to be the least efficient, where a healing ratio of almost 30% after 40 days was observed. Such findings were due to the lack of water found in cracks i.e. limited the transportation of the self-healing agent. On the other hand, Water curing specimens showed enhanced healing ratio of 80% after 14 days, while wet-dry curing specimens had healing ratio of about 43%. However, at later stage, both water and wet dry curing specimens had a healing ratio of almost 98% and 96%, respectively.

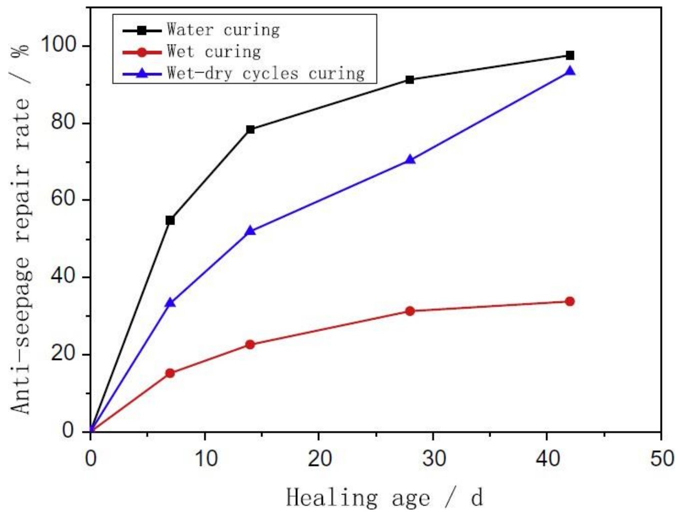


Figure 35 Crack healing ratios under different curing conditions

2.11 Encapsulation techniques for the application of self-healing mortar

Jonkers et al 2007, reported the loss of viability in bacterial cells when incorporated in fresh concrete, as a result of the mechanical forces during the mixing process and the high alkalinity of the concrete environment. Therefore, the need for encapsulating material for self-healing applications is of vital importance and prerequisites to maintain and enhance high healing efficiency over time. Immobilisation of bacillus subtilis in light weight aggregates and graphite Nano pellets was investigated (Khaliq et al 2016), where results showed that short- and long-term healing was viable when both materials are used. Other encapsulating material has also been explored such as polyurethane, silica gel and microcapsules through emulsion (Bang et al 2011; Wang et al 2014).

However, the majority of the proposed materials are relatively high in cost and limited to large-scale concrete structures. This created the demand to develop and investigate more practical techniques/ approaches for the so called ‘’bio based self-healing concrete’’. Inconsistency In reported literature in regard to the efficiency of the genotype of bacteria, type of healing agent, encapsulation material and technique used for the introduction of bacteria concrete.
Bacterial encapsulation using natural or synthetic polymers have been investigated (Zhu et al 2015). However, such material has been reported to be inadequate for self-healing systems for concrete. This is because synthetic polymers are deleterious and limit the bacterial activity to a certain degree, while natural polymers are usually hydrophilic i.e. swelling issues arise as result of the water uptake of the material.

So far, bio-mineralisation using different type of taxonomic groups of bacteria have shown promising results toward restoring the materials structural integrity of concrete structures. Experimental results revealed that in a binary healing system are able to germinate, multiply and induce calcium precipitation efficiently. However, the reported strains are yet to meet the demand of practical applications due to some shortcomings, including low mineralising capacity (Han et al 2016).

Zhang et al 2017, studied the healing capacity of concrete specimens using non-ureolytic bacteria namely, Bacillus Cohnii. Three encapsulating techniques were used to determine the effectiveness on the healing efficiency of crack specimens, namely, bacterial impregnation in expanded perlite (EP), direct addition of bacteria and bacterial impregnation in expanded clay particles (EC), as shown in figure 36.

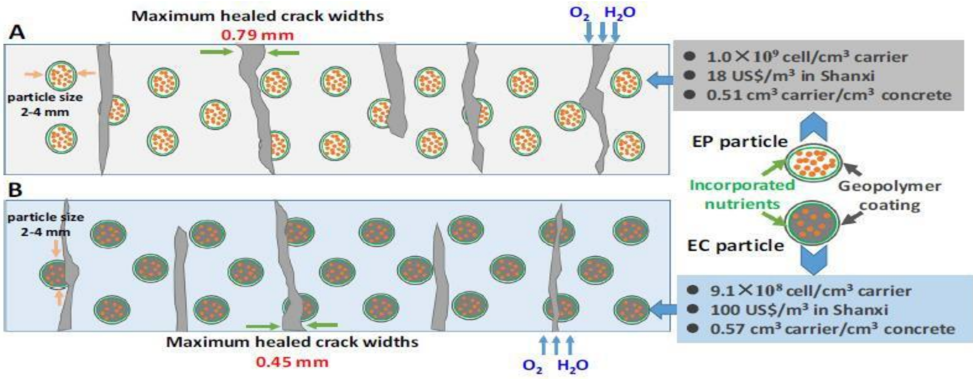


Figure 36 Schematic diagram of the healing process using immobilised expanded clay (EC) and immobilised expanded perlite (EP), *Zhang et al 2017.*

Figure 36 shows the encapsulating process used by Zhang et al 2017. Bacterial spores' solution was first injected under vacuum and oven dried at 45C for 48 hours until constant weight is achieved. The healing agent, comprising of calcium lactate (8 g/l) and yeast extract (1 g/l), was sprayed onto the surface of the particles then dried again at 45°C for 48 hours. To minimise leakage and early activation of bacterial spores, a coating layer of geopolymer that consists of metakaolin and sodium silicate (De Koster et al 2015), was applied onto the surface of the particles via high pressure nozzle spray, figure 37.

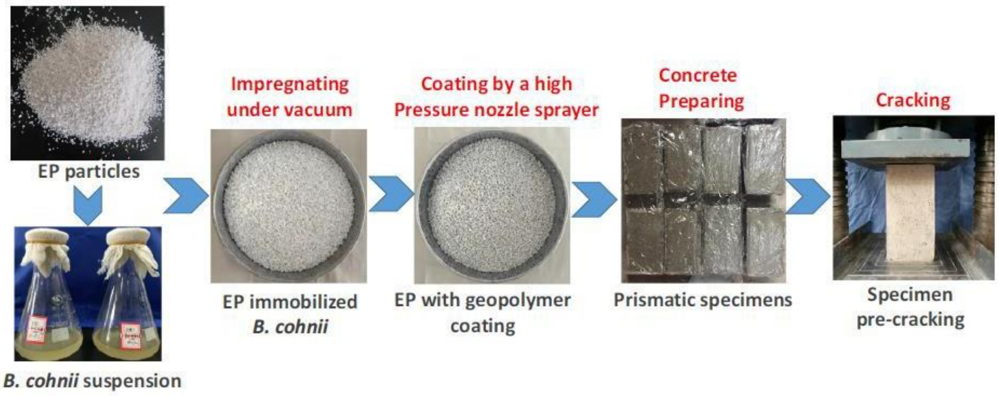


Figure 37 Encapsulating process used by Zhang et al 2017

The crack healing efficiency and morphology of healed specimens were also investigated. Mortar specimens were cracked at 28 days and submerged in tape water to monitor the healing process of cracks over 28 days. Microscopic images of 28 days healed specimens is shown in Figure 38 . Crack width of bio-based bacteria specimens with/without encapsulating material have shown healing capacity. Specimens with directly introduced bacteria (D-B) i.e. without encapsulation showed some crack healing ability but was limited. Crack healing in specimens loaded with impregnated expanded clay (EC-B) were able to bridge cracks of up 0.37mm, better healing efficiency was achieved in specimens loaded with impregnated expanded perlite (EP-B) where a crack healing of almost 0.51mm was achieved. Control specimens showed some degree of healing as a result of secondary hydration of un-hydrated cement particles, but healing was limited.

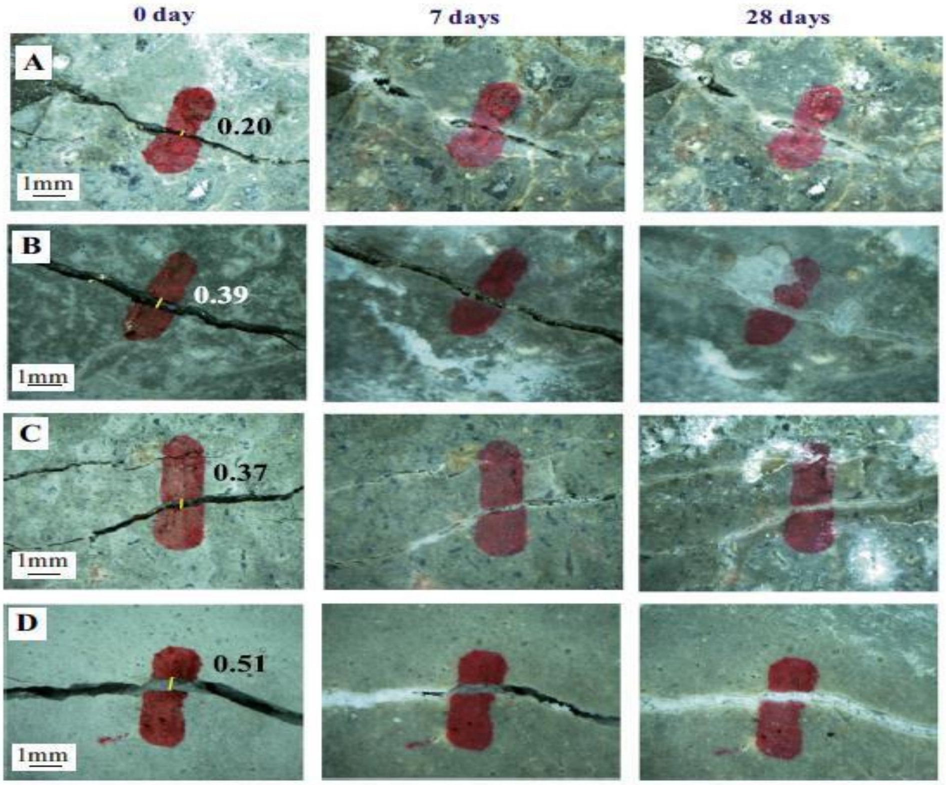


Figure 38 Crack healing for (a) control; (b) specimen with directly added bacteria i.e. without protection; (c) expanded clay bacterial impregnated specimen; (d) expanded perlite bacterial impregnated specimen, Zhang et al 2017.

Quantification of the crack healing process was done by taking measurements of crack width along the length of each crack at different intervals i.e. every 1 cm (Wiktor et al 2011). The percentage of crack healing for each specimen was calculated using the following equation:

$$\text{Healing percentage (\%)} = \frac{d_0 - d_t}{d_0} \times 100$$

Where d_0 is the initial crack width, and d_t is the width measurement at the healing time, t.

The introduction of bacteria into concrete matrix have shown great potential in enhancing the healing capacity of cracked concrete. As observed from figure 39A and B, healing of small cracks in all specimens showed some degree of healing at 7 days. However, crack healing was dramatically improved over 28 days, especially for the bacteria based concrete specimens (Figure 39C).

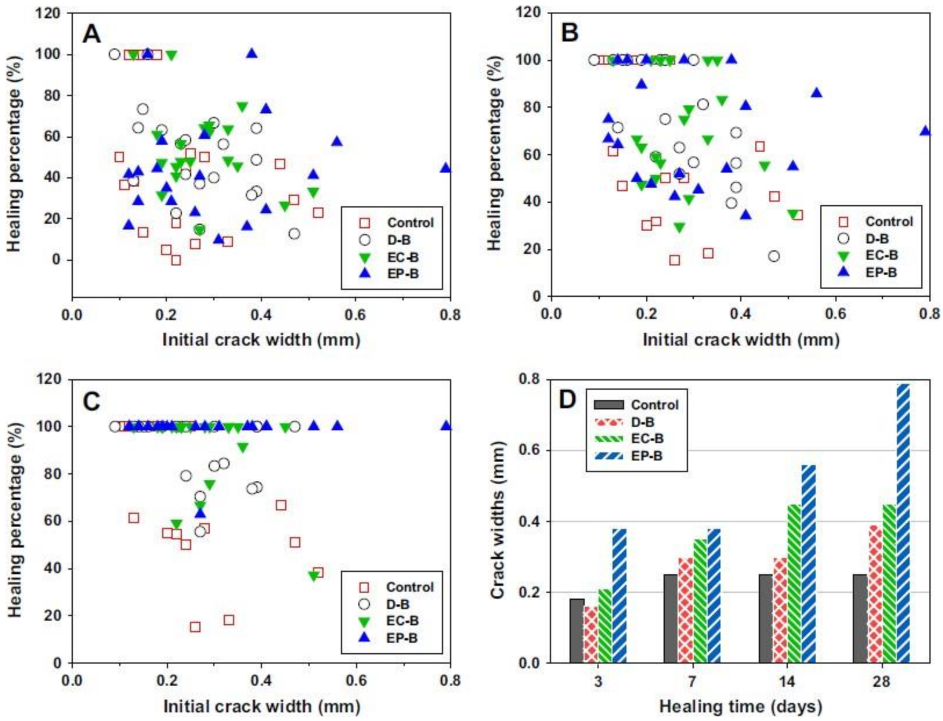


Figure 39 Crack healing percentage for concrete specimens at (A) 3 days; (B) 7 days; (C) 28 days; (D) maximum healed crack widths at different times, Zhang et al 2017.

Although specimens with direct bacterial addition have shown the capacity to heal cracks, significant or higher healing percentage was observed in concrete specimens with encapsulating material. Such phenomenon is related to the loss in viability of bacterial spores during mixing. The maximum value of healed cracks is illustrated in figure 39D. After 28 days of healing, control specimens were able to heal crack of up to 0.25mm due to the carbonate process of calcium hydroxide($Ca(OH)_2$), as shown in the following equation:

CO_2+ $((OH)_2 \rightarrow CaCO3 + H2O$ Equation (3)

Significant healing was observed for specimens loaded with impregnated expanded perlite (EP-B), which were able to heal maximum cracks of up to 0.79mm after 28 days healing. Specimens loaded with impregnated expanded clay (EC-B) were less efficient compared to the latter, where a maximum crack of 0.45mm was healed over 28 days.

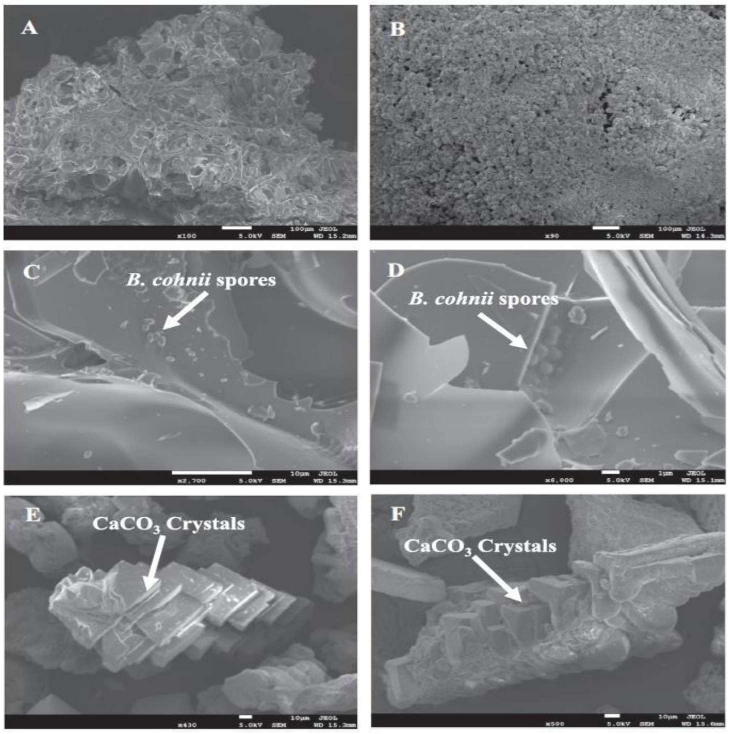


Figure 40 ESEM of expanded perlite (EP); (A) without; (B) with coating; (C-D) bacillus Cohnii spores immobilised in EP particles; (E-F) crack healing precipitation of an EP-B specimens at 28 days healing, Zhang et al 2017.

Deformed lamellar rhombohedra crystal with a needle like cluster assembled in dumbbell shapes were observed (Figure 40, E-F), similar results were observed by Wiktor et al, 2011. The improvement in crack healing capacity in specimens EP-B (i.e. specimens loaded with expanded perlite) is related to the fact that perlites (EP) are highly porous in structure and possess high water absorption capacity, compared to expanded clay particles (EC). The superiority of expanded perlite groups in terms of healing is also related to the 12 % increase in the number of cells impregnated into expanded perlite, in comparison to expanded clay particles (figure 38).

The introduction of bacteria for self-healing concrete has been investigated by Khaliq et al, 2015. The study focused on evaluating the self-healing performance of bacteria through different encapsulation materials and different introduction techniques. An alkaline bacterial strain that belongs to the gene bacillus has been used throughout the study, namely Bacillus Subtilis. Certain prerequisite characteristics or criteria that the bacteria must possess/ pass prior to being introduced for the application of self-healing concrete. According to the author, the strain was selected based on the ability to form endospores, adjust in high alkaline environments, and produce copious amounts of insoluble calcium carbonate ($CaCO_3$). The strain was also reported to be oxygen dependant, which is a favourable feature that aids in minimising corrosion of steel reinforcement in concrete structures (Jonkers et al, 2010; Gupta et al, 2013; Rao et al, 2013). Two encapsulation materials have been used in this study, namely graphite Nano platelets (GNP) and light weight aggregates (LWA). The study used four different types of mix, for the purpose of investigating the incorporation techniques of bacteria/spores to enhance the healing efficiency of cracks concrete. In this study, the control specimens were referred to as ''Mix 1'' i.e. without spores nor protective material. Mix specimens with bacterial spores added directly into the concrete matrix were referred to as ''Mix 2''. ''Mix 3'' and ''Mix 4'' was referred to specimens incorporated with bacterial spores by the use of light weight aggregates and graphite Nano platelets, respectively.

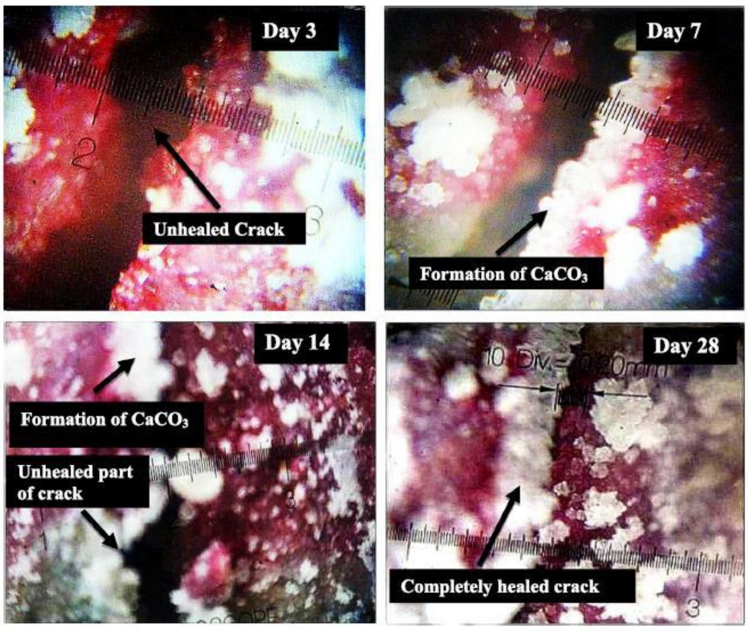


Figure 41 Monitoring crack width over time, *Khaliq et al, 2015.*

The effect of crack age on the healing efficiency was also investigated, control and bacterial specimens (with/without protective materials) were pre-cracked using compressive loading at 3, 7, 14, 28 days. After pre-cracking, samples were cured in water and the healing efficiency was monitored by measuring the crack width over 3, 7, 14, 28 days. The difference between the original crack width and the measured crack width at time (t) of healing was taken as a measure of the self-healing (Figure 41).

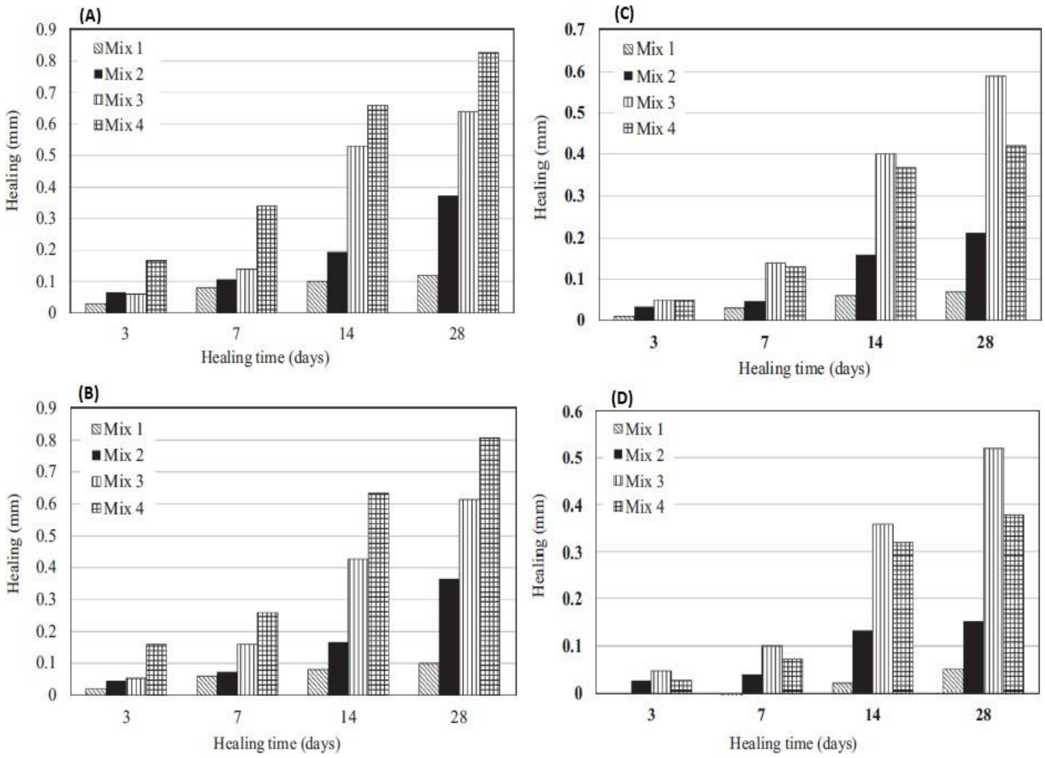


*Figure 42 M*onitoring of crack healing in specimens pre-crack at: (A) 3 days; (B) 7 days; (C) 14 days; (D) 28 days, *Khaliq et al, 2015.*

Considering the healing efficiency of specimens pre-cracked at 3 days (Figure 42A), control specimens showed some degree of healing, which could be attributed to different factors, such as autogenous healing calcium precipitation due to carbonation of calcium hydroxide in addition to the swelling in the cement matrix (Heide et al 2005). A maximum healing efficiency was observed in specimens with graphite Nano platelets (GNP) i.e. ''Mix 4'', compared to the other mixes at 3, 7, 14 and 28 days. Sixuan et al 2012, reported that graphite Nano platelets (GNP) act as filler material and can be uniformly distributed in the concrete mix due to the small particle size. Therefore, this allows better bacterial distribution and enhances the healing capacity when compared to the light weight aggregates (LWA).

Similar trend was observed for specimens pre-cracked at 7 days (Figure 42B), the maximum healing achieved by ''Mix 3'' and ''Mix 4'' was 0.61mm and 0.81mm, respectively. Direct introduction of bacteria ''Mix 2'' showed healing of 0.37mm, i.e. lower than that observed in specimens with bacterial encapsulation (''Mix 3'' and ''Mix4''). This rational has been explained by Jonkers et al 2009; 2010, where the loss of viability in bacterial spores was reported to be due to the mechanical forces resulted during the mixing phase, and the disintegration of calcium lactate into the concrete matrix.

The healing efficiency for ''Mix 4'' specimens pre-cracked at 14 days was lower in comparison to the specimens in ''Mix 3''. Khaliq et al 2015, explained that the continuous hydration reactions resulted in the formation of dense microstructure, which created a pressure on the GNP particles and resulted in loss of viability in bacteria incorporated in GNP particles. The loss in viability was also portrayed in specimens with direct incorporation of bacteria ''Mix 2'' where the value of healing at 14 days decreased from 0.37mm to 0.21mm, which could also be due to the formation of dense microstructure after 14 days of hydration.

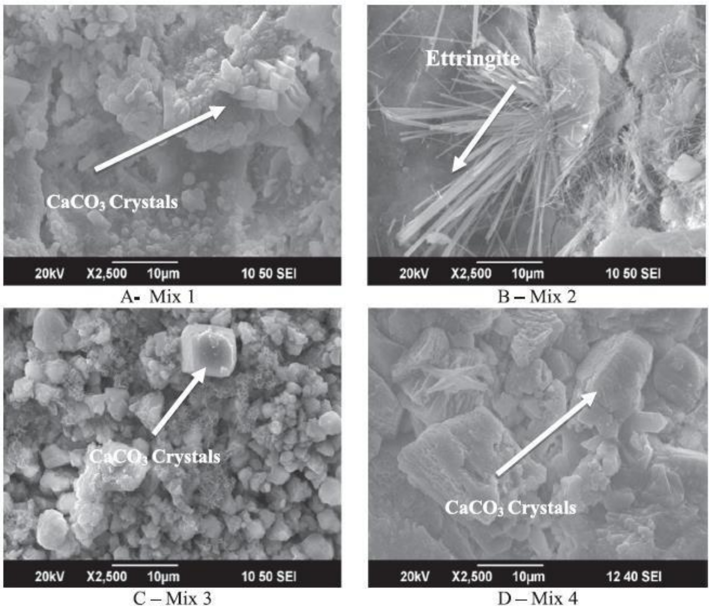


Figure 43 Scanning electron microscope (SEM) of specimens pre-cracked at 28 days, *Khaliq et al, 2015.*

Specimens pre-cracked at 28 days showed similar trend to the one's pre-cracks at 14 days. Better healing efficiency was observed for specimens with light weight aggregates ''Mix 3'', where a crack of 0.52mm was healed i.e. higher than ''Mix 2'' and ''Mix 4'', with a maximum healing of 0.15mm and 0.37mm, respectively. It is worth pin pointing that microstructural analysis (Figure 43) of all mixes confirmed the presence of calcium carbonate crystals. However, this is not surprising as the production of calcium carbonate crystals is not limited to bacterial activity only. Figure 21'' A-mix 1'', scanning electron microscopy for control specimens at 28 days, shows the formation of $CaCO_3$ in control specimens (Mix 1) which is attributed to carbonation process of calcium hydroxide.

The formation of calcium carbonate crystals in bio-based specimens was also detected. However, figure 21 shows that bio based encapsulated specimens (figure 43, C-mix3 and D-mix4) had higher amounts of $CaCO_3$ crystals in comparison to the specimens with direct incorporation (B-Mix 2) which could be attributed mainly due to loss of viability i.e. the technique used for the introduction of the self-healing concrete. The healing observed in the bio-based specimens (with/ without encapsulation), was mainly calcite crystals which are orthorhombic in nature.

The improvement in the self-healing efficiency observed in specimens with impregnated light weight aggregates (LWA) over GNP particles, can be explained in figure 43 ‘’D-Mix4’’ where early productions of calcium carbonate, have limited bacterial activity and ability to produce calcium carbonate. This is in addition to the weakness of GNP particles under multi-axial loading.

Chapter 3 Methodology

3.1 Introduction

This chapter thoroughly describes the materials and methods that have been used to achieve self-healing mortar via ureolytic bacterium species. The first stage of this study comprises of isolating bacillus species from different forms of samples taken at different locations. The protocols that have been used in isolating, purifying and characterising bacillus species are also described in details. Bacillus species were classified based on urease activity, oxygen consumption, sporulation, ability to grow in high alkaline environment (such as PH 7.5, 9 and 10) and capacity to produce copious amounts of calcium carbonate ($CaCO_3$). Five different types of calcium precipitating mediums, reported in the literature, have been investigated in order to quantify the effect on the $CaCO_3$yield. The biochemical properties of the selected strains were monitored via ammonium production, insoluble calcium, pH and cell viability at different time intervals. In a different experiment, the effect of different nutrients such as carbon source, nitrogen source and other metals on the calcium propitiation have also been studied. Qualitative assessment of the precipitate was analysed using Field emission scanning electron microscope (FESEM), X-ray powder Diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR).

The second part of this chapter, illustrates the mix design for mortar specimens. A state of art encapsulating material, namely autoclaved aerated recycled concrete aggregates (AARC) were promoted for the use of self-healing mortar. The techniques and procedures used in encapsulating bacterial spores and nutrients into the aggregates via vacuum impregnation have been thoroughly explained. Impregnated aggregates were added as part of the volume of sand used. The healing efficiency of cracked mortars with/without healing agent were monitored via microscopic images (Leica microscope) and capillary water uptake, at 7, 14 and 28 days. Qualitative assessment of the healed product was conducted using Field emission scanning electron microscope (FESEM), X-ray powder Diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR).

3.2 Materials

3.2.1 Integrated protocol for bacterial isolation and identification from soil and plant roots

a. soil associated bacteria

- Using a borer or a spatula, 4- 6 soil samples were collected by drilling into the soil, a minimum of 10 meters separation between each collection spot.
- The collected samples were then placed in a 50ml falcon tubes.
- Tubes were then labelled with site location in addition to time and date of collections.
- Samples were then stored at 4 °C until assayed.

b. Root associated bacteria

- Using scissors, around 2.5cm sample of plant root were cut from between 7.5cm and 15cm below the site surface.
- A minimum of 3-6 samples were acquired from each site and placed into 50ml falcon tubes.
- Tubes were then labelled with site isolation, time and date of collection.
- Tubes were then stored into 4 °C fridge to minimise bacterial activity.

c. Other form of samples (e.g.: concrete or feces)

- Using a spatula, a minimum of 3-6 pea sized sample of the material is collected.
- The collected samples were then placed in a 50mL falcon tubes.
- Site of isolation, time and date of collection in addition to the type of the material were recorded.

3.2.2 Growth media preparation

The growth media used throughout the study for all bacterial strains was consistent. The growth media was purchased from sigma Aldrich namely, Nutrient Broth No. 1, for Microbiology. The media consisted of (g/l):

- D (+)-glucose 1.0
- Peptone 15.0
- sodium chloride ($NaCl$) 6.0
- Yeast extract 3.0

The final PH 7.5$\pm$0.2(25°C).

The medium was then sterilised at 121°C for 15mins, then allowed to cool at room temperature until assayed.

3.2.3 Nutrient agar preparation for supporting growth

The nutrient agar used in this study was purchased from sigma Aldrich. The medium consisted of the following (g/l):

- Agar 15
- D (+)-glucose 1.0
- Peptone 15.0
- sodium chloride (NH_4Cl) 6.0
- Yeast extract 3.0

The final PH 7.5$\pm$0.2(25°C).

Bottles were then sterilised at 121°C for 15mins, then allowed to cool to temperature of 55-60°C before pouring into petri plates and allowed to cure/solidify at room temperature.

3.2.4 Isolation of bacterial colonies

a. From plant roots

- Using a sterile tweezer and pair of scissors, roots were cut into small segments and placed into 50ml falcon tubes.
- 10 ml of sterile phosphate buffer saline tween solution (PBST) was then poured into the samples as a wash buffer, to maintained constant PH and minimise possible contamination.
- Sample were then vortexed for 1minute to homogenise the solution.
- Using a sterile swab, each sample was inoculated into the first quarter of the petri plate.
- This is followed by performing streak dilution, using a 10µL loop, on the rest of ¾ of the petri plate.
- Inoculated petri plates were then incubated at 37°C for 72 hours.

b. From soil samples or other form of samples

- 10ml of previously prepared PBST was poured into the sample and vortexed for 1 minute.
- A sterile swab was then used to inoculate each sample in the first quarter of the petri plate as shown in figure 1.
- Streak dilution was then performed using a 10µL loop.
- Sample were then labelled and incubated at 37°C for 72 hours.

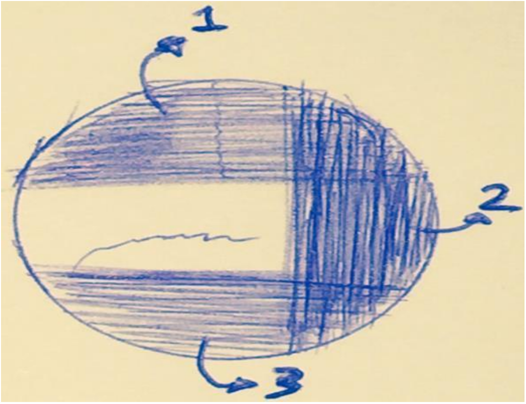


Figure 44 Sketch of the petri plate streaking pattern

3.2.5 Isolation of bacterial species

a. Gram staining

Gram staining is a common bacteriological technique that is used to distinguish between two main groups of bacteria, on the basis of the biochemical and structural differences of the cell wall components. Therefore, this technique aids in differentiating between gram positive and gram-negative bacteria by colouring the cells with either red or violet colour. Figure 45, shows the gram staining kit that have been used in this study.

Figure 45 Gram staining kit

Gram staining was done using the following steps:

- Using a P20 or P200 pipette, an aliquot of 20 µL of distilled water is placed into microscopic slide.
- A bacterial colony (obtained from the incubated petri plates) was then transferred into the microscopic slide, using a 1 µL plastic loop, and spread evenly over a circle of 2cm diameter.

Figure 46 Gram staining test

- Slides were then heat fixed at 70-80°C for approximately 30 to 90 seconds.
- Microscopic slides were then placed on a staining tray above a sink and flooded with crystal violet solution (1) and allowed to stand for 1 minute, followed by rinsing off with tap water.
- Slides were then flooded with Gram's iodine solution (2) and allowed to stand for 1minute, followed by second wash with tap water.
- Gram's differentiator i.e. acetone (3) was then applied into the slides for up to 5 seconds and washed or rinsed off with tap water.
- Slides were then flooded with carbol-fuchsin (4) solution and allowed to remain for 30 seconds before performing the final wash using tap water.
- Samples were then dried using an absorbent paper then heat fixed before microscopic examination under a 100X objective.
- Gram-positive bacteria stain deep violet to blue and negative stain pink to red.

0.28

b. Spore staining

- Similar to the initial steps of the gram staining test. A bacterial colony was spread onto a microscopic slide containing approximately 20 µL aliquot of distilled water.
- Microscopic slides were then heat fixed at 70-80°C, placed above the sink and flooded with carbol-fuchsin. The solution was allowed to stand for 5 minutes, followed by a thorough wash with tap water.
- Slides were then decolourised with ethanol to remove all traces before a final wash with tap water.
- Counterstain was then performed by applying Loeffler's methylene blue for 1-2 minutes.

- Slides were then washed using absorbent paper and heat fixed at 70-80C, before finally being subjected for microscopic observation.
- Bacterial bodies stain blue and spores stain red.

c. Bacterial cell morphology

- Following the gram staining test, cell morphology was observed via optical microscope.

Figure 47, shows the cell morphology protocol that have been used in identifying bacillus species during this study.

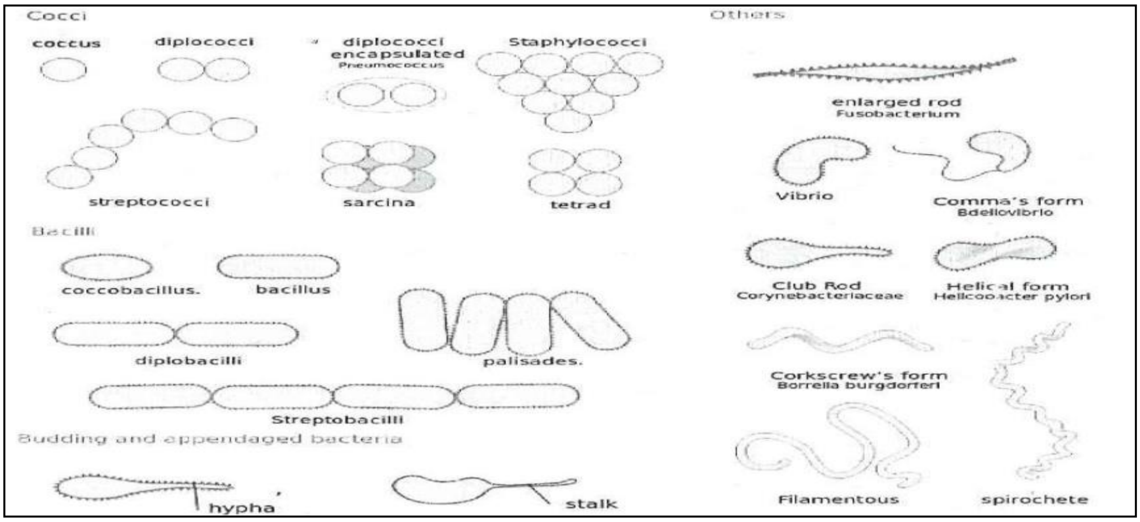


Figure 47 Various cell morphologies for different bacterial species, laboratory protocol 2018.

d. Catalase test

Bacterial cells isolated were then subject to catalase test to detect the presence of the catalase enzyme in the isolated cells. Test was conducted using the following steps:

- Using a 1 µL loop, a small amount of bacterial colony was transferred into a dry glass-slide.
- A drop of previously prepared 3% hydrogen peroxide solution (H_2O_2), was applied on the slide and mixed.
- A positive result was recorded for samples with rapid evolution of oxygen within 5- 10 seconds. Whereas, a negative result was recorded for bacterial samples with no bubbles.

e. Urease test via Urea Broth (Stuart Broth)

The presence of urease enzyme was detected using a urea broth media containing the following composition (g/l):

- Urea 20.0
- Yeast extract 0.10
- Dipotassium phosphate (K_2HPO_4) 9.5
- Phenol red 0.01
- Monopotassium phosphate ($K_2H_2PO_4$) 9.1

Final pH 6.8 $\pm$0.2 at 25°C.

To avoid precipitation, all components were firstly mixed and dissolved with distilled water, followed by filter sterilisation using 0.22 µm Sterile Millex obtained from Sigma Aldrich. Aliquots of 0.5-2 ml of the prepared solution were dispensed into small 2.5ml Eppendorf tubes. A 24-hour fresh grown culture were then introduced into the urea broth and incubated at 37°C for a period of 18-24hours. As a result of the phenol red, which is a PH indicator, positive urease samples turn the phenol red into deep-red colour i.e. confirms the presence of urease. The alkalinity of the medium is resulted from the production of ammonia during the catalysis of urea.

f. Urease test via Christensen's Urea Agar Base (UAB)

Similar to the urease test via Urea Broth, Christensen's Urea Agar Base (UAB) is a solid media for the purpose of rapid quantification and screaming of urease enzyme (Atlas et al, 2010). The media composition (g/l):

- Urea 20.0
- Sodium chloride (NaCl) 5.0
- Agar 15.0
- Phenol red 0.012
- Peptone 1.0
- Glucose 1.0

Final pH was 6.5 $\pm$0.2, all components were dissolved in distilled water except for urea and glucose which were filter sterilised using 0.22 µm Sterile Millex and added post autoclave when the media settle to temperature of 50-55°C. A fresh culture is inoculated into the solid media and incubated at 37°C for 24 hours. The presence of pink colour represents positive results i.e. confirms the presence of urease enzyme.

3.3 In-vitro assay of the total calcium precipitation in broth state

The method used in Quantification of calcium carbonate via urea hydrolysis bacterium was similar to that reported by stock-fischer et al., 19999; Okwadha et al., 2010; Achal et al., 2010; Dahmi et al., 2013 2016 ; Krishnapriya et al., 2015; Xu et al., 2015; Sharma et al., 2017; Schwantes-Cezario et al., 2017; Wei et al., 2015 and Alazhari et al., 2017. Calcium precipitation experiments were conducted in 250 Erlenmeyer flasks containing 100 ml of media with 1% inoculum of fresh bacterial culture grown overnight during the log phase. Several parameters were considered in this study, such as temperature, shaking speed, type of bacteria, type of calcium source and different pH conditions. The influence of such factors was reflected in the total amount of calcium carbonate precipitation produced over a period of 168 hours. Purification of the produced crystals was done using two methods, namely using Whatman filter paper Grade 42 (obtained from sigma Aldrich, UK) and by means of digesting enzyme.

Figure 48 In-vitro assay of $CaCO_3$experiment

Purification using the first method i.e. using Whatman filter paper is done by passing the crystallised solution into the filter paper, as result of the micro pores present, crystals will be retained on the filter paper and the supernatant including the vegetative cells will be filtered through. The Whatman filter paper containing the crystals was then dried at 50C until constant mass is achieved.

Figure 49 Purification of the $CaCO_3$crystals

The second method was also conducted to insure no crystals are lost during the purification process. The method used was in accordance to Wei et al., 2014. Lysozyme is an enzyme obtained from chicken egg white (Purchased from sigma Aldrich) that possess the capacity to digest or break the chemical bonding of the outer cell wall of bacteria, more specifically the enzyme is known to target the peptidoglycan layer that is present in the cell wall of bacteria. After incubation, the crystallised solution including the bacterial cell wall was transferred into 50ml falcon tubes and centrifuged at

8397 ×g for 2 minutes. The biomass was then suspended in a prepared 50ml TE buffer (containing 10Mm of Tris buffer, and 1mM of EDTA at pH 8.5) lysozyme was then added at a final concentration of 1mg/l. Activation of the enzyme was done by incubating the suspension at 37°C for 1 hour. The falcon tubes were then centrifuged again and the supernatant containing the digested cell wall was discarded. Washing of the crystals was performed by re-suspending and homogenising the pellets in sterile distilled water, washing was repeated at least twice until the supernatant became crystal clear. The final step is identical to the former method i.e. the collected crystals were dried at 50°C until constant mass change.

3.3.1 Effect of nutrients and calcium source on the yielding efficiency of calcium carbonate precipitation ($CaCO_3$)

Inconsistency in the reported literature in regard the composition of nutrients, including calcium source, for maximum yield of calcium carbonate precipitation. Five different Mediums, which were commonly or consistently have been used in the reported literature, have been selected for this part of the study. The composition and the make of each media are thoroughly explained. All materials and chemicals were purchased from sigma Aldrich UK.

Medium No.1 (M1)

The first media consisted of the following (g/l):

- Nutrient Broth, No1 for microbiology 25.0
- Calcium chloride 25.2mM
- Urea 20.0

Final pH 7.4 $\pm$0.2

Medium No.2 (M2)

The second media consisted of the following (g/l):

- Nutrient Broth, No1 for microbiology 3.0.0
- Calcium chloride ($CaCl_2$) 25.2mM
- Urea 20.0

Final pH 7.4 $\pm$0.2 Medium No.3 (M3)

The third media consisted of the following (g/l):

- Yeast extract 3.0
- Calcium chloride ($CaCl_2$) 25.2mM
- Peptone 4.0
- Urea 20.0

Final pH 7.4 $\pm$0.2 Medium No.4 (M4)

The fourth media consisted of the following (g/l):

- Nutrient Broth, No1 for microbiology 25.0
- Calcium acetate ($C_4H_6O_4Ca$) 25.2mM
- Urea 20.0

Final pH 7.4 $\pm$0.2 Medium No.5 (M5)

The fifth media consisted of the following (g/l):

- Yeast extract 3.0
- Calcium acetate ($C_4H_6O_4Ca$) 25.2mM
- Peptone 4.0
- Urea 20.0

Final pH 7.4 $\pm$0.2

3.3.2 pH

All components in M1, M2, M3, M4 and M5 were added and dissolved in distilled water except calcium and urea which were added post autoclave via 0.22 µm sterile filter. The solution was autoclaved/sterilised at 121°C for 20minutes. The pH of the solution was adjusted using stock solutions of either 1M of sodium hydroxide or 1M of hydrochloric acid to achieve the desired pH.

3.4 Production of Spores in broth state

The production of spores in this study was conducted in accordance to Kalfon et al., 1983. The study investigated the effect of different minerals and nutrients on the sporulation of Bacillus Sphaericus. To conclude, an optimised sporulating media was specifically designed to stress the bacteria for maximum production of spores, using toxic minerals. Mature spores were harvested using the method used by Jonkers et al, 2017. Spore solution was subjected to pasteurisation i.e. solution was placed in a water bath at 80°C to kill vegetative cells for 15 minutes. Under sterile condition, the spore solution was transferred into 250ml polyethylene centrifuging bottles for purification.

Figure 50 Sporulating mediums in a 2L flasks

Purification of the spore solution was done by washing with sterile distilled water, during each cycle the spore solution was centrifuged at a gravitational force of 8397×g for 10 minutes. The supernatant was discarded and substituted with sterile distilled water then vortexed/homogenised to wash the spores. The process was repeated at least twice until pure spores were obtained under microscopic imaging. Upon purification, the spore solution was stored at 4°C until further use.

The following media was used (g/l):

- Monopotassium phosphate (KH_2PO_4) 6.0
- Magnesium Sulfate Heptahydrate ($MgSO_4\ 7H_2O$) 0.3
- Manganese(II) sulfate ($MnSO_4$) 0.02
- Iron(III) sulfate ($Fe_2(SO_4)_3$ 0.02
- Zinc sulfate heptahydrate ($ZnSO_4\ 7H_2O$) 0.02
- Calcium chloride ($CaCl_2$) 0.2

- Tryptose 10.0
- Yeast extract 2.0

Final pH 7.2 $\pm$0.2

Stock solution of each mineral was prepared separately and added post autoclave using 0.22 µm Millipore Membrane filter. Tryptose and yeast extract were added, dissolved with distilled water and autoclaved at 121°C for 20minutes. Media was allowed to cool down to room temperature, minerals were added (v/v) and PH was adjusted using 1M of sodium hydroxide (NaOH) or 1M hydrochloric acid (HCl). To avoid dilution, the amount of sodium hydroxide added was subtracted from the total volume of the media, furthermore, Small aliquots of the sterile media were taken to check the pH.

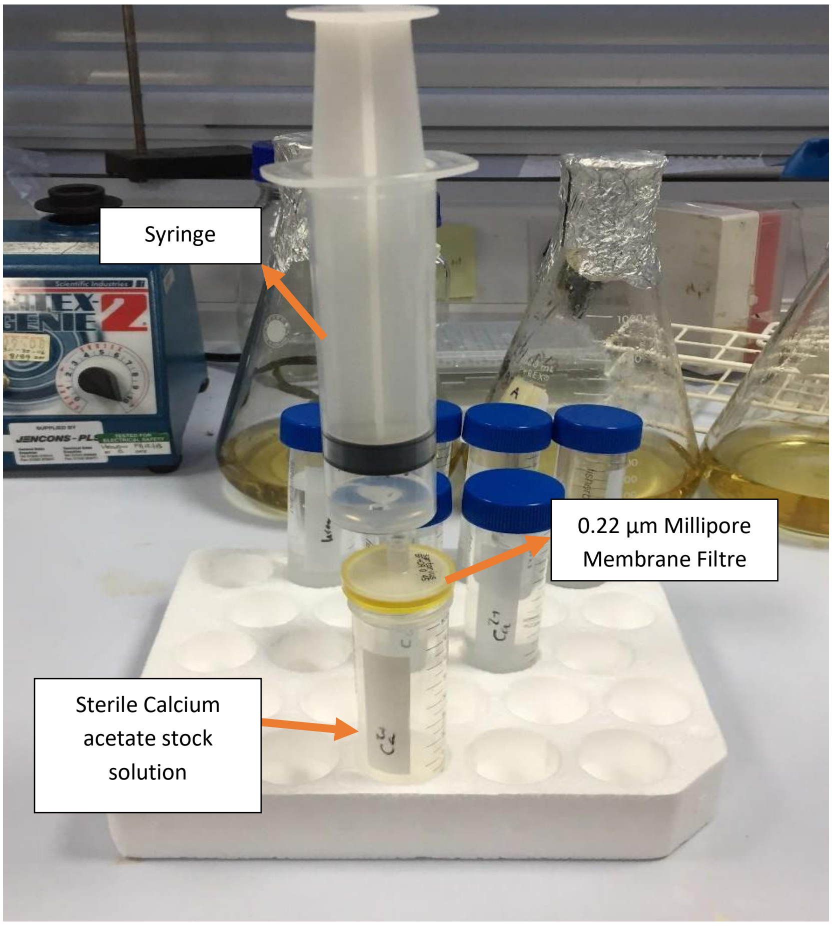


Figure 51 Filter Sterilisation

3.5 The mechanics and Biochemical properties of calcium carbonate precipitation under controlled environment

Several parameters, namely, cell viability, insoluble/soluble calcium, PH and urea hydrolysis ($Ammonium$ (NH_4) production) were placed under scrutiny and studied carefully to provide better understanding of the mechanics and the biochemical properties of calcium precipitation ($CaCO_3$) via urealytic bacterium species. Experiment was run for 120 hours, reading were taken at different time intervals i.e. 12hours, 24 hours, 48 hours, 72 hours 96 hours and 120 hours. At each time point, 10 ml of sample was taken under sterile conditions and spread into different aliquots to determine cell viability, PH, insoluble/soluble calcium and ammonium production(NH_4). It is worth pinpointing that a maximum of 2% (v/v) of the total solution was taken for sampling in order not to affect the experiment throughout the whole process. The study was adopted from Xu et al., 2015; 2018.

3.5.1 Cell viability

Cell viability was conducted by serial dilution method. Precooled autoclaved distilled water was used for dilution where a minimum of five dilutions were conducted for each reading i.e. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. Dilution was conducted in a 15ml sterile tubes (obtained from sigma Aldrich UK) which was kept in an ice bath during analysis to minimise bacterial activity. Using a pipette, 100µl of each dilution was inoculated onto pre-prepared agar Petri plates and spread using 10 µl loop. Petri plates were then incubated at 37C for 24 hours, cell viability was obtained by counting the number of colonies in relation to the dilution factor. Colony counting was done using a colony counter obtained from Stuart scientific.

Figure 52 Colony forming unit (CFU).

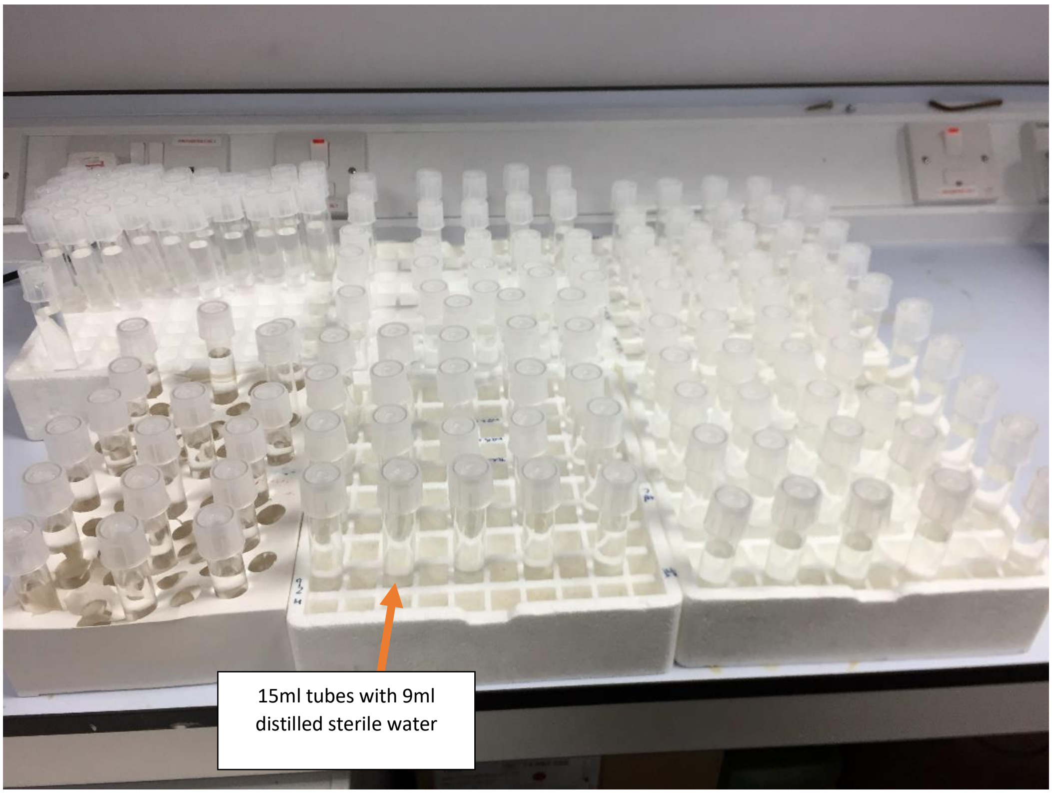


Figure 53 Cell viability using serial dilution

3.5.2 $NH3 - N$ Analysis using Nessler's Assay

The amount of urea hydrolysed by each bacterial strain was determined spectrophotometrically using modified Nessler method (Greenburg et al., 1992; Whiffin et al., 2004; Al-Thawadi et al., 2008). The urease activity is reflected in the amount of ammonium concentration found in the solution at different time intervals due to urea hydrolysis. Ammonium chloride (NH_4Cl) in the range of 0.05- 0.5mM was used as standard and the absorbance was read at 425nm using microplate reader. At each time point, samples were centrifuged to remove the cells and limit bacterial activity, the supernatant was then transferred into a clean Eppendorf tubes and frozen until assayed.

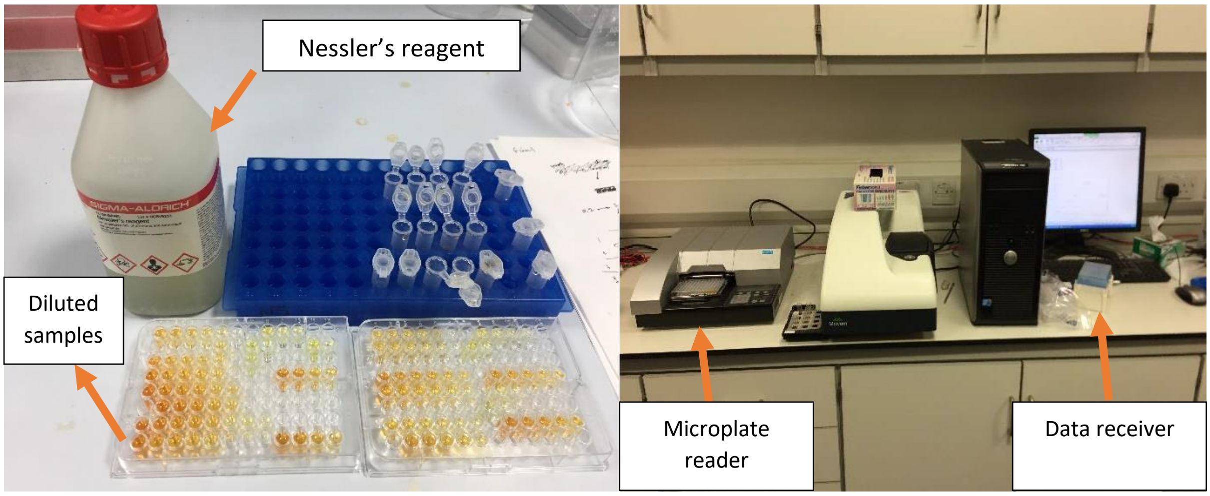


Figure 54 Nessler's Assay (left), Absorbance reading using microplate reader (right)

To minimise dilution, samples were thawed in a 4°C fridge before being brought to room temperature. Samples were then diluted in sterile distilled water to be in the range of 0-0.5Mm, 2ml of the diluted sample was mixed with 100µl of Nessler's reagent. The solution was allowed to react for exactly 1 minute before reading the absorbance at 425nm using microplate reader. The absorbance values obtained from the ammonium chloride were compared with the ones obtained from the diluted samples.

3.5.3 pH

The PH of the supernatant was also measured simultaneously at different time intervals. The increase in PH provides an indication of the urease activity during the microbial precipitation of$CaCO_3$, which is due to the production of ammonium as a result of urease enzyme which is responsible for the hydrolysis of urea. The PH readings were done using HI-5522 Research grade PH/ ORP/ ISE/ EC/ TDS/ Resistivity/ salinity Bench meter (purchased from Hannah instrument, UK). Samples of 5 ml of the obtained supernatant was used for the PH measurements.

3.5.4 Calcium analysis

The fourth parameter that was selected for analysis was the determination of the insoluble and soluble calcium during the presence of bacteria. Atomic absorption spectroscopy (AAS) which is an analytical technique for the determination of concentration of elements, was used in this study, Figure 12-3. Calcium standard obtained from Sigma Aldrich at a concentration of 1000mg/l Ca in nitric acid was used.

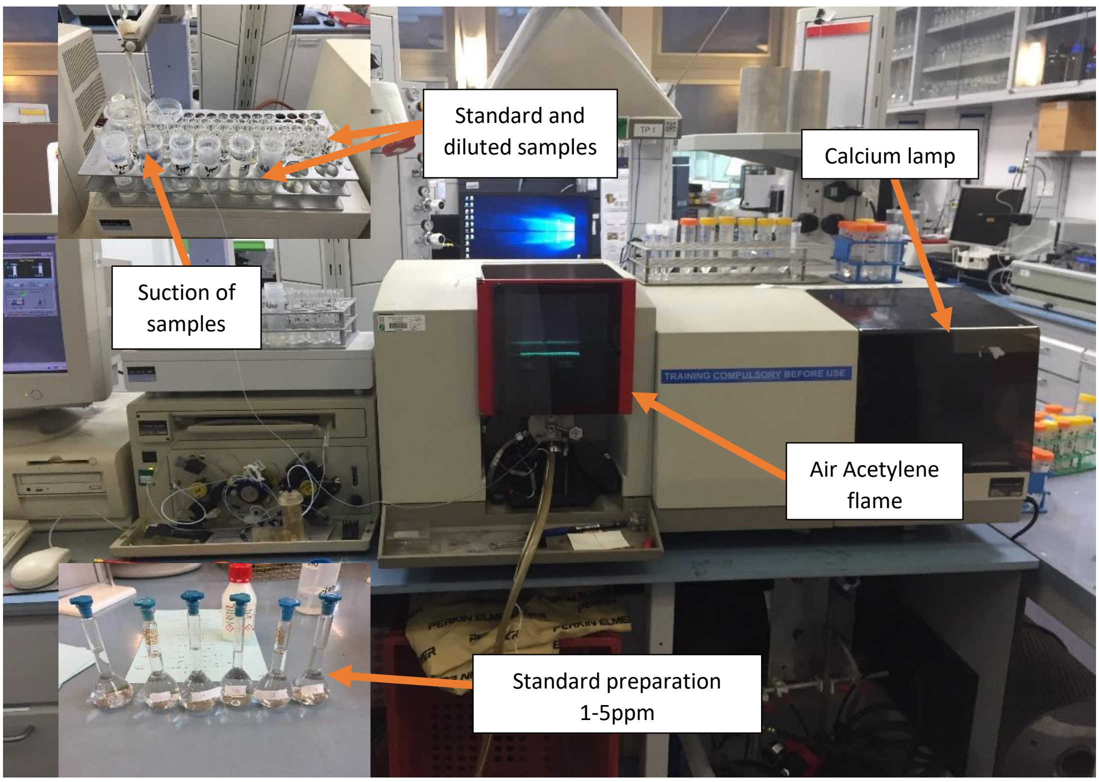


Figure 55 Atomic absorption spectrophotometer (AAS).

At each time point, under sterile conditions around 5ml of each sample was transferred into 10 sterile tubes and centrifuged and the supernatant was stored at 4c until assayed. The supernatant was then diluted to be in the range of 1-5ppm and absorbance was read at 422.7 nm. Absorbance values obtained for each diluted sample were compared to absorbance obtained from the standard.

3.6 Testing methods for mortar specimens

3.6.1 Preparation of mortar specimens

Ordinary Portland cement CEMII A-L 32.5R conforming to EN 196-1:2005 was used throughout this study. Water to cement ratio (W/C) =0.5, fine aggregate CEN sand a/c=3. For compressive strength test, specimens with a size of 40×40×40mm were used, whereas mortar specimens with dimensions of 40×40×160mm were used for flexural test. An average of three readings were taken for each test, throughout the whole study.

3.6.2 Casting and fabrication of fresh mortar

The cement II A-L 32.5R was first mixed with the water and homogenised using a Hobart mixer for about 30 seconds at low speed, sand was then added steadily and the mix was homogenised for about 30 seconds, the mixing speed was increased and the mix was further homogenised for additional 30 seconds. To ensure proper distribution of all components (i.e. cement, sand and water), the mixer was stopped for 30 seconds to scrap any excess material on the side of the stainless-steel bowel, and placed in the middle of the bowel using a spatula, this is followed by further mixing for additional 60 seconds at high speed.

3.7 Mechanical properties

3.7.1 Compressive strength

Compressive strength was conducted on specimens at the age of 7, 14 and 28 days of curing. Mortar specimens with dimensions of 40×40×40mm were tested for compressive strength in accordance to BS 1881-116:1983. Specimens were positioned between two parallel discs as shown in the figure, testing was conducted by applying uniform load at a rate of 0.30±0.05($N/mm^2.s$), until failure i.e. until no force can be sustained.

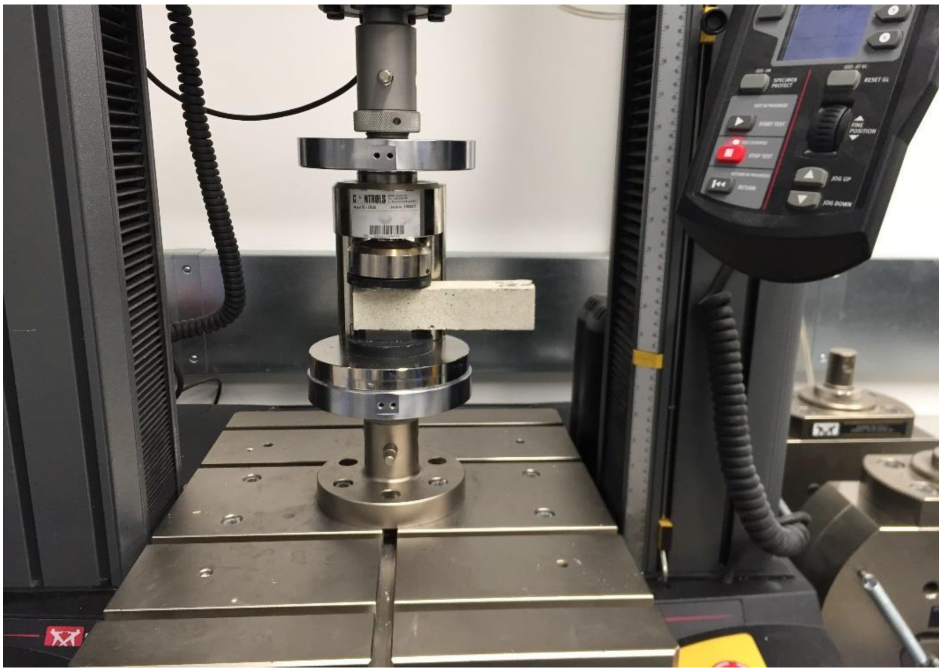


Figure 56 Compressive testing

3.7.2 Flexural strength test

Testing for flexural strength was conducted on mortar specimens conforming to BS EN 196-1:2005. Samples with dimension of 40×40×160mm were tested using three-point bending test, Figure 11. Test specimens were loaded into two steel supporting rollers, with each roller having 10.0 ±0.5 mm in diameter and a 100±0.5mm spacing in between. Load was applied using a third roller with the same diameter as the other two, at a steady speed of 0.04-0.06N/mm^2. Test was halted at failure point i.e. after the specimen has reached maximum load.

Figure 57 Flexural strength test

3.7.3 Measurement of porosity of mortar specimens

The Porosity of mortar specimens with or without bacteria was measured following steps used by Chen et al, 2012; Siddique et al, 2015; Lian et al, 2011. Firstly, specimens were cut into cubes with dimensions 40×40×40mm, then dried in oven at 40°C until no change in weight, this was recorded as the Dry weight (W_{dry}). The super saturated surface dry weight (W_s) was achieved by using vacuum technique, specimens were loaded into the vacuum chamber which was tightly sealed and pressurised at 1 bar. Once the vacuum has reached 1 bar, water was injected into the chamber under pressure. Specimens were vacuumed under pressure of 1 bar (vacuum pump was kept on during this time) for a minimum period of 6 hours to ensure that the sample has truly achieved super saturation, excess water on the surface of each specimen was removed using absorbent cloth.

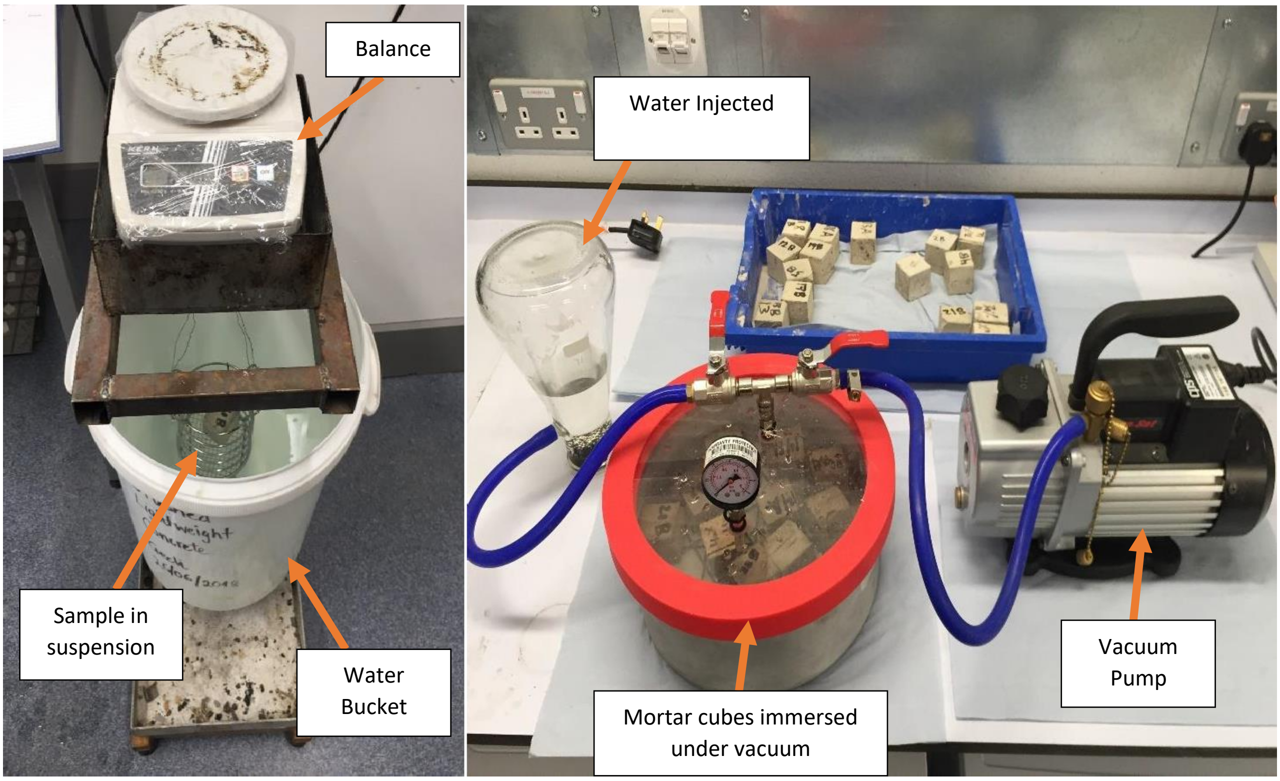


Figure 57 The suspended weight of mortar cubes, W_s (left), the saturated surface dry (W_s) using vacuum.

The suspended weight (W_s) was obtained by suspending the sample in a water tank and recording the weight in suspension. The following formula was used to calculate porosity:

Porosity (%) = $\frac{W_{ssd} - W_{dry}}{W_{ssd} - W_s}$

Where, W_{dry} is the dry weight of the sample, W_{ssd} is the super saturated surface dry and W_s is the weight in suspension.

3.7.4 Permeability via capillary absorption

Permeability of specimens with/without bacteria was measured via capillary action. The method used for this test was in accordance to ASTM D 6489, with slight modification, test specimens with dimension of 40×40×40mm were used. Dry weight of each specimens was obtained in oven at 105C until constant mass is achieved. A water proof sealant was applied on all specimens' side except the bottom and top layer to prevent ingress water, specimens were allowed to cure for almost 24hours before conducting the test.

Weight of the specimen with sealant was recorded. Test was conducted in a plastic bucket with a water level not exceeding 5±1mm, where the unsealed face is rested on a stainless-steel mesh to allow water circulation. Readings were taken at regular time intervals 1h, 2h, 4h, 8h, 24h and 48 hours. Water uptake/absorption (%) of the tested specimens was calculated using the following formula:

Absorption (%) = $\frac{W_2 - W_1}{W_1}$ ×100

Where, W_2 is the weight of the immersed sample at time t and $W_1$is the dry weigh of the tested specimen.

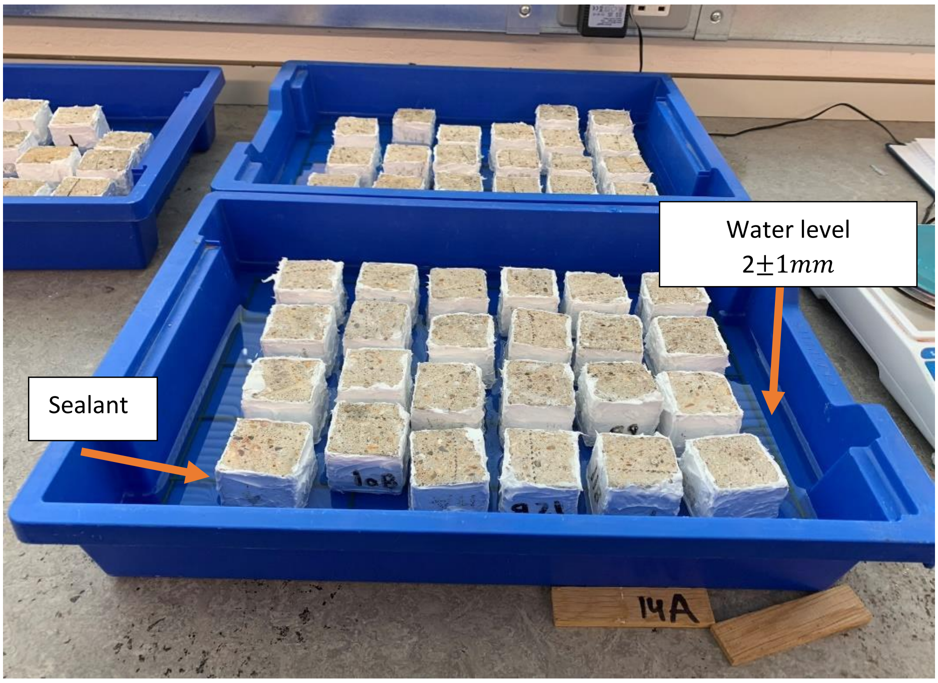


Figure 58 Water uptake of mortar cubes.

3.8 Encapsulation of healing agent (spores + nutrients)

To achieve self-healing concrete without the need for human intervention led the study to explore possible carriers for the application of self-healing mortar. Therefore, autoclaved aerated recycled concrete aggregates (AARC) were investigated for this study. Impregnation of the healing agent was conducted following methods used by Jonker et al 2010, 2011, 2012; Wiktor et al 2011; Alghamri et al 2016, with slight modification. Aggregates ranging from 0.05-2 mm were used in this study. Impregnation under vacuum was conducted twice, where the first process involves injecting bacterial spore solution under 1 bar into the vacuumed aggregates, aggregates were then oven dried at 45C until constant weight change is achieved.

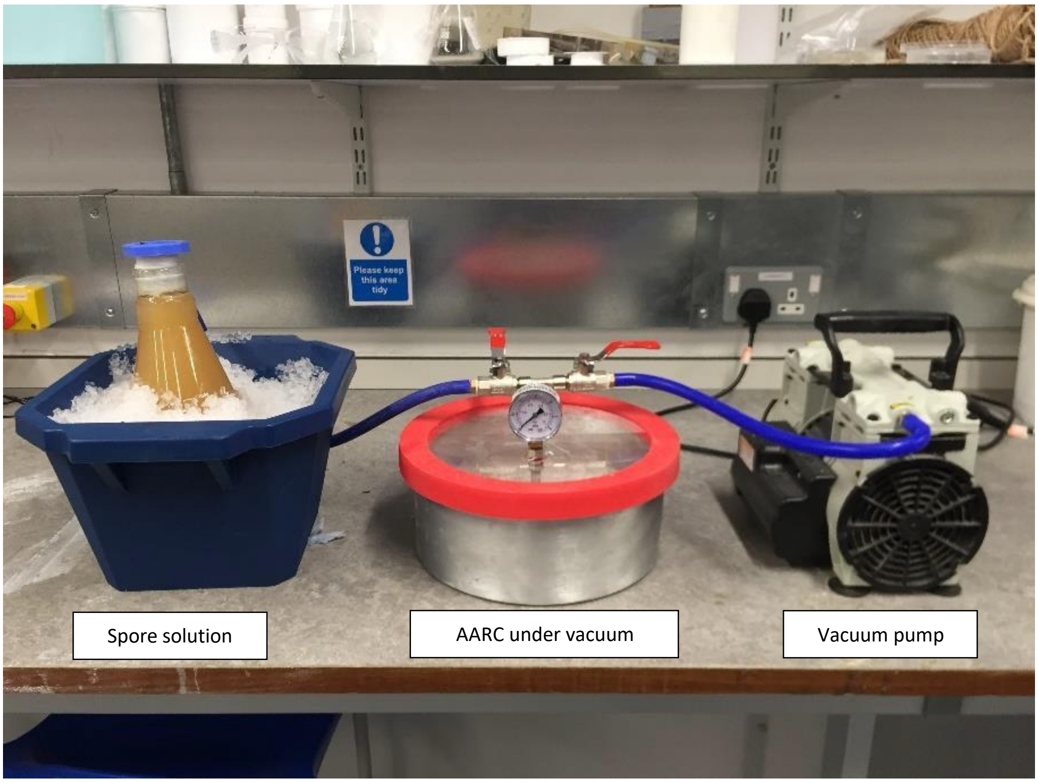


Figure 59 The introduction of bacterial spores, first cycle of impregnation

The second cycle of impregnation consisted of injecting nutrient solution composing of (g/l): Urea 150, calcium acetate 112.5 and nutrient broth 7. At each cycle the solution level, in both cases, was maintained to a minimum of 10±2mm above the immersed aggregates.

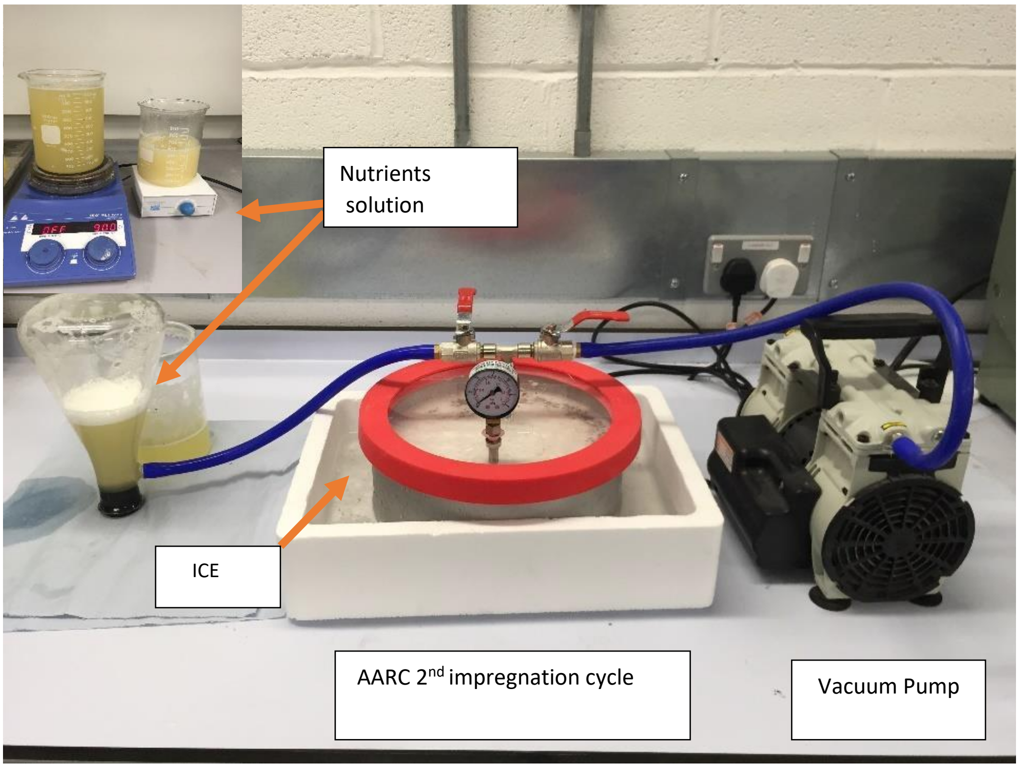


Figure 60 2nd cycle of impregnation i.e. medium encapsulation.

In order to avoid possible germination of bacterial spores during the drying stage of the aggregates, drying was conducted by means of freeze-drying method. Up on the completion of the first cycle of bacterial spore impregnation, incorporated aggregates were placed in a fabricated foil mould which was positioned on an aluminium tray with a thickness of 3mm and four bottom supports. Liquid nitrogen was then injected into the aluminium tray to allow freezing of the aggregates. Freeze drying was conducted using Alpha 1-2 LD chamber with a maximum ice condenser of 2kg/24 hr and ice condenser temperature of -55C. The freeze-dryer was set to pre-freezing 1 hour before the main drying to reach -55C. The frozen aggregates were then loaded into the freeze-drying chamber and subjected to high vacuum for a minimum period of 7 days.

Figure 61 Freezing of the aggregates using liquid nitrogen, before loading into the freeze dry chamber.

Several studies stressed the need for a suitable coating material to minimise the effect of the healing agent on the cement matrix. Therefore, to minimise leakage of the impregnated aggregates, a spray coating material consisting of 30% sodium silicate solution (v/v) was used. Furthermore, simultaneously sprinkles of fly ash were applied on the aggregates to enhance the bonding between the impregnated aggregates and the cement matrix, effectiveness of the coating material is depicted in chapter 4, following method used by Al-Azhari et al., 2017.

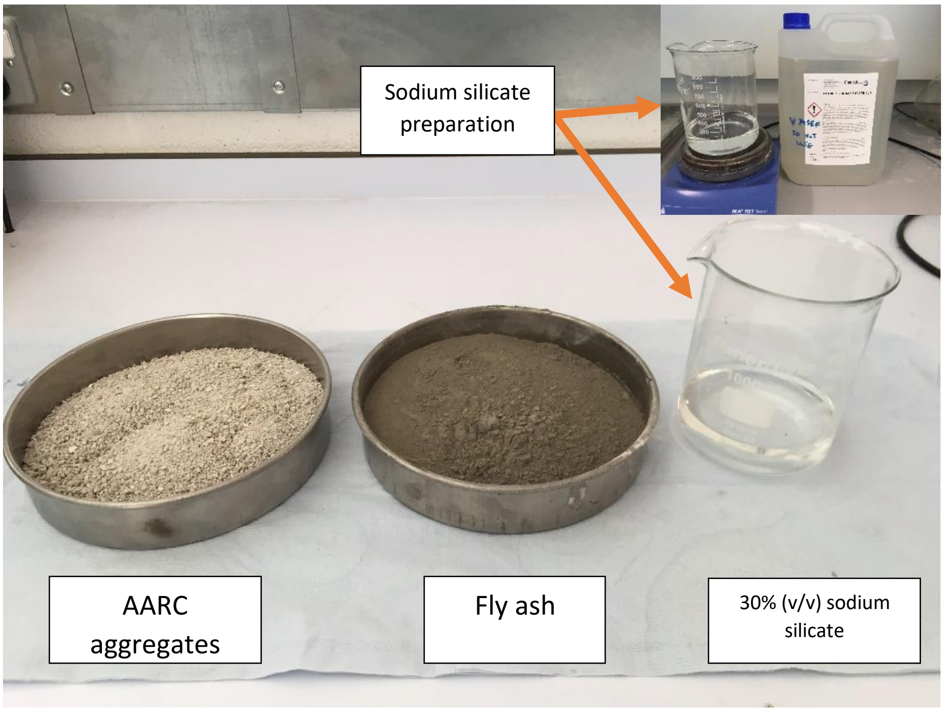


*Figure 62 T*he application of the coating material

3.8.1 Quantification of the cell viability of the impregnated autoclaved aerated recycled concrete aggregates (AARC)

The viability of bacterial spores and the Incorporation in a suitable carrier were investigated in this study. Previous studies by Jonkers et al., 2010, reported the loss in viability of bacterial spores when incorporated/ applied into the cement matrix, where cement past containing bacteria was crushed using hand held mortar. One factor that should be noted down is that the use of such crushing technique exerts high temperature due to the shear force from the rotational speed of the mortar, resulting in more loss in cell viability in addition to other factors such as high pH.

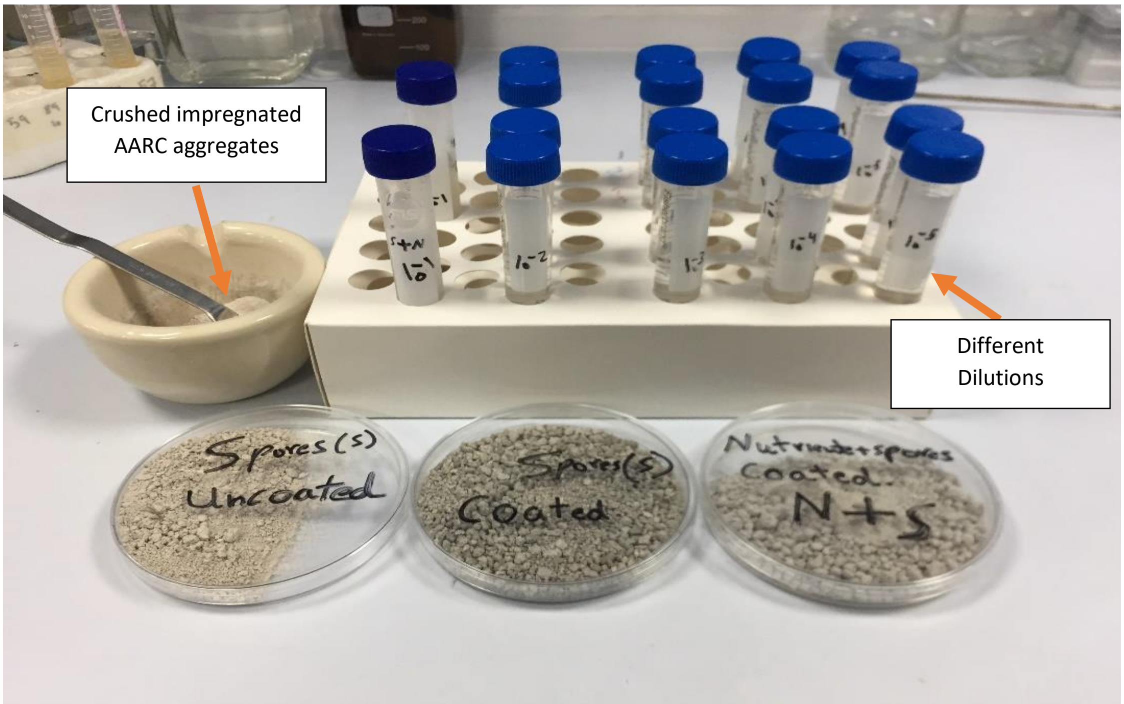


Figure 63 Evaluating the amount of CFU per gram, using serial dilution.

Therefore, in this study simply uses mortar and a pistol to reduce the amount of heat and physical forces created. Sterile conditions were followed as careful as possible; however, contamination is reduced during the increase of dilution factor. A minimum of 10 dilutions were used for the determination the number of viable spores. A total amount of 10 grams of the impregnated AARC aggregates were crushed in the mortar, the mix was then homogenised and 1 gram was taken and suspended in a 50ml flacon tube containing only 9ml of sterile distilled water i.e. 10^{-1} dilution, this was performed until 10^{-10} has been achieved. Using a pipette, a 100µl was taken from each dilution and inoculated onto separate agar plates. Plates were then incubated overnight at 37°C.

Table 8 Mix design for crack healing mortar, stage 1.

Sample NO.	Material	Impregnation	Amount of sand (%)	Strain Type	Live Cells (Cfu/ml)	Nutrients (v/v of water)	Cement(g)	Sand (g)	Water(g)	Amount of LWA (g)	crack size (microm)
Stage 1											
1	LWA	NON	20%	N.A	N.A	N.A	346.6670	1029.7206	187.7780	96.9464	
4	LWA	Spores only (S)	20%	N.A	N.A	N.A	346.6670	1018.8867	187.7780	107.7803	
10	LWA	Spore + Nutrients (S+N)	20%	N.A	N.A	N.A	346.6670	1014.9413	187.7780	111.7257	
2	LWA	NON	30%	N.A	N.A	N.A	346.6670	981.2474	187.7780	145.4196	
5	LWA	Spores only (S)	30%	N.A	N.A	N.A	346.6670	964.9966	187.7780	161.6704	
11	LWA	Spore + Nutrients (S+N)	30%	N.A	N.A	N.A	346.6670	959.0784	187.7780	167.5886	
3	LWA	NON	40%	N.A	N.A	N.A	346.6670	932.7742	187.7780	193.8928	
6	LWA	Spores only (S)	40%	N.A	N.A	N.A	346.6670	911.1064	187.7780	215.5606	
12	LWA	Spore + Nutrients (S+N)	40%	N.A	N.A	N.A	346.6670	903.2156	187.7780	223.4514	

Table 8 Mix Design for crack healing mortar, Stage 2 and 3

Sample NO.	Material	Impregnation	Amount of sand (%)	Strain Type	Live Cells (Cfu/ml)	Nutrients (v/v of water)	Cement (g)	Sand (g)	Water(g)	Amount of LWA (g)	crack size (microm)
Stage 2											
15	LWA	NON	30%	89	10^7	N.A	346.6670	981.2474	187.7780	145.4196	
16	LWA	NON	30%	89	10^8	N.A	346.6670	981.2474	187.7780	145.4196	
21	LWA	S+N	30%	89	10^7	N.A	346.6670	959.0784	187.7780	167.5886	
22	LWA	S+N	30%	89	10^8	N.A	346.6670	959.0784	187.7780	167.5886	
Stage 3											
13	LWA	NON	40%	89	10^7	N.A	346.6670	932.7742	187.7780	193.8928	
14	LWA	NON	40%	89	10^8	N.A	346.6670	932.7742	187.7780	193.8928	
17	LWA	S+N	40%	67	10^7	N.A	346.6670	903.2170	187.7780	223.4514	
18	LWA	S+N	40%	67	10^8	N.A	346.6670	903.2170	187.7780	223.4500	
19	LWA	S+N	40%	89	10^7	N.A	346.6670	903.2170	187.7780	223.4500	
20	LWA	S+N	40%	89	10^8	N.A	346.6670	903.2170	187.7780	223.4500	

3.9 Crack healing experiment

3.9.1 Crack creation and incubation

For crack healing experiment, Mortar specimens were embedded with an alkaline mesh sheet which was added during the mixing stage. The method used for this test was in accordance to that used in Al-Azhari et al 2017, a layer of cement paste in the range of 10-15mm was first poured into the mould, followed by placing the mesh sheet on top. The subsequent steps were the same as the steps explained in section 4.7.1 Casting and fabrication of fresh mortar. The purpose of such approach is to ensure that the sample is intact during cracking stage, in addition to maintaining uniform crack line along the middle position of the specimen. After 24 hours of casting, specimens were demoulded and cured at 20°C±2 and 95% humidity for 28 days. Crack creation was done by means of flexural strength, load was applied at a constant rate by adjusting the load drop in the setting menu by 0.04-0.06N/mm^2. Load was further applied manually to achieve higher crack widths when required. After 28 days of cracks, specimens were subjected to wet-dry cycles, during one cycle specimens were submerged in water for 12 hours and allowed to breath at room temperature for another 12hours.

3.9.2 Monitoring of crack width

Crack widths were monitored microscopically at regular time intervals 3, 7, 14 and 28 days of cracking, at each time point pre-cracked specimens were measured using an Olympus SZX16. The microscopic imaging software was provided by Leica Microsystems, scale was adjusted using 10mm long graticule with a 0.1mm spacing division, as shown in the figure. Once the scale has been confirmed, each cracked specimen was loaded into the microspore base carefully for crack width measurements.

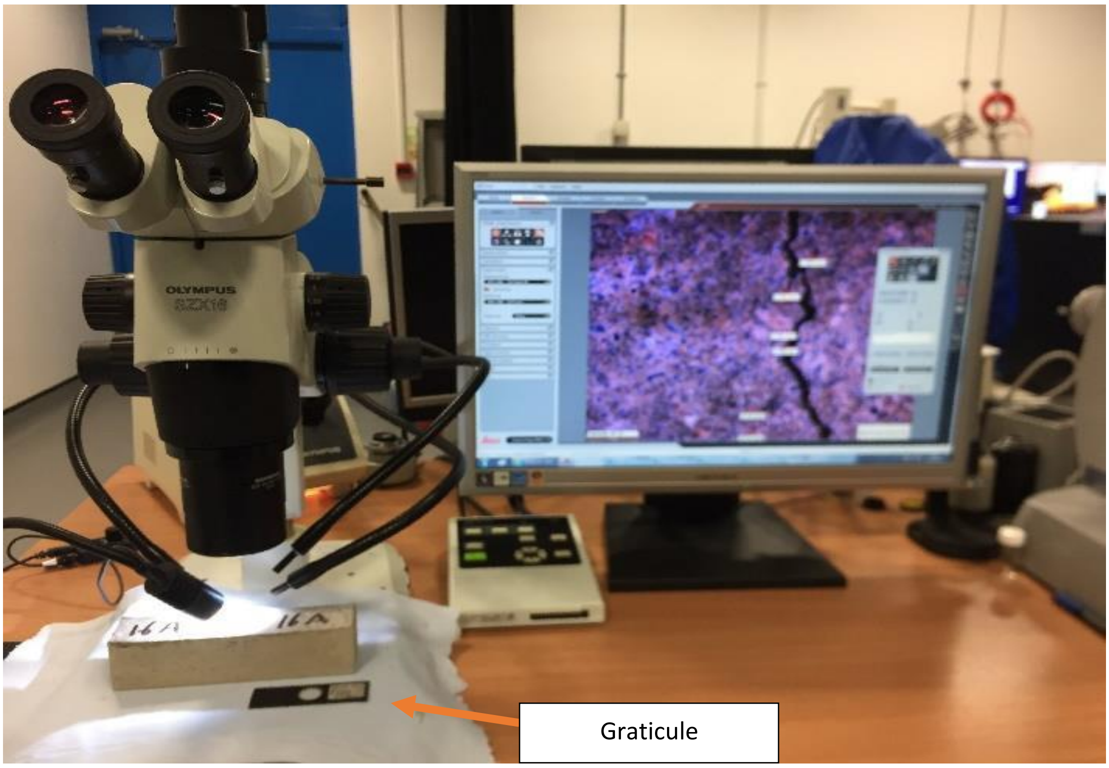


Figure 64 Microscopic analysis for measuring Crack width

3.9.3 The evolution of the healing efficiency

The healing evolution of pre-cracked mortar prims was determined using the method used by Wiktor et al., 2011; Wang et al, 2014; Jonkers et al 2011. Using microscopic imaging at regular time intervals (7, 14, and 28 days), the healing efficiency of pre-cracked mortar specimens was quantified by measuring the crack width before and after healing. A minimum of 5 readings at regular intervals along the crack line were taken. The healing efficiency was determined using the following formula:

Healing percentage (%) = $\frac{W_i - W_f}{W_i} \times 100$

Where $W_i$the initial is crack width and $W_f$is the final crack width at time t.

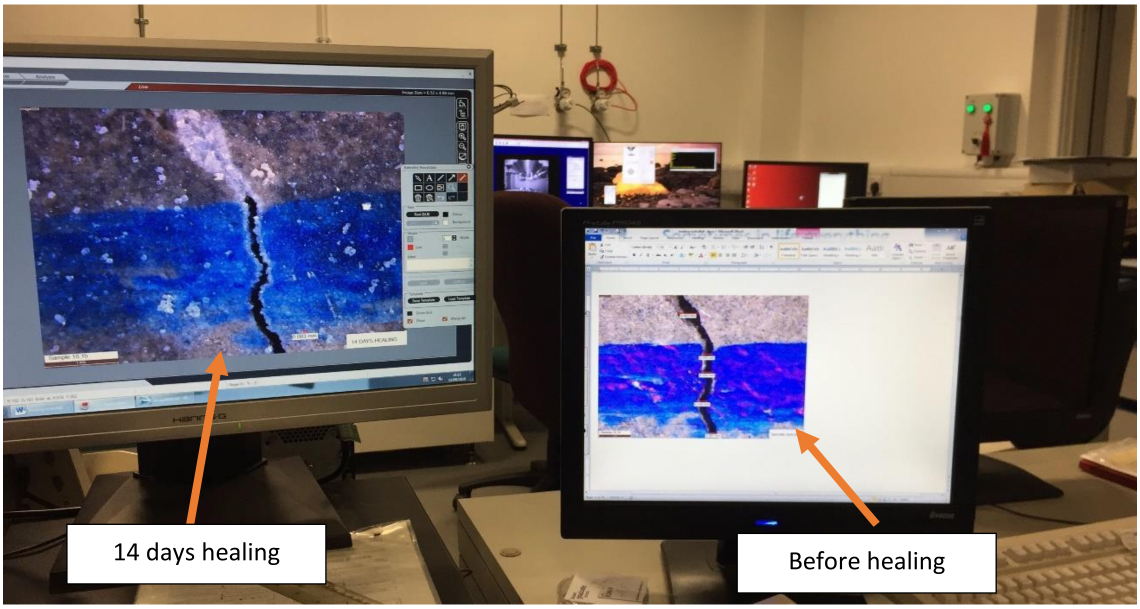


Figure 65 Monitoring of the healing process

3.9.4 The efficiency of healed mortar via capillary water absorption

The efficiency of healed mortar was examined by means of capillary water absorption, test was conducted following method used in assessing the durability via determination of capillary absorption EN 13057:2002. Mortar prims specimens with dimension of 40×40×160mm were first dried in oven at 40°C for a minimum period of 7 days to obtain constant weight change. The following procedure were followed for sample preparation:

- All samples were covered with a double layer of a water proof tape/sealant except an area of 20×40mm centred at the crack zone.
- The dry weight (W_1) including the water proof tape/sealant was obtained prior to conducting the test.
- Sample were immersed in a water tank with crack face/healed are on the bottom. Sample were positioned on a stainless-steel mesh with a water level not exceeding 2.0±1mm.
- The weight increase at regular time intervals was recorded i.e. 0.25 hour, 0.5hour, 1 hour, 2 hours, 4 hours, 6 hours and 8 hours. Excess water on the surface was removed using absorbent cloth before each weighing.

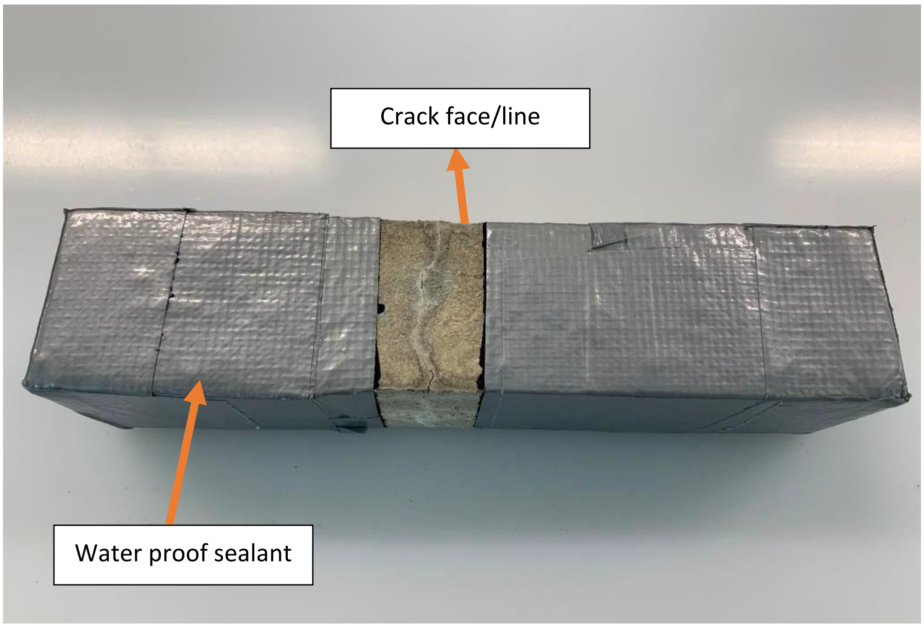


Figure 66 Sample preparation for permeability test.

The data obtained from the capillary water absorption were used to calculate and plot the water uptake (i) against $t^{0.5}$. The sorption coefficient (SC) was calculated using the formula adopted from Hall et al 1989, for testing materials with little suction. The following formulas have been used:

$$i = \frac{\Delta W}{\rho A}$$

Where,

ΔW: Is the change in weight (g) at time t, with the dry weight being W_1 and the wet surface dry weight is W_2.

ρ: the density of water (g/mm^3) A : the tested are (mm^2)

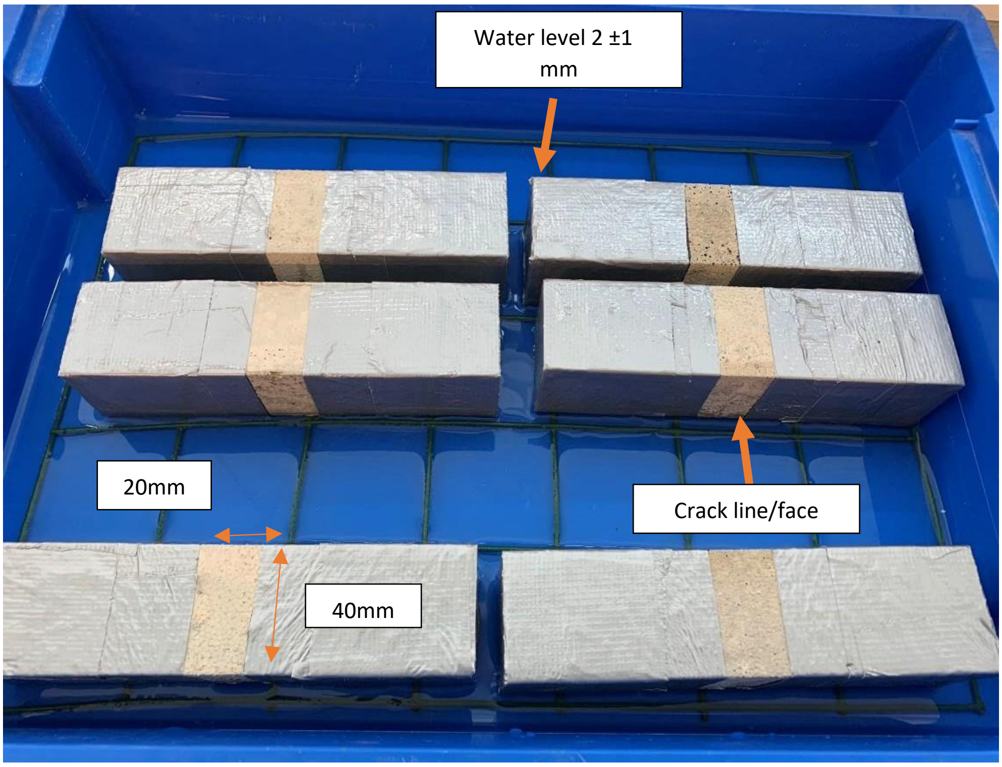


Figure 67 Permeability test healed mortar via water uptake

The line of best fit was applied using excel to obtain the below equation to help calculate the sorption coefficient (SC) i.e. the gradient:

$$i = SC\ t^{0.5} + A \qquad (5)$$

Where:

i: the water uptake (mm)

A: constant value or the intersection at the y-axis t: time in hours

SC: the sorption coefficient in

3.9.5 Analytical techniques

3.9.5.1 Fourier infrared spectroscopy (FTIR)

The functional groups of the in-vitro precipitation and that produced at the crack mouth (including control specimens) were analysed through Fourier infrared spectroscopy (FTIR) using Perkin Elmer Spectrum one. Samples were dried at 40C until constant weight is achieved then crushed into homogenised fine powder and kept in a desiccator (with silica beads) to avoid excess moisture absorbance, until analysis. Samples were then positioned on the ATR equipped with 3 × bounce diamond crystal detector.

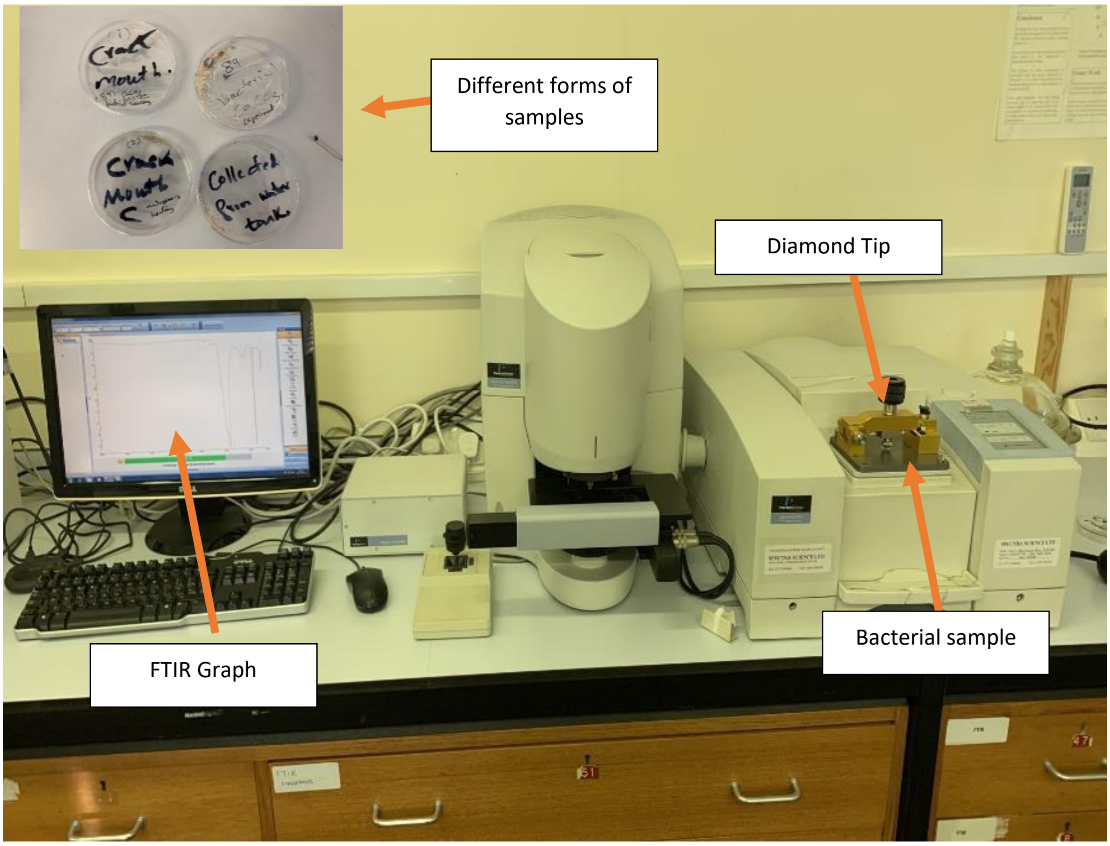


Figure 68 FTIR analysis.

3.9.5.2 X-ray powder diffraction (XRD)

The collected precipitation was further analysed using X-ray powder diffraction to confirm the type of crystals formed or responsible for self-healing concrete. XRD pattern were recorded via X'pert ray D8 advanced Burker AXS diffractometer with CuKα radiation source (λ=0.15406 nm), graphite monochromator and a 3-d-area detector GADDS system. 2 θ was scanned from 5-100 (2×30minutes) with a resolution of 0.02 at rate of 1/min. mortar samples were ball milled to obtain a fine powder.

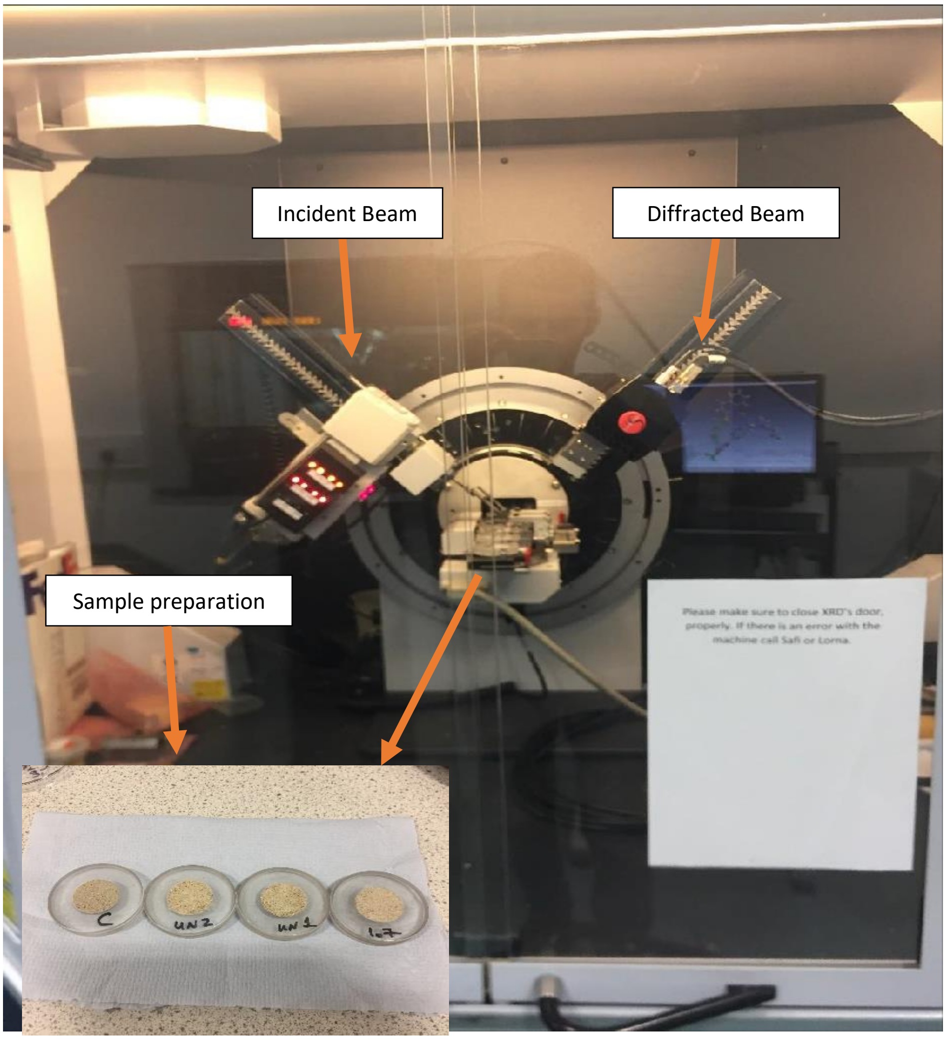


Figure 69 X-ray Powder Diffraction.

3.9.6 Microstructure characterisation

3.9.6.1 Scanning electron microscope

Further analysis of the microstructure of the collected precipitate was also conducted. Analysis was done through field emission gun-scanning electron microscopy, LEO 143VP SEM. Prior to analysis, samples were fixed into the holders using double carbon tape then sputter coated twice with a thin gold layer using Polaron-SC640 for 90 seconds.

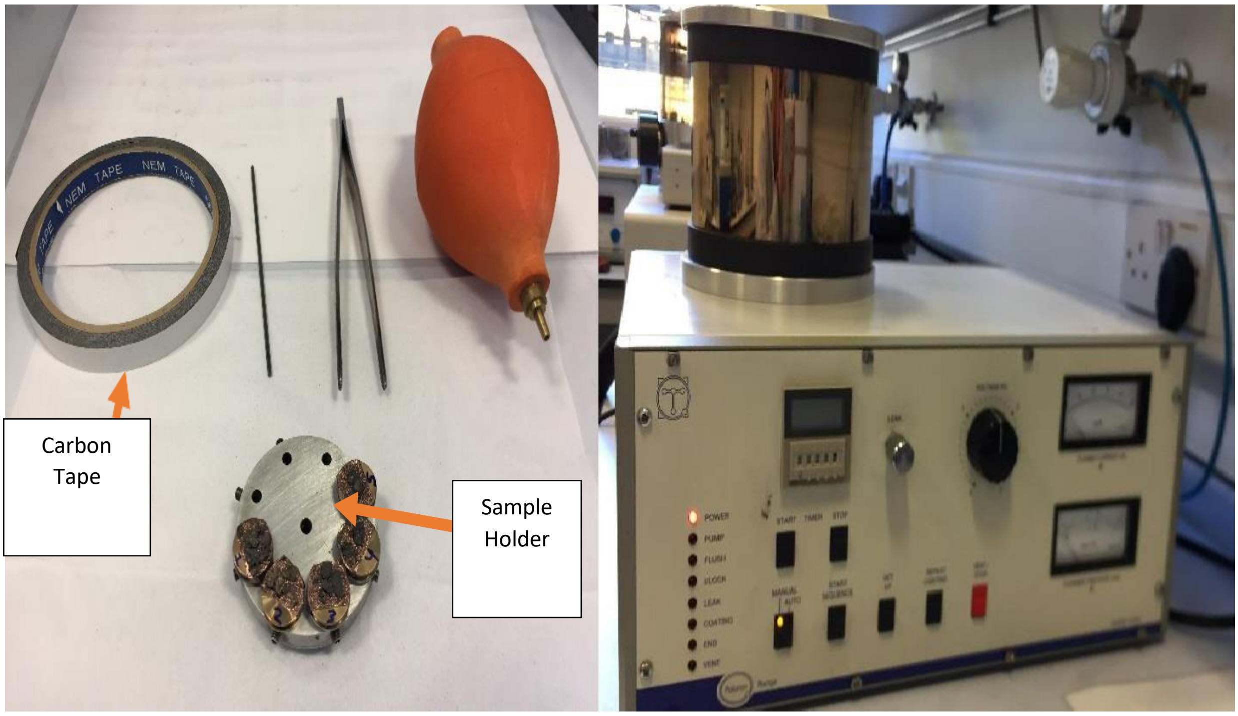


Figure 70 Sample preparation (left), and gold coating (Right).

Figure 71 LEO®1430VP FESEM.

The sample holder was then placed into the SEM chamber and vacuumed. Imaging was conducted using SE2 detector at scanning speed of 9±2, the electron high tension (EHT) was adjusted at 10KV. Different parameters such as distance, brightness, stigmatisms and contrast were all adjusted to the desired level for better imaging.

3.10 Ethics

All the research and innovation activities carried out during the project will comply with ethical principles and relevant national, Union and international legislation, including the Charter of Fundamental Rights of the European Union and the European Convention on Human Rights and its Supplementary Protocols. Concerning the protection of individual privacy (personal data), participants will respect the national legislation concerning the processing of personal data and the free movement of such data from one subject to another. Personal and professional contact data collected at the occasion of workshops, meetings, etc. will not be released or distributed without the consent of the owners.

3.11 Health and safety

It is considered essential to be aware of the different environmental aspects involved around the project (especially real scale demonstration and testing work), from both a design and a construction point of view. An environmental impact assessment is obligatory for projects which are considered as having significant effects on the environment (for example: due to the handling of biological samples, waste disposal installations for hazardous waste, waste disposal installations for non-hazardous waste, waste water treatment plants). The principal environmental aspects may include atmospheric emissions (dust, particles, smoke and gas, noise, vibrations), discharges, waste management, raw materials and natural resources (water catchment, occupation of land and waterways, water, electricity and fuel consumption) and others (changes in accessibility, impact of services on heritage, flora and fauna and natural spaces). These criteria have been assessed and it hasn't been identified any significant negative environmental impacts for the project. Health and safety issues has been taken in account at the project design and planning stages, as well as during the construction phase. During real scale demonstration and testing work the European standards of occupational safety and health in construction has been be deployed.

3.12 Identification of errors and repeatability of the experimental work

Conducting the experimental work on the biological samples i.e. live cells was a key challenge in this project. all bacterial strains that have been used in this study are clearly labelled, categorised and conserved. All experimental work reported in this study have performed at least twice, with each run having at least three replicates for each sample, therefore, the final reported result represents the average of three runs. All the methodology used throughout this project have been clearly stated step by step i.e. substantial expertise and training was provided prior to conducting the experiment, specially the bioengineering part. when performing the repeatability test, one must ensure that the same method, same operator, same equipment, same environmental conditions and the same chemical and items used for conducting the test.

3.13 Summary of the methodology

The main methodologies used throughout this study have been briefly described in Figure 72. Several reported results and studies have indeed had a great influence on achieving and understanding the mechanisms of self-healing concrete. Delft university is the leading institute in self-healing materials, where Jonker et al 2010;2011 was the first to use bacterial species in self-healing concrete via the metabolic conversion of calcium lactate into insoluble calcium carbonate, within the same institute, Wang et al 2017 also conducted a thorough investigation on the suitability of using a spore-forming bacterium via urea hydrolysis. The methodologies used in obtaining spores, cell isolation, characterisation and assessing the self-healing properties have been greatly adopted from those aforementioned studies.

Furthermore, the In-vitro calcium carbonate precipitation experiment was inspired by the study conducted at Bath university though Al-azhari et al 2017, where they have designed and optimised a calcium carbonate precipitating medium for the use of self-healing concrete. Studies conducted by Paine et al at Bath university have been a great inspiration in identifying the current gaps and obstacles towards self-healing concrete. Where the need for a suitable bacterium and medium for maximum calcium carbonate precipitation have been clearly raised.

Finally, the use of autoclaved aerated recycled concrete as an encapsulating material for the use of self-healing concrete have been inspired by Dr Kastiukas through working on construction waste materials (CDW).

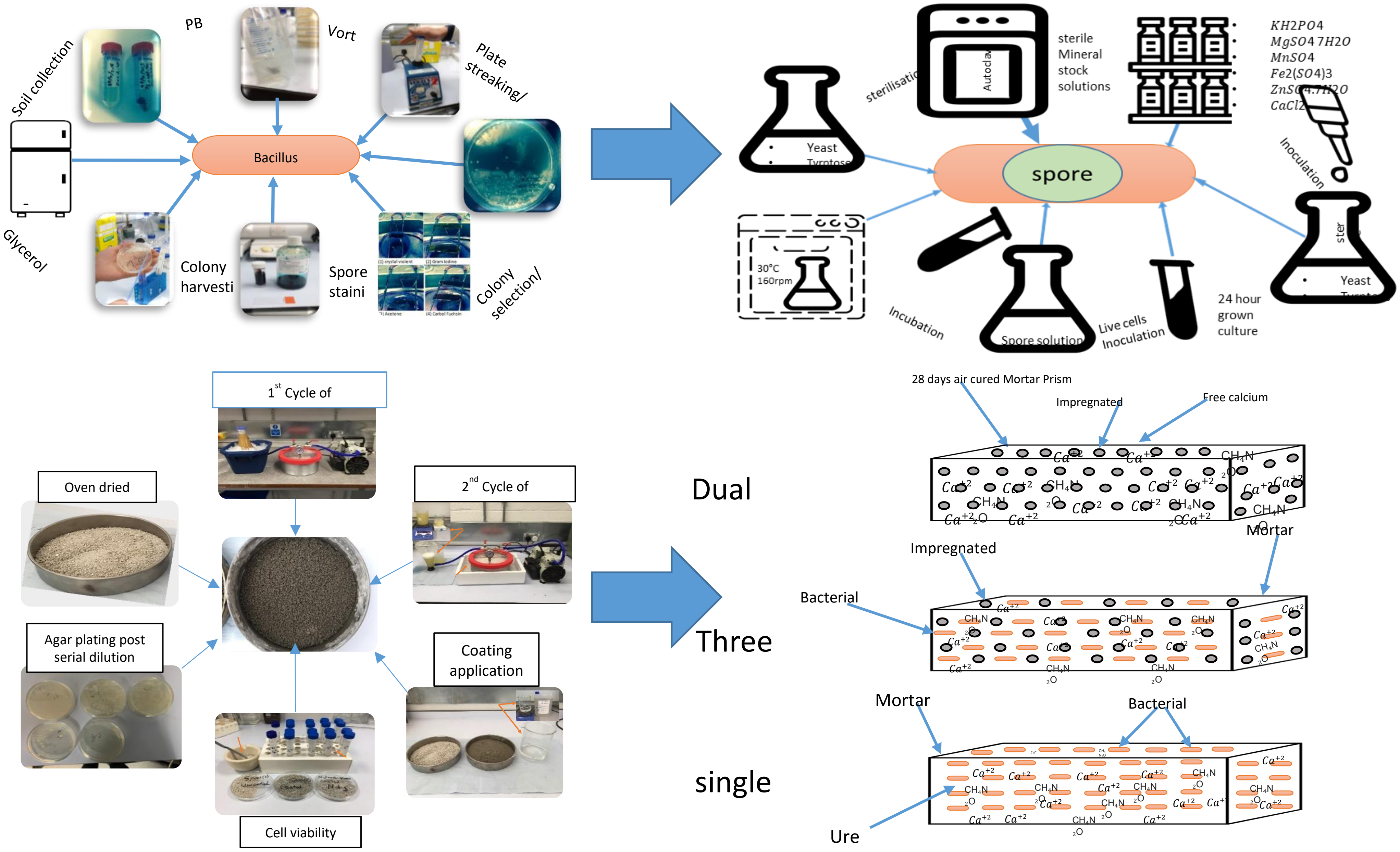


Figure 72 Summary of the main methodologies used.

Chapter 4 Characterisation of various bacillus Sphaericus strains for the use of self-healing mortar

4.0 In-vitro assay of the total calcium precipitation using different bacterial strains

4.1 Introduction

This part of the study investigates the efficiency of different bacterial strains in producing calcium carbonate precipitation ($CaCO_3$) using five different media compositions that were reported in the literatures. Quantification of total calcium carbonate precipitation has been reported by several studies (Stock-fischer et al., 19999; Okwadha et al., 2010; Achal et al., 2010; Dahmi et al., 2013; 2016; Krishnapriya et al., 2015; Xu et al., 2015; Sharma et al., 2017; Schwantes-Cezario et al., 2017; Wei et al., 2015 and Alazhari et al., 2017). As reported in the literatures, the use of bacteria for the application of self-healing material follows at least three different approaches, namely, via urea hydrolysis bacterium, denitrification and through the conversion of inorganic calcium lactate into insoluble calcium carbonate ($CaCO_3$). Extensive study of the reported literatures led to the conclusion that self-healing concrete via urea hydrolysis bacterium is indeed the most efficient and easiest approach towards self-healing concrete. One can argue that such approach can lead to nitrogen loading, however, this problem can be tackled by the incorporation of mixed culture for the application of self-healing concrete. Ersan et al., 2014 used a denitrifying bacterium for the application of self-healing concrete, namely, Diaphorobacter nitroreducens. The strain showed promising results in inducing calcium carbonate precipitation through the oxidation of organic matter by using nitrate (NO^-) as an electron acceptor in the absence of oxygen (O_2), i.e. it reduces the amount of fixed nitrogen. Therefore, incorporation of a mixed culture can enhance the healing efficiency of healed mortar and tackle problems in regard to nitrogen loading.

Characterisation of bacterial strains for the application of self-healing smart material was conducted by different means, i.e. the presence of urease enzyme, ability of the strain to survive in the alkaline environment of the concrete matrix, ability to produce copious amounts of calcium carbonate precipitation and finally ability to produce spores. However, Inconsistency exists in the reported literatures in regard to the optimum nutrients/healing agent for maximum healing efficiency. It is understood that each bacterial strain behaves differently when subjected to different environments, some may thrive and multiply, while other may be stressed and sporulate or even die. The same can be applied in regard to the optimum nutrients, as some bacteria may utilise glycerol as a carbon source, while other maybe more efficient in utilising calcium acetate as a carbon source. Jonkers et al., 2010; 2017 stressed the use of yeast extract as a carbon source for urea hydrolysis pathway and as a nitrogen source for the metabolic pathway, however, such component has been shown to greatly affect the mechanical properties of the cement matrix when incorporated at higher percentage than 0.5% w/w of cement, Cunniffe et al., 2011. Different calcium salts have been used in the reported literature, the choice of the calcium compound have been reported to play a vital role toward the morphology and rheology of the formed calcium carbonate crystal (Stock-fischer et al., 19999; Okwadha et al., 2010; Achal et al., 2010; Dahmi et al., 2013 2016).

Therefore, it can be concluded that all reported literature agrees to the fact that bacteria is indeed the most important component in self-healing bacterial concrete. Prior to the application of concrete, bacteria must be tested in laboratory scale, i.e. in conditions that mimic the concrete environment. Therefore, this part of the study characterises 130 different bacterial strains that belong to bacillus species, namely, Bacillus Sphaericus. Characterisation was done initially to check the presence of the urease enzyme using the Stuart assay in broth state. Bacterial strains with positive urease enzyme were grown in an alkaline nutrient agar and incubated at 37°C for 48 hours to examine the capacity of bacterial strains to grow in alkaline environment. The strains were then checked for the ability to produce spores. In-vitro calcium precipitation experiment was conducted on strains with weak to strong urease activity to understand the biochemical properties responsible for calcium carbonate precipitation. Five different Medias were selected from the reported literatures, where each medium uses different types and amount of nutrients.

On the basis of the aforementioned parameters, this chapter encompasses the following

- Characterisation and screening of different Bacillus Sphaericus strains for the application of self-healing mortar.
- Understanding the biochemical properties of the selected strains for the application of self-healing mortar, through the measurement of ammonium production during the calcium carbonate precipitation.

4.2 Research program

The first part involves in screening 100 bacterial strains belonging to bacillus species namely, bacillus Sphaericus. The presence of urease enzyme in the selected strains was determined using the urease test adopted from Stuart broth and the test was further confirmed in agar state, i.e. using Christensen's Urea Agar (UAB). Both composition and the make of each media are presented in Section 3.2.3 e and f. In both cases, the inoculated samples were incubated over night at 37°C to observe the change in colour intensity due to the presence of phenol red in the medium. Positive results were obtained for samples with deep red colour.

The outcome of the urease test is a key factor in self-healing mortar using urea hydrolysis, the bacterial strains with positive results, i.e. weak to strong colour intensity of deep Red colour, were further analysed for pH tolerance. Nutrient agar plates with pH 10 were prepared, the preparation of nutrient agar was similar to that explained in Section 3.3.1. All components in the media such as nutrient broth, calcium source and urea were added and dissolved in distilled water, followed by adjusting the pH, then the media was toped up to the mark in order to avoid dilution.

The third part of the experiment investigates the effect of five different mediums, which were reported in the literature, namely, M1 (composition (g/l): 25.0 nutrient broth, 25.2mM calcium chloride, 20.0 urea); M2 (3.0 nutrient broth, 25.2mM calcium chloride, 20.0 urea); M3 (composition (g/l): 3.0 yeast extract, 25.2mM calcium chloride, 4.0 Peptone, 20.0 urea); M4 (composition (g/l): 25.0 nutrient broth, 25.2mM calcium acetate, 20.0 urea); and M5 (composition (g/l): 3.0 yeast extract, 25.2mM calcium acetate, 4.0 peptone, 20.0 urea), Chapter 3 Section 3.3.1. Details of the experimental program in regard to the type of media is shown in table 8, a minimum of three reading were taken throughout the whole study. Calcium precipitation experiment in this study was conducted using 250ml Erlenmeyer flask containing 100 ml of media, 1% inoculum was kept constant at all time, where bacteria were inoculated into the media during the log phase.

Table 9 In-vitro assay of calcium carbonate precipitation using different mediums

Strain	Media Type	Initial pH	Rpm	temperature (°C)	Inoculum (v/v)	Cells/ml	Duration (hours)
control	M1	7.4	140	37	1%	0	168
57	M1	7.4	140	37	1%	4.30E+08	168
58	M1	7.4	140	37	1%	2.50E+08	168
59	M1	7.4	140	37	1%	6.30E+08	168
60	M1	7.4	140	37	1%	5.15E+08	168
63	M1	7.4	140	37	1%	3.55E+08	168
64	M1	7.4	140	37	1%	8.20E+08	168
67	M1	7.4	140	37	1%	3.55E+08	168
89	M1	7.4	140	37	1%	4.35E+08	168
i	M1	7.4	140	37	1%	1.20E+08	168
57	M2	7.4	140	37	1%	4.30E+08	168
58	M2	7.4	140	37	1%	2.50E+08	168
67	M2	7.4	140	37	1%	3.55E+08	168
89	M2	7.4	140	37	1%	4.35E+08	168
I	M2	7.4	140	37	1%	1.20E+08	168
57	M3	7.4	140	37	1%	4.30E+08	168
58	M3	7.4	140	37	1%	2.50E+08	168
67	M3	7.4	140	37	1%	3.55E+08	168
89	M3	7.4	140	37	1%	4.35E+08	168

I	M3	7.4	140	37	1%	1.20E+08	168
57	M4	7.4	140	37	1%	3.05E+08	168
58	M4	7.4	140	37	1%	3.10E+08	168
59	M4	7.4	140	37	1%	3.00E+08	168
63	M4	7.4	140	37	1%	3.25E+08	168
64	M4	7.4	140	37	1%	3.75E+08	168
67	M4	7.4	140	37	1%	2.95E+08	168
79	M4	7.4	140	37	1%	4.80E+08	168
89	M4	7.4	140	37	1%	2.75E+08	168
57	M5	7.4	140	37	1%	3.05E+08	168
58	M5	7.4	140	37	1%	3.10E+08	168
64	M5	7.4	140	37	1%	3.00E+08	168
89	M5	7.4	140	37	1%	3.25E+08	168

In addition to the type of media, several key parameters that affects the bacterial activity and in turn impacting the final calcium precipitation yielding, such as temperature, rotational speed and the final amount of precipitation under high pH was also conducted in this study. The Calcium precipitation experiment was conducted under various temperatures, such as 37°C, 30°C and room temperature 20°C.

Table 10 The effect of temperature on the efficiency of the total calcium carbonate precipitation

Strain	**Media Type**	**Initial pH**	**Rpm**	**temperature (°C)**	**Inoculum (v/v)**	**Cells/ml**	**Duration (hours)**
control	M1	7.4	140	37	1%	0	168
57	M1	7.4	140	37	1%	4.30E+08	168
58	M1	7.4	140	37	1%	2.50E+08	168
59	M1	7.4	140	37	1%	6.30E+08	168
60	M1	7.4	140	37	1%	5.15E+08	168
63	M1	7.4	140	37	1%	3.55E+08	168
64	M1	7.4	140	37	1%	8.20E+08	168
67	M1	7.4	140	37	1%	3.55E+08	168
89	M1	7.4	140	37	1%	4.35E+08	168
i	M1	7.4	140	37	1%	1.20E+08	168
CONTROL	M1	7.4	140	30	1%	0	168
57	M1	7.4	140	30	1%	4.30E+08	168
58	M1	7.4	140	30	1%	2.50E+08	168
67	M1	7.4	140	30	1%	3.55E+08	168

89	M1	7.4	140	30	1%	4.35E+08	168
I	M1	7.4	140	30	1%	1.20E+08	168
57	M1	7.4	140	ROOM	1%	4.30E+08	168
58	M1	7.4	140	ROOM	1%	2.50E+08	168
67	M1	7.4	140	ROOM	1%	3.55E+08	168
89	M1	7.4	140	ROOM	1%	4.35E+08	168
I	M1	7.4	140	ROOM	1%	1.20E+08	168

The effect of shaking conditions was also considered as an important parameter, this is due to the fact that bacteria spores will remain in almost static condition when incorporated into the mortar. The test was done in order to observe the difference, if present, in the total amount of calcium carbonate precipitation when the shaking conditions became static.

Table 11 The efficiency of calcium carbonate precipitation under static conditions.

Strain	**Media Type**	**Initial pH**	**Rpm**	**Temperature (°C)**	**Inoculum (v/v)**	**Cells/ml**	**Duration (hours)**
control	M1	7.4	140	37	1%	0	168
57	M1	7.4	140	37	1%	4.30E+08	168
58	M1	7.4	140	37	1%	2.50E+08	168
59	M1	7.4	140	37	1%	6.30E+08	168
60	M1	7.4	140	37	1%	5.15E+08	168
63	M1	7.4	140	37	1%	3.55E+08	168
64	M1	7.4	140	37	1%	8.20E+08	168
67	M1	7.4	140	37	1%	3.55E+08	168
89	M1	7.4	140	37	1%	4.35E+08	168
i	M1	7.4	140	37	1%	1.20E+08	168
Control	M1	7.4	STATIC	37	1%	0	168
57	M1	7.4	STATIC	37	1%	4.30E+08	168
58	M1	7.4	STATIC	37	1%	2.50E+08	168
67	M1	7.4	STATIC	37	1%	3.55E+08	168
89	M1	7.4	STATIC	37	1%	4.35E+08	168
I	M1	7.4	STATIC	37	1%	1.20E+08	168

In a different set of experiment, in-vitro calcium carbonate precipitation was conducted under different pH levels, i.e. pH 7.4, 9.0 and 10.0. Several authors quantified the amount of calcium carbonate precipitation under optimum pH for the selected bacterial strain, however, the limited data in regard to the effect of pH on the bacterial activity in high alkaline environments, therefore, it is important to understand the bacterial metabolism and behaviour when subjected to unfavourable conditions such as high alkaline conditions i.e. mimicking the concrete environment.

Table 12 The effect of total calcium carbonate precipitation when subjected to pH 9, pH 10, and pH 7.4

Strain	Media Type	Initial pH	Rpm	Temperature (°C)	Inoculum (v/v)	Cells/ml	Duration (hours)
control	M1	9	140	37	1%	0	168
57	M1	9	140	37	1%	4.30E+08	168
58	M1	9	140	37	1%	2.50E+08	168
67	M1	9	140	37	1%	3.55E+08	168
89	M1	9	140	37	1%	4.35E+08	168
I	M1	9	140	37	1%	1.20E+08	168
Control	M1	10	140	37	1%	0	168
57	M1	10	140	37	1%	4.30E+08	168
58	M1	10	140	37	1%	2.50E+08	168
67	M1	10	140	37	1%	3.55E+08	168
89	M1	10	140	37	1%	4.35E+08	168
I	M1	10	140	37	1%	1.20E+08	168
control	M1	7.4	140	37	1%	0	168
57	M1	7.4	140	37	1%	4.30E+08	168
58	M1	7.4	140	37	1%	2.50E+08	168
59	M1	7.4	140	37	1%	6.30E+08	168
60	M1	7.4	140	37	1%	5.15E+08	168
63	M1	7.4	140	37	1%	3.55E+08	168
64	M1	7.4	140	37	1%	8.20E+08	168
67	M1	7.4	140	37	1%	3.55E+08	168
89	M1	7.4	140	37	1%	4.35E+08	168
i	M1	7.4	140	37	1%	1.20E+08	168

4.3 Results and discussion

4.3.1 Urease activity

A total of 100 different bacterial strains belonging to the bacillus species were screened for the urease enzyme. Using the urease test in broth state explained in chapter 3, Section 3.2.3 e, a total of 14 strains, showed urease positive ranging from weak to strong, the test was conducted for 24 hours and the change in colour intensity to deep red colour was continuously monitored over the test period. A noticeable change in colour was observed in strain 89 during the first 12 hours of testing, where a strong deep red violet colour was observed i.e. provides strong indication in regard to the urease activity in comparison to all other strains. Strains 57, 58, 59, 60, 61, 62, 64 showed strong urease activity but the change in colour was observed at around 18 hours of testing.

Table 13 Observation from the urease test

Strain number	Name	Urease test	comments
control	negative	Media with no inoculum	-
57	B. Sphaericus	Strong positive	-
58	B. Sphaericus	Strong positive	-
59	B. Sphaericus	Strong positive	-
60	B. Sphaericus	Strong positive	-
61	B. Sphaericus	Strong positive	-
62	B. Sphaericus	Strong positive	-
64	B. Sphaericus	Strong positive	-
67	B. Sphaericus	Medium -strong positive	-
79	B. Sphaericus	Weak Positive	-
81	B. Sphaericus	Weak Positive	Very similar to control
82	B. Sphaericus	Weak positive	-
85	B. Sphaericus	Strong positive	-
88	B. Sphaericus	Weak Positive	Very similar to control however a slight change in colour observed, hence weak positive
89	B. Sphaericus	Strong positive	The strongest out of all strains as the colour changed during 12 hours.

No change colour intensity in sample with no inoculum i.e. control. Strain 67 was indeed the only strain that showed medium-strong urease activity observed at 16 hours. However, strains with weak positive colour intensity was detected for samples 81, 82, 79 and 88 indicating limited urease activity over a 24 hours period as shown in Figure 73.

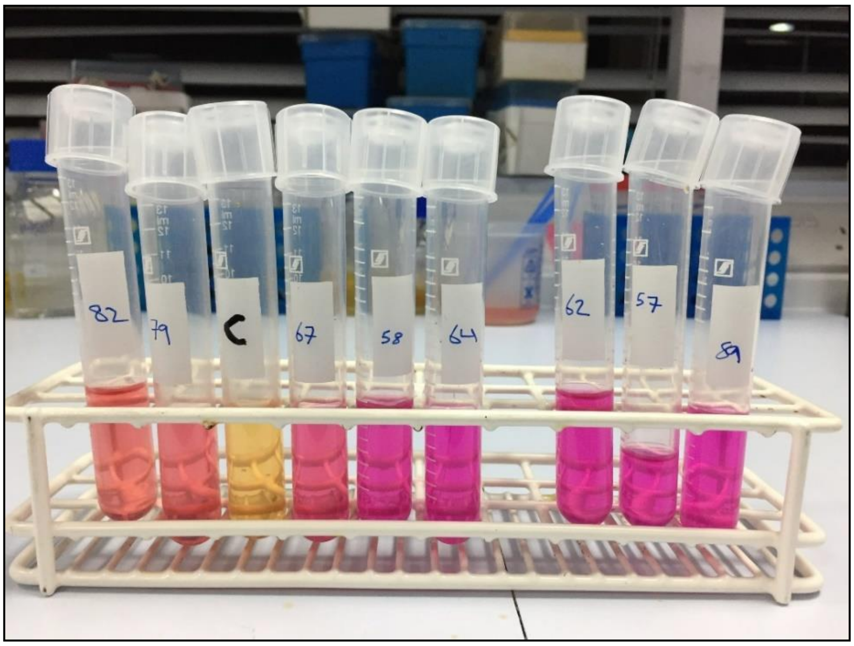
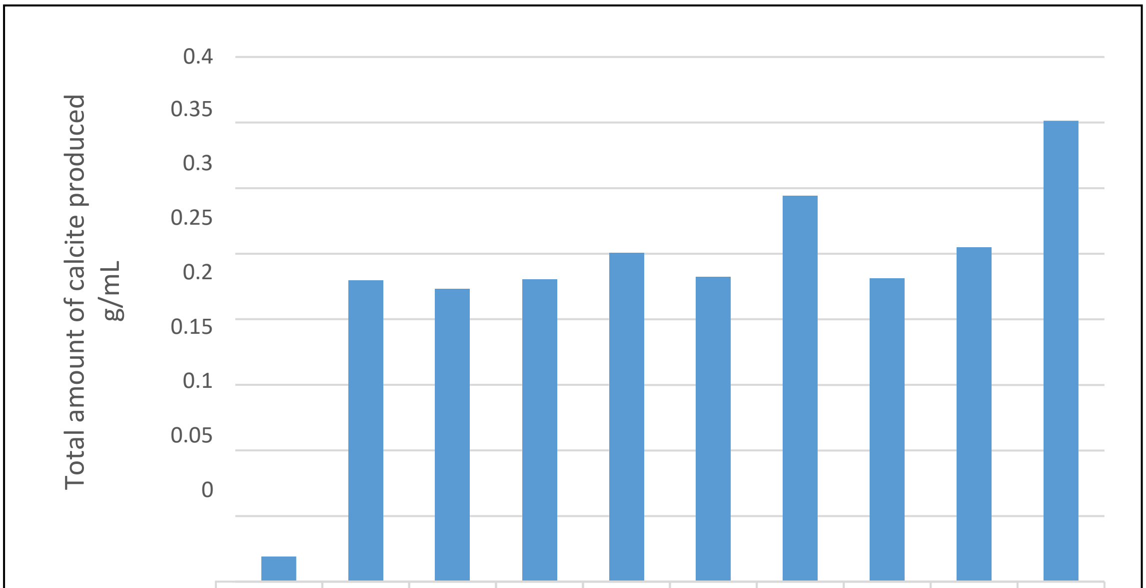


Figure 73 A 24 hours urease test using Stuart Broth.

4.3.2 The effect of different media on the efficiency of total calcium carbonate precipitation

Incorporation of nutrients and minerals is of vital importance in facilitating the precipitation of the healing compound. When considering the appropriate nutrients, two factors should be taken into consideration. First, it should aid in the germination of spores and be an efficient source for the growth of the bacterial cells. On the basis of the urease activity test using Stuart broth method, a total of 9 bacterial strains were selected for calcium precipitation experiment. Test was done over a period 168 hours, media with no inoculum or cells was taken as a reference for comparison. Quantification of the amount of calcium carbonate precipitation ($CaCO_3$) was done using the method explained in Chapter 3, Section 3.3.0.

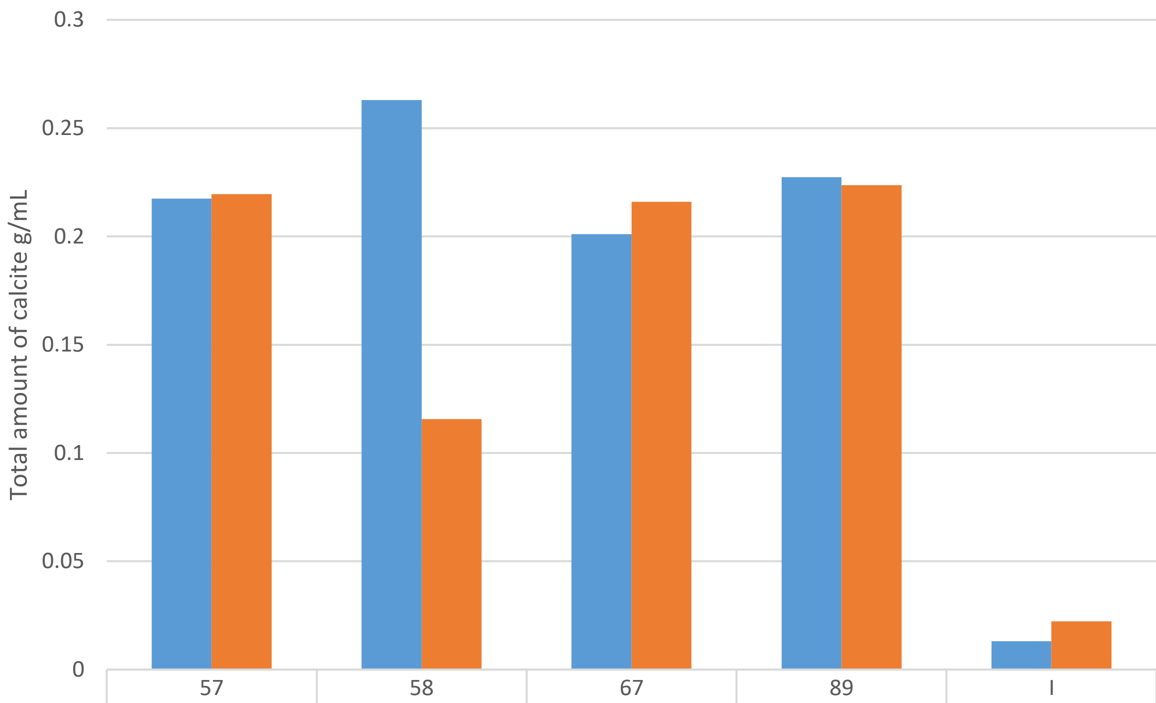
Figure 74 shows the calcium precipitation yield of different bacterial strains when cultivated in media M1. Cell counting was done by plate counting method. All bacterial strains showed promising calcium precipitation yielding, with strain (i) being the most efficient, where an inoculum of 1.2×10^{8} cells/ml resulted in a 0.351g of $CaCO_3$ precipitation. Almost negligible calcium precipitation was observed in control sample, suggesting that the biochemical process may have resulted into little precipitation, which was observed during the introduction of calcium at the reparation stages. The yielding efficiency of strains 57, 58, 63 were very close, 0.2297, 0.2235 and 0.2323 g/ml, respectively i.e. almost similar when taking into considerations the number cell/ml used. Strains 59, 60 and 64 were the least efficient in regard to the yielding of calcium carbonate, as high incorporation of cells resulted in only 0.2307, 0.2507 and 0.2941 g/ml, respectively. Strain 89 and 67 were indeed more efficient in comparison to the former strains, where the yielding of calcium carbonate was found to be 0.2549 and 0.2315 g, respectively.



	contr ol	57	58	59	60	63	64	67	89	i
TOTAL (g/100ml)	0.0191	0.2297	0.2235	0.2307	0.2507	0.2323	0.2941	0.2315	0.2549	0.3514

Figure 74 Total amount of calcium precipitation ($CaCO3$) produced by different bacterial strains using media M1.

The presence of calcium carbonate crystals in all bacterial samples indicate that the bacteria served as a nucleation site during the bio-mineralisation process as reported by Achal et al., 2009. It is should be note the nutrient broth in M1 contained (g/l); D(+)-glucose, 1.0; peptone, 15; sodium chloride, 6 and yeast extract, 3.0. Based on the values obtained from the calcium precipitation, it suggests that only strain i was efficient in utilising the peptone as a source of amino acid during the growth stage in the calcifying media.



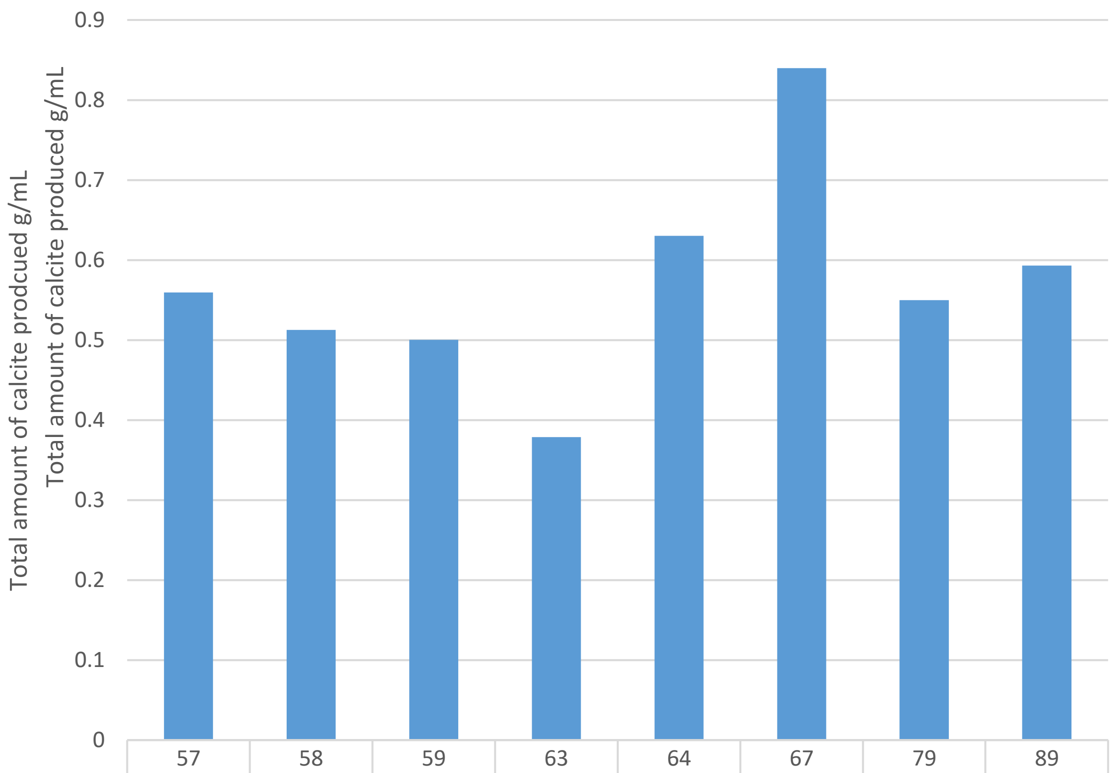
	57	58	67	89	I
M2	0.2174	0.263	0.2011	0.2274	0.0131
M3	0.2196	0.1156	0.216	0.2236	0.0223

Figure 75 Total amount of calcium carbonate produced by different bacterial strain using media M2and M3.

The production of calcium carbonate precipitation using strain i was greatly reduced when the nutrient broth was reduced from 25.0 to 3.0 g/l. this supports the analysis that higher introduction of the components of the nutrient broth was indeed in the favour of strain i. No major drop in the amount of precipitation was observed in the case of strain 57, 58, 67 and 89 as values were within deviation. This suggests that calcium chloride was an effective electron acceptor (Ca^{2+}) as reported by Seifan et al., 2016. Similar pattern was observed in media M3 to that in M2, expect a drop-in strain 58 where a drop of 0.1474 was observed in the production of calcium carbonate ($CaCO_3$).

The effect of calcium source on the production of calcium carbonate precipitation was investigated in this study. Calcium acetate was used at a concentration of 25.2mM, the yielding of calcium carbonate precipitation was greatly increased for all bacterial strains compared to media M1, M2 and M3, where the yielding of calcium carbonate value was almost double to that observed in previous medium, this supports the analysis that the calcium source is key factor in the microbial induced calcium carbonate precipitation. Significant increase was observed in strain 79 where the total amount of calcium carbonate produced was 0.8398 g/ml, followed by strain 64 and 89 where the total yield for each strain was 0.6302 and 0.5932 g/ml, respectively.

Alazhari et al., 2017 used calcium acetate for the production of calcium carbonate, it was reported that the total amount of precipitation was increased with the amount of acetate used in the media. Therefore, this suggests that bacillus Sphaericus successfully utilised acetate as an effective carbon source, which increase the cell growth rapidly during the log phase.



	57	58	59	63	64	67	79	89
Series1	0.5595	0.5126	0.5004	0.3785	0.6302	0.8398	0.5498	0.5932

Figure 76 Total amount of calcium carbonate produced by various bacterial strains using media M4.

The introduction of calcium acetate to induce calcium carbonate precipitation was indeed effective, however, the increase in the yield of precipitation was less to that produced in the medium M4 but higher than that in the medium M3, this is suggests that yeast extract and peptone were not as effective as the incorporation of nutrient broth.

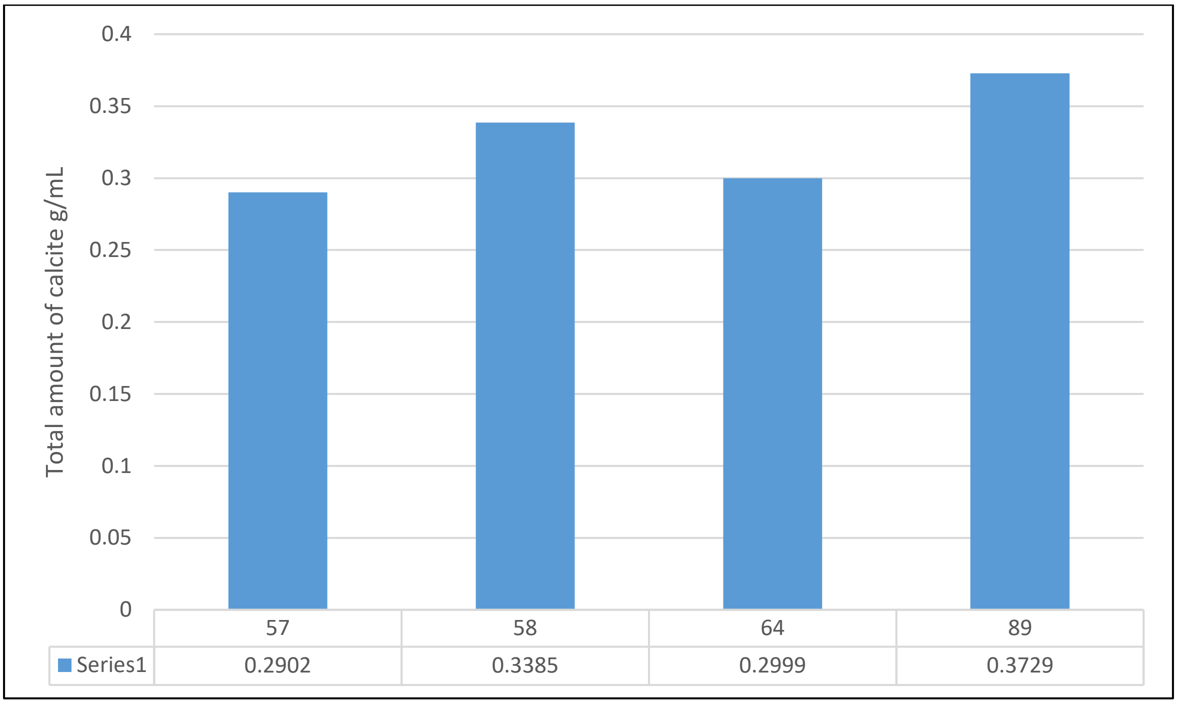


Figure 77 Total amount of calcium carbonate produced by various bacterial strains using media M5

The effect of shaking conditions was investigated in this study, flasks were incubated in static conditions to examine the effect on the capacity of bacterial strains in producing calcium carbonate without agitation.

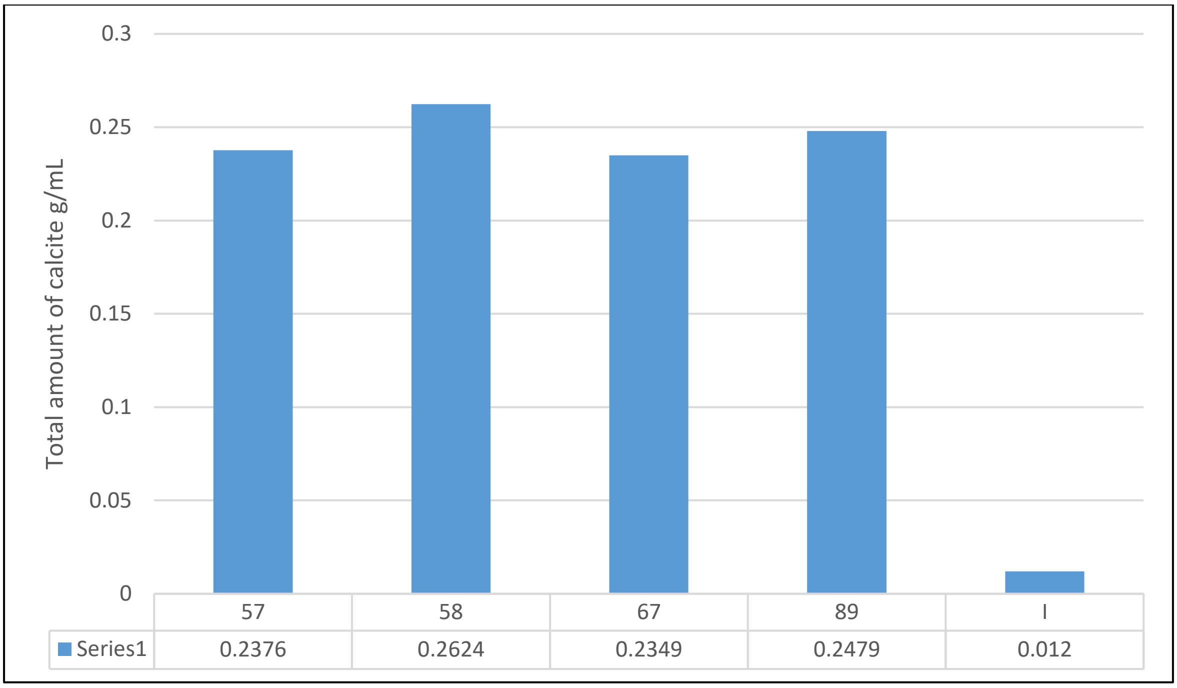


Figure 78 Total amount of calcium carbonate produced by different bacterial strains in static state

Seifan et al., 2016 reported that agitation aids in the circulation of oxygen which promotes bacterial growth in addition to the enhancing the interactions between negatively charged bacterial cells and the electron acceptors present in the media. However, no significant change was observed in this study, Figure 7.

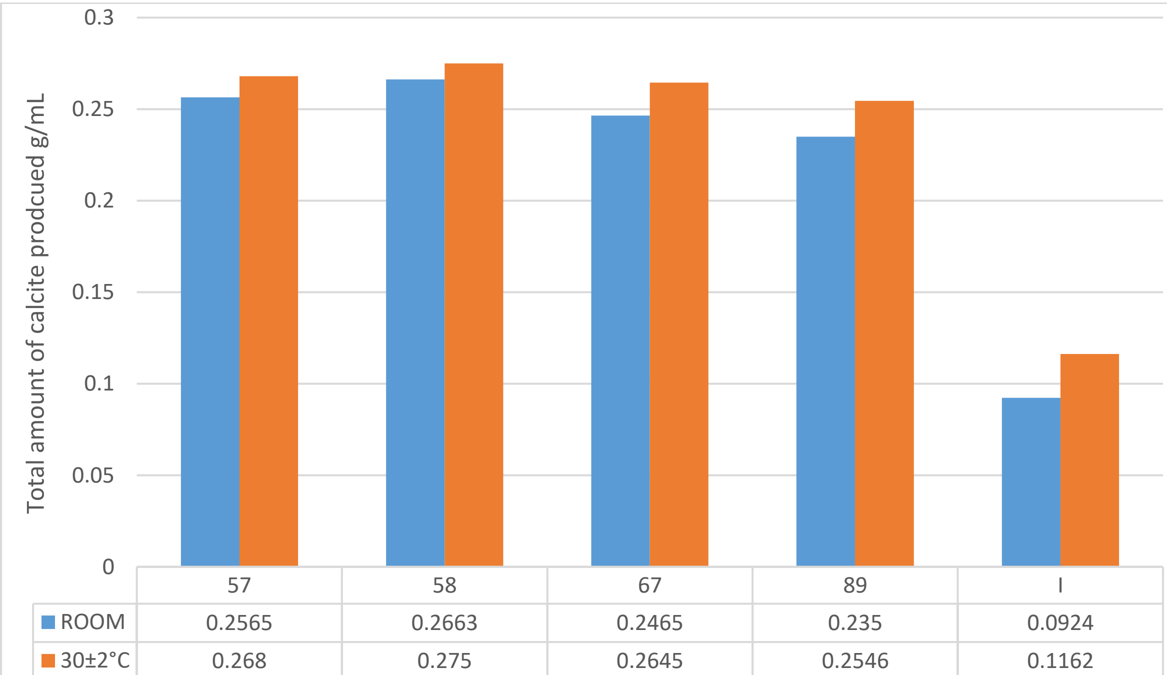


Figure 79 Total amount of calcium carbonate produced by different bacterial strains at room temperature and 30±2°C

No significant change was observed when the temperature dropped to 30°C, however a slight expected decrease in the precipitation capacity for all strain when the temperature was dropped 20°C±2. This was not surprising as the optimum temperature for strain 89 was found to be 37°C and strain 58 was 30°C. Jonker et al., reported a delay in the germination time from 12hours at 28°C to 6 days at 10°C.

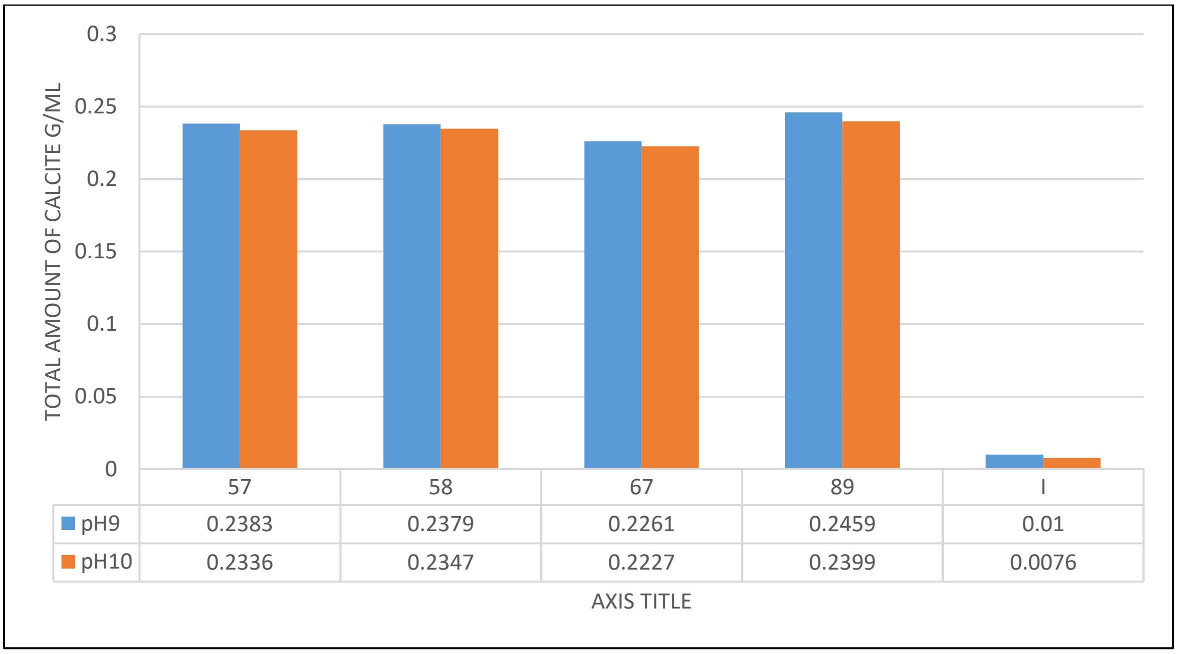


Figure 80 The effect of pH on the total amount of calcium carbonate by various strains.

The effect of pH on the precipitation capacity was evident in strain I, where the activity/precipitation was greatly limited with increasing the pH. Slight decrease was observed in all strains as the pH increases, however strain number 89 showed the maximum tolerance in comparison to strain 57, 58 and 67.

Figure 81 In-vitro calcium carbonate precipitation.

4.3.3 Biochemical properties via ammonium production of the selected bacterial strains

The pathway of urea hydrolysing bacterium has been already used in concrete repair systems other than self-healing concrete. Hydrolysis of urea is catalysed into ammonium and carbonate by urease that is produced by the bacteria. Bang et al 2001; Stock-fischer et al 1999; Wang et al 2014; are studies that reported rapid production of calcium carbonate precipitation as a result of the increase in pH due to ammonium production due to the metabolic activity of the bacterial species. In regard to the self-healing efficiency, ureolytic bacterial species have been reported the highest among all other microbial pathways due to the high production of calcium carbonate precipitation rate i.e. the most favourable approach for self-healing concrete, Wang et al 2016.

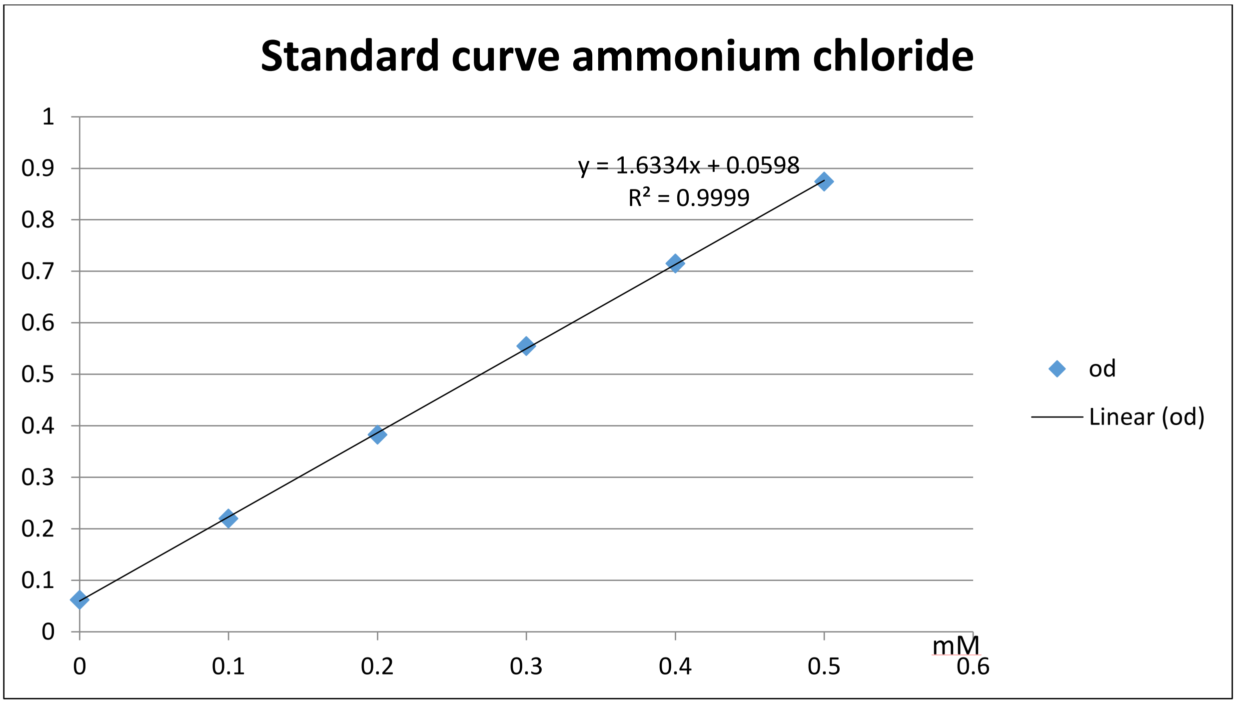


Figure 82 Standard curve of ammonium chloride

Figure 82 shows the calibration curve used to obtain the measure of ammonium production as a result of the urease activity. Understanding the rate at which the urease degrades and breaks down the urea is a key factor in selecting the right bacteria for the application of self-healing concrete. The production of ammonium due to the urea hydrolysis can be seen in the following chemical reaction:

$$\left.\begin{array}{l} CO(NH_2)_2 + 2H_2O \longrightarrow 2NH_3 + H_2CO_3 \\ 2NH_3 + 2H_2O \longrightarrow 2NH_4OH \quad \textbf{pH increase} \\ H_2CO_3 + 2NH_4OH + Ca^{2+} \longrightarrow CaCO_3 + 2NH_4 + 2H_2O \end{array}\right\} CaCO_3 \text{ due to bacterial activity}$$

Therefore, the ammonium production for strains 89, 67 and 98 has been measured over a period of 12, 36 and 60 hours. It is worth mentioning that strain 98 has a weak urease activity which was observed from the urea broth medium experiment conducted in section 4.3.1 Urease activity.

12 hours

Control	
Dilution	OD
900	0.194
100	na
50	na
40	na
30	

89	
Dilution	OD
100	0.479
50	0.28
40	0.234
30	
20	

67	
Dilution	OD
900	0.279
100	0.078
50	
40	
30	

88	
Dilution	OD
900	0.548
100	0.132
50	
40	
30	

36 hours

Control	
Dilution	OD
900	0.263
100	0.092
50	
40	

89	
Dilution	OD
100	2.134
50	1.2
40	0.94
30	0.6963
20	0.45
10	0.251

67	
Dilution	od
100	2.273
50	1.263
40	1.049
30	0.723
20	0.558
10	

88	
Dilution	od
100	0.253
50	0.161
40	na
30	na
20	na
10	na

60 hours

Control	
Dilution	OD
900	0.278
50	na
40	
30	

89	
Dilution	OD
100	2.662
50	1.512
40	1.168
30	0.938
20	0.677
10	0.562

67	
Dilution	OD
100	2.7
50	1.585
40	1.202
30	0.988
20	0.676
10	0.289

88	
Dilution	OD
100	0.319
50	0.194
40	
30	
20	
10	

Table 14 Urease activity by colour intensity (OD).

Table 14 presents the urease activity measurement using the colour intensity (OD), control has been used as medium but with no inoculum i.e. no bacteria added. It is quite evident that the rate of urea hydrolysis was the most efficient in the case of strains 89 and 67, where most of the urea provided to the medium (20g/l) was degraded at 36 hours and fully degraded at 60hours. The former further supports the analysis in the previous section and promotes the two strains to be used and incorporated in the subsequent section as an integral part in self-healing concrete. The control samples have shown slight ammonium production, this could be due to the addition of urea at the initial stage of the experiment which resulted in some cloudiness in the medium. In regard to strain 98, the OD measurement at 12 hours was 0.548 which is not far from the control value which was 0.194 at 12 hours i.e. suggesting very low and weak urease activity. Strain 67 has indeed showed high urease activity; hence it has been selected for further studies but as third component i.e. in the direct addition to the cement matrix due to its limitations to produce spores.

4.3.4 Stat graphic of experiment

The composition of the medium to induce calcium carbonate precipitation due to the bacterial activity is also important, the effect of increasing or decreasing the amount of calcium source and urea in the medium has been explored. Several studies have reported the use of metals and minerals to enhance the yielding of calcium precipitation, different sources of carbon, nitrogen and metals have been explored with each having a maximum value and a minimum value, Table 15, and the amount of calcium carbonate produced by each set have been measured.

Urea and calcium are the most important components in calcium precipitating medium, however, it can be seen that higher amounts of urea i.e. 70 g/l had a negative impact on the yielding of calcium precipitation, this phenomenon could be explained due to the toxic dose of urea added which limited the bacterial activity and hence cell were unable to grow and multiply and ultimately utilise the calcium and hydrolysis of urea. Similar results were reported by Wang et al 2017, where high dosages of urea have been reported to limit bacterial activity.

BLOCK	urea(g/l)	ca-acetate(g/l)	yeast extract(g/l)	glucose(g/l)	nh4cl(g/l)	nh4so4(g/l)	nahco3(g/l)	kh2po4 (g/l)	caco3(mg/100ml)
1	20	50	15	15	2	4	1	0	3.5247
2	20	5	15	2	2	4	0	0.1	0.699
3	70	50	5	15	2	1	0	0.1	3.2476
4	20	5	5	15	2	1	1	0	0.5778
5	70	5	5	2	6	4	1	0	0.6786
6	70	50	5	15	6	1	1	0	3.6981
7	70	5	5	15	2	4	1	0.1	0.9382
8	20	50	5	2	2	4	1	0.1	3.223
9	20	50	5	2	6	1	1	0.1	3.1883
10	20	50	15	2	6	1	0	0	3.351
11	20	50	5	15	6	4	0	0	3.2162
12	70	5	5	2	6	1	0	0.1	0.68
13	20	50	15	15	2	1	0	0.1	3.4507
14	70	50	5	2	2	4	0	0	3.7476
15	70	5	15	2	2	1	1	0.1	0.6366
16	20	5	15	15	6	1	1	0.1	0.7834
17	70	50	15	15	6	4	1	0.1	2.7508
18	70	5	15	15	6	1	0	0	0.7072
19	20	5	5	2	2	1	0	0	0.4404
20	20	5	5	15	6	4	0	0.1	0.5024
21	20	5	15	2	6	4	1	0	0.6666
22	70	50	15	2	6	4	0	0.1	3.4503
23	70	50	15	2	2	1	1	0	3.4415
24	70	5	15	15	2	4	0	0	0.7408
28	70	50	5	0	0	0	0	0	3.2902
29	70	50	10	0	0	0	0	0	2.9501
30	70	50	15	0	0	0	0	0	2.8754
31	70	50	5	5	0	0	0	0	3.364
32	70	50	5	10	0	0	0	0	3.3884

33	70	50	5	15	0	0	0	0	3.4741
34	70	40	5	5	0	0	0	0	2.8995
35	70	60	5	5	0	0	0	0	3.8808
36	70	30	5	5	0	0	0	0	1.7848
37	60	50	5	5	0	0	0	0	3.2988
38	80	50	5	5	0	0	0	0	3.4261
39	50	50	5	5	0	0	0	0	3.4446

Table 15 In-vitro calcium carbonate precipitation using stat graphics

Jonker et al 2010, reported that yeast extract is vital for the cell growth and the most vital component for cell growth. This study however, acetate was found to be the most important component due to the fact that bacteria have used the acetate as a carbon source which promoted further higher growth and induced higher yielding of calcium precipitation.

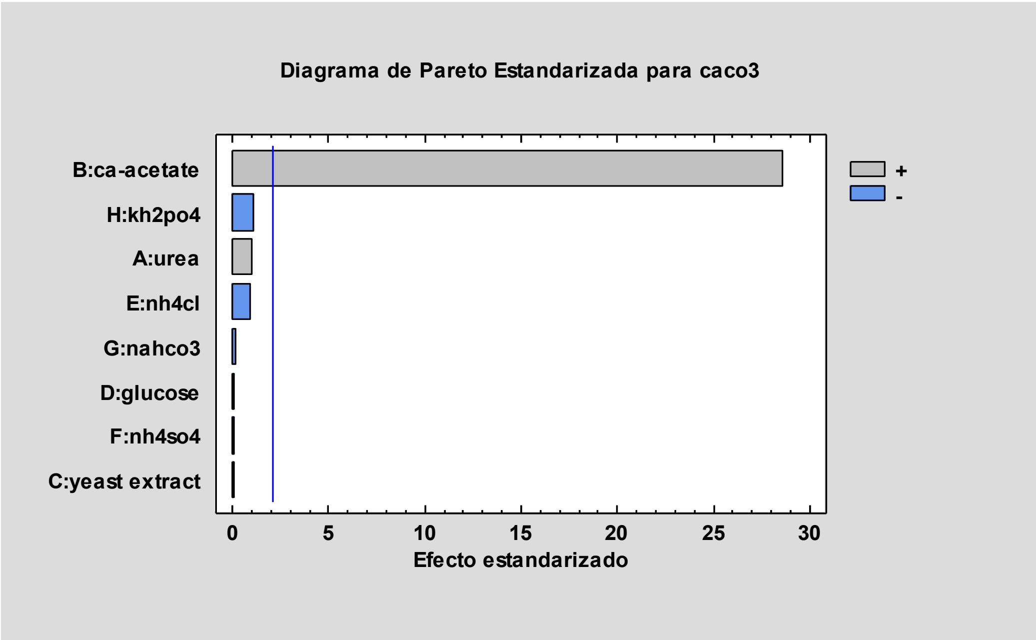
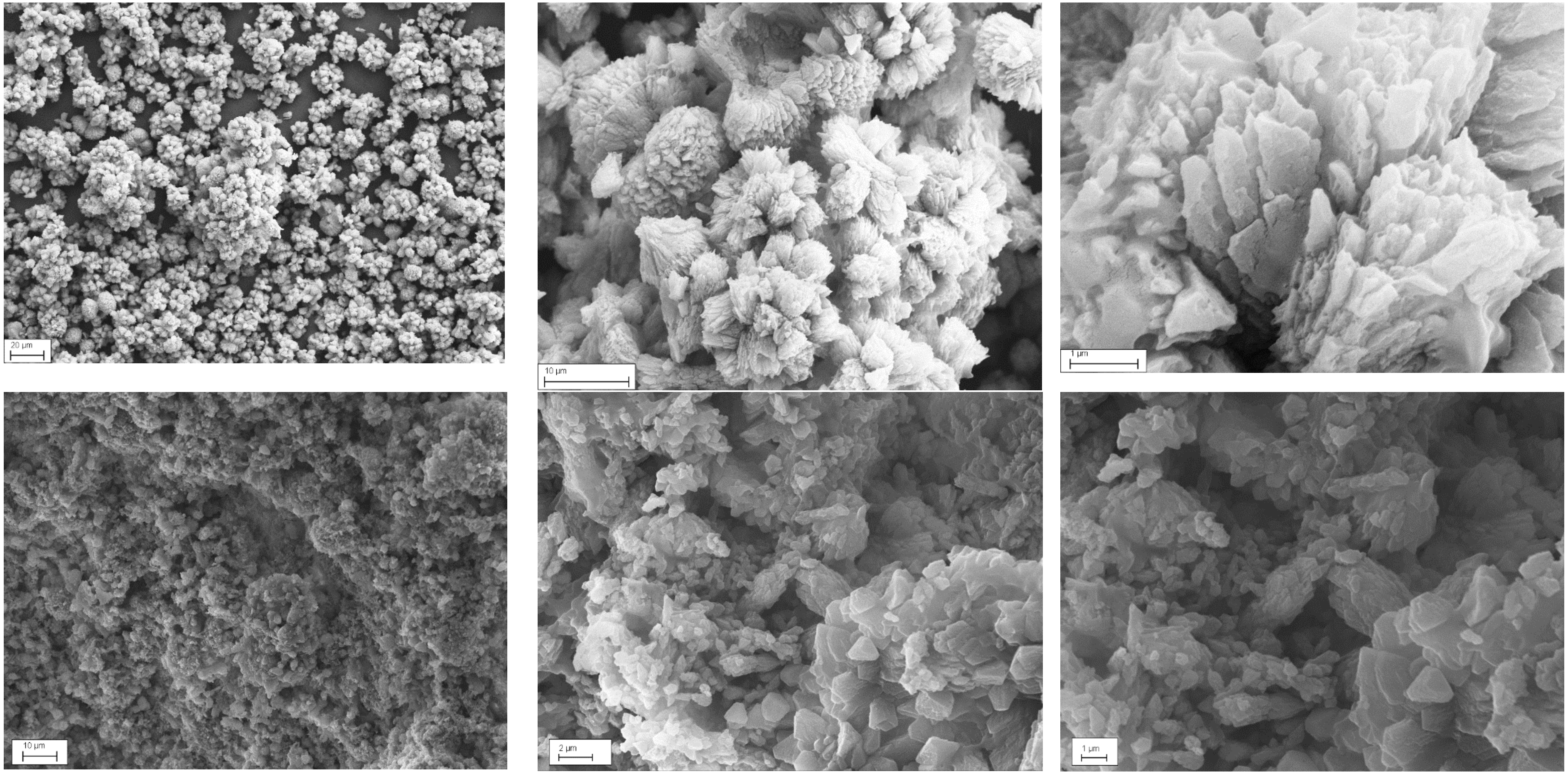


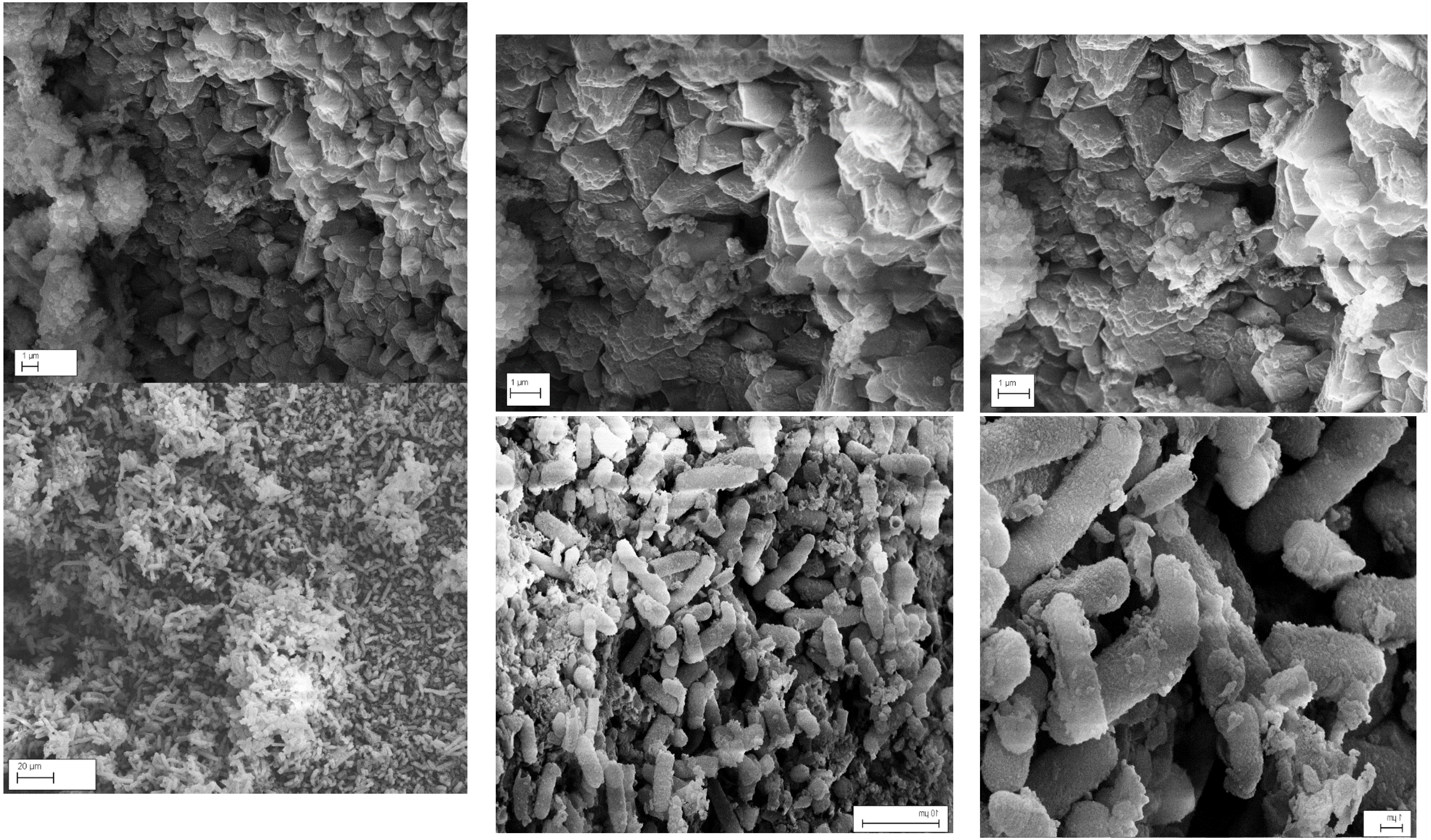
Figure 83 Effectiveness of each component on the yielding of calcium carbonate.

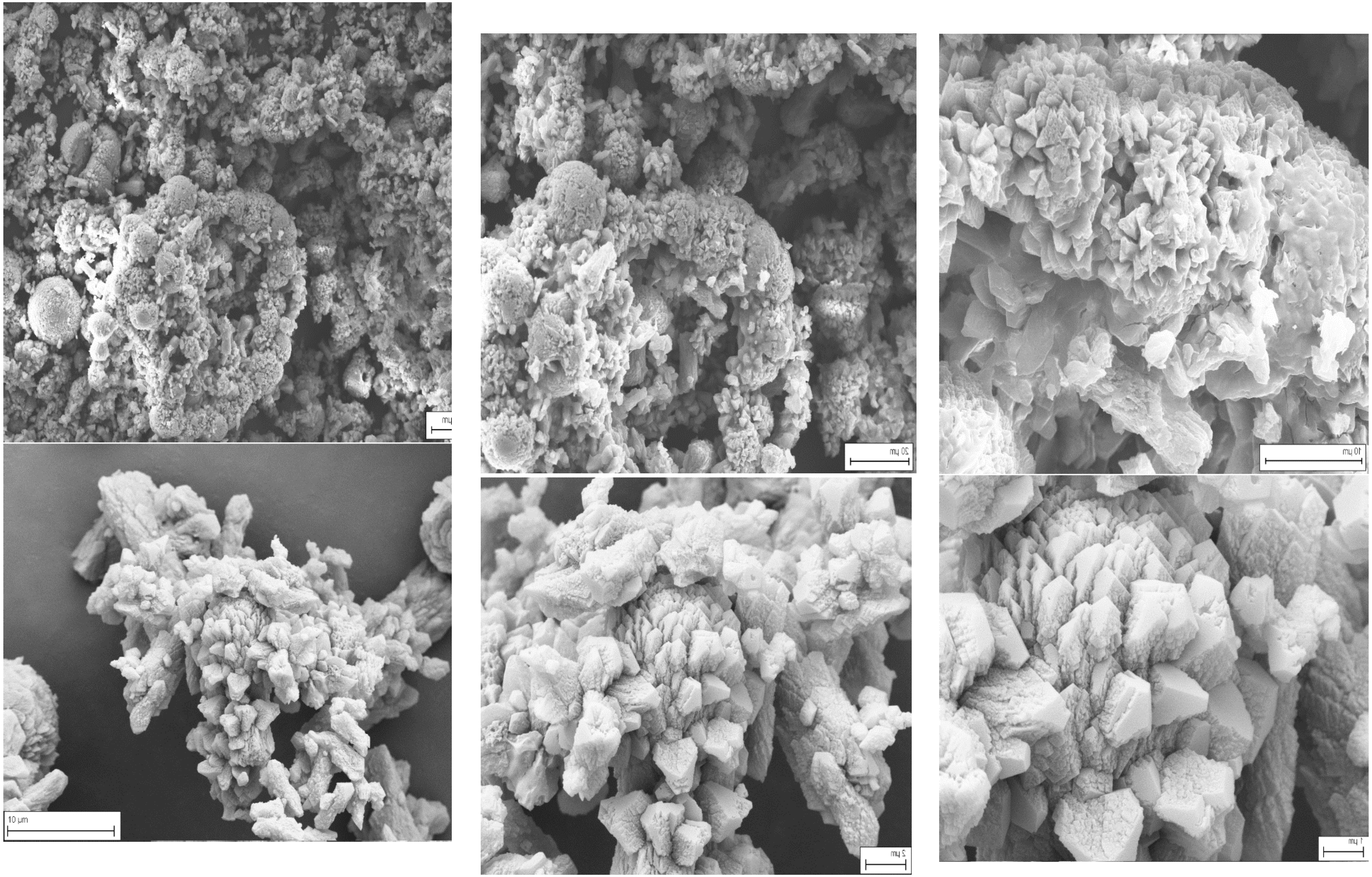
The highest yielding of calcium carbonate was observed in set 35 at 3.698 g/100ml, due to the incorporation of glucose into the medium at 15g/l. higher incorporation of yeast extract didn’t result in higher yielding of calcium precipitation. this is suggesting that bacteria were more efficient utilising acetate as a carbon source.

4.3.3 Microstructure analysis

4.3.3.1 Scanning electron microscope




1 µm
1 µm
1 µm
20 µm


20 µm
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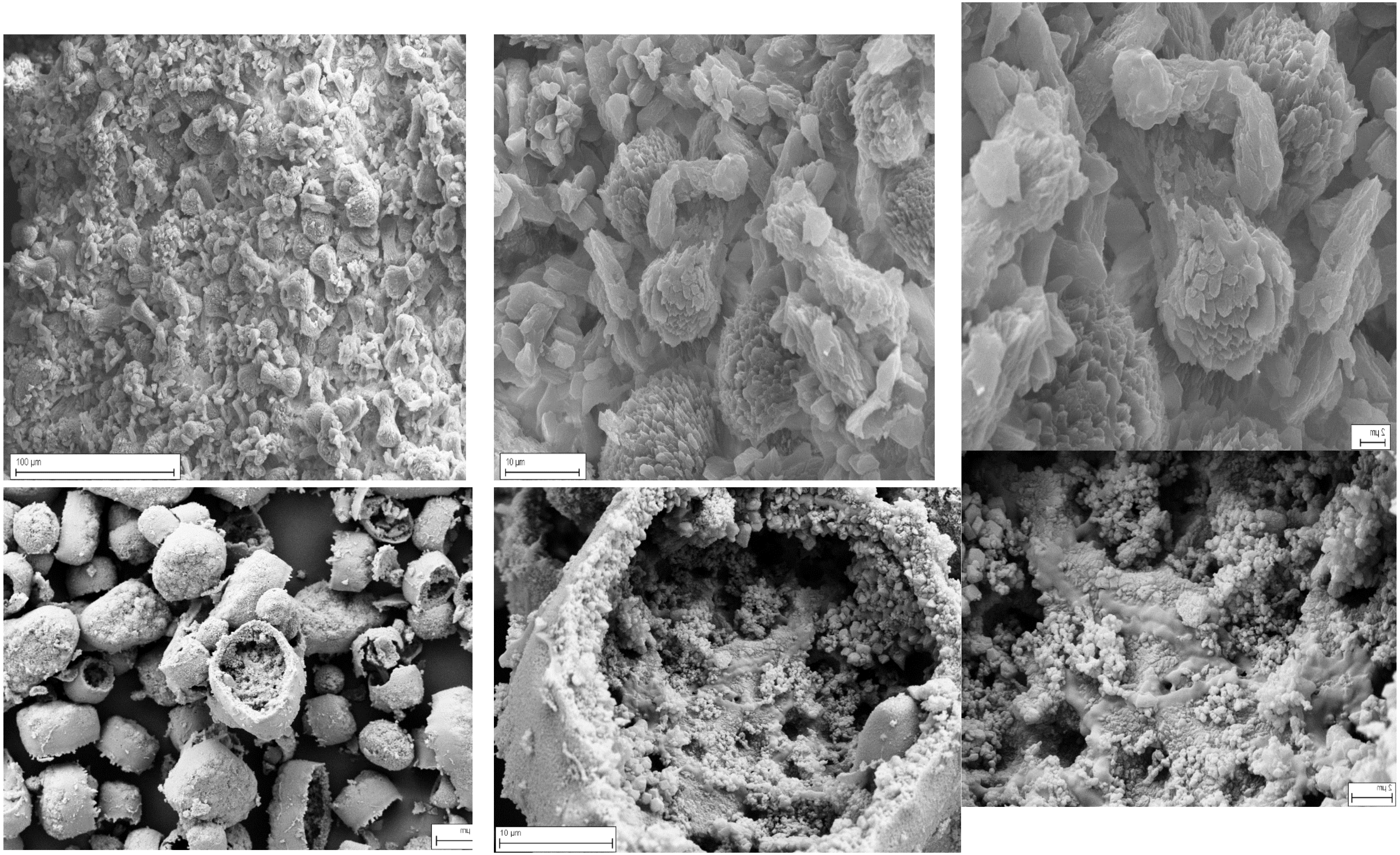
100 µm
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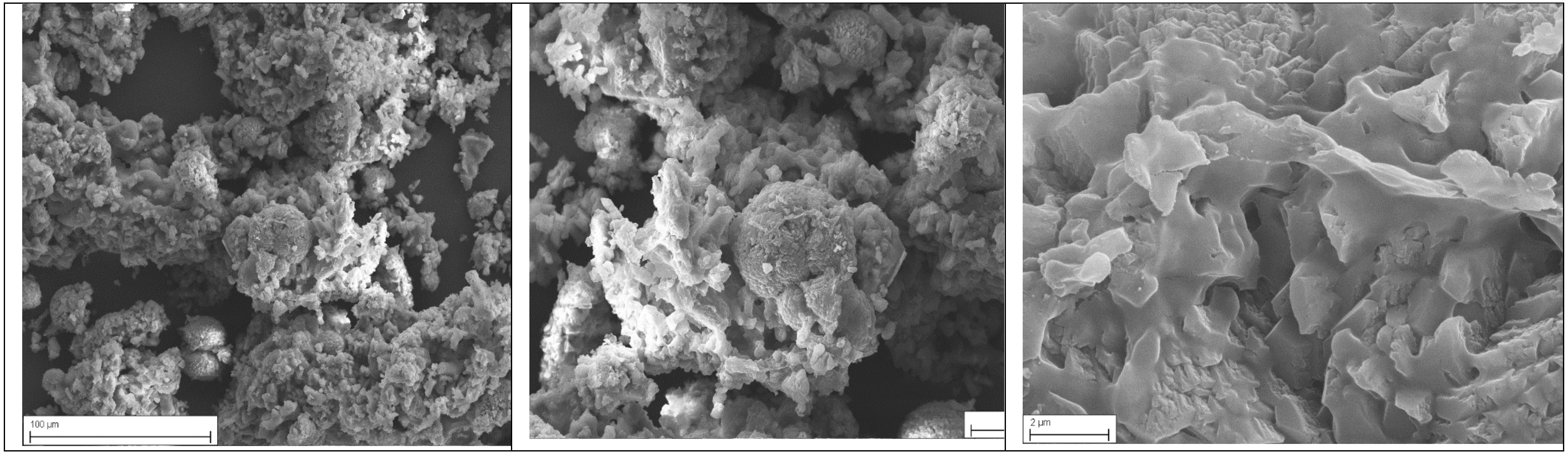


Figure 84 In-vitro calcium carbonate crystals by different bacterial strain i.e. strain 57, 58, 59, 60, 64, 67, 89, 79 and 98.

4.3.4 Analytical techniques

Fourier Transform Infrared (FTIR)

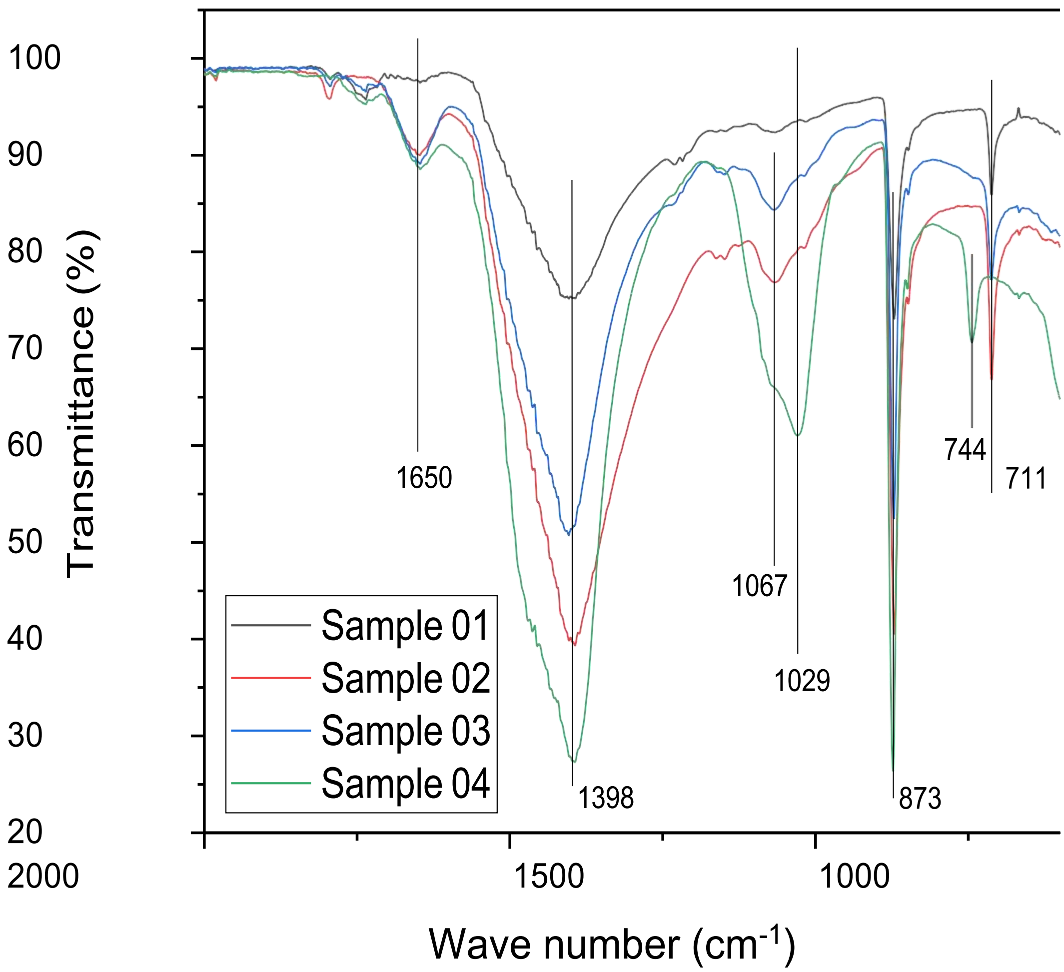


Figure 85 Fourier Transform Infrared (FTIR) spectra of calcite produced by different bacterial strains.

FTIR spectra of the in-vitro calcium carbonate precipitation was performed in order to confirm the functional groups in the precipitate. FTIR spectra of the precipitate was revealed by a broad band σ(O-H bending vibration) between 1645 and 1650cm^{-1}.

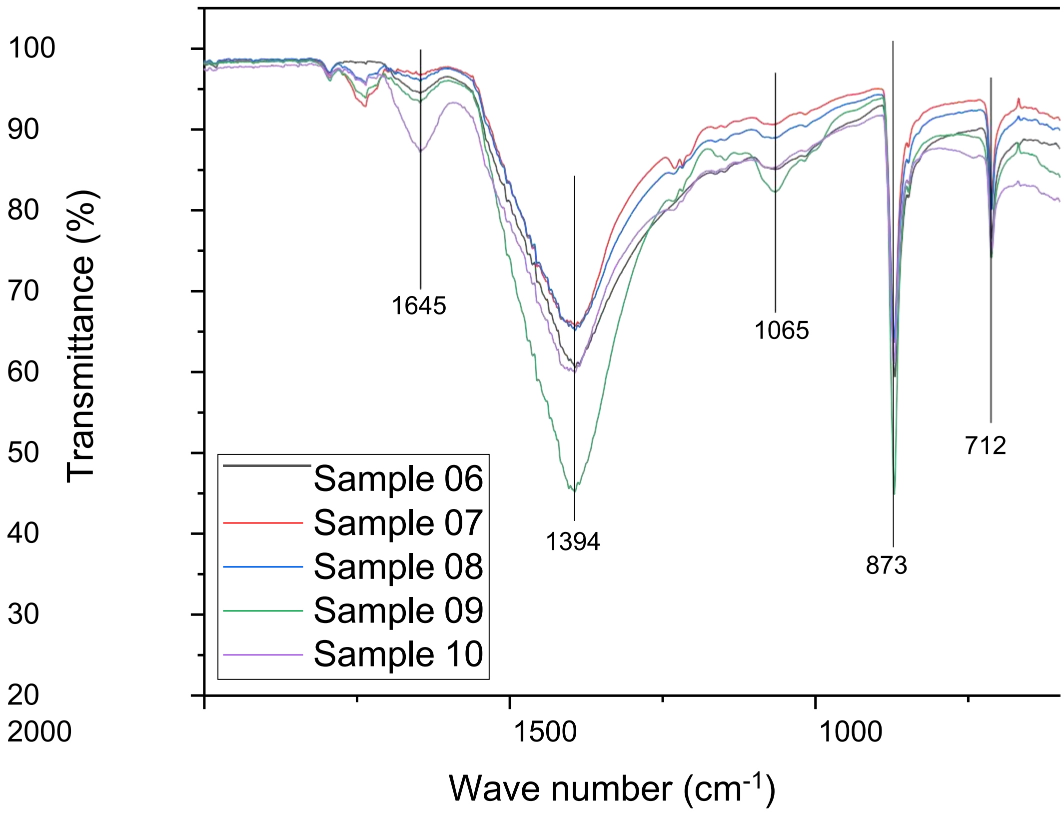


Figure 86 Fourier Transform Infrared (FTIR) spectra of calcite produced by different bacterial strains.

Four distinctive vibrations that confirm Calcite due to bacterial activity. Calcite was confirmed by the presence of shoulder v_3 asymmetric stretching vibration of CO_3at 1394 and 1398cm^{-1}. In addition to v_1 asymmetric stretching vibration CO_3 at 1065cm^{-1}, 1029 cm^{-1} and 1067cm^{-1}, v_2 asymmetric stretching vibration of CO_3at 873 cm^{-1}. The fourth main medium-strong peak was v_1 asymmetric stretching vibration of CO_3 at 712cm^{-1}.

The shift in sample 4 at 1029 cm^{-1}, could be due to the effect of different bacterial metabolism and structure which suggest the presence of other functional group that impacted the calcium carbonate precipitation.

Chapter 5 Understanding the mechanisms of the self-healing mortar using autoclaved aerated recycled concrete (AARC)impregnated aggregates

5.1 Introduction

Autoclaved aerated recycled concrete (AARC) is a light weight cellular material with adequate insulating properties that have been used for more than 80 years, Bergmans et al. 2015. This chapter investigates the use of autoclaved aerated recycled concrete aggregates (AARC) as a protection material for the use in self- healing mortar as part of a two-component healing system, where the healing system is composed of spores and precursor. The main objective of this study is to evaluate the self-healing capacity of the proposed system using ureolytic bacterium namely, Bacillus Sphaericus. Furthermore, different incorporation techniques of the self-healing agent were investigated. In other words, incorporation of the self-healing agent directly into the cement pastes was conducted to examine the effectiveness of the healing capacity of the two systems. H=the healing efficiency was continuously monitored and the nature of the minerals produced inside the crack mouth was analysed by means of Field emission scanning electron microscope (FESEM) and Fourier- transform infrared spectroscopy (FTIR).

5.2 Experimental work

For crack healing experiments, two strains have been selected on the basis of urease activity, survival in high pH, production of spores, the first part of the experiment involves encapsulating the healing agent, consisting of bacterial spores and precursor. The precursor was based on the results obtained from chapter 4, where the precursor contained (g/l): calcium acetate, 350; Urea, 400; and nutrient broth, 8. Encapsulation of the healing agent was performed via vacuum impregnation technique as explained in Chapter 3, Section3.9.0.

In the second part of the experiment, the density of the non-encapsulated autoclaved aerated recycled concrete aggregates with and without coating material have been calculated. The same was also performed for the coated encapsulated aggregates with spores only and the coated encapsulated aggregates with spores and nutrients.

The introduction of the coated encapsulated aggregates with/without bacterial healing agents, was performed by substituting it from the total volume of sand (v/v) of sand used, at 20%, 30% and 40%.

For crack healing experiment, different crack widths were induced by means of flexural testing, as explained in Chapter 3, Section 3.9.1. The healing efficiency of each mix was analysed via microscopic analysis as depicted in Chapter 3, Section 3.9.2. In addition to the former, crack healing efficiency was also performed by testing the permeability properties of healed mortar via water uptake following the formula adopted from Hall et al., 1989; Chapter 3, Section 3.9.4.

The fourth part of the experiment involves introducing a third healing component added directly into the cement matrix along with the coated impregnated aggregates with spores plus nutrient. This part of the experiment also introduces a second type of bacteria which showed high yield in terms of the calcium precipitation when performed in-situ.

In another set of experiment crack healing by means of introducing the bacteria directly into the mix was also performed in order to investigate the healing capacity of such approach. The testing methods used for this part of the experiment was the same as in the previous stages i.e. stage 1-4.

The effect of incorporating the coated impregnated aggregates with spores and nutrients, on the mechanical properties of mortar specimens was also conducted. The specific compressive strength and porosity of each mix was determined in addition to the water permeability via water uptake. The testing methods for porosity, water permeability via water uptake and specific compressive strength are presented in Chapter 3, Section 3.8.3; 3.8.4 and 3.8.1.

The final stage of the experiment involves identifying the composition of the healed material at the crack mouth in both bacterial specimens and control specimens. This was done via analytical techniques i.e. Fourier infrared spectroscopy (FTIR) and X-ray powder diffraction (XRD).

5.3 Results and discussion

5.3.1 Total of viable CFU and nutrient following the Impregnation of the AARC aggregates

Al-azhari et al., 2017, used a different approach in regard to the application of the impregnated aggregates towards self-healing mortar, the study encapsulated spores and nutrients separately in order to minimise the germination of the spores. On the other hand, Khaliq et al., 2015; Zhang et al., 2017; Ersan et al; Wiktor., 2011; Jonker et al., 2010; 2010; 2012, are all studies related to self-healing mortar using bacterial approach, where both nutrients and spores were encapsulated together. In all of the aforementioned studies, drying of the aggregates was done either at room temperature or oven dried at 40°C. One important factor that must be noted and is of paramount importance, is the germination of the encapsulated spores during the impregnation process and the drying process of the encapsulated aggregates. The amount of the impregnated spores i.e. spores viability, indeed greatly affects the efficiency and the capacity of the healing process.

Figure 87 Impregnated AARC particles subject to 24 hours oven drying at 40°C.

Figure 87 shows the end product of drying process of the AARC aggregates. One can observe that calcium carbonate crystals were evident in the dried aggregates which suggests that calcium precipitation due to bacterial activities indeed took place during the drying process. In addition to the former, the toasting effect on the surface layer of the aggregates was observed, some aggregates on the top layer were burnt during the drying process i.e. it could have contributed towards the loss of viability of the impregnated bacterial spores. Spores have been reported to stand temperatures up to 80°C, however, during the drying process some spores may have germinated and tuned into live cells when the temperature was in the range of 25- 30°C. It is worth pinpointing that even though the oven's temperature was set to 40°C and no germination of spores should take place at such temperatures, it will take some time for the aggregates to reach that temperature. Therefore, the aggregates in the core layer of the Pan may have reached optimum temperatures for spores to germinate.

Drying of the aggregates using the freeze-drying method was more efficient, neither the toasting effect nor the calcium precipitation crystals were observed, Figure 88. This suggests that the spore germination was greatly limited in comparison to the oven drying method.

Figure 88 Impregnated AARC aggregates subject to freeze drying.

Encapsulation process is an integral part in self-healing concrete. For this purpose, autoclaved aerated recycled aggregates were selected due to its high-water absorption, water retention and mechanical properties. furthermore, the coating layer that resides on the outer wall of the aggregates is equally important post incorporation as it provides protection for the bacterial spores during the mixing process and minimises spore leakage when embedded into the mortar specimens.

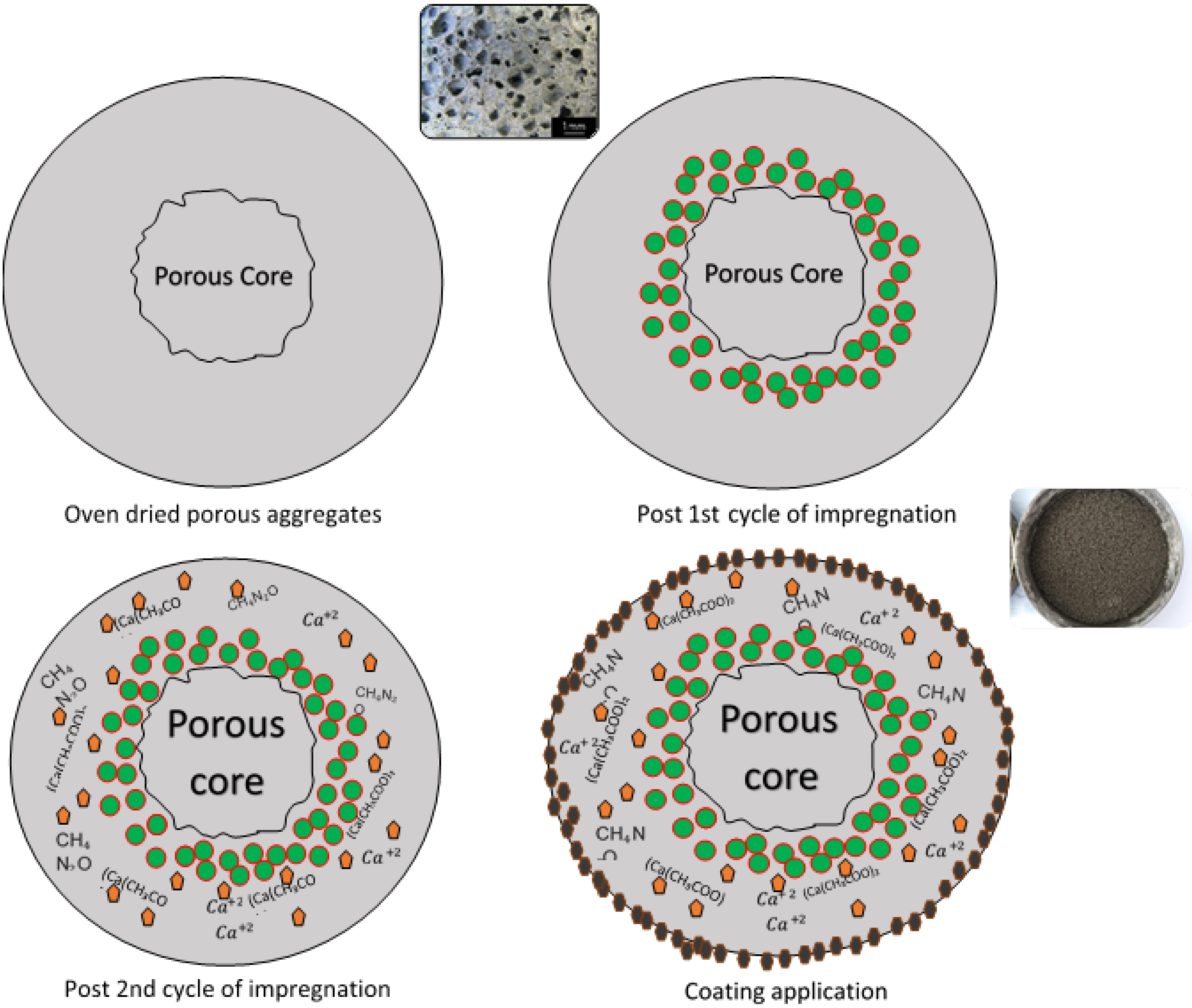


Figure 89 Shows a schematic diagram of the encapsulation and application of coating layer.

During each cycle, freeze drying process proved to be efficient in terms of reducing germination and calcium carbonate precipitation. The introduction of the healing agent into the AARC aggregates was performed twice under vacuum, to minimise possible germination, the spore solution was kept in an ice box during the impregnation process, this was the case in both cycles i.e. the first and the second cycle. To obtain the amount of nutrients present in the impregnated particles, the density of each patch of the impregnated AARC aggregates was determined. The density of the coated aggregates without healing agent (non) and the density of the coated aggregates with spores(spores), this is in addition to the density of the coated (Spores nutrient) aggregates are depicted in Table 16, the presented values are per gram of the AARC aggregates.

Table 16 The obtained densities of the impregnated AARC aggregates.

Coated (Spores)		
	Mass (g)	Density(g/cm^3)
Sand	80.7	1.614
AARC (Spores)	38.6	0.772
Coated (Spores +Nutrients)		
	Mass (g)	Density(g/cm^3)
Sand	80.7	1.614
AARC (S+N)	42.013	0.80026

Quantification of the number of spores present in the encapsulated aggregates was determined using the plate counting method, Chapter 3, Section 3.8.1. To minimise the amount of heat and friction generated during the crushing process of the aggregates, which could affect spore's quantification, an amount of 10 grams of the encapsulated aggregates was taken and crushed using a mortar and a pistol. The sample was then mixed and homogenised, followed by taking 1 gram of the crushed sample and suspended in a 9 ml sterile distilled water. A minimum of 10 dilutions were taken to determine the number of viable spores, a 100 µl of each dilution was inoculated and streaked into pre-prepared nutrient agar petri plates.

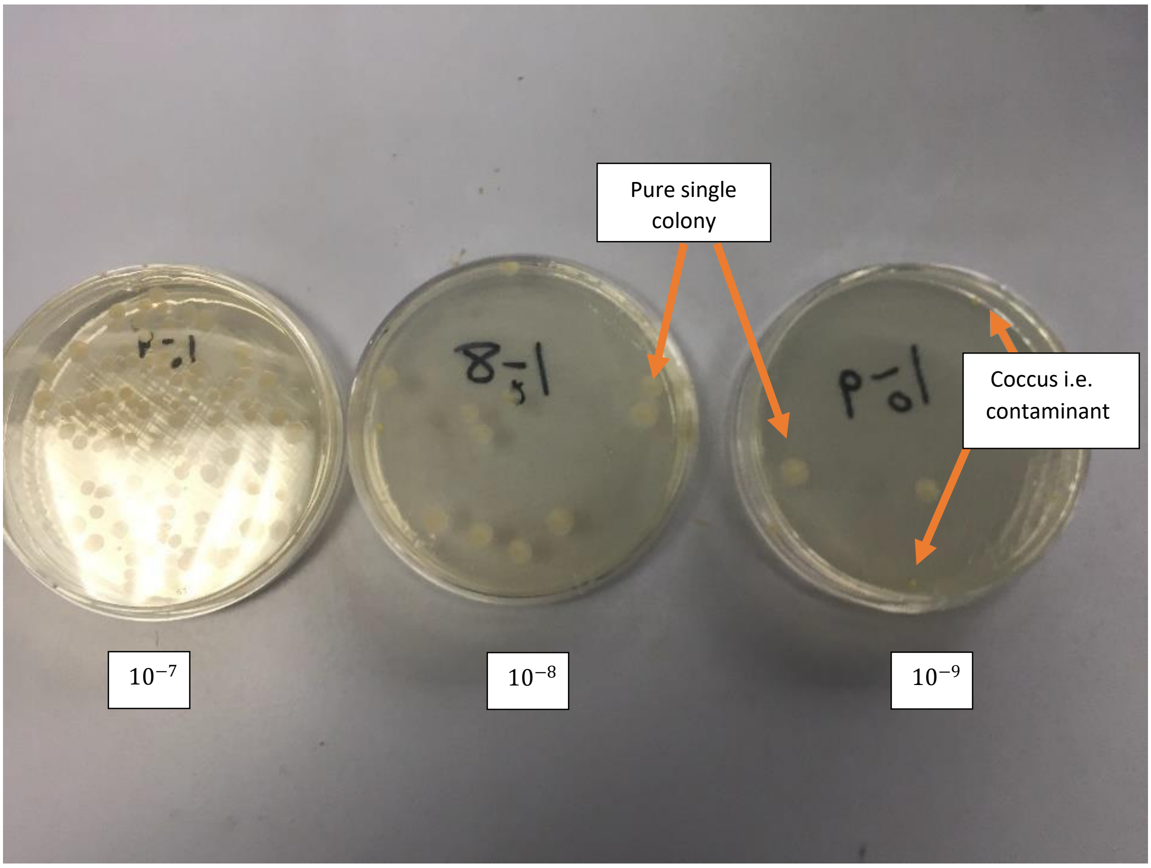


Figure 90 Determination of the number of cells viable in the impregnated AARC aggregates.

Following 24 hours of incubation at 37°C, the number of colony forming unit (CFU) of each dilution was counted to be 2.0×10^9 cells/gram, Figure 90. It is worth pinpoint that despite all efforts and precautions taken, contamination i.e. coccus was observed. However, due to the nature of the experiment, it was difficult to work under 100% sterile conditions, but contamination was minimised as much as possible using 70% (v/v) diluted ethanol and the serial dilution method when counting cells viability. Therefore, contamination was neglected in this case as it did not affect the readings of this part of the study.

For self-healing mortar using Uerolytic bacterium, the incorporation of a suitable carbon source, nitrogen source and calcium source is an absolute must in regard to the healing mechanisms. Bacteria uses the carbon source such as, acetate and glucose to grow and multiply, whereas nitrogen source such urea and yeast extract are used for the metabolic pathway. However, one factor that must also be taken into consideration and equally important during the makeup of the calcium precipitating media, is the effect on the cement matrix. Although self-healing mortar is moving towards the encapsulation of nutrients, much research is still incorporate such components directly into the cement matrix.

Several studies on Uerolytic bacterium species reported the use of calcium chloride to enhance calcium precipitation, however, the introduction of chloride ions into the cement matrix have been proven to detrimental to the steel reinforcement when used above 0.4% (w/w) of cement, Angst et al., 2009. Although some of the chloride ions will react or bind with the hydration products such as calcium aluminium silicate hydrate (C-A-S-H), some ions will still be remained free in the solution i.e. harmful for the steel reinforcement, Florea et al 2014. Calcium nitrate and lactate have been reported to be used in concrete as an admixture for antifreeze and setting accelerator. Karagöl et al., 2013 observed calcium hydroxynitrate in the form of a sharp needle-like crystals when calcium nitrate was added into the cement matrix, such formation was reported to be due to the direct reaction with the calcium hydroxide present in cement. Wang et al., 2014 recommend a urea dosage of 4% (w/w) of cement, however, studies by Bunder et al., 2015 observed very little effect when urea added at a dosage of 0.5 % by weight of cement. In another study, Jonkers et al., 2010 reported a loss in compressive strength when yeast extract is used at 1% of the cement weight.

The aforementioned studies led this experiment towards applying a coating material to aid minimise any effect on the cement matrix and limit the possible germination of bacterial spores, in addition to the availability of the nutrients which enhance and facilitate bacterial activity for the metabolic pathway.

As stated earlier, the density of each patch of the impregnated aggregates was calculated in order to determine the amount of nutrients present per gram of the encapsulated aggregates, Table 2-5. The amount of calcium acetate, urea and nutrient broth was found to be 0.015, 0.017 and 0.00336 per gram of the AARC aggregates. These values are low in comparison to the study reported by Al-azhari et al., 2017; where the amount of calcium and yeast extract was found to be 0.3 and 0.03 per gram of the encapsulated aggregates, this is due to the fact that spores and nutrients were encapsulated separately i.e. allowing more nutrients to be available for bacterial spores.

Table 17 The Composition of the coated AARC, per gram of AARC, after 2× impregnation cycles with bacterial healing agent

	Calcium acetate, g	**Urea, g**	**Nutrient broth, g**	**Spores (B.Sphaericus)**
Coated AARC with nutrients + spores	0.015	0.017	3.36×10^{-3}	2.0×10^{9}

Therefore, the aggregates were lightly sprayed with a healing agent (g/l), calcium acetate 5.0, and urea 6.0, prior to applying the coating material which composed of 30% sodium silicate (v/v) and sprinkles of fly ash. The application of the coating material used in this study was based on previous work by Zhang et al., 2017 and Al-azhari et al., 2017. In both studies' leakage of the healing agent and the encapsulated spores was observed. The effect of incorporating bacteria directly into the cement have been reported to either adversely or positively affect the matrix of the cement. Bundur et al., 2015, reported the decrease in compressive strength at early ages as a result in the delay of hydration caused by the incorporation of bacteria directly into the cement, namely S. pasteuri. Ghosh et al., 2005, reported an increase of 25% in compressive strength when bacteria belonging to Shewanella species was incorporated. The effect of the healing agent on the cement matrix have also been extensively studied. The healing agent incorporated often consists of components that are vital and directly related to the calcium precipitation process.

5.3.2 Two component healing system

5.3.2.1 Crack healing quantification via optical microscope

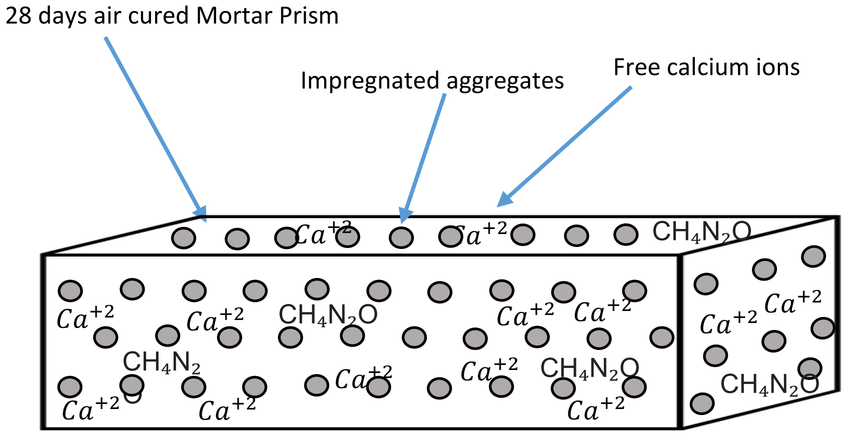


Figure 91 Schematic diagram of the two-component healing system.

The healing efficiency of the pre-cracked mortar specimens was obtained following the method used in Chapter 3, Section 3.9.2 i.e. by taking measurements via microscopic images at 3, 7, 14 and 28 days. Although monitoring of the crack's width was performed at 3 and 7 days of healing, the results were not presented as no healing was observed. The experimental work regarding the crack healing experiment was divided into three main stages, the first stage investigates the introduction of Autoclaved Aerated recycled concrete aggregates (AARC) impregnated with either spores only or spores and nutrients. Pre-cracked mortar specimens incorporated with AARC aggregates with no healing agent i.e. no spores nor nutrients, served as abiotic control. The phenomenon of self-healing in concrete structures has been known and extensively studied in recent years. Several studies have reported the healing capacity of concrete structures without the need for human intervention, such phenomenon have been linked to several parameters such as the swelling and rehydration of cement pastes, blockage due to water impurities and the formation of calcium carbonate due to the natural carbonation of cement pastes.

However, Jacobsen et al 1996; Reinhardt et al 2003; Li et al 2007; Neville et al 1995; Ter et al 2005; Qian et al., 2010 reported that autogenous healing in concrete structures is limited to cracks between 0.1 to 0.3mm. Due to the former, mortar specimens were induced with different crack widths ranging from 0.127 to 0.875mm in order to examine and understand the healing mechanism/ efficiency and capacity of pre-cracked specimens as a result of abiotic healing and bacterial healing.

Microscopic Monitoring of the healing process of the induced cracks is depicted in Figure 92 and Figure 93 Both autogenous and bacterial healing can be observed in all mortar specimens i.e. those with and without bacteria incorporated, where the healing compound of calcium carbonate crystals was clearly visible for specimens incorporated with bacteria. Considering the first patch of the pre-cracked mortar specimens Figure 92 A, B and C, one can observe that incorporation of AARC aggregates at 20% by volume of sand did not show good distribution across the cement matrix as little calcium carbonate precipitation was observed in specimens in group C, however the healing is evident in comparison to specimens in group A and B, where crack closure is thought to be due to autogenous healing i.e. secondary hydration of un-hydrated cement particles. The introduction of the AARC aggregates impregnated with bacteria and spores at 30% by volume of the sand used did show improvement in regard to microbial healing when compared 20% AARC incorporation, suggesting that the aggregates were better distributed across the cement matrix and were able to hit the crack zone, however, microscopic images did not portray complete crack closure.

Microscopic images of the pre-cracked mortar specimens incorporated with 40% AARC by volume of the sand used, indeed enhanced the distribution of aggregates across the cement matrix, this is shown in Figure 4I-5, where complete crack closure was observed for specimens incorporated with spores and nutrients. The healing compound observed is thought to be due to both autogenous healing and microbial healing, with the latter being the more dominant factor towards the healing. The results obtained agree with Jonkers et al., 2010; Khaliq et al., 2015; Al-Azhari et al, 2017; Zhang et al. 2015 and Wiktor et al., 2011, where the healing compound was visible on the surface of pre-cracked mortar specimens.

The healing efficiency as a function of time is not a surprising phenomenon, this due to the alkaline environment inside the concrete matrix where bacteria needs time to adjust to the harsh concrete environment by creating nucleation sites that aid in the microbial healing. On the contrary, pre-cracked specimens incorporated with spores only impregnated AARC exposed the need for better coating material, as the leakage of the spores resulted in the formation of the calcium carbonate crystals despite the fact that no nutrients were incorporated. This could be explained by previous studies by Ersan et al., 2015 where a denitrifying bacterium species were efficient in utilising concrete admixtures as a carbon source to aid in the microbial formation of calcium carbonate, such as calcium nitrate and calcium formate.

Therefore, in addition to the contamination that may have resulted during that water curing stage, bacterial spores were able to germinate into live cells and utilise the available calcium inside the cement matrix to ultimately produce calcium carbonate crystals. Around 70% of the cement hydrates over the period of 28 days, the results presented in Figure 92 E and Figure 929 H suggest that bacterial spores were able to utilise some of the hydration products such as calcium silicate hydrate(C-S-H) and calcium hydroxide ($Ca(OH)_2$), for the formation of calcium carbonate crystals ($CaCO_3$). The phenomenon was observed in all specimens incorporated with spores only i.e. Figure 92 B, E and Figure 93 I.

In addition to the dipicolonic acid which is present in the core of the spore, micronutrients such as proteins, vitamins, micro minerals and trace minerals are indeed present within the shell of the spore. Upon the availability of optimum conditions, the spore utilises these germinants or molecules via germinant receptors that are located in the inner membrane of the spore shell. Stewart et al., 1980 and Cowan et al., 2004 reported that the spore membrane increases in fluidity during the early stages of germination, where cations such as H^+, K^+ Na^+ are released into the supernatant, followed by Ca^{2+} and dipicolinic acid (DPA) from the spore core. Further to germination by micronutrient, there are non-physiological treatments that can induce germination including exposure to high concentration of CaDPA, high pressure or even alkylamines as reported by Reimann and Ordal 1961; Gould and Sale 1970; Rode and Foster 1961.

It is worth pinpointing that based on previous studies in regard to microbial healing recommended wet-dry cycle curing for microbial mortar specimens. Wang et al., 2014 investigated the effect of curing conditions on the healing efficiency of ordinary and bacterial concrete. It must be noted that water is a crucial component for both microbial and autogenous healing, where the former needs water for the spores to germinate and the latter needs water to promote the occurrence of the secondary hydration of the un- hydrated cement particles, precipitation of $CaCO_3$ and the swelling of the hydration products.

In another study, Jonkers et al, 2017 stressed the importance of oxygen as an important factor during the growth stage i.e. log phase of Bacillus Sphaericus when incorporated in mortar. In addition to mimicking the real-life condition such as rain, the benefit of wet dry cycle towards bacterial concrete lies in providing water and oxygen which are two important factors that are required for the metabolic conversion of insoluble calcium carbonate.

One can observe from the microscopic images Figure 92 that precipitation was found on the surface of the specimens incorporated with bacterial spores. This phenomenon suggests that during the 12-hour immersion in water tank, bacterial spores escaped the capsule seeking for oxygen and nutrients, where crystals were able adhere to the surface of the mortar specimens during the other 12 hours of drying at room temperature. Therefore, some of the precipitation was lost in the curing tank which could have affected the sealing efficiency in the specimens loaded with bacterial spores' impregnated aggregates.

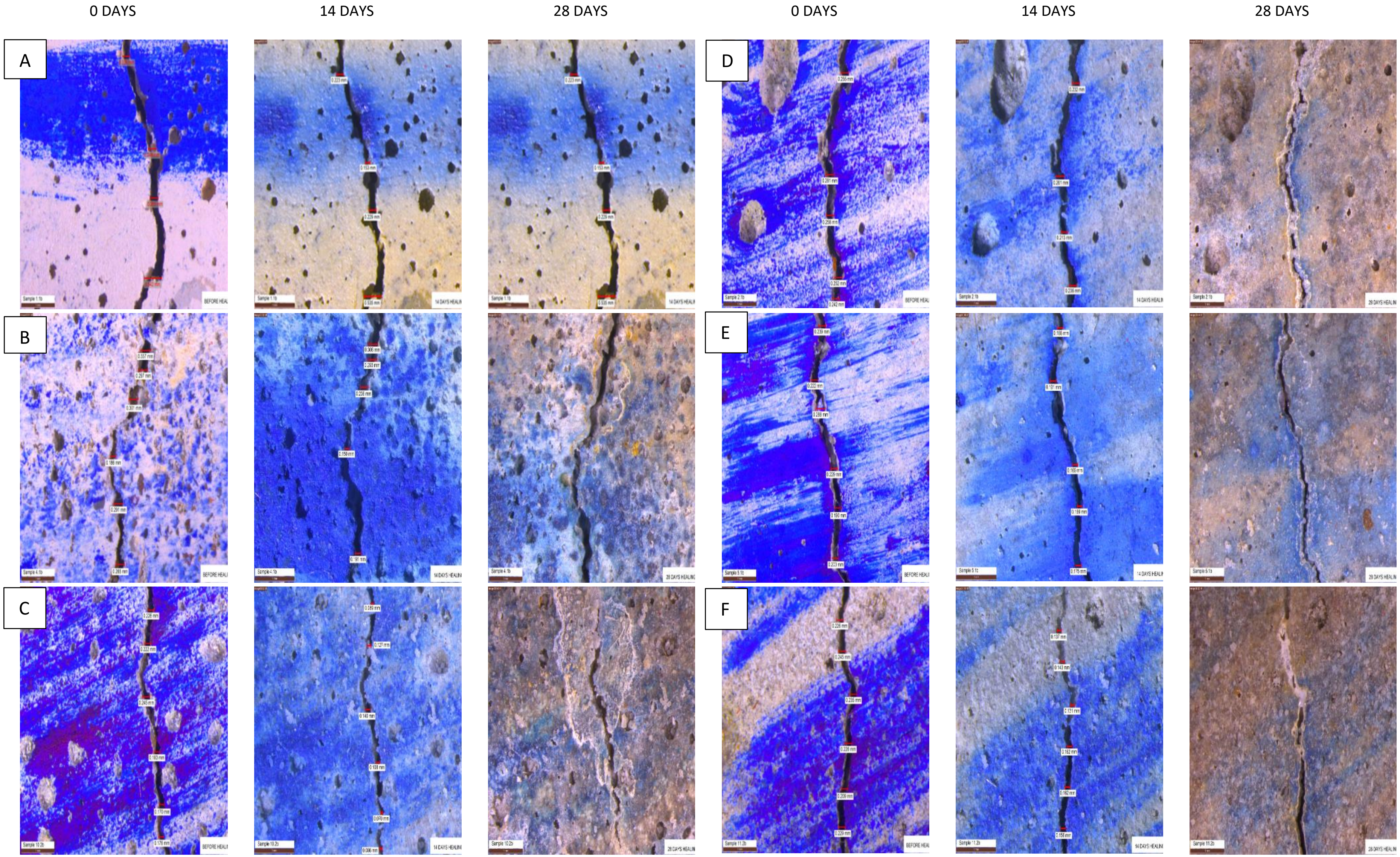


Figure 92 Microscopic images of the crack healing process: (A) 1B; (B) 4B; (C) 10.2b; (D) 2B; (E) 5B; (F) 11.2b, at 14 and 28 days. Specimens in set A-C with 20% AARC and set D-F with 30% AARC.

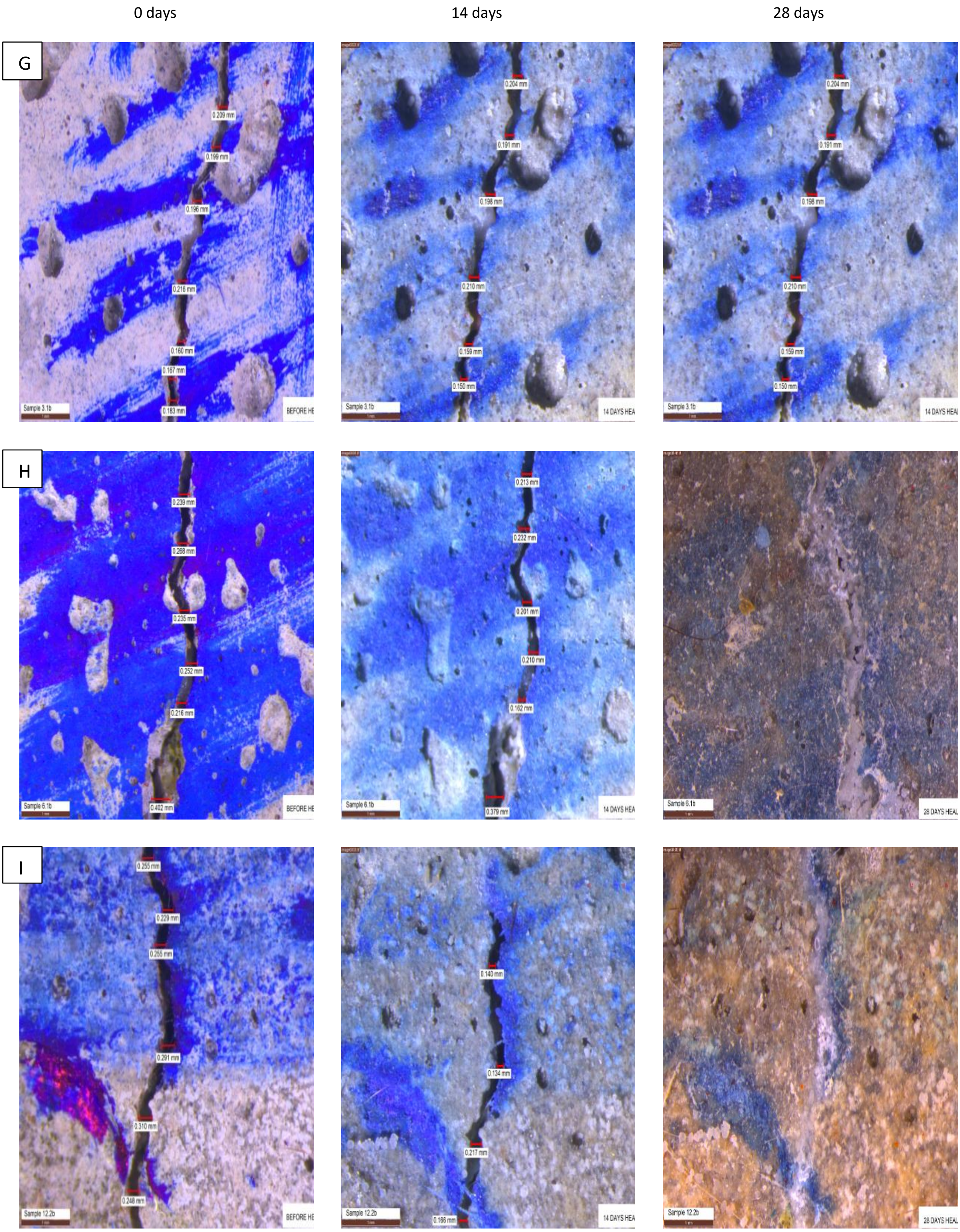


Figure 93 Microscopic images of the crack healing process: (G) 3b; (H) 6b; (I) 12.2b, at 14 and 28 days with 40% ARRC aggregates.

5.3.2.2 Crack healing percentage -The two-component healing system

As explained earlier, mortar specimens were cracked at 28 days of curing under the condition of 20±2 C and 95 % relative humidity, followed by the inducing crack by means of flexural loading until crack were visible. The nature of the experiment made it very difficult to induce the exact crack width for each specimen. Figure 6-5 presents the healing efficiency as a percentage for mortar specimens with/without impregnated healing agent added as a replacement of the sand used (v/v). Various crack widths in the range of 0.127- 0.875mm were induced, monitoring of the crack to determine the healing percentage was conducted by monitoring the crack closure at different intervals along the crack face with a minimum of five points along the crack were monitored. The healing percentage was calculated based on the formula used in Chapter 3, Section 3.9.3.

The higher the crack healing capacity that bacteria incorporated specimens possess, the large the crack areas covered as a result of bacterial precipitation for the same healing time. Figure 94, presents the healing percentages for all mortar specimens with/without healing agent impregnated AARC aggregates. All specimens with or without healing agent portrayed some healing capacity due to autogenous healing and bacterial metabolism, with the latter being the more dominant factor.

The healing percentage as a function of the initial crack showed proportional relationship in regard to the healing percentage and the amount of AARC aggregates incorporated, in other words higher incorporation of AARC aggregates loaded with spores only or spores and nutrient, resulted in higher healing percentage in comparison to the abiotic control. This is not surprising, as higher incorporation of bacteria is expected to result in higher precipitation capacity. Considering the specimens with 20% AARC aggregates at 14 days, specimens with spores and nutrient was efficient in sealing 57.9% of 0.226mm cracks, while abiotic control and spores only specimens were able to seal 38.3% of 0.248mm cracks and 27.9% of 0.265 mm cracks, respectively. The increase in duration of the healing time was in favour of bacterial healing at 28 days where 72.3% of 0.226mm cracks were sealed, in comparison to the 40.3% of 0.239mm cracks for abiotic control and 47.5% of 0.265mm cracks for specimens with spores only, were sealed.

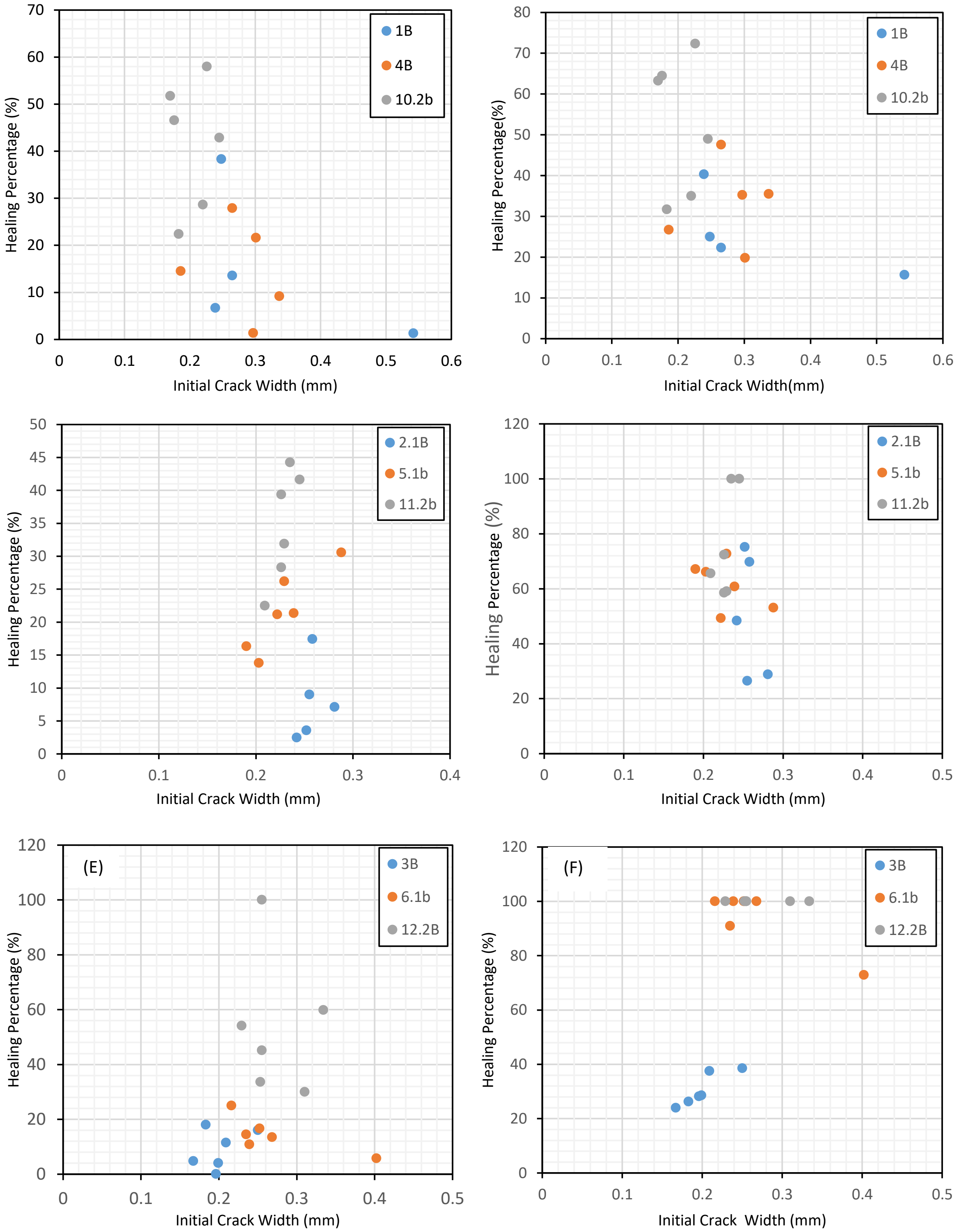


Figure 94 Crack healing Percentage as a function of the initial crack width for mortar specimens at 14- and 28-days healing time (A-B) 20% AARC, (C-D) 30% AARC and (E-F) 40% AARC.

Incorporation of 30% AARC aggregates loaded with spores and nutrients showed better healing percentages where 100% crack closure was observed for cracks of 0.235 and 0.245mm, which is higher than the 20% AARC incorporation. Considering the abiotic control at 30% AARC, 75% and 69% of cracks closure was observed for 0.252 and 0.258mm, respectively. This is higher than the abiotic control with 20%, which could be due to particles size of the AARC aggregates incorporated i.e. more fine particles were incorporated at 30 % when compared to the 20% AARC which could have acted as a filler material, similar findings were reported by Sixuan et al., 2012. Similar pattern was observed for mortar specimens loaded with spores only, where cracks of 0.239 and 0.203mm were closed by 72.7% and 66.1%, respectively.

An enhanced healing percentage was observed for specimens loaded with spores and nutrient at 14 days, where 100% crack closure of 0.255mm was observed, this suggest that bacterial healing occurred within the 14 days of curing. Poor performance in terms of the crack sealing capacity at 14 days was observed for abiotic control and specimens with AARC aggregates incorporated with spores only, which could be due to the wet dry cycle which was not serving in favour of the secondary hydration of un-hydrated cement particles. Over the period of 28 days of healing, specimens with 40% AARC aggregates impregnated with spores and nutrients were able to completely heal cracks of 0.253, 0.31, 0.334 and 0.255mm. Similar pattern was observed for the spore only specimens at 40% AARC aggregates, which suggests the occurrence of spore leakage due to the poor coating material applied and supports the analysis that spores contain micronutrient along with using the available calcium and nutrients provided by the concrete environment i.e. feeding on the available calcium from the hydration products.

The results shown in Figure 94 agree with the literature, as similar findings were observed by Jonkers et al., 2011 and 2011; Alazhari et al., 2017; Wang et al., 2013 and 2014; Khaliq et al., 2015 and Zhang et al., 2017, in regard to the ability for bacteria to seal cracks over the period of 28 days. However, the availability of micronutrients within the spore shell along with its role as a nutrient supply in addition to the feeding on the hydration products have not yet been explored.

5.3.2.2 Permeability via capillary action of healed mortar

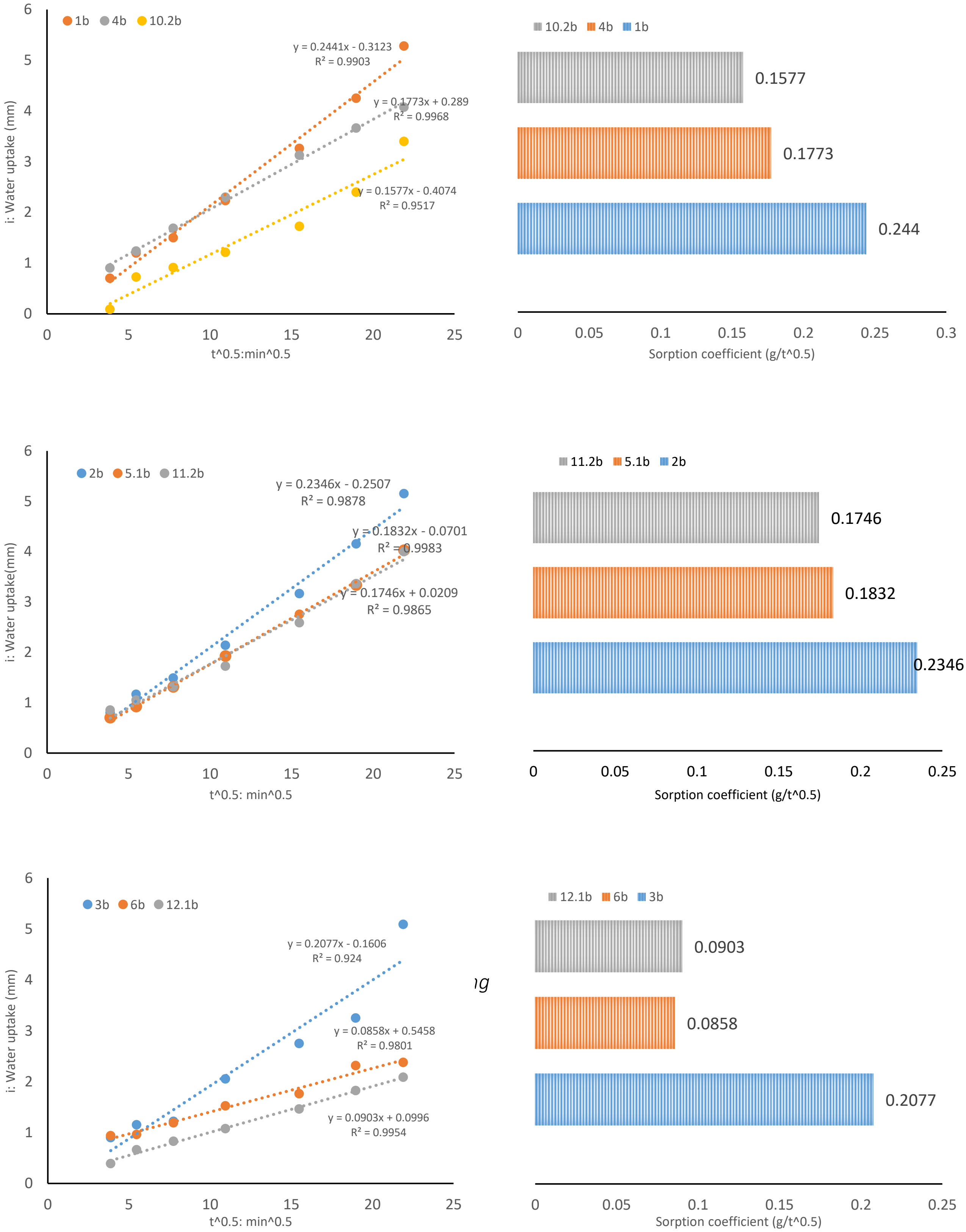
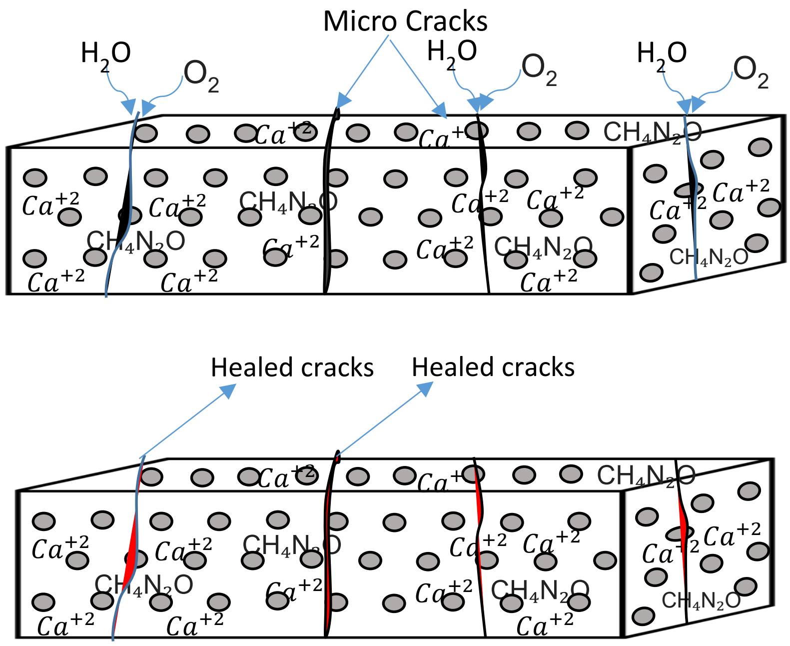


Figure 95 Permeability via capillary action of healed mortar.



- Cracks of 0.34mm 60% healed at 14 days and completely sealed at 28 days,

Figure 96 Schematic diagram of the two-component healed system.

The healing mechanisms of the two-component healing system is depicted in Figure 96. Upon the formation of micro cracks, ingress water and oxygen will seep into the cracks. Embedded spores will then re-activate, due to the micro nutrients present in the spore's shell, and start growing into live cells providing the right temperature that is suitable for microbial growth. The live cells will then feed on the available nutrients such as acetate, nutrient broth and urea.

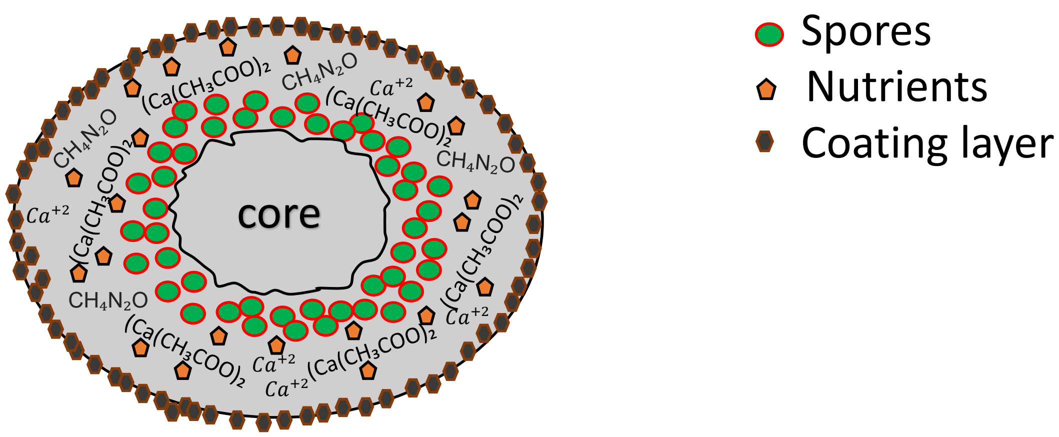


Figure 97 Impregnated aggregates –cross section view.

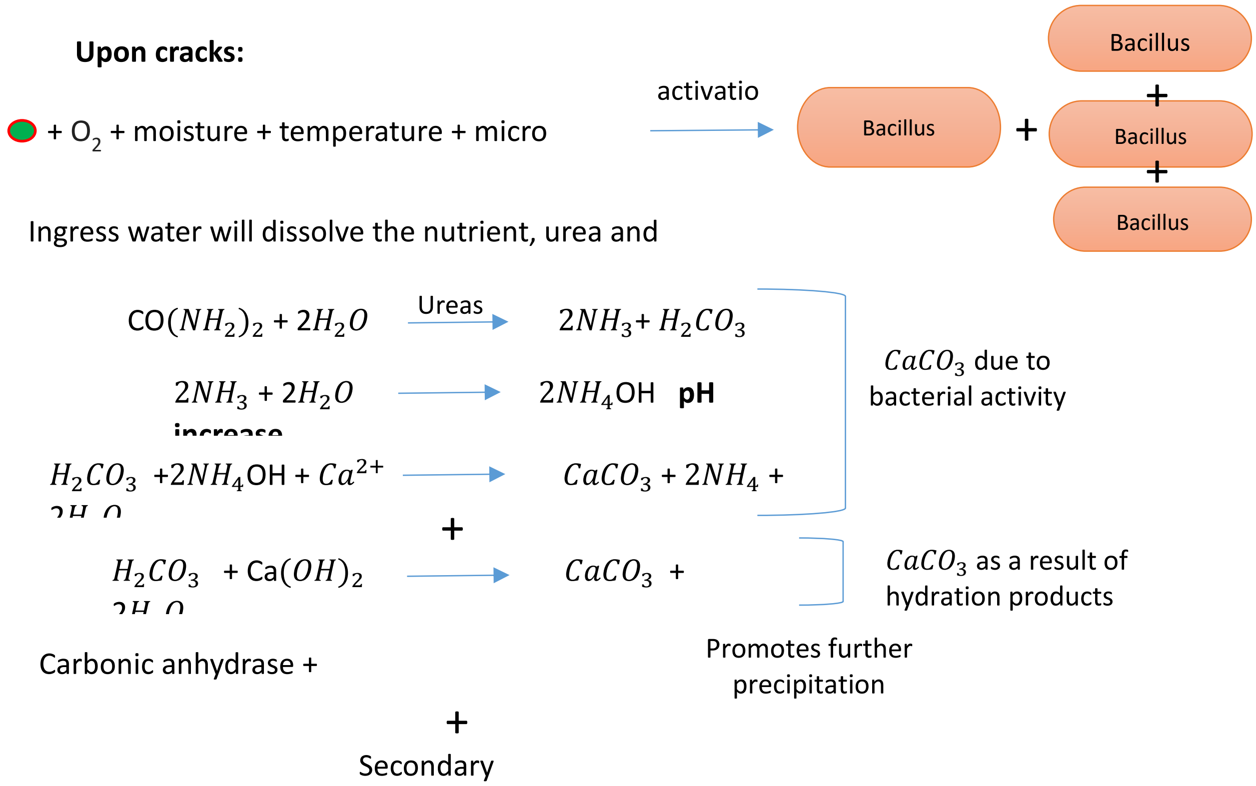


Figure 98 Illustration of the different mechanisms that responsible for the healing and formation of $CaCO_3$.

As a result of the bacterial growth, urease enzyme will feed on urea i.e. it breaks it down into two moles of ammonia and carbonic acid. The two moles of ammonia then further react with the two molecules of water resulting in a pH increase and production of $2NH_4OH$. The produced carbonic acid then reacts with the available calcium i.e. resulting into the formation of calcium carbonate precipitation. Simultaneously, the presence of carbonic anhydrase, which is a zinc enzyme that captures CO_2 and produce carbonic acid which further reacts with the available calcium and result into the formation of $CaCO_3$. In addition to the two mechanisms, secondary hydration also plays a role into the healing and production of further $CaCO_3$ due to secondary hydration and the reaction of carbonic acid with the calcium hydroxide.

5.3.3 Optimised three component healing system

5.3.3.1 Crack healing quantification via optical microscope

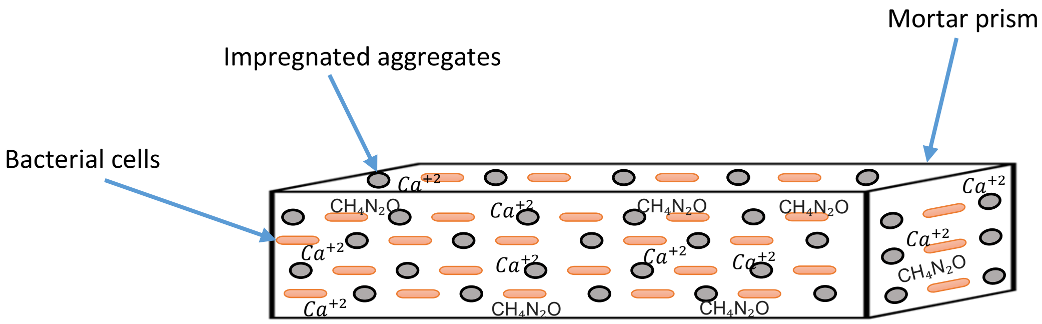


Figure 99 Schematic diagram of the optimised three component healing system.

The introduction of bacterial spores along with the healing agent via AARC aggregates indicated promising results towards self-healing applications as cracks were successfully healed over a period of 28 days of curing. In addition to other parameters such as cell concentration, amount of nutrients available, the crack size plays an important factor toward the healing capacity of mortar specimens. A three-component healing system comprising of AARC impregnated with spores and nutrients along with the third component being the fresh cells introduced into the cement matrix to provide better reinforcement for the cement matrix and enhance the capacity for bacterial specimens to seal larger cracks.

Previous studies in regard to direct incorporation of bacterial cells into the cement matrix have been reported. Jonkers et al., 2010; Wang et al., 2014; Al-zhari et al., 2017 used Bacillus pseudofirmus as part of a two-component healing system, comprising of where the proposed healing system provided promising results in regard the sealing of cracks. However, the loss of viability was reported when fresh cells/ spores were introduced directly into the cement matrix, where a decrease in compressive strength of mortar specimens was observed. It is of vital importance to note that the production of calcium carbonate proposed by Jonkers is via the metabolic conversion of calcium lactate, whereas the production of calcium carbonate via urea hydrolysis bacterium species is suggested to be due to enzymatic activity. Therefore, it is important to note the even though the cell might be dead the enzyme is still however active.

In-vitro studies by Dhami et al., 2012 and 2016; Krishnapriya et al., 2015; Sharma et al., 2017; Achal et al., 2010 and 2011; Okwadha et al., 2010; Cezario et al., 2017; Stocks-Fischer et al., 1999; Wei et al., 2015 are all studies that indirectly linked enzymatic activity to the bio-mineralisation process of bacterial concrete via ureolytioc hydrolysing bacterium species. Ezymatic aactivites such urease acitivity and cvarbonic anhydrase are two key enzymes in regard to the bacterial concrte via urea hydrolysis, where the former catalysis or hydrolysis urea to produce carbonate and the latter is a zinc enzyme that utilises the Co2 result from the urease activity. In-situ soil cementation via ureolytic bacteria was conducted by Achal et al 2011, where the calcite producing bacteria such as S.Pasteurii proved to be effective in improving the permeation properties and the tolerance against ingress substances when used in sand plugging applications. In another study, Achal et al., 2011 reported the use of strain CT-5, belonging to bacillus species, for the application of enhancing the durability of mortar specimens, where 36% increase in compressive strength at 28 days was observed.

Krishnapriya et al., 2015 isolated five alkali resistant bacteria for the application of self-healing concrete, where the strains isolated were tested for their ability to produce endospores, $CaCO_3$ precipitation in broth state. Direct incorporation of bacterial strain Namely, B. megaterium proved to be effective in enhancing the mechanical properties of the concrete specimens when compared to control.

Ramachandran et al., 2001 explained that the improvement in compressive strength by inclusion of bacteria was most probably die to the microbial deposition of caco3 on the surface and within the pore of the cement sand matrix. In another study, Gosh et al., 2006 reported that the enhancement in compressive strength could be due to the deposition of a filler material as a by-product of the bacteria metabolism that reduced the pore size and modified the microstructure of concrete.

Based on the aforementioned studies, incorporation of the bacteria directly into the cement matrix have shown to be viable in the case of ureolytic hydrolysis bacterium. It is worth pin pointing that the selection of the strains in this study was based on several vital parameters such as the ability to produce endospores, capacity to endure and grow in alkaline and extreme environments and produce copious amount of calcium carbonate. Based on the results shown in Chapter 4, two strains were selected for this part of the study namely strain 89 and strain 67 where both strains belong to bacillus Sphaericus. Experimental work in regard to the ability to produce endospores, no spores were produced by strain 67 while strain 89 was able to achieve over 90% sporulation over a period of 5-6 days. However, in-vitro calcium precipitation experiment in broth state showed that strain 67 produced the maximum yield in regard to the calcium precipitation when compared to the other strains. Both strains showed endurance when grown in alkaline conditions.

The mix design for stage 2 and stage 3 for this part of the experiment is depicted in Chapter 3, Section 3.8.1, Table 2-3. Direct incorporation of bacteria was performed as part of the makeup water where bacterial cell concentration equivalent to $10^7$and 10^8 cells/ml was directly added into the fresh cement during the mixing stage. Mortar specimens incorporated with 30% and 40 % (v/v of sand) non-impregnated AARC aggregates were used as control. Figure 8-5 shows microscopic images obtained at 14 and 28 days of healing. The process in inducing cracks and incubation was the same as reported in Chapter 5, Section 5.3.2.1., however higher cracks ranging from 0.17-0.769mm were induced in order to test the effectiveness and capacity of such approach to heal higher crack widths.

Microscopic monitoring showed similar pattern in regard to the healing process portrayed stage 1, bacterial specimens were able to produce calcium carbonate within 14 days of wet dry cycle curing, however, the production of calcium carbonate was prominent after the 14 days period in the case of fresh cells. Considering the introduction of bacteria at different concentrations i.e. $10^7$and 10^8 cells/ml with 30% AARC non-impregnated aggregates, both incorporations showed the tendency to heal cracks of 0.17 to 0.769 but the calcium precipitation was not sufficient to provide complete crack closure. Figure 100 A and Figure 100 B, shows the microscopic images for both concentrations, the sealing material produced by the former mainly comprised of white precipitate with orange colour material, which could be due to bacterial metabolism and the incorporation of the media. Similar observation was reported by Mohammad et al., 2017 where direct injection of media (SHA-2) and B.halodurans bacteria produced orange-like colour with calcium precipitation crystals i.e. excess amount of media results in the formation of poor orange-like sealing material.

Higher incorporation of bacterial cells at 10^8 cell/ml, Figure 100 B indeed yielded higher precipitation as higher cracks were completely sealed, the orange-like material was also present in this patch however, was very minimal in comparison to the ones in set 15.1b. This suggests that higher incorporation of bacterial cells was able to utilise more of the media incorporated, which explains the fading of orange-like colour when compared to the specimens with 10^7cells/ml.

To enhance the healing mechanisms of bacterial mortar, the introduction of a third component healing system which acts as a reinforcement within the cement matrix showed promising results in terms of contributing towards the overall healing efficiency.

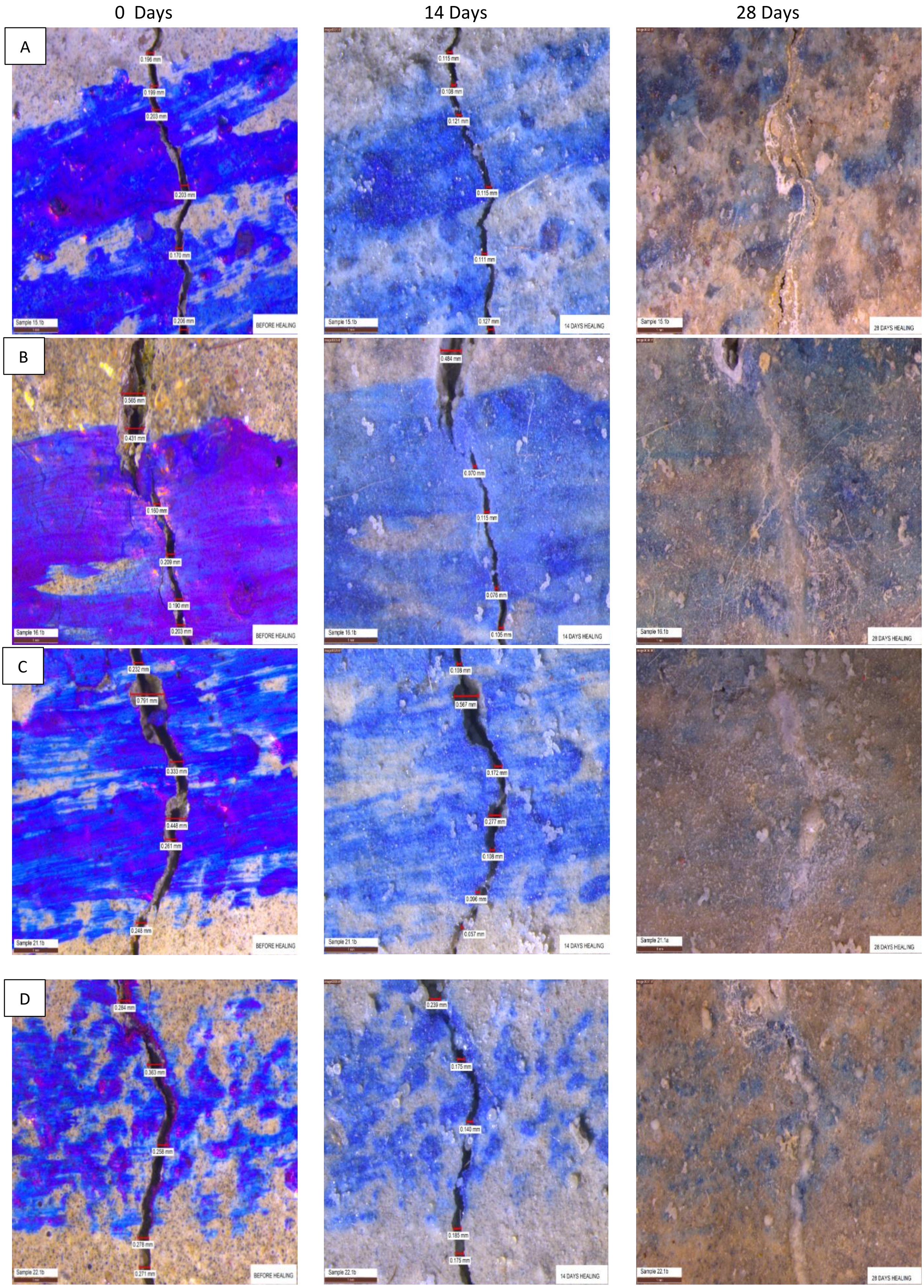


Figure 100 Microscopic images of the crack healing process: (A) 15.1b; (B) 16.1b; (C) 21.1b; (D) 22.1b, at 14 and 28 days with 30% AARC. The red bar in the bottom left corner corresponds to 1mm.

Figure 100 C and Figure 100 D shows microscopic images of a three-component healing system with 30% AARC aggregates impregnated with spores and nutrients. One can observe that the healing was very aggressive in both sets, suggesting that AARC aggregates were suitable carrier for the bacterial spores. It's worth noting that crystals were observed in the curing tank during the wet-dry cycle and on the surface of the mortar specimen, which suggests that some of the precipitation was lost during the curing process. The wet-dry cycle performed in this study could have had an impact on the lost in precipitation, during one cycle specimens were immersed in water for 12hours then allowed to breath for another 12 hours at room temperature 95%RH. Similar observations were reported by Mian et al., 2015 were wet-dry curing proved to be more efficient in healing cracks up to 0.8mm, when a 24-hour bacterial culture, corresponding to 10^9cells/ml, was introduced directly into the cement mix.

The introduction of bacterial cells (strain 89) was also performed for the mortar specimens with 40% AARC aggregates, non-impregnated and impregnated with spores and nutrients. This was performed in order to determine the efficiency of the three-component healing system when introduced to higher amounts of AARC aggregates. The introduction of fresh bacterial cells at 10^7 and 10^8cells/ml, Figure 101E and Figure 101F, showed similar performance to the ones incorporated at 30% impregnated AARC aggregates. The orange-like sealing material was apparent when media and cells 10^7cells/ml incorporated, suggesting excess amounts of media incorporated. On the contrary, 10^8cells/ml was able to utilise more of the media incorporated which could explain the disappearance of the orange-like sealing material and the presence of impermeable white crystallising material. The introduction of the three-component healing system using mixed culture, with strain 67 in the cement matrix, was also performed in this study. Figure 100 G and Figure 100 H shows the three-component healing system using mixed fresh culture at 10^7 and 10^8cells/ml with 40% AARC aggregates impregnated with spores and nutrients. The introduction of a mixed culture showed promising results in the regard to enhancing the healing efficiency of cracked mortar specimens.

In the case of a three-component healing system with strain 89 acting as a reinforcement within the cement matrix, also showed the tendency to seal and successfully heal higher cracks when incorporated with 40% AARC impregnated aggregates which proves the efficiency of such healing system towards the application of self-healing materials, Figure 101I and Figure 101.Furthermore, the inclusion of fresh bacteria as a third component healing system indeed enhanced the precipitation capacity produced. One can observe from the microscopic images that the appearance of a white precipitate was in abundance at 14 days of healing when compared to the ones with two component healing system which showed that precipitation was prominent after the 14 days period, Figure 92, Figure 93 and Figure 94.

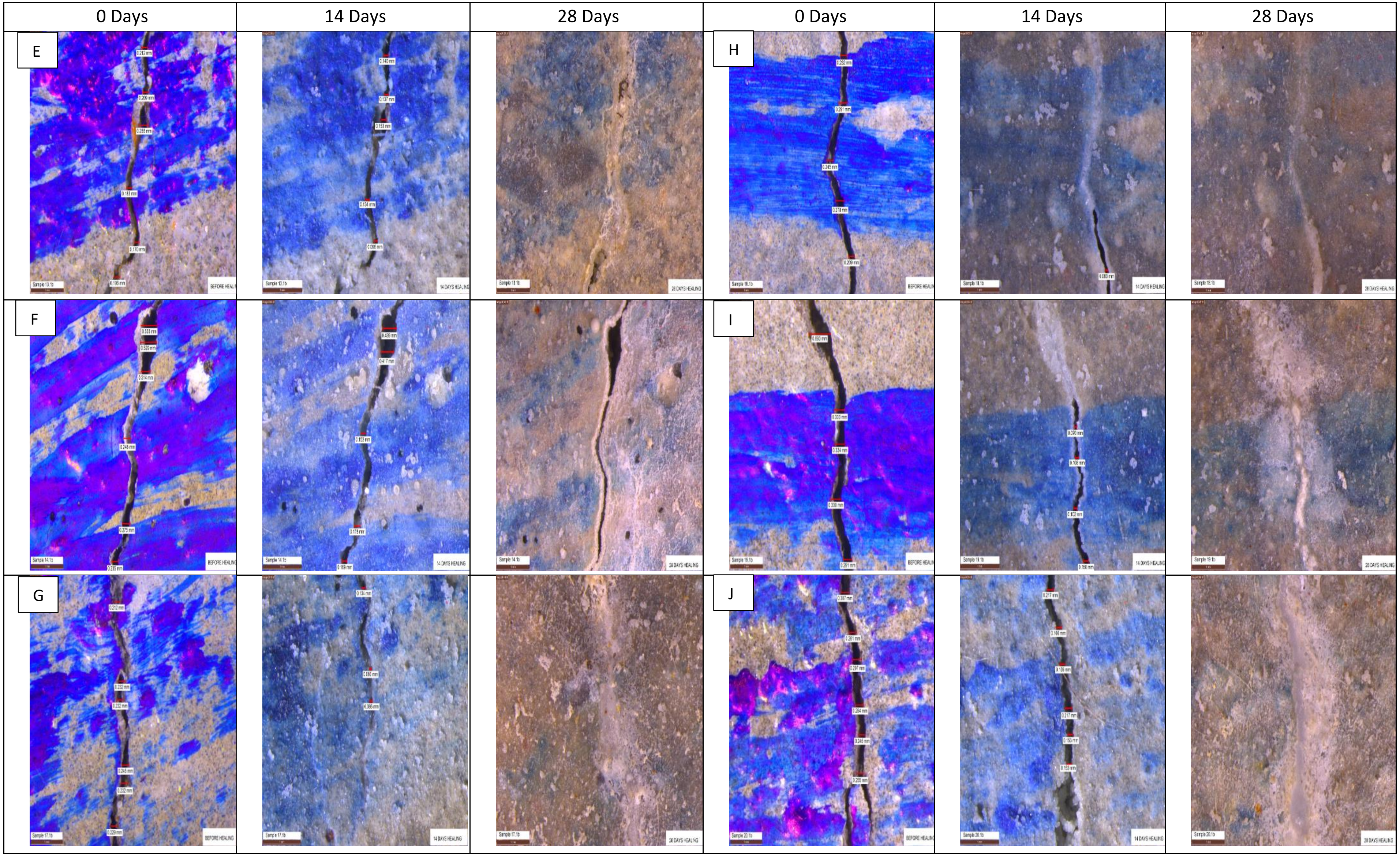


Figure 101 Microscopic images of the crack healing process: (E) 13.1b; (F) 14.1b; (G) 17.1b; (H) 18.1b; (I) 19.1b; (J) 20.1b, at 14 and 28 days with 40% AARC. The red bar in the bottom left corner corresponds to 1mm.

5.3.3.2 Crack healing percentage –Optimised three component healing system

The smaller the cracks the higher the healing percentages, Figure 102. Direct incorporation of bacterial cells at 10^7 cells/ml showed inconsistency in regard to the healing percentage, at 14 and 28 days, as different cracks of 0.2 mm across the same crack line had different healing percentages i.e. suggesting that the cells were not uniformly distributed across the cement matrix, Figure 102A-5 and Figure 102B.

Higher incorporation of bacterial cells (10^8 cells/ml) portrayed better healing percentages over the period of 28 days when compared to the 10^7cells/ml when incorporated with 30% AARC aggregates, where the former was able to seal 92.2% of 0.206mm cracks and the latter was able to seal crack of 0.5383mm by almost 100%. The introduction of higher cracks for the same group of specimens showed less percentages when the crack was higher, this was observed in the case of specimens with 10^8 cells/ml where 54.11 % of 0.769mm cracks was healed. The enhanced healing is attributed to higher incorporation of the number of cells which resulted into higher calcium carbonate yielding, this is in addition to the autogenous healing factor which contributed to the sealing off the cracks.

The healing percentages observed for specimens with 30% AARC impregnated aggregates with strains 89 acting as a reinforcement showed the capacity to heal higher cracks. Direct Incorporation of 10^7cells/ml with 30% impregnated aggregates showed the capacity to partially heal cracks of 0.791mm by 28.3% over 14 days and 100% over 28 days healing period. Increasing the number of cells showed expected higher healing efficiency, specimens in set 22.1B were able to completely seal cracks of 0.875mm by 99.085 % over the period of 28 days.

The incorporation of bacteria directly with 40% non-impregnated AARC aggregates, Figure 102Cand Figure 102D, showed similar behaviour in terms of the healing percentage when compared to the specimens in group 15.1b and 16.1b, Figure 102Aand Figure 102B. Inconsistency in regard to the healing percentage was also observed at 10^7cells/ml i.e. implying poor distribution across the cement matrix, this is shown in specimens in group 13.1b at 14 and 28 days, Figure 102C and Figure 102D, respectively.

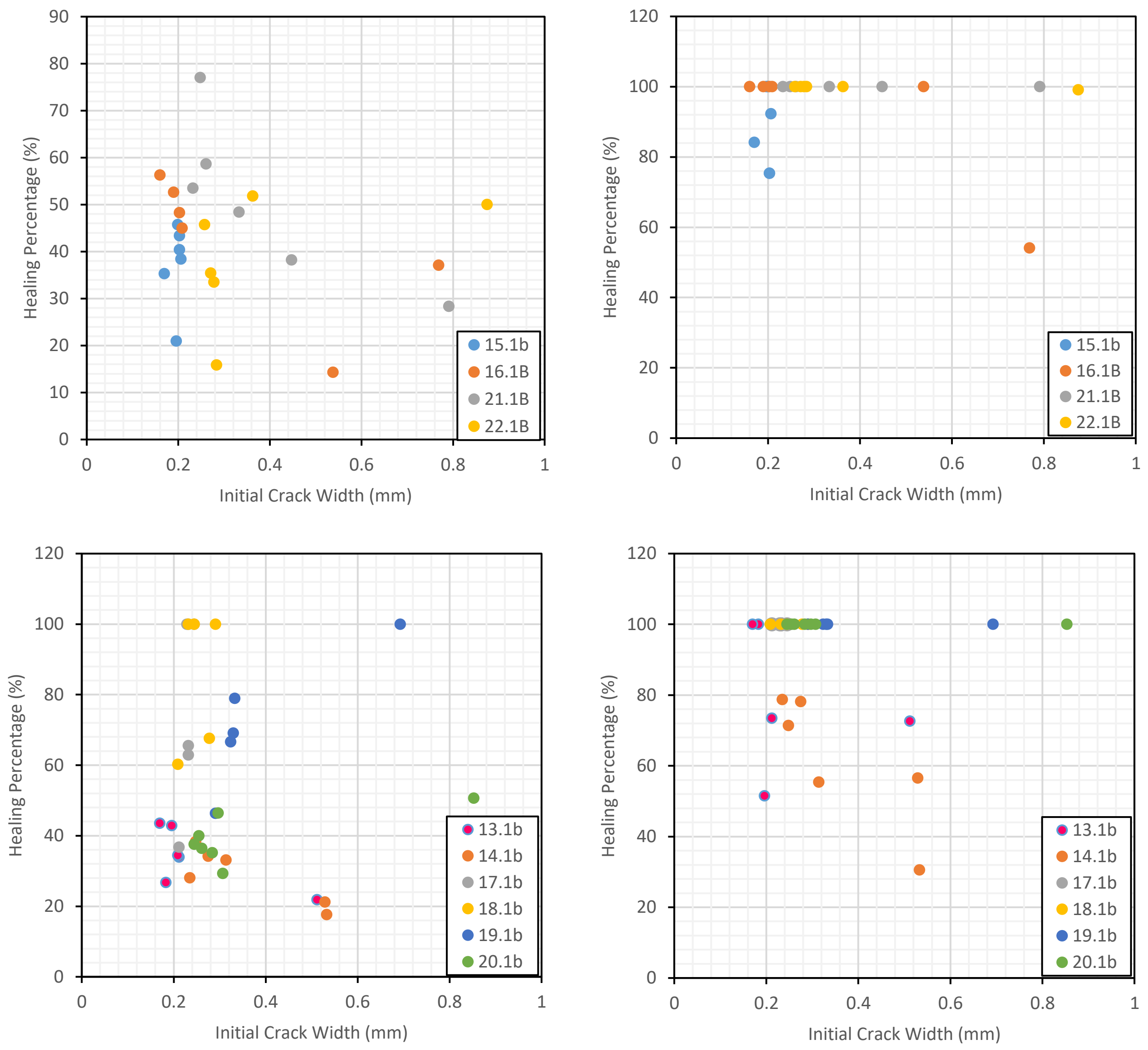


Figure 102 Crack healing Percentage as a function of the initial crack width for mortar specimens at 14- and 28-days healing time (A-B) 30% AARC, (C-D) 40% AARC with bacterial reinforcement.

The small drop in the healing percentage in regard to specimens in group 14.1b in comparison to 13.1b could be attributed to the formation of a dense micro-structure due to the continuing hydration of cement in the concrete matrix at early ages which limited bacterial activity to produce more calcium carbonate crystals, similar findings were reported by Jonkers et al 2010 and Zhan et al., 2017.

The highest crack healing percentage at 40%AARC impregnated aggregates was observed for specimens in group 20.1b where maximum crack of 0.853mm were completely healed over the period of 28 days. Which suggests that autoclaved aerated recycled concrete is indeed and suitable carrier for the application of self- healing mortar.

The introduction of a mixed culture with 40% impregnated AARC aggregates showed great potential for complete sealing off maximum cracks of 0.212mm and 0.291mm, at 10^7cells/ ml and 10^8cells/ml over the period of 28 days, respectively. Therefore, an enhanced healing mechanism were observed in the case of strain 67 when introduced as part of three component healing system.

5.3.3.3 Permeability via capillary action of healed mortar

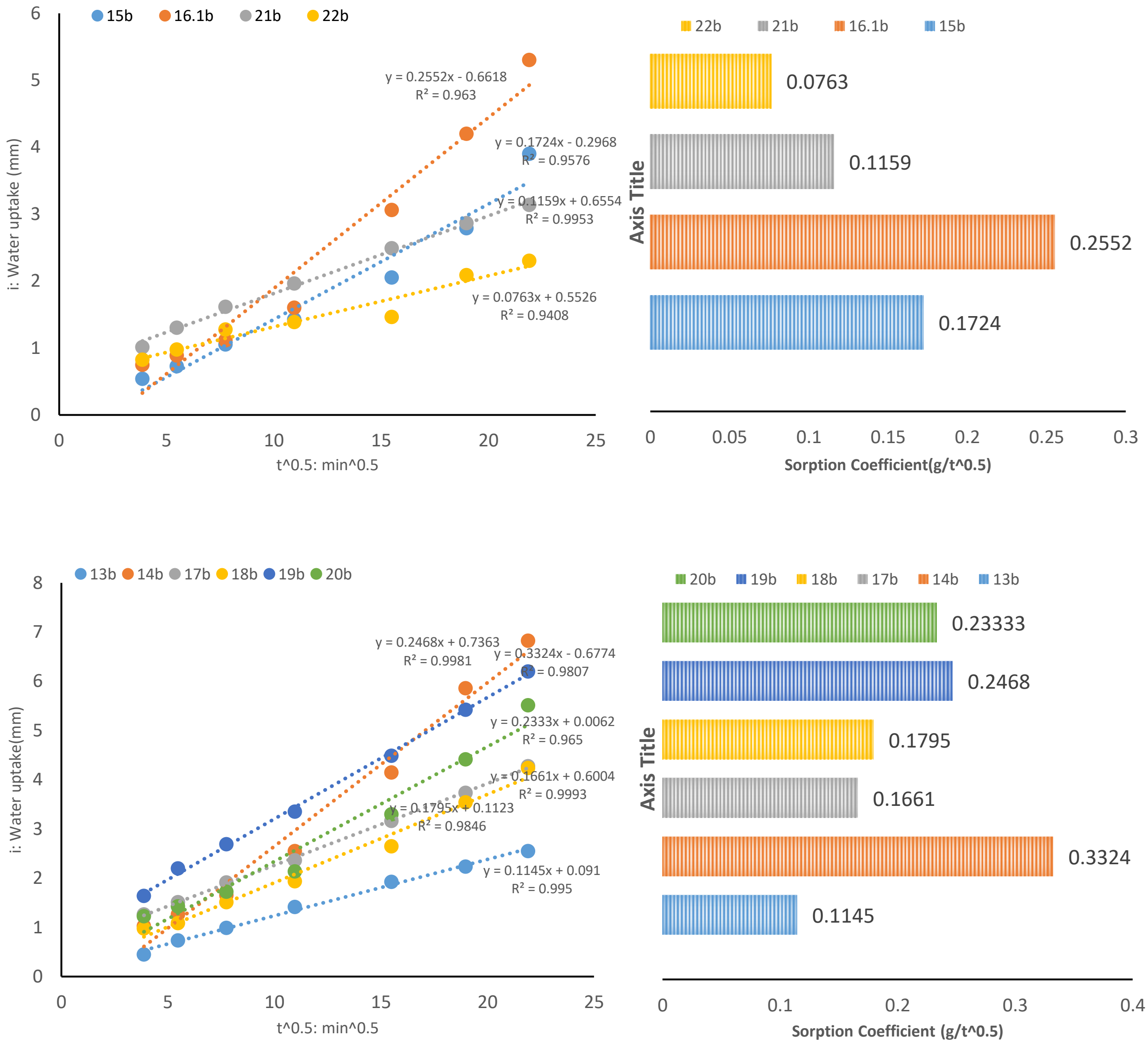
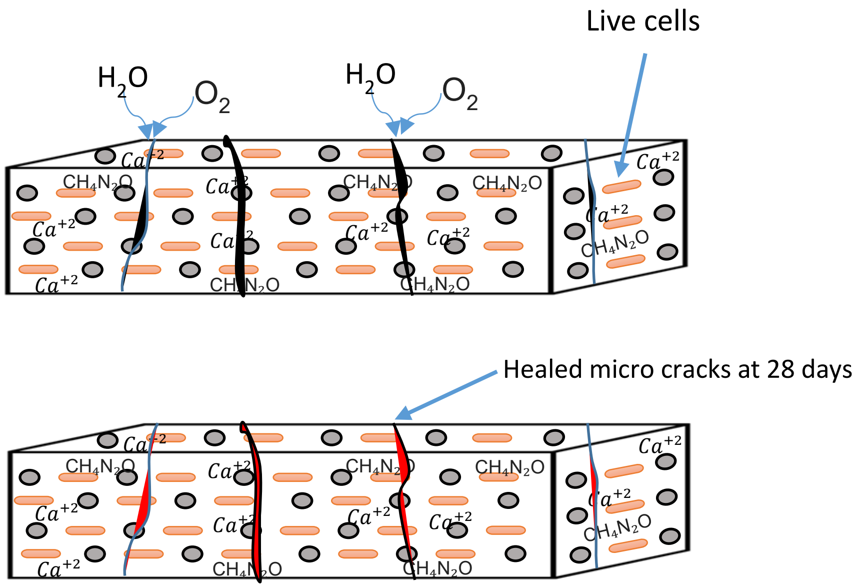


Figure 103 5 Sorptivity plots for the mortar specimens (Left) and Sorption coefficient (right) at 28 days of healing.

5.3.3.4. Healing mechanisms - optimised three component healing system



The three-component healing system promotes further production of calcium carbonate precipitation by introducing a third component, namely, live cells that act as reinforcement in the cement matrix. The live cells are added or introduced directly into the cement matrix during the mixing process as part of the makeup water, and the they are activated upon the occurrence of cracks and the right conditions to promote microbial growth. Figure xxx, shows a cross section view of the impregnated aggregates and incorporated live cells.

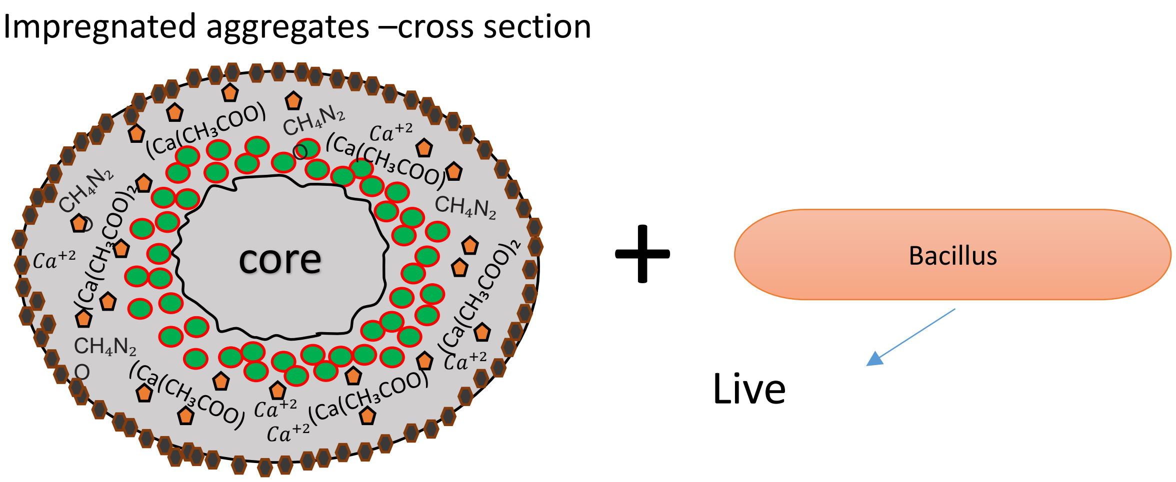


Figure 104 Schematic diagram of the aggregates and live cells

$$CO(NH_2)_2 + 2H_2O \xrightarrow{\text{Urease}} 2NH_3 + H_2CO_3$$

$$2NH_3 + 2H_2O \longrightarrow 2NH_4OH \quad \textbf{pH increase}$$

$$H_2CO_3 + 2NH_4OH + Ca^{2+} \longrightarrow CaCO_3 + 2NH_4 + 2H_2O$$

$CaCO_3$ due to bacterial activity

$$+$$

$$H_2CO_3 + Ca(OH)_2 \longrightarrow CaCO_3 + 2H_2O$$

$CaCO_3$ as a result of hydration products

$$\text{Carbonic anhydrase} + CO_2 \qquad H_2CO_3$$

Promotes further precipitation

$$+$$

Secondary hydration

Figure 105 illustration of the healing mechanisms in a three-component healing system.

The survival of live cells into the cement matrix is a key factor for the promotion of further calcium carbonate precipitation and hence higher healing to crack ratio. The functionality of the live cells in the cement matrix is similar to the spores incorporated in to the AARC aggregates, where they both stay dormant and activate upon on the availability of nutrients and temperature. Incorporation of live cells in the cement matrix is indeed a novel approach, and it helps in the healing of early aged cracks upon formation.

5.3.4 Direct incorporation of bacteria for the recovery of self-healing mortar

Despite the fact the previous studies have reported the loss in viability of bacterial spores when introduced directly into the cement matrix Jonkers et al 2010; al-azhari et al 2017, it must be noted that the approach used by the aforementioned studies is different t that used via urea hydrolysis. The former relies on the conversion of calcium lactate to produce calcium carbonate while the latter have been linked to enzymatic activity i.e. mainly due to the urease enzyme and carbonic anhydrase enzyme.

To the knowledge of this study, one distinctive test that reported literature lacked towards the self-healing via urease hydrolysing bacterium is the treatment of bacterial cells with gamma radiation at different doses to try limit and the life of the cell while maintaining the enzymatic activity. Such test should be definitive towards understanding and identifying the real mechanisms that are responsible for the precipitation of calcium carbonate.

The results obtained from the three-component healing system directed the study to investigate the healing mechanisms when bacterial cells added directly into the cement matrix without the need for an encapsulating material. Although it is of vital importance to use a suitable carrier in order to achieve self- healing smart material without the need for human intervention, direct incorporation of bacterial cells attracts several civil engineering applications where the technology can provide several advantages. One application that could be of a great potential is in the pavement sector, where bacterial cells are introduced during the subgrade, capping and sub-bas layers to provide reinforcement and prevent ravelling. Another potential application that can be of great attraction is toward the geotechnical sector, where bacteria can be used in soil consolidation as type of reinforcement for infrastructure applications.

To enhance the healing capacity of pre-cracked mortar specimens, incorporation of nutrients was essential in regard to providing enough precipitate to seal cracks. For this part of experiment strain 67 and strain 89 were selected for their capacity to tolerate and endure alkaline condition while utilising the nutrients in the surrounding environment to ultimately produce calcium carbonate as a by-product. The nutrient along with the fresh cells were introduced as part of the makeup water at 20% and 30%, the media used composed (g/l) of Calcium acetate 15.0, Urea 26.5 and nutrient broth. Introduction of the bacterial during the log phase corresponding to 10^7 and 10^8cells/ml was performed for both bacterial strains.

5.3.4.1 Crack healing quantification via optical microscope

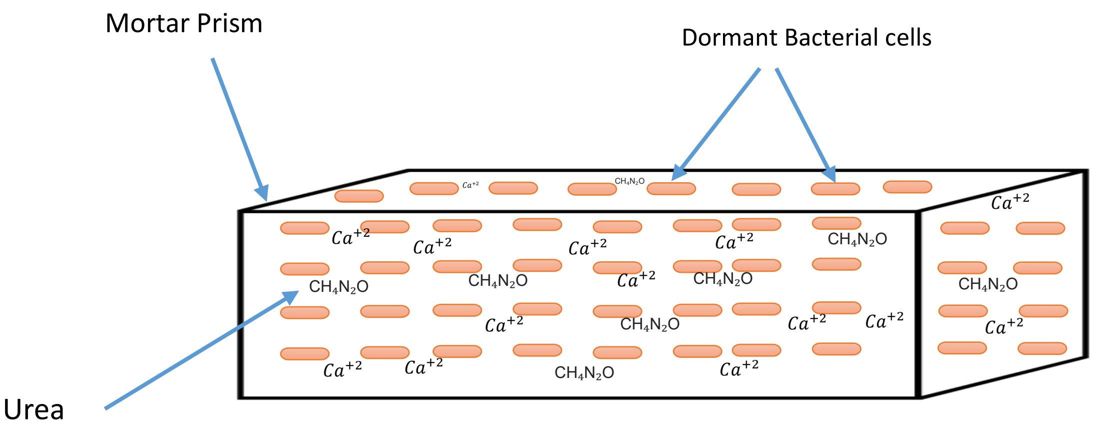


Figure 106 Ssingle component healing system.

The results obtained from the microscopic images are not surprising, observation of what could be calcium precipitation due to bacterial activity was in abundance not only in the crack zone but all over the mortar specimens. The loss in precipitation in the curing tank during the wet dry cycle resulted in less precipitation to be utilised for enhancing the properties of self-healing mortar, Figure 107.

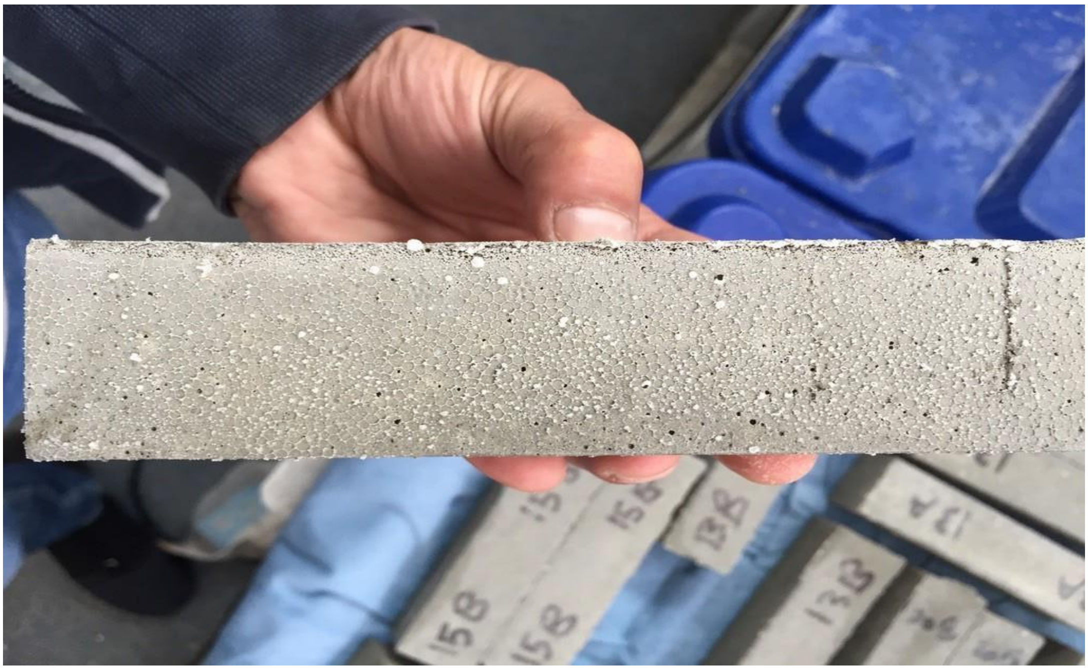


Figure 107 Loss of the calcium carbonate crystals during the wet dry cycle.

Direct incorporation of the bacterial cells into the cement matrix resulted in higher crack healing capacity at 14 days in comparison to the ones with encapsulated in AARC aggregates. Suggesting that the AARC aggregates were able to provide suitable environment to protect and limit bacterial activity from early activation. In addition to the formation of calcium carbonate as result of bacterial activity, other factors such as hydration of un-hydrated cement particles and the carbonation of mortar specimens all could have contributed towards increasing and enhancing the crack healing efficiency.

Although not tested, but in-vitro experiments indicated the presence of carbonic anhydrase enzyme, which is a zinc enzyme that catalysis or aid in the interconversion of carbon dioxide to carbonic acid and bicarbonate ions which contributes toward the yielding efficiency of the produced calcium carbonate precipitation.

Microscopic monitoring of the healing evolution of pre-cracked mortar specimens showed the capacity both bacterial strains in enduring the high alkalinity to utilise and produce calcium carbonate in abundance. Figure 108 A Figure 108 B shows the healing evolution of strain 89 at different concentrations, increasing the cell 10^7cells/ ml from 10^8cells/ml resulted in better crack healing efficiency. On the contrary strain 67 showed less capacity to produce calcium carbonate crystals at 10^7cells/ ml when compared to strain 89, suggesting that strain 89 was better in enduring the high alkaline and extreme harsh conditions in the cement matrix. Another factor that could have contributed to this is the loss of cells during the mixing process and the loss of precipitation during the wet-dry cycle. Similar results were obtained by Mian et al 2015 where bacterial mortar specimens pre-cracked at 21 were able to successfully seal cracks of up 0.3mm over 28 healing period.

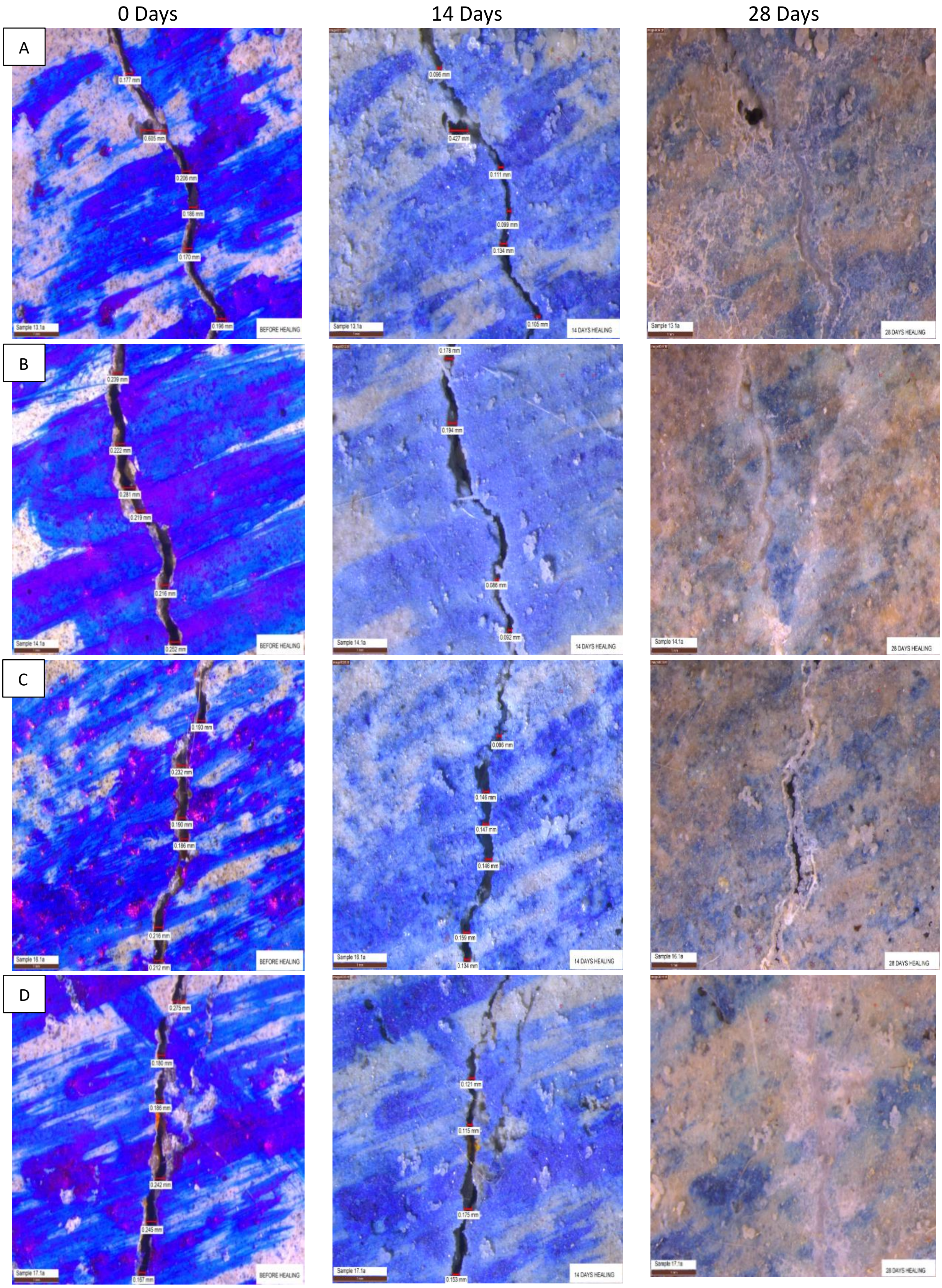


Figure 108 Microscopic images of the crack healing process: (A)-(B) strain 89 at 10^7 *cells/ ml and* 10^8 *cells/ml; (C)-(D)) strain 67 at* 10^7 *cells/ ml and* 10^8 *cells/ml.*

5.3.4.2 The evolution of the crack healing capacity

The results obtained from the crack healing percentage were indeed in harmony with the microscopic images, increasing in cell concentration from 10^7cells/ ml to 10^8cells/ml resulted in higher tendency to heal bigger cracks. In the case of strain 89 at 10^7cells/ml cracks of 0.206 and 0.737mm were healed by 100% and 42.23%, respectively. Higher healing percentages were observed when strain 89 incorporated with 10^8cells/ml as crack of 0.252mm and 0.486mm were completely sealed over a period of 28 days of healing. For the specimens incorporated with strain 67 at cracks of 0.186mm and 0.212mm were only healed by 54.3%and 84.91%, respectively. Similar pattern to strain 89 at 10^8cells/ml was observed in the case of strain 67 at the same concentration, where cracks of 0.18 and up to 0.58 were completely healed over the period of 28 days of curing.

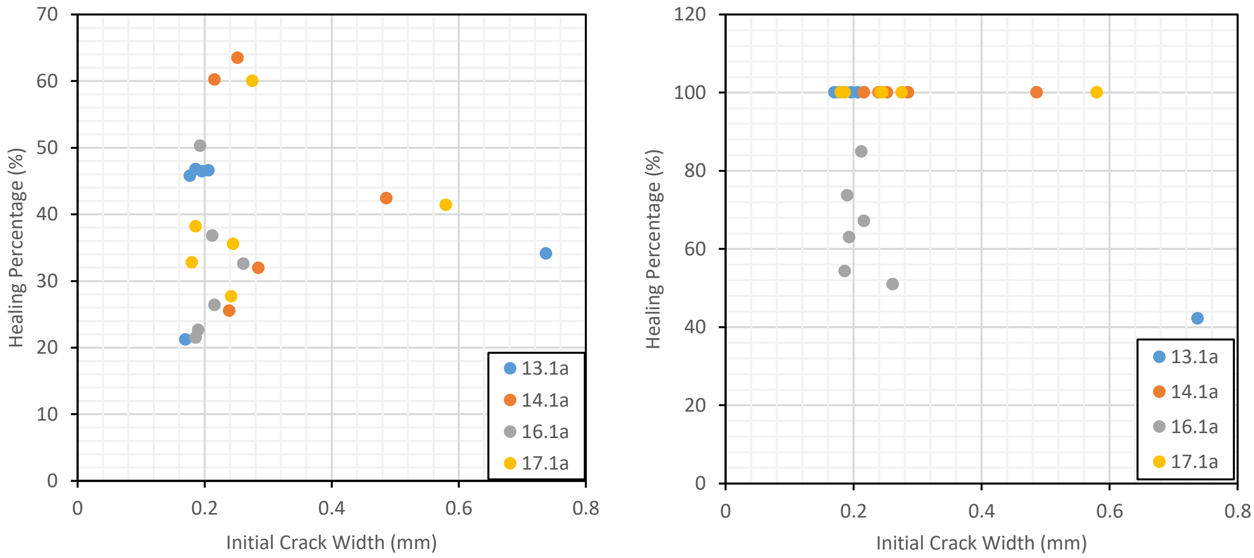


Figure 109 Crack healing Percentage as a function of the initial crack width for mortar specimens at 14 and 28 days healing times; 13.1a and 14.1a correspond to strain 89 at 10^7cells/ml 10^8cells/ml, respectively and 16.1a and 17.1a correspond to strain 67 at 10^7cells/ml 10^8cells/ml, respectively.

The result obtained from Figure 109 suggest that strain 89 was faster in utilising the available nutrients in the surrounding environment to produce calcium carbonate over the period of 28 days, by adjusting and creating nucleation site i.e. microenvironment for the calcium precipitation to occur. On the other hand, strain 67 was slow in the metabolic conversion nutrients to produce calcium carbonate precipitation at the initial stages of the healing process, suggesting that higher period over healing is required for maximum yielding when incorporated into the cement matrix.

Therefore, the enzymatic activity i.e. the carbonic anhydrase and urease enzyme could be higher or stronger in the case of strain 89 than strain 67 when subjected to alkaline environments. Similar results were obtained by Dhami et al., 2012 and 2016; Krishnapriya et al., 2015and Achal et al., 2011 where the carbonic anhydrase and urease activity of different bacterial strains have supported direct relationship between the enzymatic activity and the final yielding of calcium carbonate by different bacterial strains.

5.3.4.3 Permeability via water uptake: Direct incorporation

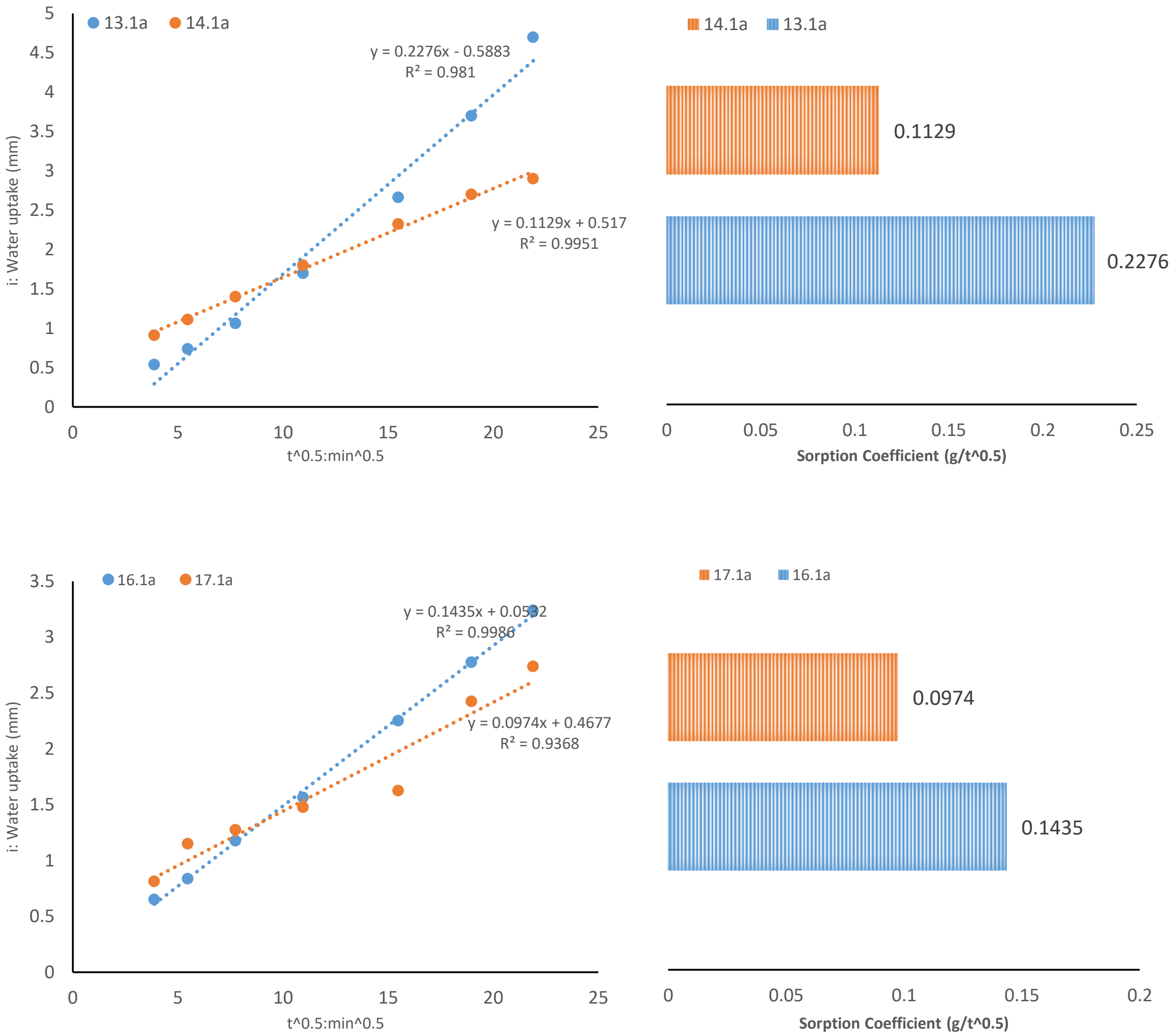


Figure 110 Sorptivity plots for the mortar specimens (Left) and Sorption coefficient (right) at 28 days of healing.

5.3.4.4 healing mechanisms – single component healing system

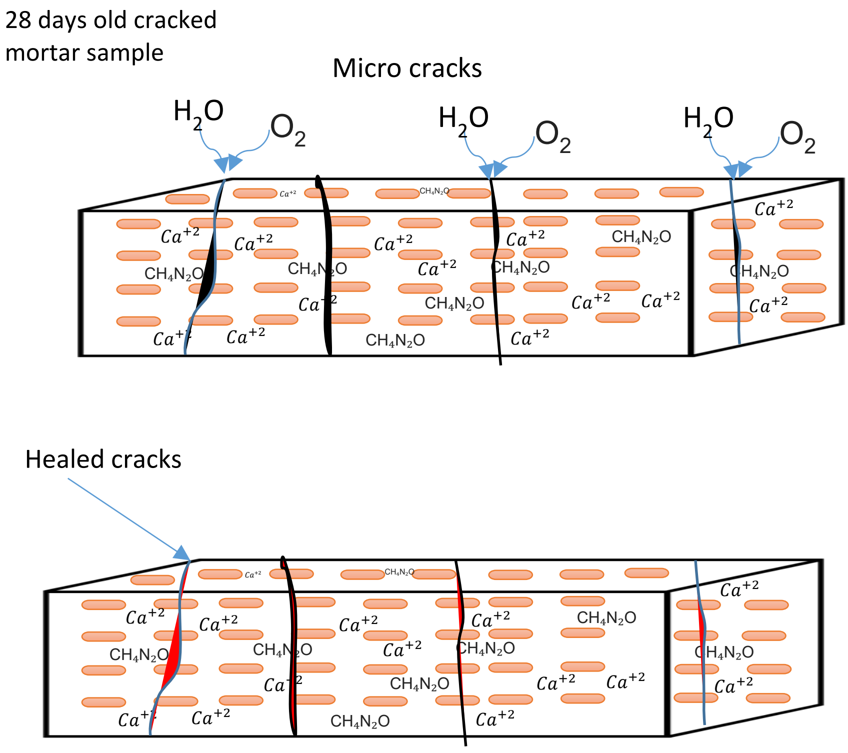


Figure 111 Single component healing system.

Direct incorporation of the live cells(Figure 111) into the cement matrix proved to be a viable approach due to the fact that the healing mechanisms is enzymatic process, where they are able to survive the mechanical forces and the harsh and alkaline environment of the concrete matrix, Figure 112shows the cell wall of a bacillus species with the presence f carbonic anhydrase and urease enzymes within the cell.

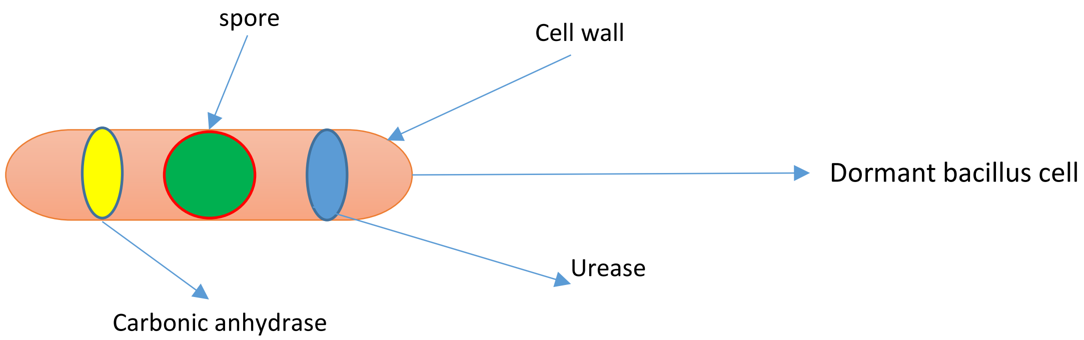


Figure 112 Schematic diagram of the bacterial cell wall.

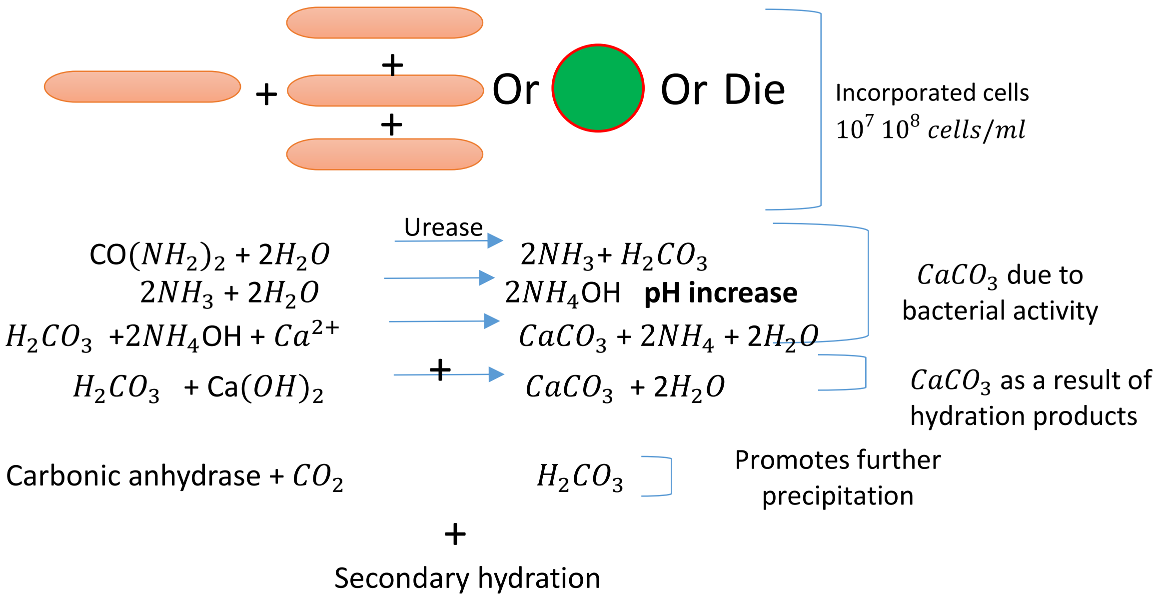


Figure 113 Mechanisms of a single component healing system.

Upon incorporation of the live cells as part of the makeup water, cells either remain dormant, sporulate or die. Even though the cell may die due to the mechanical forces during the mixing process, enzymes such as urease and carbonic anhydrase may still be active and feed on micronutrient and available calcium from the surrounding resulting into the formation of bacterial calcium carbonate as by-product of the bacterial metabolisms. The chemical reaction behind the calcium precipitation in the case of a single component healing system is similar to the dual and three component healing systems, except the fact that bacterial precipitation is limited when compared to the other healing systems.

5.3.3.6 Microstructural analysis

5.3.3.6.1 Scanning electron microscope

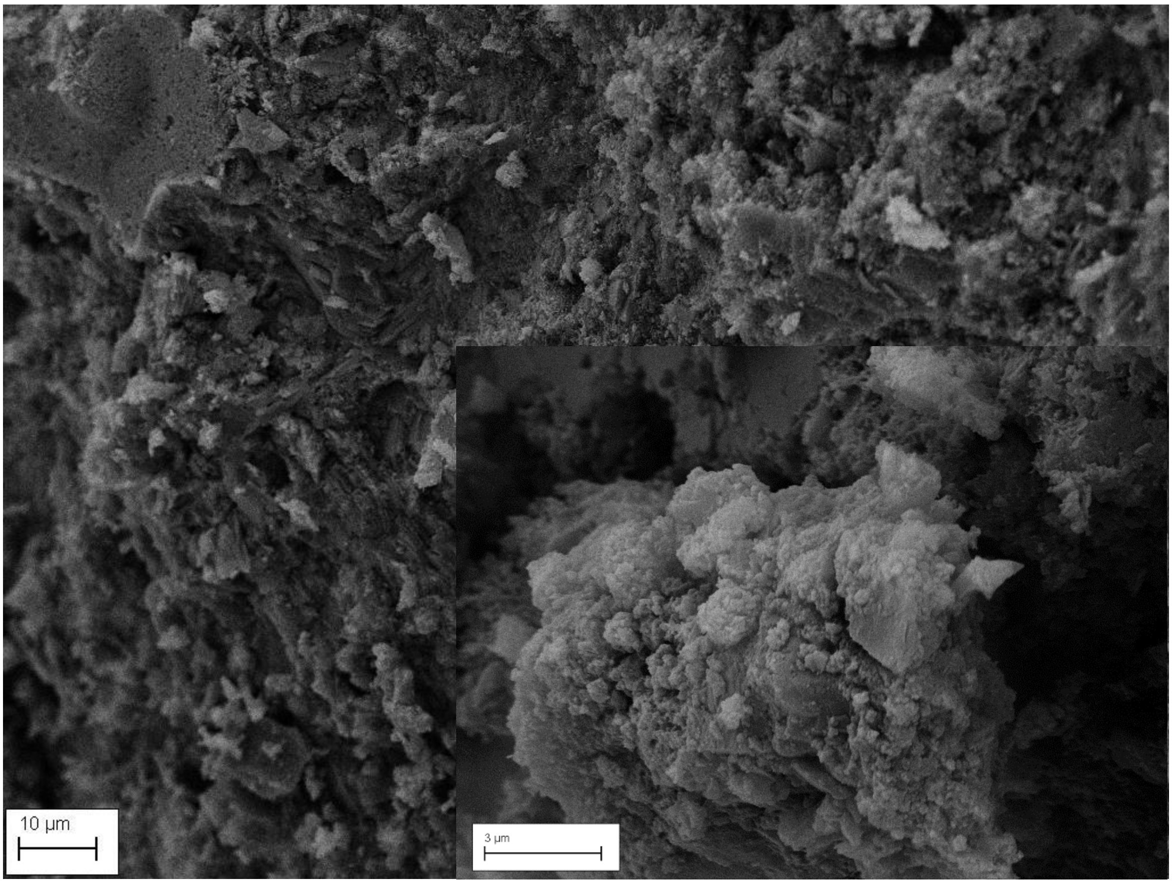


Figure 114 Shows the calcium precipitation as a result of bacterial activity. The precipitates had calcite crystals that composed mainly of C, O and Ca.

5.3.3.6.2 FTIR

The healing material produced at the crack mouth was analysed to confirm the presence of calcium carbonate crystals i.e. calcite. The calcium carbonate produced in-vitro was used as control. Weak peak of σ(O-H) bending vibration at 1645 cm^{-1}, The calcite produced was identified by four main peaks, v_3 asymmetric stretching vibration of CO_3at 1410cm^{-1} , v_1 asymmetric stretching vibration of CO_3at 1042cm^{-1}, sharp-strong peak of v_2 asymmetric stretching vibration of CO_3at 870cm^{-1} and medium to strong peak of v_4 asymmetric stretching vibration of CO_3at 710 cm^{-1}.

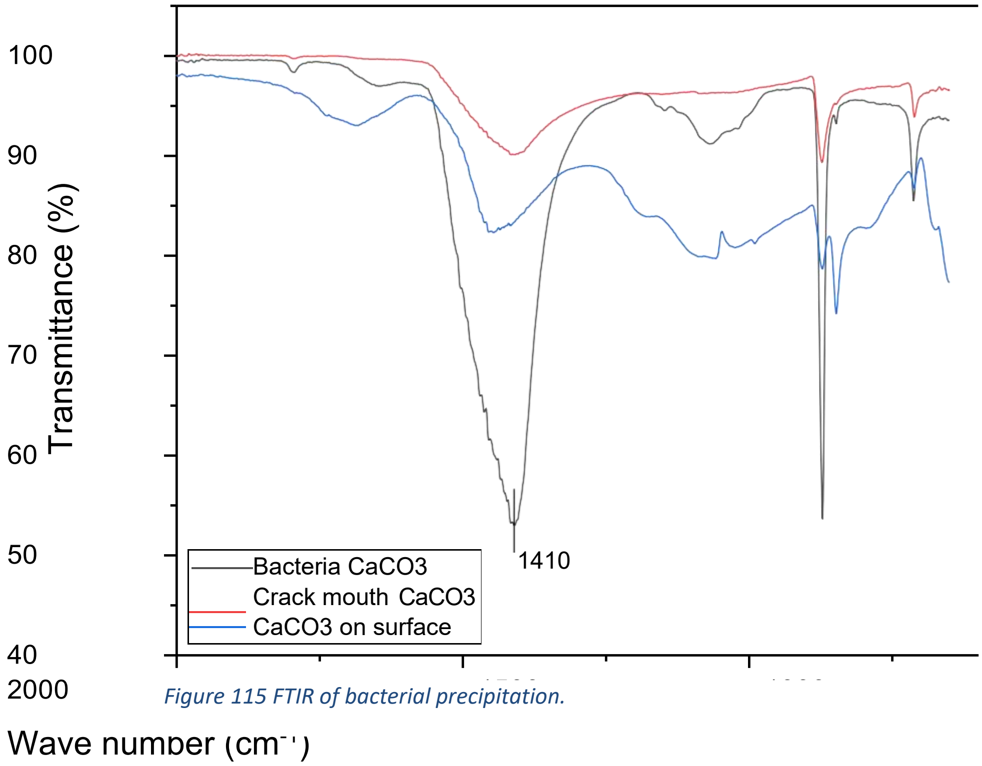


Figure 115 FTIR of bacterial precipitation.

Figure 116 FTIR of bacterial precipitation collected from different healed samples

Therefore, this confirm that the application of bacteria in sealing cracks is viable in addition to autogenous healing as a result of the hydration products.

Chapter 6 Final appraisal and future work

6.1 Summary of the research

Inspired by the biological systems, the application of self-healing mortar has shown promising results toward the partial/total recovery of at least one of the materials properties, such as mechanical strength and durability. The recovery of the material structural integrity via bacterial approach follows two concepts: the first concept considers that an alkaline tolerant bacterium belonging to bacillus species, namely Bacillus pseudofirmus, have the capacity to heal cracks through the metabolic conversion of calcium lactate to finally produce calcium carbonate; and the second concept of bacterial concrete relies heavily on the enzymatic activity, such as urease enzyme and carbonic anhydrase enzyme. However, inconsistency in the reported literature towards distinguishing between the two approaches and understanding the real mechanisms behind the two approaches was clear and evident. Several studies reported the loss in compressive strength when bacteria incorporated at different concentration, while other studies reported an increase in the compressive strength.

In-vitro calcium carbonate precipitation experiment showed 12 strains, belonging to bacillus species, with the capacity to produce insoluble calcium carbonate, namely, strains 57, 58, 59, 60, 63, 67, 89, i. When subjected to high pH (pH 9 and 10) Strains 67, 58 and 89 yielded the highest calcium carbonate when compared to all other strains. The selected strains were also tested for the production of spores, strain 57 and 89 were very efficient in producing spores, in contrast to strain 67 where no production of spores was observed. Therefore, strain 67 has been used as a reinforcement in the cement matrix to provide further healing capacity.

The encapsulating material is of vital importance for self-healing concrete, the material has to be porous, with high absorption capacity, water retention and a suitable mechanical property i.e. able to withstand the mechanical forces during the mixing process and provide protection to the incorporated spores without compensating for the overall mechanical properties of the end product. Therefore, autoclaved aerated recycled concrete aggregates were found to be a suitable carrier or host for the use of self-healing concrete, coating material composed of sodium silicate(30%v/v) and fly ash were used to minimise leakage of the spores.

Incorporation of the impregnated autoclaved aerated recycled aggregates (AARC) was done at 20% 30% and 40% (v/v) of sand. Incorporation of 30% AARC aggregates loaded with spores and nutrients showed better healing percentages where 100% crack closure was observed for cracks of 0.235 and 0.245mm, which is higher than the 20% AARC incorporation. Considering the abiotic control at 30% AARC, 75% and 69% of cracks closure was observed for 0.252 and 0.258mm, respectively. Over the period of 28 days of healing, specimens with 40% AARC aggregates impregnated with spores and nutrients were able to completely heal cracks of 0.253, 0.31, 0.334 and 0.255mm.

The introduction of a three-component healing system showed enhanced healing capacity, Direct Incorporation of 10^7cells/ml with 30% impregnated aggregates showed the capacity to partially heal cracks of 0.791mm by 28.3% over 14 days and 100% over 28 days healing period. Increasing the number of cells showed expected higher healing efficiency, specimens in set 22.1B were able to completely seal cracks of 0.875mm by 99.085 % over the period of 28 days.

Simplicity is the key in this thesis, the study starts self-healing mortar from scratch, i.e. bacteria/spore's production, screening and characterisation, in order to provide better understanding of the real mechanisms that are directly/indirectly responsible for the production of calcium carbonate. The work truly brings and combines bioengineering and civil engineering together as the new generation towards the civil engineering applications.

6.2 Major conclusions

The major conclusions towards the scientific community from this study are summarised as follows:

(1) Pilot study on 120 different bacterial strains was performed. Initial characterisation was performed by screening for the presence of urease enzyme, followed by growing the positive strains under high pH environment to examine the tolerance of the selected strains. The selection process was then confirmed by conducting in-vitro calcium carbonate precipitation in broth state to provide better understanding between the enzymatic activity and the yielding of calcium carbonate crystals.

(2) The calcium source played an important role in enhancing the yielding of calcium carbonate precipitation, three types of calcium were investigated, namely, calcium lactate, calcium chloride and calcium acetate. Calcium lactate was efficient towards the yielding of calcium carbonate precipitation, bacterial strain was able to use the acetate as a carbon source for maximum growth and to finally increase the yielding of precipitation.

(3) The biochemical properties of the selected bacterial strains were further investigated, experimental work revealed direct correlation between several parameters and the amount of calcium carbonate produced. Such parameters were pH, production of ammonium, the presence of insoluble and soluble calcium, and the number of viable cells.

(4) In-situ analysis, i.e. XRD of the produced crystals confirmed that the produced precipitate was composed mainly of calcite crystals which is the most stable form of calcium carbonate. Further analysis of the functional groups that are responsible for the calcium carbonate process revealed four distinctive bands that were in good agreement with the reported literature. The main bands that were identified were at 1645 (O-H bending vibration), 1394 (V_3asymetiric stretching of CO_3), 1065 (V_1asymetiric stretching of CO_3), 873 V_2 asymmetric stretching of CO_3) and 712 (V_1asymmetric stretching of CO_3).

(5) On the basis of in-vitro calcium carbonate precipitation in broth state, several parameters were under scrutiny, such as temperature, shaking conditions, type of media and calcium source. Three strains were promoted for the use of self-healing application, strain 89, 67 and 57 all showed copious amounts of calcium carbonate yielding with strain 67 being the highest as it yielded 0.84 g/100ml, the second being strain 89 as it yielded 0.59 g/100ml and finally strain 57 with a yielding of 0.56 g/100ml of calcium carbonate

(6) Sporulation experiment in a broth state showed that the strain 89 was able to produce spores over 90% in a period of five days of incubation in MBS medium containing elements that are designed specifically to stress the bacteria for maximum production of spores. Although strain 67 was not able to produce spores but showed tolerance toward alkaline condition, therefore, the strain was promoted forward to be used a third component healing system that acts as a reinforcement within the cement matrix.

(7) The use of a suitable encapsulating material has also been explored in this study. Autoclaved aerated recycled concrete (AARC) aggregates have been promoted for the application of self-healing mortar. The healing agent composing of nutrient and bacterial spores was impregnated under vacuum impregnation, two cycles were performed where the former introduced bacterial spore solution and the latter nutrient solution.

(8) Dying of the AARC impregnated aggregates in an oven at 40°C resulted in a loss in viability as spore germination and the production of calcium carbonate during the drying stage were observed. The freeze-drying method proved to be efficient in limiting the cell activity and maintaining the cell viability.

(9) Although a coating material comprising of sodium silicate (30% v/v) and fly ash was introduced to limit the leakage of spores and healing agent, leakage of the spores was observed when specimens incorporated with AARC impregnated aggregates with spores only. Therefore, this suggests the need for a better coating material to efficiently utilise the maximum number of spores and nutrient for better healing efficiency.

(10) The leakage observed in specimens with AARC aggregates impregnated with spores only, revealed that spores were able to utilise the micronutrient present in the spore's shell to germinate in addition to utilising the calcium from the hydration products to finally produce calcium carbonate which resulted in sealing off crack of up to 0.268mm.

(11) The introduction of AARC aggregates impregnated with spores and nutrients was efficient towards the application of self-healing mortar at 30% and 40% by volume of the amount of sand used.

(12) The introduction of a two-component healing system comprising of spores and nutrient encapsulated in AARC aggregates proved to efficient in healing cracks of up 0.334mm at 40% and while less efficient when incorporated at 30% where crack of up 0.226 mm were partially healed.

(13) The introduction of bacterial cells directly into the cement matrix showed the tendency to heal cracks of 0.512mm by 72.65% at a cell concentration of 10^7cells/ml when added with 40% AARC aggregates. Loss of viability was observed when higher cell concentration of 10^8cells/ml in the case of 40% AARC non-impregnated aggregates which could be due to the loss of precipitate during the immersion in water, this is in addition to the formation of a dense micro-structure at early ages which limited the cell viability.

(14) Wet- dry cycle curing proved to be the most efficient curing as bacteria needs water and oxygen which are two essential components for support the outgrowth of bacterial activity to produce calcium carbonate crystals.

(15) The introduction of a three-component healing system with the bacterial cells added into the cement matrix directly showed higher healing capacity as cracks of 0.875mm were completely healed.

(16) The use of a three-component healing system have indeed enhanced the durability of mortar specimens in regard to the permeability properties of pre-cracked mortar specimens. Where the direct addition of bacteria into the cement matrix provided an extra source of precipitation to maximise the impermeable properties of mortar specimens.

(17) The introduction of mixed culture, i.e. strain 67 showed better healing capacity when compared to the strain 89. The improvement in the healing capacity was not surprising as in-vitro $CaCO_3$ in

broth state showed that the strain 67 had the maximum heling capacity.

(18) The introduction of bacterial cells with micronutrient into the cement matrix enhanced the mechanical properties of mortar specimen as an increase of 32% in compressive strength was observed. The enhancement in compressive strength could be attributed to the clogging of pores with $CaCO_3$ crystals.

(19) In addition to the healing via hydrolysing bacteria, autogenous healing due to the hydration of un-hydrated cement particles and the carbonation of cement mortar were all factors that aided in healing the crack with the $CaCO_3$ due to bacterial activity being the more dominant factor.

(20) Experimental work has indeed supported the analysis that the self-healing via urea hydrolysis could be mainly due to enzymatic activity. Two important enzymes that were directly linked to the production of calcium carbonate are urease enzyme and carbonic anhydrase. The former utilises urea to produce carbonic acid and bicarbonate to finally produce calcium carbonate as a by-product. The role of carbonic anhydrase lies in the interconversion of carbon dioxide into carbonic acid and bicarbonate, i.e. contribute towards the final reaction to enhance the yielding of calcium precipitation.

6.3 Future work

This study has portrayed great understanding towards identifying the mechanisms of the self-healing mortar. However, for the application of such technology in the construction industry, several parameters must be optimised based on the previous findings:

(1) The use of autoclaved aerated recycled concrete aggregates has proved to be an efficient carrier for the use of self-healing concrete. Further encapsulating materials for the use of self-healing concrete are yet to be explored, such as Nano graphene, cellulose and brick dust. The material selected should also consider the impact when introduced into the cement matrix i.e. without having to compensate for the mechanical properties when incorporated at high amounts to achieve higher healing to crack ratio.

(2) Calcium source is very important, the need for a cheap calcium source is hidden in bones. Extraction of calcium from bones can be performed by immersing the recycled bones in an acid solution until it is dissolved. Extraction of calcium from the dissolved solution should be explored further, however, such solution should provide carbon source and calcium source due to the reaction between the bones and the acid.

(3) The effect of introducing live cells and spores directly into the cement matrix needs to be further explored, by examining the different approaches of adding bacteria on the mechanical properties of both early aged concrete and harden concrete.

(4) Incorporation of mixed cultures such as denitrifying bacteria and ureolytic bacteria provides a promising solution to tackle the nitrogen loading resulted from the use of urea hydrolysing bacterium solely.

(5) Further research on the healed material to find its suitability in the case of creep, stress and properties of the healed sections such as net area, moment of inertia and centroid.

(6) Further research for the development of a unified international standard in assessing the healed compound and the healed material is highly recommended.

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