# Identification of genes in the bacterial isolates involved in microbial induced calcite precipitation (MICP) and investigation of various calcium sources for enhanced calcite precipitation and improved mechanical properties


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It is confirmed that the MS thesis entitled **"Identification of genes in the bacterial isolates involved in microbial induced calcite precipitation (MICP) and investigation of various calcium sources for enhanced calcite precipitation and improved mechanical properties"** of **Ms. Tahreem Nadeem,** registration no. **00000317958** has been examined by me. I undertake that:

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# DECLARATION

I certify that this research work titled **"Identification of genes in the bacterial isolates involved in microbial induced calcite precipitation (MICP) and investigation of various calcium sources for enhanced calcite precipitation and improved mechanical properties"** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged/ referred to.

**Tahreem Nadeem**

Master of Science in Industrial Biotechnology

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**Dedicated to**

**My Baba and Mama**

**Who supported and motivated me to remain steadfast**

**during the entire course of my degree.**

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In the name of Allah, the most merciful, and compassionate all praises be to Allah, the lord of the worlds, and prayers and Peace be upon Muhammad his servant and messenger.

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# Abstract

Microbially induced calcite precipitation (MICP), has an important place in construction industry. It is a modern technique which is very cost effective and environment friendly. MICP uses naturally occurring bacteria to strengthen soil and to enhance the properties of concrete. This modern persistent healing system is at risk to microbial depletion in the highly alkaline cementitious environment. Therefore, only alkali resistant calcifying microbes are used and these alkaliphilic microbes can be isolated from soil. In the present study, the genes which are responsible for calcite production were identified by performing 16S rRNA sequencing. Gel electrophoresis confirmed presences of calcifying genes (*IcfA*, *etfA* and *etfB)* in *B.subtilis* and *B.safensis*. Calcium carbonate was formed using five different calcium sources (calcium hydroxide, calcium gluconate, calcium formate, calcium nitrate and calcium chloride) at three different pH values (pH 7, pH 10 and pH 12). Precipitated calcite was further confirmed via SEM and EDX analysis. Bacterial inoculation and calcium sources were investigated in cementitious system through determination of compressive and flexural stresses. Furthermore, mortar specimens were prepared consists of bacterial solution and calcium source and pre-cracked. After 7 days of curing, self-healing of mortar cement cracks was achieved in mortar sample with a crack width of 0.7 mm consist of calcium hydroxide and *B.safensis*. After 16S rRna sequencing Bacillus sp. were most common in identified strains and also gave higher calcite precipitation than rest of the species.Among all calcium sources, calcium hydroxide possessed higher calcite secretion potential and comprises higher mechanical strength. Presence of *etfA, etfB* and *icfA* genes are confirmed in *B. safensis* same like in *B. subtilis.* The study reveals that Calcium hydroxide is a potent source for self-healing in concrete. *B. safensis* along with calcium hydroxide holds greater calcite secretion potential and comprises higher mechanical strength.

# Chapter 1

# Introduction

Cement is the most common material utilized in construction owing to its great strength, durability and low cost. There are so many advantages of cement but there are also some disadvantages. With passing time, cracks start to appear, due to which it loses its strength and also cause corrosion of steel. These cracks requires maintenance (Mehta & Monteiro, 2014). Many healing treatments have been applied but was not proven environmentally safe. Recently a technique known as Microbial Induced Calcite Precipitation (MICP) has been presented which is also known as self-healing concrete system. This technique have been applied in various fields mainly for the improvement of concrete strength and durability (Ng, Lee, & Hii, 2012). MICP contains bacteria which is present everywhere on earth, on surface of earth and inside the surface of earth. These bacteria can survive in extreme conditions.

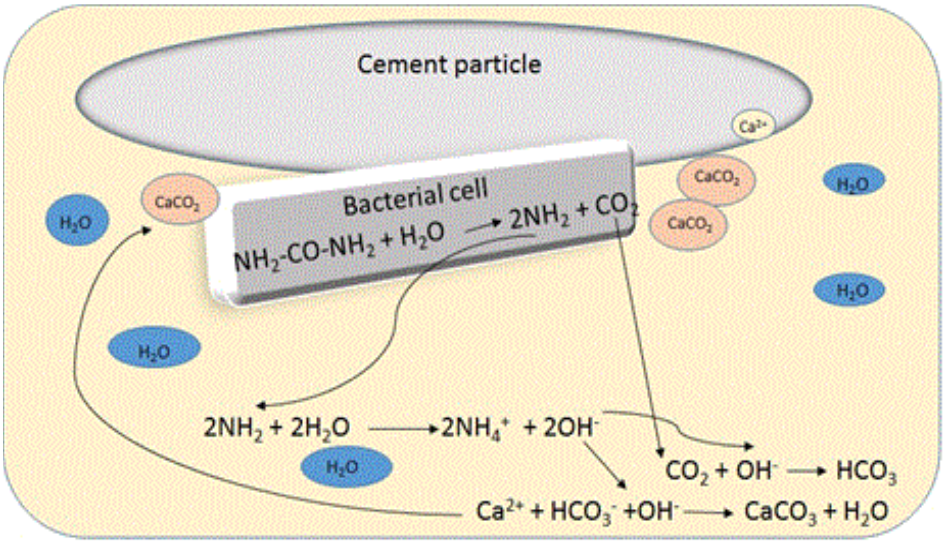



Figure 1.1 Self-healing procedure of cracks through bacteria

MICP's mechanism of action depends on the type of bacteria used in the procedure. In ureolytic and non-ureolytic bacteria, calcite precipitation is entirely dependent on urea hydrolysis and respiration. In MICP, the enzyme urease catalyzes the hydrolysis of urea to the substrate urea, precipitating carbonate ions in the presence of ammonium. These carbonate ions present as a source of calcium led to the formation of calcium carbonate. This raises the pH of the surroundings, leading to self-healing of cracks (Kendall, 1998).

There are many possible uses of MICP reported in different engineering applications like in bio mineralized concrete, self-healing of cracks, biological mortar, soil stabilization and dust control (Mathur, Bhatt, & Patel, 2018). Some studies also proved that MICP is effectively involved in soil improvement, improving its shear strength, MICP is also involved in many engineering applications like bio clogging and bio concrete. Bio-concrete has been very useful in construction industry. Bio-concrete has the ability to self-heal when cracks are formed. This technique has been very popular lately in field of construction (Soon, Lee, Khun, & Ling, 2013). Bio concrete is composed of suitable bacteria and calcium which acts as an energy source for bacteria. This technique has been very useful in construction as it is environmentally friendly and helpful in increasing compressive strength of concrete. MICP has headed to many applications like crack remediation of concrete, soil improvement and restoration of ancient buildings (Vekariya & Pitroda, 2013).

In addition, there are many factors which are influencing microbial induced calcite precipitation like type of bacteria used, concentration of bacteria, shape and size of bacteria, particle size of bacteria, chemical solutions applied to increase calcite production and also nutrients involved, which acts as energy source for bacteria (Mathur et al., 2018).

Bacterial strains precipitate calcite via active and passive pathways. Passive pathways consist of

urea hydrolysis, amino acid ammonification, denitrification and dissimilatory sulphate reduction. A drawback of the nitrogen and sulfur cycle is the production of ammonia and elemental sulfur products that are harmful to both climate and concrete (Seifan & Berenjian, 2019).

Microcracks are naturally present in concrete. This causes deterioration of the concrete and leads to the penetration of harmful substances into the concrete, which leads to corrosion of the structure (Basheer, Kropp, & Cleland, 2001).

Addition of urease-producing bacteria along with a calcium source which precipitates calcite in concrete. Biomineralization techniques show optimistic results in closing microcracks in concrete. Newly formed microcracks can be sealed by the continuous hydration process of concrete (Vijay, Murmu, & Deo, 2017).

Scientists have proved that people who are frequently exposed to cement dust have reduced ventilator functions. Composition of cement involves heavy metals which are harmful for environment, animals and humans. Cement dust can lead to chronic impairment of lung function in humans also it damages the natural water quality. Bio-cementation is a solution and have many advantages over conventional cementation process. Bio-cementation or MICP is cost effective and environmentally safe. MICP is used in dust control, biological mortar and also improves the load bearing ability of concrete (Mathur et al., 2018).

In this process bacteria in dormant stage are introduced along with nutrient compound and they remain inactive until cracks are formed. When water penetrates in concrete it causes the bacteria to activate and then bacteria start to feed on nutrient compound. Calcium carbonate is then produced which heals the cracks while increasing the durability of concrete (Vekariya & Pitroda, 2013).

Bio mineralization occurs through bacterial activity. It is characterized in three classes know as, Biologically Mediated Mineralization (BMM), Biologically Induced Mineralization (BIM), and Biologically Controlled Mineralization (BCM). Bacteria precipitates calcite with help of two different pathways known as active pathway and passive pathway. The passive pathway comprises of urea hydrolysis, amino acid ammonification, denitrification, and sulphate reduction. Active pathway consists of oxidation of organic matter and is more suitable than other methods which includes nitrogen and Sulphur cycle. It is proven to be eco-friendly (Vijay et al., 2017).

In this study 10 morphologically different isolates were identified by 16SrRNA sequencing and further tested for quantification of calcite precipitation. Sequenced microorganisms were identified as Bacillus, Arthrobacter, Planococcus, Chryseomicrobium and Corynebacterium species. Calcite precipitation quantification was performed in presence of five different calcium sources at three different pH values (pH 7, pH 10 and pH 12). A non-ureolytic pathway was chosen for calcite precipitation to eliminate the formation of toxic ammonia. For this purpose, calcium nitrate $Ca(NO_3)_2$, calcium formate $Ca(HCOO)_2$, calcium chloride $CaCl_2$, calcium hydroxide $Ca(OH)_2$ and calcium gluconate $C_{12}H_{22}CaO_{14}$ were used as CaCO3 precipitation precursors. Furthermore, to study precipitated CaCO3, scanning electron microscopy (SEM) was performed to analyse the presence of caco3 followed by Electron dispersive X ray (EDX) was done to check the elemental composition of calcite. Among these five calcium sources, three were selected (calcium hydroxide, calcium nitrate and calcium gluconate) along with the most calcite producing strain *(Bacillus safensis)* for more analysis on cement and effect on its mechanical properties. Mechanical evaluation was performed by evaluating the effect of bacterial inoculation and three different calcium sources in cementitious system and further investigated through determination of compressive and flexural stresses. Moreover, genes were identified that were involved in calcite

precipitation. Presences of calcifying genes were confirmed in *Bacillus subtilis, Bacillus safensis* and *Bacillus australimaris*. This study will aid in crack healing process and diminish the limitations of MICP.

# Chapter 2

# Literature Review:

### 2.1 Background:

Concrete is widely used material all over the world most popular in construction industry. Main ingredients used to make up concrete are sand, cement, water, and gravel. Concrete possesses good compressive and low tensile strength. Concrete is cheap and certainly available material. There are many applications of concrete in construction. Concrete is used in building roads, bridges, concrete dams, commercial buildings, fences, walls and foundations.

Every year, above 10 billion tons of concrete are utilized worldwide, and it is predicted that demands will grow to 16 billion tons by 2050. Existing technology used by construction industry causes a negative effect on environment and economy. Consumption of concrete is increased is due to exposure of substructures to physical, chemical, and biological factors for example temperature variations, exposure to radioactive substances, natural disasters, and microbial activity (Castro-Alonso et al., 2019).

These factors lead to the formation of micro-cracks, which effects compressive, flexural, and mechanical strength of concrete. Thus, the useful life of concrete is reduced, and repair cost of infrastructures is multiplied (Achal, Mukherjee, Kumari, & Zhang, 2015).

## 2.2 Causes of Crack Appearance:

A crack is a full or half separation of concrete into two or more parts formed by breaking. Appearance of cracks is a common problem in construction because it affects the building structure

and also reduces its durability. Cracks may also occur due to corrosion or reinforcement bars, temperature changes, poor construction and many more such as:

### I. Shrinkage

Many of the building materials enlarge in presence of moisture and shrink when dehydrated. Factors which cause shrinkage in cement mortar are due to the excessive use of water and excessive use of cement. Materials like brickwork or concrete shrinks in the start. This shrinkage is to some extent irreparable. Shrinkage cracks in buildings can be decreased by using not so rich cement mortar in the masonry and by quitting use of plaster on the brickwork surface (Thagunna, 2014).

### II. Thermal Movement

One of the most common cause of cracks in building are because of expansion and contraction of structures due to change in temperature. Materials expand when heated and contract when chilled. This thermal movement generates cracks in buildings. Daily changes in temperature are quicker and have greater damaging effect.

### III. Chemical Reaction

Chemical reactions happen because of the materials used for composition of concrete. Concrete may crack slowly because of gradually rising expansive reactions between aggregate comprising active silica and alkalis derived from cement hydration, admixtures, or external sources.

### IV. Poor Construction

Lack of good construction practices is the main cause of appearance of cracks. To ensure a well-constructed building, good quality products and proper supervision is necessary. Some more examples of poor construction practices are Inappropriate selection of materials, poor quality materials, incorrect proportioning of constituents of concrete or mortar and excess of water in concrete or mortar.

### V. Cracking caused by Vegetation

Presence of vegetation can be a cause of cracks in the walls of a building. This is commonly because of spread-out of roots growing under the foundation. Roots of trees in a wall can create cracks. The cracks appear in clay soil because of moisture contained by roots.

### VI. Natural Forces

Cracks can appear due to natural forces like earthquakes, flooding, rains, and winds. A quick shift in lower layer of earth due to earthquake can be a big cause of cracks (Velumani et al., 2020).

## 2.3 Pre-existing Treatments:

### I. Epoxy Injections

The 0.05-millimeter narrow cracks were filled with help of epoxy injections. Cracks which on concrete surface are sealed injecting under the concrete. Before injecting it is necessary to repair the root cause of cracks or else it will keep emerging. Skillful workforce is required to execute this procedure successfully.

### II. Routing and Sealing

It is a common and simple method used for sealing cracks. It is only used in case remedial crack repairing and not structural crack repairing. In this method the cracks are enlarged first and then sealed with the help of joint sealant.

### III. Stitching Cracks

It is a long-lasting method for repairing cracks. In this method drilling is done to produce holes first at entry and exit points, then U-shaped metallic staples are passed through these holes and then fixed toughly in the holes with a grout.

### IV. Drilling and Plugging

This method is used for repairing vertical cracks runs in straight lines. This method is cost effective and not time taking. Vertical holes are drilled in holes first then a key is made passing down a grout. This grout key aids in avoiding leakages.

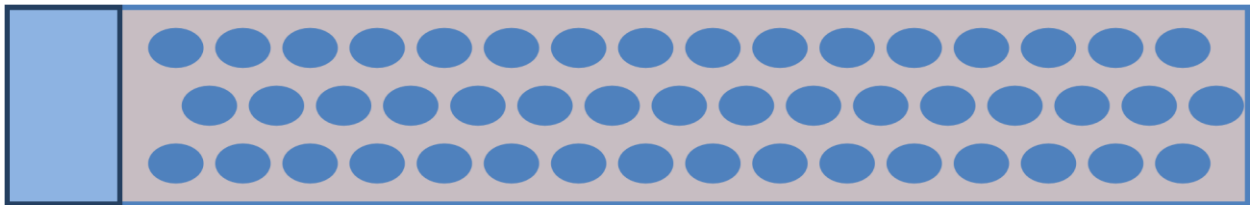
There are many other methods which are useful in healing cracks like Gravity filling, dry packing overlay and surface treatments etc. Treatment depends upon the nature of crack if it requires remedial or structural repair (Thagunna, 2014). Scientists has used these techniques to heal cracks but most of them were discovered to be unsuited with concrete, unsafe to environment and costly (Vekariya & Pitroda, 2013).

## 2.4 Bio Influenced Self-Healing Concrete:

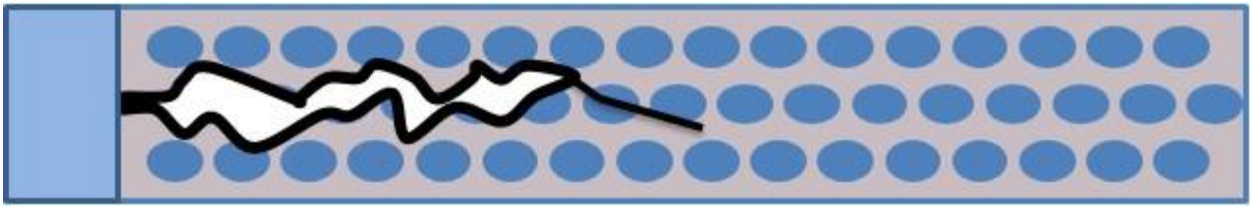
Lately, another method came into light known as Microbial Induced Calcite Precipitation (MICP) also known as Bio concrete. This method is environmental-friendly and cost effective.

It is emerging as a possible solution for preventing crack formation in concrete structures. MICP not only assists in self-healing but also improves the mechanical properties of concrete.

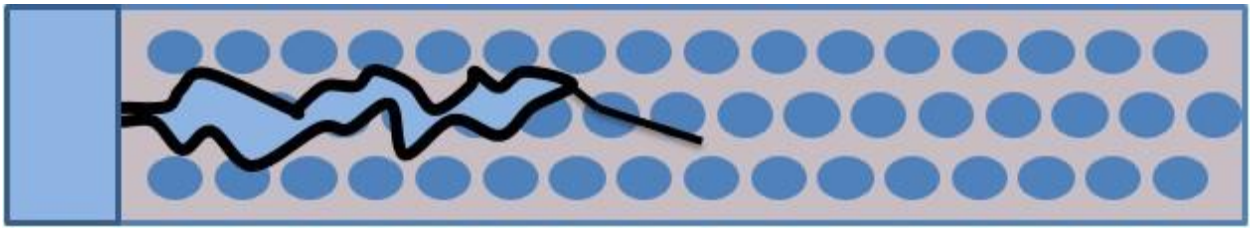
Bio concrete is the combination of two components which aids in self-healing concrete, consisting suitable bacteria and calcium based nutrient compound (Zhang, Bundur, Mondal, & Ferron, 2015). Normally, bacteria are integrated in a dormant phase with a calcium source in concrete. Calcium carbonate is formed as the product of metabolic action of bacteria on calcium and heals the crack, limiting the water access in concrete and improving durability of concrete.



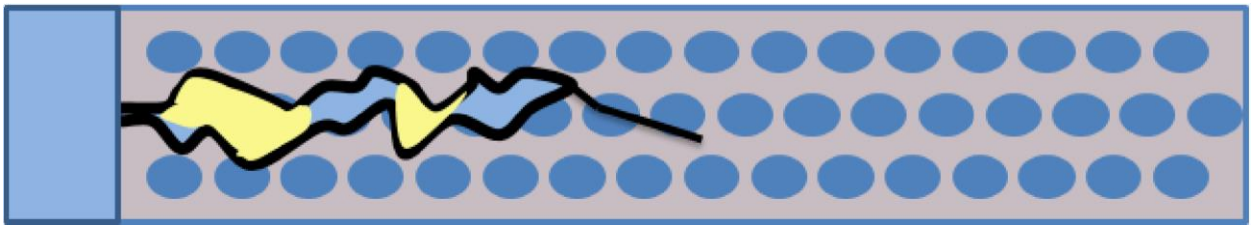
(a) Addition of bacteria in concrete



(b)- Crack formation because of tensile stresses



(c)- Water is purposely added in the cracks



(d)- $CaCO_3$ is formed which aids in healing cracks

Figure 2.1 Self-healing procedure of cracks through bacteria

## 2.5 Factors Influencing MICP:

Factors influencing MICP are:

### I. Type of bacteria

Bacteria plays the most important part and have maximum significant factors in precipitation process. The type of bacteria being used could affect the efficiency of precipitation process. Bacteria type which is appropriate for MICP process have the ability to catalyst urea hydrolysis, and they normally urease positive bacteria (Nemati & Voordouw, 2003).

### II. Bacterial concentration

Concentration of bacteria matters as it helps in increasing the calcite precipitation from MICP process. Higher the bacterial cell concentration, higher will be the calcite precipitation (Okwadha & Li, 2010).

### III. Particle size of Soil

Particle size of soil influences MICP process because size of soil granules should be enough to transport bacteria with size 0.5-3.0 μm in length. For effective MICP treatment two most important factors are bacterial cell size and soil particle size because grain size produces soil pores which helps in bacterial movement (Murphy & Ginn, 2000).

### IV. Bacteria size and shape

Classification of bacteria is greatly influenced by the size of bacteria. Bacterial size usually ranges from 0.5 to 3.0 μm. Microbial cell transportation in soil depends upon size of bacterial cell, cell structural properties and physiological state of cell (Mitchell & Santamarina, 2005).

### V. Chemical solution

Chemical solutions are adopted for enhancing calcite precipitation. Chemical solutions are injected in cracks which then produces calcite. Many chemical solutions are reported during MICP research such as sodium carbonate, sodium acetate, calcium sulphate, ammonia and alcohol (Osinubi, Sani, Eberemu, Ijimdiya, & Yakubu, 2018).

### VI. Nutrients

In this process bacteria are only organism which needs energy source to start the metabolic processes which then helps in calcite precipitation. In culture stage and soil treatment stage, nutrients are delivered to bacteria which acts as an energy source for bacteria. Nutrients which are

commonly used for bacteria are, Fe, N, K, Ca, P, etc. But all of these nutrients are costly so to reduce the cost, other nutrients are proposed that are also environmental-friendly (Mathur et al., 2018).

**VII. Temperature**

Change in temperature affects the calcite precipitation. Ultimate temperatures are beneficial for calcite precipitation. Calcium carbonate of calcite can be stable at room temperature (Whiffin, Van Paassen, & Harkes, 2007).

## 2.6 Mechanism of Action:

In MICP, by urea hydrolysis, the enzyme urease catalyses urea and precipitates carbonate ions in existence of ammonium. These carbonate ions with help of calcium source directed to the formation of calcium carbonate. Due to which the pH is increased causing self-healing of cracks (Kendall, 1998).

But the calcite precipitation by this process has consequences. It causes environmental pollution due to presence of ammonia which restricts the efficiency of ureolysis. Therefore, non-ureolytic bacteria are used to get environment-friendly outcomes. Non-ureolytic bacteria use organic compounds such as lactate or acetate in place of urea. Carbon dioxide which is then released from calcium source to react with calcium hydroxide in cement causing calcite precipitation (Mathur et al., 2018).

## 2.7 Bacterial metabolic procedures involved in MICP:

This process includes many different pathways named as Nitrogen cycle, Sulphur cycle, Photosynthesis, Methane oxidation and Fatty Acid Catabolism.

## ❖ The Nitrogen Cycle

- **Urea Hydrolysis**

The enzymes that force the complicated reactions in hydrolysis of urea are referred to as Urease (UE) and Carbonic Anhydrase (CA). First of all, Urease (UE) hydrolyses one mole of urea to one mole of carbonate and ammonia, followed with the aid of spontaneous hydrolysis of carbonate to at least one mole of carbonic acid and ammonia. The enzyme Carbonic Anhydrase (CA) in addition changes the carbonic acid to bicarbonate at the side of the formation of two moles of ammonia and hydroxide because of hydrolysis of ammonia (Wang et al., 2014).

- **Amino acid ammonification**

Amino acid ammonification is another bacterial mechanism concerned in MICP. In this method, amino acids metabolism is enabled by using microbial interest which produce ammonia and CO2. Ammonia hydrolysis effects inside the manufacturing of OH- and NH4+ ions which collect within the cell. This reasons the super saturation of ions which effects in the calcite participation (Zhu & Dittrich, 2016).

Table 2 1 Amino acid ammonification steps

| Reaction Steps | Reaction Equation |
|---|---|
| Step 1 | $Amino\ acids + O_2 \rightarrow NH_3 + CO_2 + H_2O$ |
| Step 2 | $NH_3 + H_2O \rightarrow NH_4 + OH^-$ |
| Step 3 | $CO_2 + OH \rightarrow HCO_3^-$ |
| Step 4 | $Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$ |

(Rodriguez-Navarro, Rodriguez-Gallego, Ben Chekroun, & Gonzalez-Munoz, 2003).

- **De-nitrification**

This technique includes using NO3- as final acceptor of electron which oxides the organic content and therefore supports calcium carbonate precipitation. In existence of soluble Ca2+ ions, bacteria produce an alkaline environment with the aid of the concentration of H+ ions and by formation of $OH^{-}$, $CO_2$ and $NO_2$ (Ruan et al., 2019).

Table 2. 2: De-nitrification reaction

| **Reaction Steps** | **Reaction Equation** |
|---|---|
| **Step 1** | $(CH_3COOH)_2\ Ca + NO_3^- \rightarrow CaCO_3 + N_2 + 3CO_2 + 3H_2O + OH^-$ |

## ❖ The Sulphur Cycle

- **Dissimilatory Sulphate reduction**

In dissimilatory sulphate reduction procedure, sulphate reducing bacteria (SRB) encourages the production of calcium carbonate in anaerobic environments**.** Elimination of sulphates from gypsum in *Desulphovibrio sp*. is accountable for calcite precipitation via the three distinctive mechanisms named as: diffusion, dissolution and calcium carbonate precipitation. Because of the elimination of sulphide in alkaline surroundings, a sulphate reduction reaction is caused between $Ca^{2+}$ ions, which leads to precipitation of Calcium carbonate (Seifan, Ebrahiminezhad, Ghasemi, Samani, & Berenjian, 2018).

### ❖ Photosynthesis

In aquatic surroundings, the main microorganisms involved in MICP are the photosynthetic Cyanobacteria and Micro-algae. In these microorganisms, precipitation occurs because of the exchange of HCO-3HCO3- and CO2-3CO32-. First, through the cell membrane, HCO-3HCO3-

diffuses and once inside the cytosol, it separates into $OH^-$ and $CO_2$. It causes the increase in pH because OH- is produced (Al Qabany, Soga, & Santamarina, 2012).

Table 2. 3 Photosynthesis reaction steps

| Reaction Steps | Reaction Equation |
|---|---|
| **Step 1** | $Ca^{2+} + 2HCO^{3-} \rightarrow CaCO_3 + CO_2 + H_2O$ |
| **Step 2** | $Ca^{2+} + HCO_2^- + OH^- \rightarrow CaCO_3 + CO + H_2O$ |
| **Step 3** | $2HCO_2 \leftrightarrow CO_2 + CO_2^{3-} + H_2O$ |

- **Methane Oxidation**

To maintain the carbon dioxide concentration in aerobic conditions in fresh water and marine, methane oxidizing bacteria are useful. In case of aerobic conditions, enzyme named as methano mono-oxygenase changes methane to methanol (Achal, Pan, & Zhang, 2011).

Table 2 4 Methane oxidation reaction steps

| Reaction Steps | Reaction Equation |
|---|---|
| **Step 1** | $CH_4 + O_2 \leftrightarrow CH_3OH + H_2O$ |
| **Step 2** | $CH_3OH \rightarrow CHOH$ |
| **Step 3** | $CHOH + H_2O \rightarrow HCOO^- + H^+$ |
| **Step 4** | $HCOO^- + H_2O \leftrightarrow HCOOH + OH^-$ |
| **Step 5** | $HCOOH \rightarrow CO_2$ |
| **Step 6** | $Ca^{2+} + CO_2 + 2OH^- \leftrightarrow CaCO_3 + H_2O$ |

- **Catabolism of Fatty Acids**

The process to facilitate the bacterial induced calcite mineralization (BICM) in B. subtilis, has been labeled into three ways liable to presence or absence of urea. One of this method occurs

through urea hydrolysis whereas the other takes place in urea-deprived medium (Zhang et al., 2015).

## 2.8 Bacterial gene cluster involved in calcium carbonate Bio-mineralization:

In a study, formation of calcium carbonated by *B. subtilis,* and its genes were identified that were involved in bio mineralization. *lcfA* operon consist of five genes named as *lcfA, ysiA, ysiB, etfB,* and *etfA* was seemed to be involved in calcite precipitation (Barabesi et al., 2007).

Further investigation stated that all strains carrying altered *lcfA* operon were incapable of forming $CaCO_3$ crystals, with elimination of non-mutated ones (Tojo, Satomura, Matsuoka, Hirooka, & Fujita, 2011).

It has been informed that the last gene named as *etfA* gene plays the most important role in *lcfA* operon. All the genes of *lcfA* operon are putative in nature. Studies shows a relation between calcium carbonate precipitation and fatty acid metabolism (Wang et al., 2014).

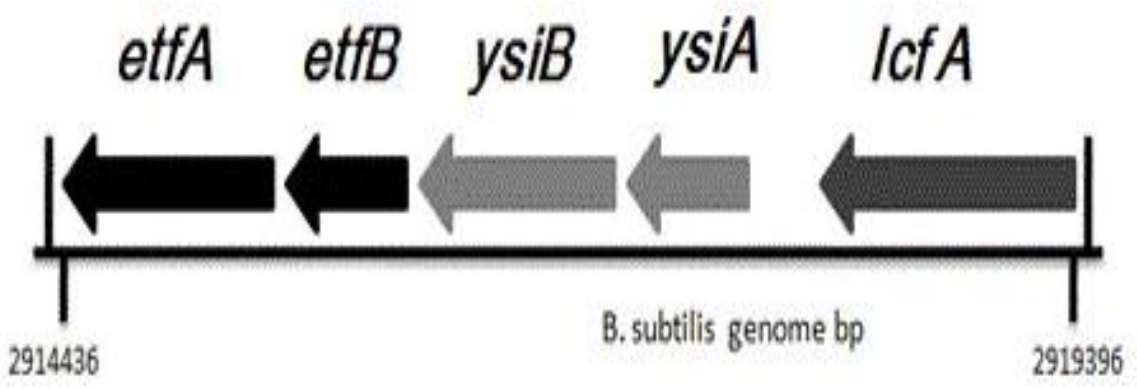



Figure 2.2 lcfA operon gene cluster
(Perito et al., 2014)

The *B. subtilis* primers applied for running PCR in this research are enlisted below:

Table 2. 5 *lcfA* operon genes primers for B. subtilis

| Gene | Primer name (forward or reverse)[a] | Genome location (nt)[b] | Sequence (5′–3′)[c] |
|---|---|---|---|
| *lcfA* | A2 (f) | 2918663–2918681 | CCGCTTGGATGTATGACG |
| | A3 (r) | 2918112–2918130 | CTCTCAGCACTGCGGCGG |
| | A4 (f) | 2918561–2918577 | GCGGGATCCGGCTTCGAAATGATTC (A) |
| | A5 (r) | 2918384–2981401 | AAAACTGCAGCCTCCACGGGGAGCGCG (B) |
| *ysiA* | B1 (r) | 2917528–2917545 | GTGACTGGTGGTAGCCG |
| | B2 (f) | 2917555–2917573 | GATGCAGCAGTAGAAGTC |
| | B3 (r) | 2917074–2917092 | AGCGCAACGAGATCGTAC |
| | B4 (f) | 2917259–2917277 | GCGGGATCCTGGCGCAGCTCCAAGTTG (A) |
| | B5 (r) | 2917394–2917412 | AAAACTGCAGGAGCGGATGGAAGAGGAC (B) |
| | B6 (f) | 2917155–2917174 | GGCCTCGATGTCCGCCTCG |
| | B7 (f) | 2917617–2917631 | TGCTCTAGACTATAGGAAGGAACG (C) |
| | B8 (r) | 2917409–2917424 | CCCATCGATCTCAATAAATTGACC (D) |
| *ysiB* | C1 (r) | 2916867–2916887 | GCTTCGTACGCCTGCATCTG |
| | C2 (f) | 2916945–2916963 | GATTCACAATCCGCCGGC |
| | C3 (r) | 2916459–2916477 | TGCCTCATCTTTCGCTCC |
| | C4 (f) | 2916776–2916792 | GCGGGATCCGAGGATTCCTCATTGC (A) |
| | C5 (r) | 2916547–2916561 | AAAACTGCAGGCTTTGGCTGTACC (B) |
| | C6 (f) | 2916376–2916396 | GGCATCTTTGCTTGAACTTC |
| | C7 (f) | 2917008–2917028 | TGCTCTAGAGTAAAGGAGTTCTGTCC (C) |
| | C8 (r) | 2916815–2916832 | CCCATCGATCTTTAATATCAGCGCCG (D) |
| *etfB* | D1 (r) | 2916022–2916039 | TCTCCGCTTCTTCACCG |
| | D2 (f) | 2916133–2916151 | GATTCAGGATGACGGAGC |
| | D3 (r) | 2915576–2915594 | TCAGCATCCTCTTCGTCC |
| | D4 (f) | 2915846–2915865 | GCGGGATCCGCCATTGACGGAGGATCAGG (A) |
| | D5 (r) | 2915644–2915664 | AAAACTGCAGCATAATTCCCGGAAGCGATG (B) |
| | D6 (f) | 2915592–2915610 | CTTGATGATCTTGATCTG |
| | D7 (f) | 2916217–2916236 | TGCTCTAGAAGGGGATATGATCATG (C) |
| | D8 (r) | 2916014–2916029 | CCCATCGATCGCAATTCTTTCTCCG (D) |
| *etfA* | E1 (r) | 2915286–2915306 | CCGATGAGAACACCGATGAC |
| | E2 (f) | 2915155–2915173 | TGAGAGGTGTTATCGAGC |
| | E3 (r) | 2914633–2914651 | GAGTAACGACTTTCCCGG |
| | E4 (f) | 2915029–2915047 | GCGGGATCCGTAAGCGTTACCGGAGGG (A) |
| | E5 (r) | 2914849–2914867 | AAAACTGCAGGATAACAGTGCGGAGGTC (B) |
| | E7 (f) | 2915445–2915431 | TGCTCTAGACTTGGACATTCAAAG (C) |
| | E8 (r) | 2915206–2915222 | CCCATCGATCAGCTTCGGGTCCTCAG (D) |

[a] f, forward primer; r, reverse primer.
[b] According to the *B. subtilis* genome numeration used at http://www.pasteur.fr/Bio/SubtiList. nt, nucleotides.
[c] Underlined sequences correspond to the following restriction sites: A, BamHI; B, PstI; C, XbaI; D, ClaI.

(Barabesi et al., 2007).

# Chapter 3 (a)

# Materials

It includes all the software, tools, equipment, enzymes, chemicals, bacterial strains, buffers and media used during this research.

## 3.1. Software and online tools used:

Table 3. 1 List of all online tools used during this research

| **Name** | **Function** | **Link** |
|---|---|---|
| NCBI | Retrieval of sequence,<br><br>Blast | https://www.ncbi.nlm.nih.gov/ |
| Primer3 Input<br><br>(version 0.4.0) | Primer designing for PCR | http://bioinfo.ut.ee/primer3-0.4.0/ |
| *In silico* PCR | To check the amplification of the<br><br>primers | http://insilico.ehu.es/PCR/ |

## 3.1.1 Equipment used:

Table 3. 2 List of all the equipment used during this research followed by their manufacturers:

| **Equipment** | **Manufacturer** |
|---|---|
| 2720 Thermal Cycler | Applied Bio systems |
| Shaking Incubator | JSR |
| Gel Electrophoresis Tank | Cleaver Scientific Ltd |
| Ultraviolet Viewing cabinet | Extra Gene |

| | |
|---|---|
| Hot Plate | Velp-Scientifica |
| Centrifuge Machine | Hermle |
| pH Meter | WTW inoLab |
| Microwave Oven | Haier |
| Laminar Flow cabinet | Esco |
| Microcentrifuge | Sigma |
| Vortex Mixer | Heidolph |
| Spectrophotometer | Optima |
| Incubator | Memmert |
| Gel Dolphin Doc | Weal Tech ELITE 300 Plus |
| Genomic DNA Extraction/Purification Kit | Thermo Fisher Scientific |
| Refrigerator | Caravell |
| Automatic Mortar mixer | Matest |
| Dual chamber | ELE International |

### 3.1.2. Chemicals used:

Table 3. 3 Chemicals along with their manufacturers are enlisted that are used during this research:

| **Chemicals** | **Manufacturer** |
|---|---|
| Agar | Bioworld USA |
| Agarose | Bioworld USA |
| Nutrient Broth | Lab M UK |
| Tryptone | Bioworld USA |

| | |
|---|---|
| Yeast extract | Lab M UK |
| Sodium chloride | BDH Laboratories |
| Nuclease Free Water | Caisson labs |
| Crystal Violet | Daejung |

### 3.1.3. Enzymes used:

Table 3. 4 List of enzymes used during this research:

| **Enzymes** | **Manufacturer** |
|---|---|
| RNase A | Thermo scientific |
| Proteinase K | Thermo scientific |
| lysozyme | Sigma |

### 3.1.4. Molecular biology grade Markers:

Table 3. 5 List of molecular biology markers used during this research:

| **Marker** | **Manufacturer** |
|---|---|
| GeneRuler 1kb DNA Ladder | Thermo scientific |

### 3.1.5. Bacterial strains:

Table 3. 6 List of all bacterial strains used during this research.

| **Strain** | **Bacteria** | **Source/Reference** |
|---|---|---|
| *BS* | *Bacillus subtilis* | AntiBacter research lab, ASAB, NUST. |

| | | |
|---|---|---|
| 1G | *Bacillus safensis* | AntiBacter research lab, ASAB, NUST. |
| 4A | *Bacillus pumilus* | AntiBacter research lab, ASAB, NUST. |
| 4B | *Bacillus australimaris* | AntiBacter research lab, ASAB, NUST. |
| 2C | *Glutamicibacter mysorens* | AntiBacter research lab, ASAB, NUST. |
| 6C | *Arthrobacter koreensis* | AntiBacter research lab, ASAB, NUST. |
| 2D | *Planococcus plakortidis* | AntiBacter research lab, ASAB, NUST. |
| 1E | *Corynebacterium efficiens* | AntiBacter research lab, ASAB, NUST. |
| 6E | *Arthrobacter luteolus* | AntiBacter research lab, ASAB, NUST. |
| 3A | *Chryseomicrobium amylolyticum* | AntiBacter research lab, ASAB, NUST. |
| 4F | *Chryseomicrobium imtechense* | AntiBacter research lab, ASAB, NUST. |

### 3.1.6. Gram staining solution:

Table 3. 7 Components used for gram-staining process.

| **Sr. No** | **Components** |
|---|---|
| 1 | Crystal violet |

| | |
|---|---|
| 2 | Iodine |
| 3 | Safranin |

### 3.1.7. Media used for bacterial culturing:

Media used for culturing bacteria are enlisted below. All the media were prepared using distilled water.

Table 3. 8 Components used for preparing Luria Broth (L Broth)

| Sr. No | Components | Quantity (g/L) |
|---|---|---|
| 1 | Tryptone | 10 |
| 2 | Yeast extract | 5 |
| 3 | Sodium chloride | 10 |

Table 3. 9 Components used for preparing Luria Agar (L Agar)

| Sr. No | Components | Quantity (g/L) |
|---|---|---|
| 1 | Yeast extract | 5 |
| 2 | Tryptone | 10 |
| 3 | Sodium chloride | 10 |
| 4 | Agar | 14 |

### 3.1.8. Media used for calcite precipitation test:

Table 3. 10 Components used for composition of CPM media

| Sr. No | Components | Quantity |
|---|---|---|

| | | |
|---|---|---|
| 1 | Calcium source | 120 g |
| 2 | Yeast extract | 9.6 g |
| 3 | Distilled water | 2400 ml |

## 3.1.9. Solutions used in DNA Extraction

**Solution I (Lysis buffer)**

Table 3. 11 Components used to prepare solution I (lysis buffer)

| Sr. No | Components | Quantity |
|---|---|---|
| 1 | Tris-HCl | 0.02 M (pH 8) |
| 2 | 0.002M EDTA | 0.002 M (pH 8) |
| 3 | 1.2% Triton X100 | 1.2% |

## 3.1.10. TE Buffer used in calcite precipitation test:

Table 3. 12 Components used for preparing TE Buffer

| Sr. No | Components | Quantity |
|---|---|---|
| 1 | 0.5 M EDTA (pH 8) | 2 ml |
| 2 | 1 M Tris-Cl (pH 8) | 10 ml |
| 3 | Lysozyme | 1mg/mL |
| 4 | Distilled water | 988 ml |

### 3.1.11. Mortar specimen composition for compressive strength test:

Table 3. 13 Materials required for mortar specimen preparation for compressive strength evaluation

| Sr. No | Components | Quantity |
|---|---|---|
| 1 | Sand | 1.54 kg |
| 2 | Cement | 0.77 kg |
| 3 | Water | 320 ml |
| 4 | Super plasticizer | 8 ml |
| 5 | Bacterial solution | 15 ml |
| 6 | Calcium source | 15.4 g |

### 3.1.12. Mortar specimen composition for flexural strength test:

Table 3. 14 Materials required for mortar specimen preparation for flexural strength evaluation

| Sr. No | Components | Quantity |
|---|---|---|
| 1 | Sand | 2.95 kg |
| 2 | Cement | 1.47 kg |
| 3 | Water | 637 ml |
| 4 | Super plasticizer | 14.75 ml |
| 5 | Bacterial solution | 26 ml |
| 6 | Calcium source | 29.4 g |

# Chapter 3 (b)

# Methods

## 3.2.1 Isolation of alkaliphilic bacterial strains:

For isolation of alkaliphilic bacteria, samples were collected from six different locations in Pakistan from highly alkaline cementitious environment. From which 51 isolates were selected after serial dilutions. Then 24 of them were selected for further screening. Quantitative method was used then to recover 10 calcite producing bacterial isolates. Then 16s rRNA sequencing was performed. Strains were then stored on -80°C freezer.

## 3.2.2 Bacterial culturing:

Streak plate method was performed to attain pure bacterial colonies selected 11 different bacterial strains. Bacteria was streaked on autoclaved LB Agar plates. The plates were then placed in a 37°C incubator for 24 hours. After 24 hours of incubation, plates were examined. Using a sterile inoculating loop, single pure colony was picked and then streaked on a LB agar plate and further incubated for 16 hours at 37°C. Bacterial strains used for culturing are enlisted below:

Table 3. 15 different calcifying bacterial strains were used for bacterial culturing:

| **Strain** | **Bacteria** |
|---|---|
| BS | *Bacillus subtilis* |
| 1G | *Bacillus safensis* |
| 4A | *Bacillus pumilus* |

| | |
|---|---|
| 4B | *Bacillus australimaris* |
| 2C | *Glutamicibacter mysorens* |
| 6C | *Arthrobacter koreensis* |
| 2D | *Planococcus plakortidis* |
| 1E | *Corynebacterium efficiens* |
| 6E | *Arthrobacter luteolus* |
| 3A | *Chryseomicrobium amylolyticum* |
| 4F | *Chryseomicrobium imtechense* |

### 3.2.3 Gram staining:

Gram staining was performed to identify Gram-positive bacteria. Using a sterile inoculating loop, single colony was picked from LB agar plate. Bacterial smear was made using a single drop of autoclaved distilled water on a microscopic slide. Then it was heat-fixed by passing it over the flame 2-3 times. Few drops of crystal violet were poured on smear for 45 seconds then it was washed with water. After that, few drops of gram iodine were poured on smear for 1 minute and washed off with water. Decolorize until water flows colourlessly from slide. In the end safranin was added for 30 seconds. After washing with water, cover the slide with coverslip by putting a drop of immersion oil and observe it under (100x) light microscope.

### 3.2.4: Bacterial stock preparation:

To preserve all the bacterial strains for a longer period, stocks were prepared. 1ml was taken from an overnight grown bacterial culture in an Eppendorf tube and was spun at 5000 rpm for 5 minutes. After that supernatant was pipetted out. Suspend the resulting media in 1 ml LB media. After that transfer it to 2ml cryovial containing 0.5ml of 80% glycerol. Place the cryovials in liquid nitrogen for 2-3 minutes and then store them at -80 °C freezer.

### 3.3.1: Quantification of Calcite precipitation under different calcium sources

Five different types of calcium sources named as Calcium Chloride, Calcium Hydroxide, Calcium Nitrate, Calcium Gluconate and Calcium Formate were used for the calcification evaluation of eleven bacterial strains at three different pH value (pH 7, pH10 and pH 12). This test was performed separately for each calcium source in triplicates, three sets of 100ml flasks containing 22 flask each were autoclaved and labelled. After that CPM media was prepared for each set. For preparation of CPM media 120 g of calcium source along with 9.6 g yeast extract was added in reagent bottle and topped up with 2400 ml of distilled water. Three different sets were set at three different pH value (pH 7, pH 10 and pH 12) and autoclaved. 48 ml of CPM media was added in each autoclaved flask with 2 ml of bacterial culture grown overnight with OD (optical density) of 0.5. After that flask were placed in shaking incubator at 37°C with speed of 200 rpm for 7 and 15 days. Repeat this test for rest of calcium sources. After the completion of incubation time, flasks were taken out and the media was transferred to labelled sterile 50 ml falcons and centrifuged at 4000 rpm for 15 minutes. Pellet was formed which was then re-suspended in 25 ml TE buffer (pH 8). Falcons were placed at room temperature for 1 hour and then again centrifuged at 4000 rpm for 15 minutes to form pellet. Supernatant was discarded and pellet was dried at 65°C overnight.

### 3.3.2: Chemical verification via acetic acid method

To confirm the presence of calcite, chemical verification was performed of the dried calcite powder. For this test few drops of acetic acid was added on dried calcite powder. If it causes effervescence, CaCO3 is present.

### 3.3.3: Characterization via SEM and EDX

Scanning electron microscopy and Electron dispersive X ray was performed of dried calcite powder. SEM was performed to analyze the presence of CaCO3 and to examine the crystal morphology. Followed by Electron dispersive X ray was performed to check the elemental composition of calcite.

### 3.4.1: Mechanical evaluation of cementitious system:

The effect of bacterial inoculation of strain 1G (*Bacillus safensis*) and three different calcium sources named as Calcium Hydroxide, Calcium Gluconate and Calcium Nitrate in cementitious system was investigated through determination of compressive and flexural stresses.

### 3.4.2: Sieve analysis of fine aggregates

For mechanical evaluation, sieve analysis is performed first. This test method is done for determination of the particle size distribution of fine aggregates. It is performed according to ASTM standard C136/C136M. To perform this test, sand sample is weighed and then passed through series of sieves and shacked. After that sample from each sieve is weighed and overall recovered sample is calculated.

### 3.4.3: Mortar specimen preparation:

Mortar specimens were casted according to ASTM standard C348, with dimensions of (40×40×160 mm) for prism and (50 $mm^3$) for cube. Four different types of mix were prepared for the study. In mix proportion, ordinary Portland cement (OPC) type – I conforming to ASTM C 150 was used. Mix proportion for specimens (1:2) was applied with 0.34 of water to cement ratio and with 1% of super plasticizer by weight of cement and replacing 4% of water with bacterial solution. Three specimens were casted for one strain named as 1G (*Bacillus safensis*) and with three different calcium sources and tested at $7^{th}$ and $15^{th}$ day for compressive and flexural strength.

In this process, at first fine aggregates were prepared for experiment through sieve analysis test. For mortar specimen preparation, dry powders like cement and calcium source were mixed with hand for 2 minutes. Then water, bacterial solution and sand were added, and mechanical mixing was done at high speed (speed 4) for 5 minutes. Samples were then casted. After 24 hours samples were demolded and were placed in water for curing. Cured samples after 7 and 15 days of curing were tested for compressive strength, flexural strength and self-healing test.

### 3.4.4: Compressive strength test:

Three specimens were casted for each formulation for $7^{th}$ and $15^{th}$ day for compressive strength. This test was performed in a machine known as dual chamber. It was performed on cube specimen with a diameter of 50 $mm^3$. To perform this test, a sample was placed in a compression tester with a compression fixture. Weight was then applied until the sample crushed.

### 3.4.5: Flexural strength test:

Three specimens were casted for each formulation for $7^{th}$ and $15^{th}$ day for flexural strength tests. This test was performed in a machine known as dual chamber which is also suitable for

compression strength test. It was performed on prism specimen with diameter of (40×40×160 mm). To perform this test, sample is laid horizontally and then force is applied to the top of the sample until the sample fails.

### 3.4.6: Self-healing test:

Cube specimens were used for self-healing test. After demoulding and curing of mortar specimens. Samples were pre cracked and were observed at day 1 and day 7 to observe any changes in healing process. 2 samples were pre cracked. One specimen was designated as CF (control formulation) without bacteria and calcium source present. Other specimen was designated as OH with addition of bacteria (*B. safensis*) and calcium source.

## 3.5: Identification of genes in bacterial strains that are involved in calcite precipitation

For idenetification of genes *icfA* operon was used which is composed of five genes named as *etfA, etfB, ysiA, ysiB, IcfA.* From this operon we took 3 genes for b subtilis and b.safensis.

### 3.5.1 Primer designing

### Methodology for In-silico Analysis:

Sequences of genes were retrieved from NCBI in FASTA format. Gene sequences of *lcfA, etfA, etfB* of *Bacillus subtilis* were took to perform blast against the whole genome sequences of *Bacillus safensis* and *Bacillus australimaris*. For primer designing, sequences were downloaded with similarities. In case of *Bacillus subtilis,* primers were taken from a research paper. Furthermore *In-silico* PCR was performed to check amplificcations of primers.

### Methodology for Wet lab Analysis:

### 3.5.2 DNA Extraction:

Thermofisher kit was used to separate DNA of three strains named as BS (*Bacillus subtilis*), 1G (*Bacillus safensis*), 4B (*Bacillus australimaris*). At first working solution of Lysis Buffer was prepared containing detergent Triton X100, Tris HCl, and EDTA.

For DNA extraction, bacterial cells were harvested in a 2ml microcentrifuge tube. Centrifugation was done for 10 minutes at 5000 xg. Supernatant was discarded and pellet was re suspended in 180 µl gram positive lysis buffer. Solution was incubated then for 30 minutes at 37°C. 200 µl lysis solution and 20 µl proteinase was added and mixed thoroughly through vortexing. Solution was again incubated at 56°C for 30 minutes while vortexing occasionally. After that 20 µl of RNase A enzyme was added and mixed through vortexing. Incubation was done for 10 more minutes at room temperature. 400 µl of 50 % ethanol was added and mixed by vortexing. Then prepared lysate was transfered to DNA purification column inserted in a collection tube and column was centrifuged for 1 minute at 6000 xg. Collection tube containing flow through solution was discarded and DNA purification column was placed into a new 2ml collection tube. 500 µl of wash buffer 1 (ethanol added) was added and centrifuged for 1 minute at 8000 xg. Flow through solution was discarded and purification column was placed back into collection tube. 500 µl of wash buffer 2 (ethanol added) was added in a GeneJet Genomic DNA purification column and centrifuged for 3 minutes at maximum speed (> 12000 xg). Collection tube containing flow through solution was then discarded and DNA purification column was transferred to sterile 1.5 ml micro centrifuge tube. 200 µl elution buffer was added to centre of purification column membrane to elute genomic DNA and then incubated for 2 minutes at room temperature and centrifuged for 1 minute at 8000 xg. (for maximum yield; elution step can be repeated with 200 µl of elution buffer). In the end Purification column was discarded and DNA was stored at -20 °C freezer.

### 3.5.3 PCR

After DNA extraction, PCR was performed for amplification of DNA. Gradient PCR was performed, and temperature range from 55-65°C was set for annealing temperature. Extension temperature was adjusted to 72°C. 30 cycles were completed in 1 hour and 30 minutes. After completion of PCR, Gel electrophoresis was done to examine the results.

### 3.5.4 Gel electrophoresis

To prepare 1% agarose gel, 0.5g of multipurpose agarose and 50 ml TAE buffer was poured in a flask and heated until clear solution. After cooling down, 3 µl of ethidium bromide was added and poured slowly in a gel casting tray with adjusted combs. After the Gel hardens, combs were removed, 4 µl of sample along with 3 µl of loading dye were loaded on gel with 4 µl of 1 kb ladder. Gel was run for 45 minutes at 80 volts. In the end gel was visualized on a UV transilluminator.

# Chapter 4

# Results

## 4.1 Phylogenetic analysis (16S rRNA sequencing):

In order to identify the bacterial isolates at strain level, 16srRNa gene sequencing was performed. Analysis of these 16S rRNA gene sequences of 10 strains were conducted through MEGA X. Here the phylogenetic tree shows the diversity in genes of calcifying strains isolated from local soil. Individual phylogenies are represented in the form of rootless trees with two superhulls, as shown in Fig. 4.1. Among strains, Bacillus species were the most common, with other common species being Arthrobacter and Chryseomicrobium. Other species belong to Planococcus, Glutamisibacter and Corynebacterium. There are two super clads covering three sub clads each. First super clad consist of *Arthobacter, Glutamicibacter,* and *Corynebacterium*. Other clad includes *Bacillus, Planococcus* and *Chryseomicrobium*. This study demonstrates the diversity among strains involved in the production of calcite isolated from soil.

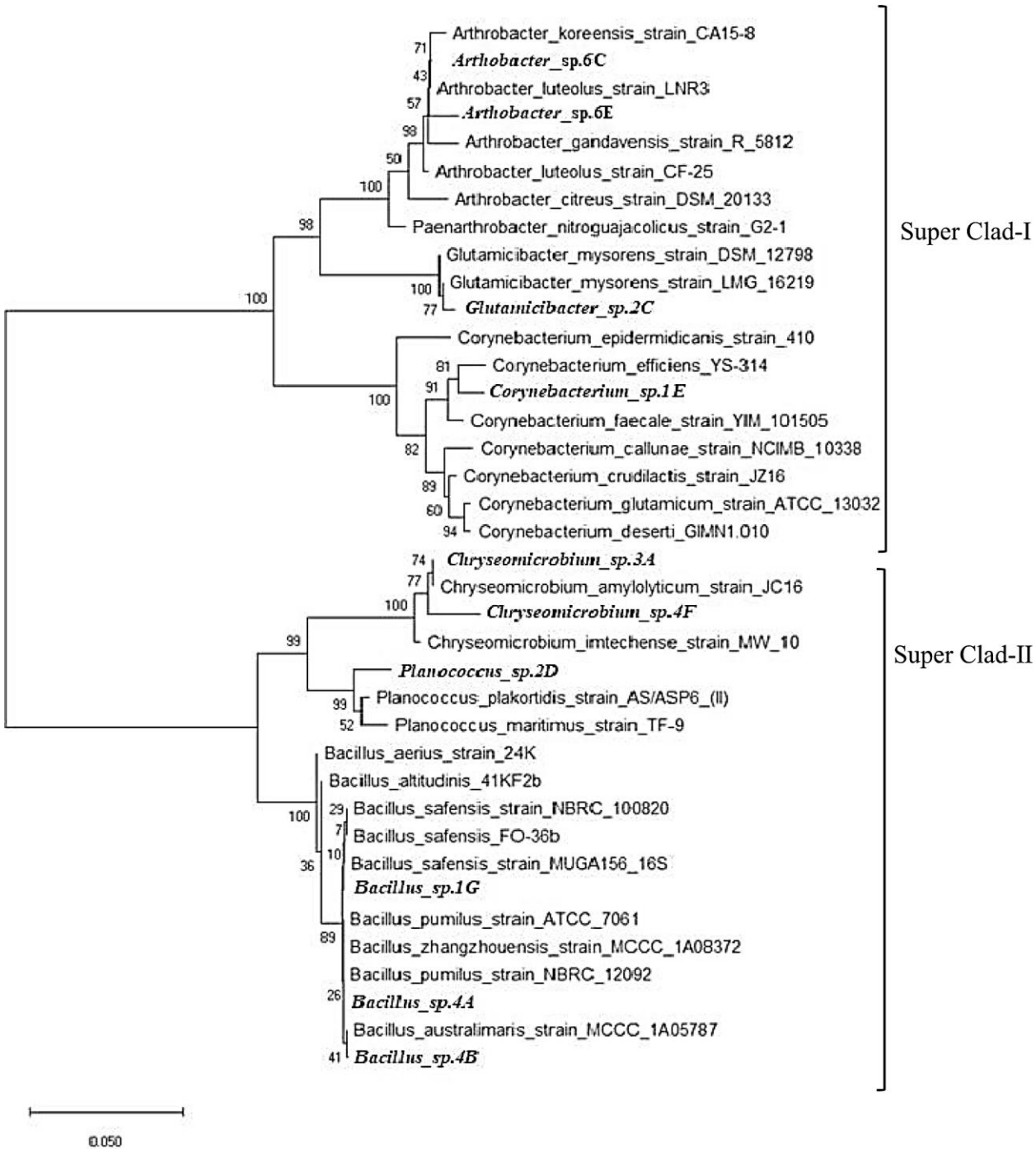



Figure 4. 1 Mega X was used for constructing phylogenetic tree

## 4.2 Maximum calcite was produced by Calcium hydroxide as calcium source

After 15 days of incubation, formation of calcite was confirmed. Calcite was formed using *B. safensis* in addition to five different calcium cources at ph 12. Fig 4.2 shows the amount of precipitated calcite. Calcium hydroxide formed (1420 mg) of calcite, Calcium gluconate formed (460 mg), Calcium formate formed (260 mg), Calcium chloride formed (142 mg) and calcium nitrate formed (130 mg) of calcite. Among all the calcium sources, calcium hydroxide was the potential calcium source for producing maximum calcite whereas calcium nitrate proved to be least susceptible.

Figure 4 2 Precipitated calcite left to right: Calcium hydroxide (1420 mg), Calcium gluconate (460 mg), Calcium formate (260 mg)

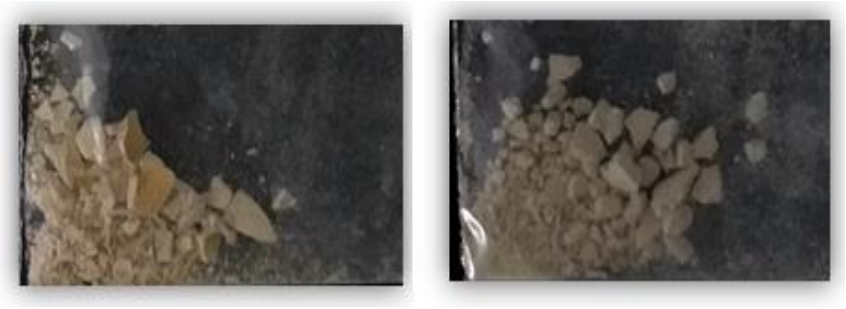


Figure 4 3 Precipitated calcite left to right: Calcium chloride (142 mg), Calcium nitrate (130 mg)

## 4.3 Chemical verification proving $CaCO_3$ presence:

To confirm the presence of $CACO_3$ chemical verification was performed using addition of acetic acid. Few drops of acetic acid on calcite powder caused effervescence which proved $CaCO_3$ is present in calcite produced with all five different calcium sources.

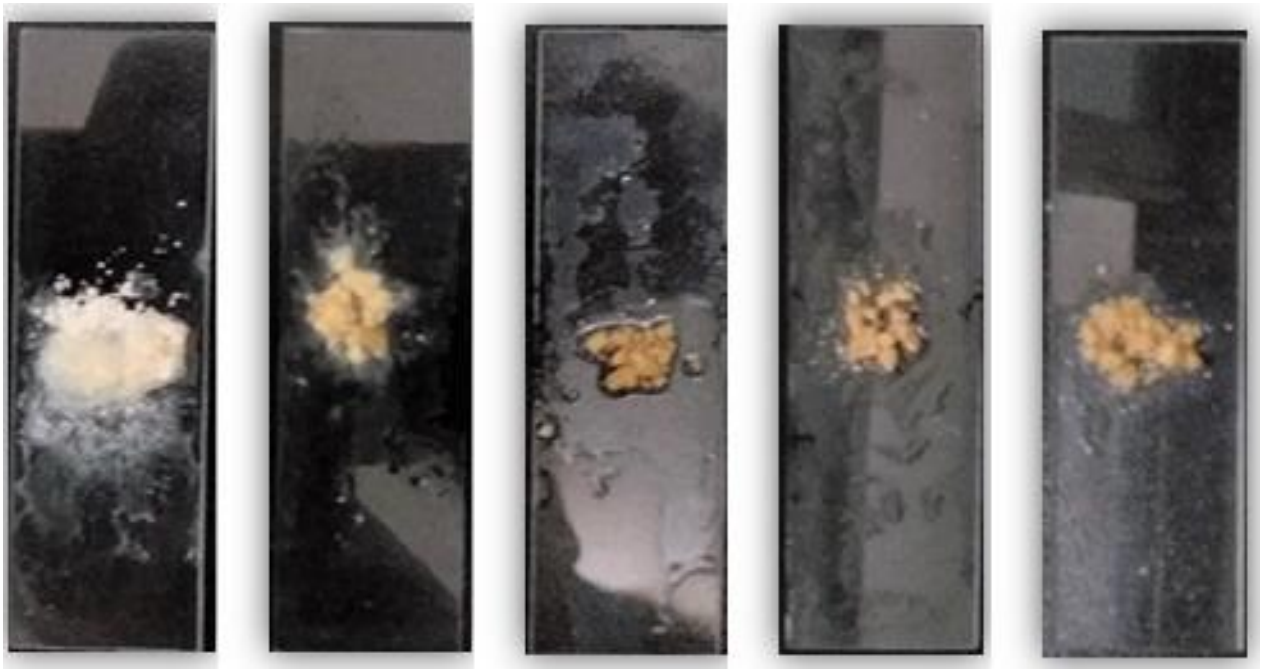


Figure 4 4 Addition of acetic acid on calcite proved the presence of calcite

## 4.4 Quantification of Calcite precipitation under different calcium sources

Five types of calcium sources calcium chloride, calcium hydroxide, calcium gluconate, calcium nitrate and calcium formate were used for the calcification evaluation of bacterial strains at pH 7, pH 10 and pH 12 after 7 and 15 days of interval. Calcification potential of bacterial strains and calcium sources are represented in figures below.

## 1. Calcite production under Calcium Nitrate

After performing calcite precipitation under calcium nitrate it was observed that only few bacterial strains in combination with calcium nitrate precipitated good amount of calcite, Higher calcite precipitation was observed at pH 7 and 1G (*B. safensis*) and 4A (*B.pumilus*) possessed higher calcite formation potential at pH 12 after 7 and 15 days of incubation.

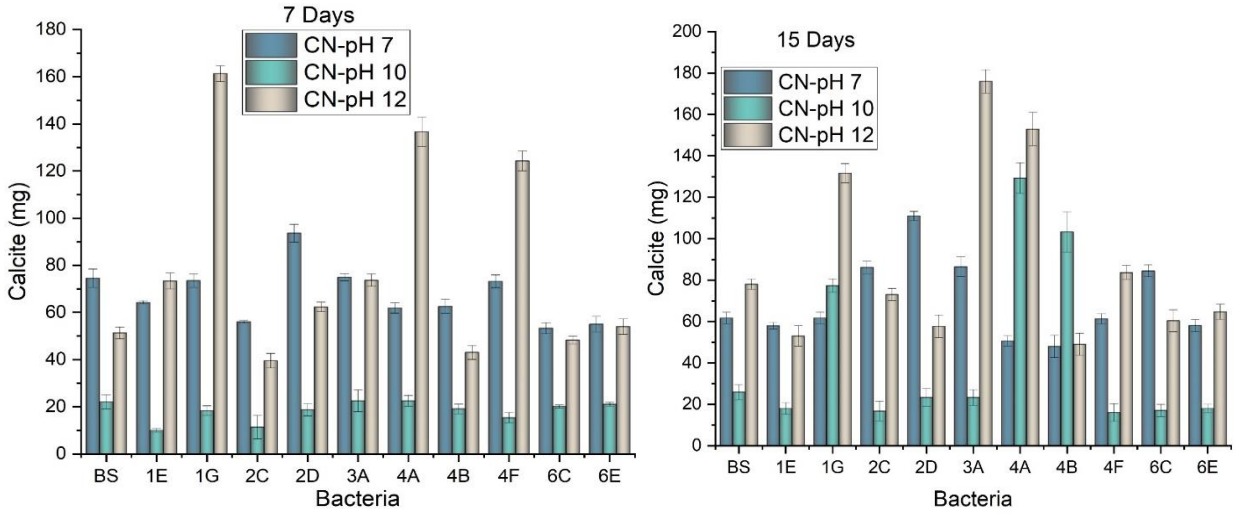



Figure 4. 5 Quantification of calcite production under calcium nitrate showed that only few bacterial strains in combination with calcium nitrate precipitated good amount of calcite.

## 2. Calcite production under Calcium Chloride:

In case of calcium chloride, after 7 days of incubation higher calcite precipitation was observed at pH 10 and pH 12 and after 15 days of incubation higher calcite precipitation was observed at pH 7 and pH 12. Calcium Chloride is an optimal calcium supplement that is useful for generating calcium carbonate therefore more calcite was formed after 15 days. After 7 days of interval 4A (*B.pumilus)* and 4B (*B.australimaris)* showed more calcite production. After 15 days of interval 1G (B.safensis) and 4B (*B.australimaris)* showed more calcite production. Below figures represents the quantification evaluation calcite production.

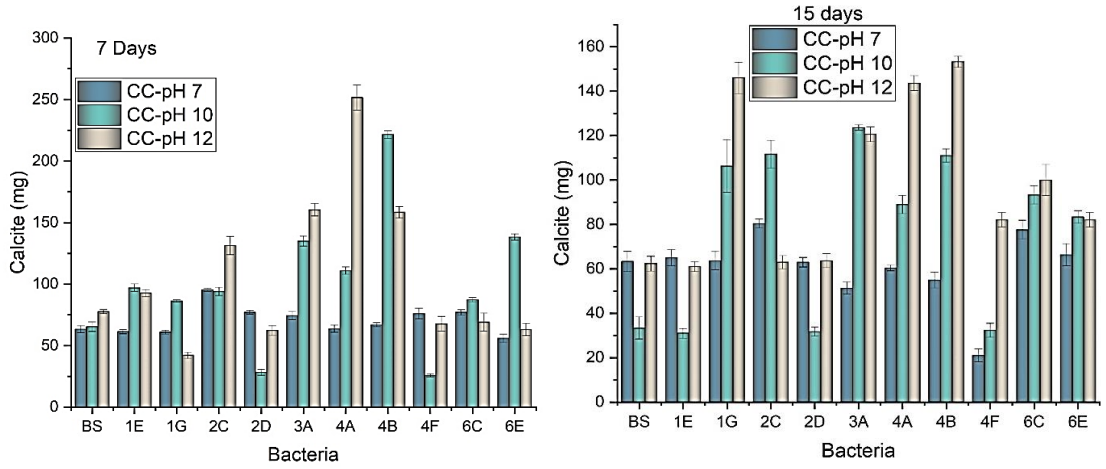



Figure 4. 6 Quantification of calcite production under calcium chloride proved that Calcium chloride is an optimal calcium supplement that is useful for generating calcium carbonate therefore more calcite was formed after 15 days

## 3. Calcite production under Calcium Formate:

In case of calcium formate only few bacterial strains were actively participating.4A (*B. pumilus*), 1G (*B. safensis*), 4B (*B. australimaris*) and 2C (*G. mysorens*) possessed higher calcite formation potential at pH 10 and pH 12. Below figures represents the evaluation of calcite production with calcium formate.

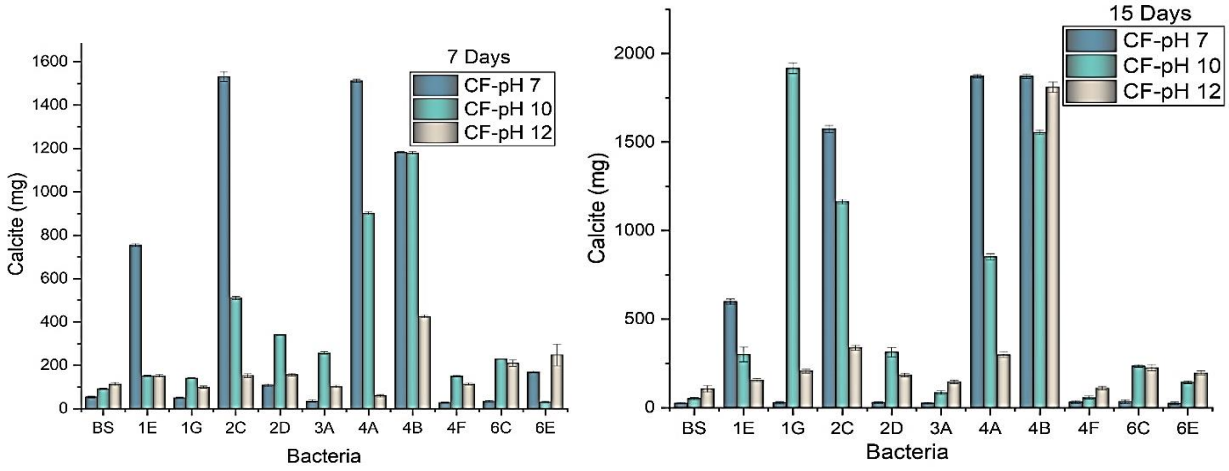



Figure 4.6 1 Quantification of calcite production under calcium formate showed least favourable results as it is known to prevent growth of bacteria in growth medium.

## 4. Calcite production under Calcium Gluconate:

Calcium gluconate was the second highest producer of calcite among all sources. Higher calcite precipitation was observed at interval of 15 days as compared to 7 days of incubation with all bacterial strains. Graphs represent the evaluation of calcite precipitation in fig 4.6.3

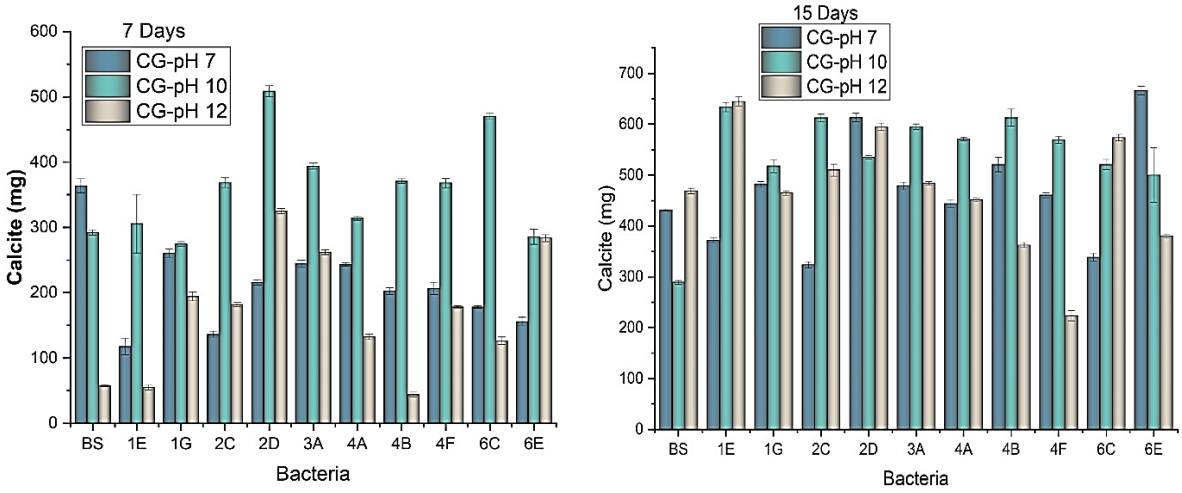



Figure 4.7 Quantification of calcite production under calcium gluconate showed good results

## 5. Calcite production under Calcium Hydroxide:

Calcium ions speed up the formation of calcium hydroxide in cement mortar. Among other sources calcium hydroxide possessed higher calcite secretion potential. All bacterial strains showed more calcite precipitation with calcium hydroxide at pH 10 and pH 12 after 7 and 15 days of interval. Among other sources calcium hydroxide possessed higher calcite secretion potential.

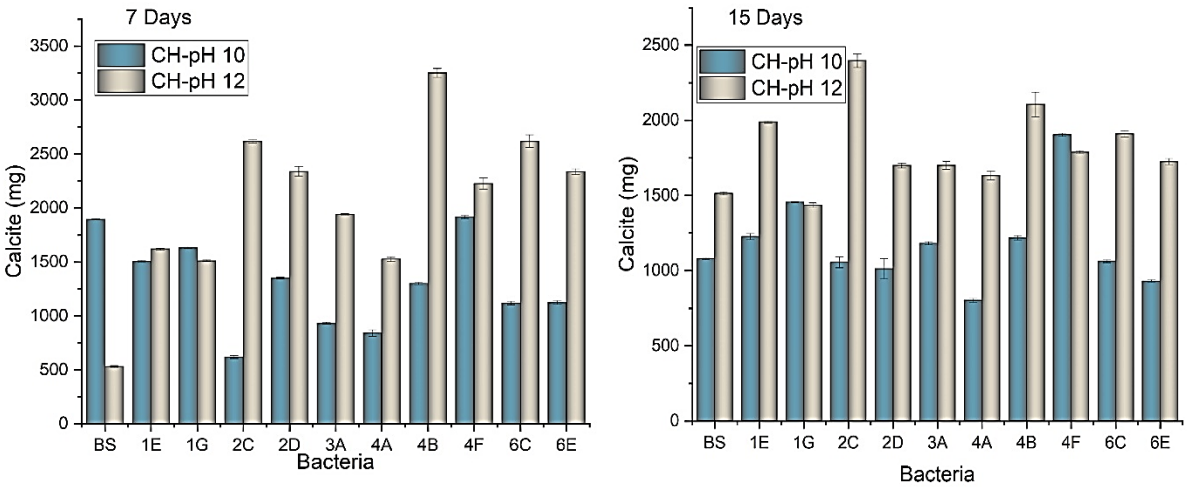



Figure 4 7 Quantification of calcite production under calcium hydroxide showed promising results and considered as the higher producer of calcite among all different calcium sources.

## 4.5 Crystal morphology of Calcite via SEM:

To observe the crystal morphology of Calcite, SEM was performed. Objective of scanning electron microscopy was to analyse the presence of Calcium Carbonate. Crystal morphology of precipitated calcite was confirmed and examined through SEM. SEM micrograph was matched with literature reported calcite pictures. Figures below represent the crystal morphology of precipitated calcite under two different calcium sources, Calcium gluconate and Calcium hydroxide at pH 12, and pH 10.

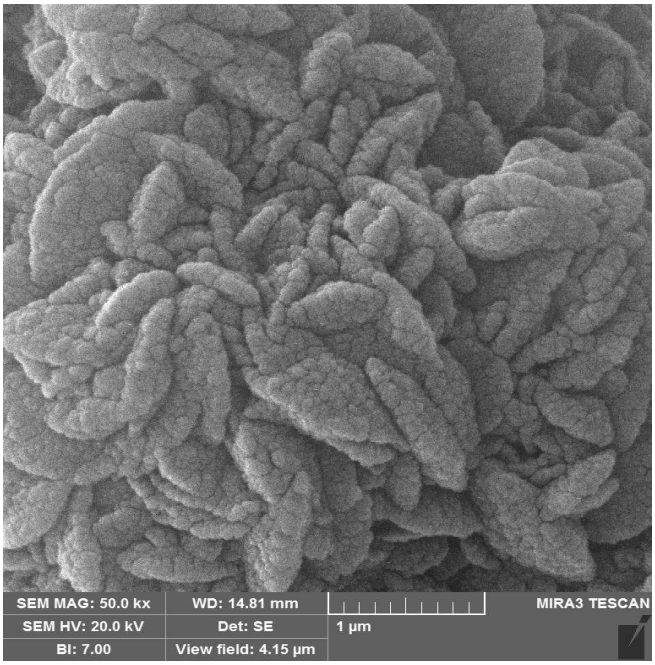



Figure 4 8 Image represents crystal morphology of precipitated calcite under Calcium gluconate at pH 12 (scale bar: 1 µm). It is quite visible that calcium source affects the morphology of calcite.

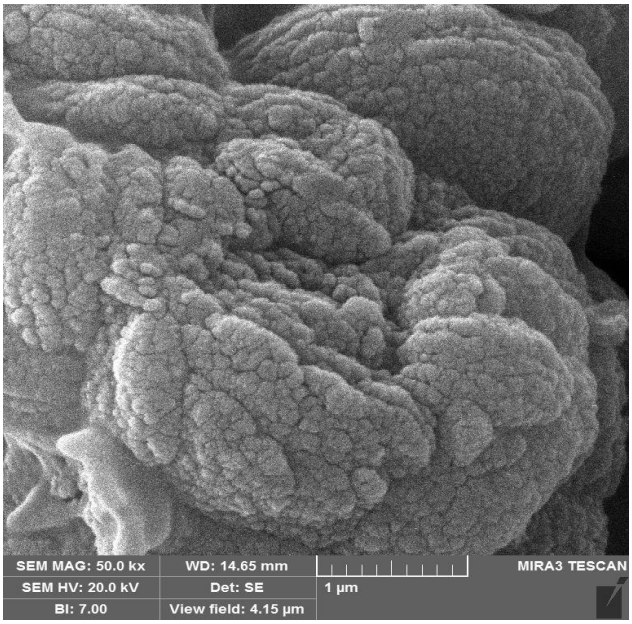



Figure 4 9 Image represents crystal morphology of precipitated calcite under Calcium gluconate at pH 10 (scale bar: 1 µm). Morphology of calcite is greatly affected by presence of calcium gluconate.

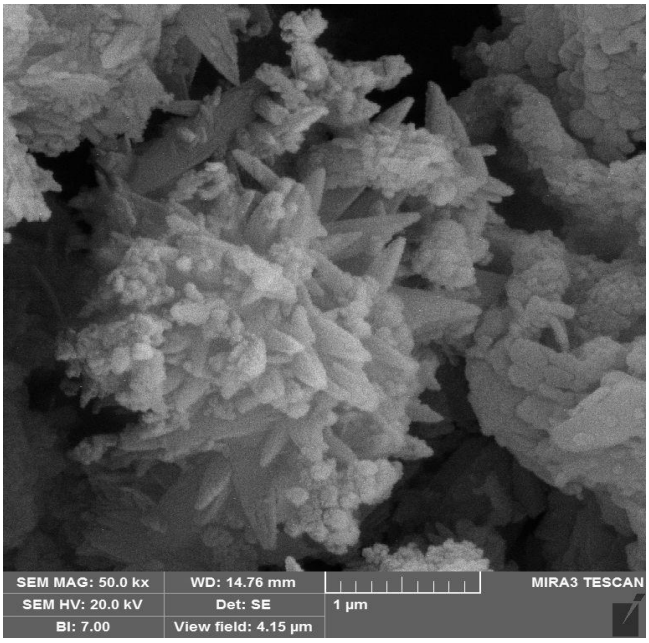



Figure 4. 10 Image represents crystal morphology of precipitated calcite under Calcium hydroxide at pH 12 (scale bar: 1 µm). Sharp crystalline form confirms calcite formation proving bacterial type and Calcium source influence crystal morphology.

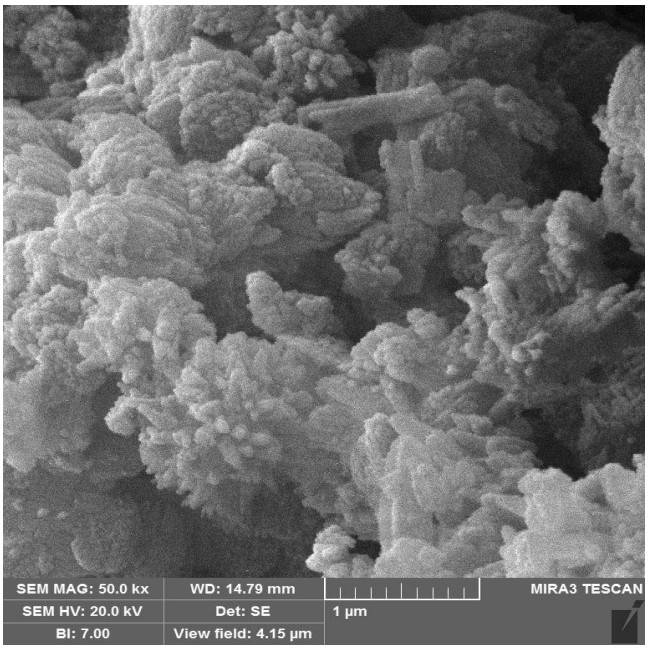



Figure 4. 11 Image represents crystal morphology of precipitated calcite under Calcium hydroxide at pH 10 (scale bar: 1 µm). Crystalline form confirms calcite formation.

- **Crystal Morphology of Crystals**

There are three crystalline polymorphs of caco3 present known as Calcite, Vaterite, Aragonite and crystal morphology is greatly influenced by bacterial type and calcium source. In this study, in case of Calcium Gluconate, spherical crystals are Vaterite while rhombohedral crystals are Calcite and in case of Calcium Hydroxide, Aragonite crystals are formed. Images shown below represents the crystal morphology of calcium Gluconate and calcium Hydroxide.

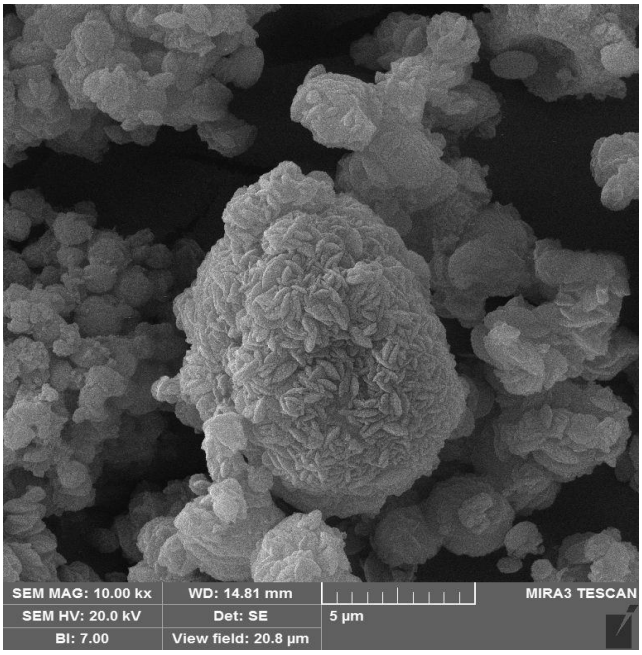



Figure 4 12 In this figure, Calcium Gluconate shows two types of crystals. Spherical crystals are vaterite while rhombohedral crystals are calcite.

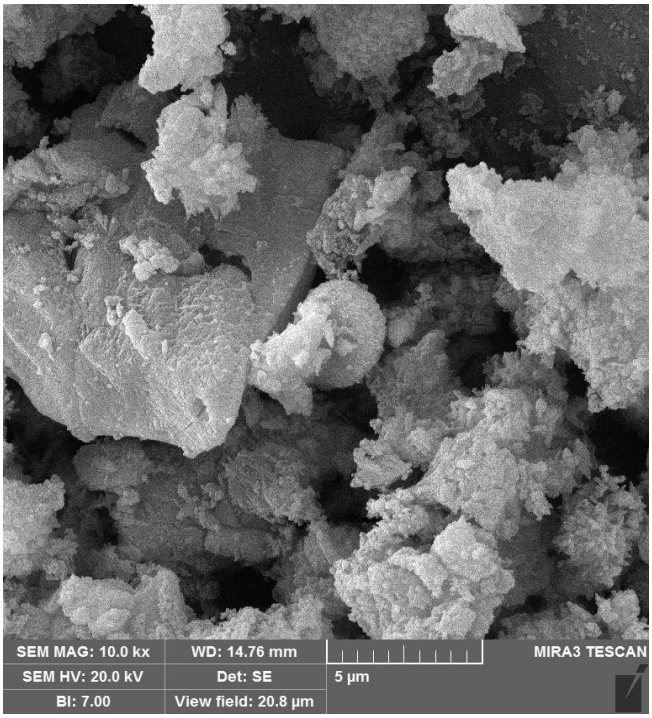
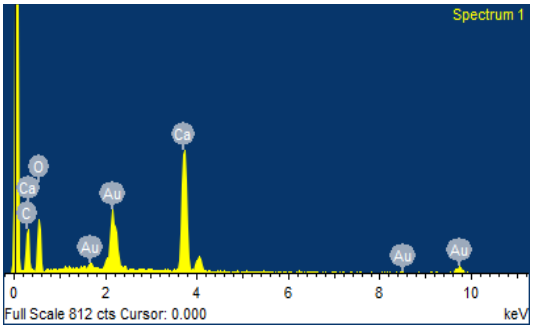


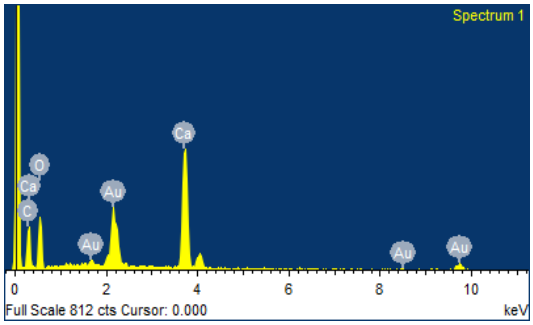

Figure 4. 13 In this figure, Calcium Hydroxide shows only one type of crystals known as Aragonite crystals.

## 4.6 Confirmation of calcite formation via EDX

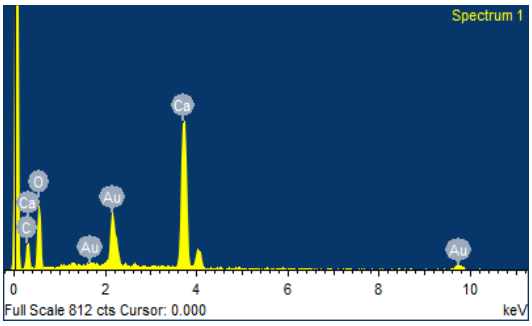
For qualitative analysis as well as quantitative analysis ofcalcite precipitates formed, EDX was performed. Electron dispersive X ray was done to check the elemental composition of calcite and elemental composition of sample confirms the presence of calcite. Sharp peaks represent the good crystallinity of particles. Presence of Ca, C and O confirms the formation of CaCO3. Below figures represents EDX graphs of calcium gluconate and calcium hydroxide at pH 10 and pH 12.



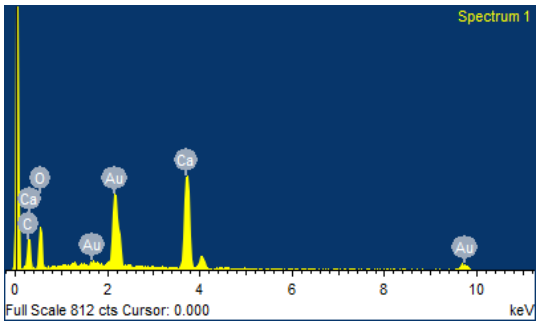

(a) Calcium Gluconate pH 12




(b) Calcium Gluconate pH 10




(c) Calcium Hydroxide pH 10




(d) Calcium Hydroxide pH 12

Figure 4. 14 Calcite formation via EDX

Table 4. 1 Elemental composition of calcium hydroxide and calcium gluconate via EDX

| Calcium source | Carbon % | Oxygen % | Calcium % |
|---|---|---|---|
| Calcium Hydroxide pH 10 | 12.45 | 43.49 | 27.32 |
| Calcium Hydroxide pH 12 | 18.21 | 31.70 | 20.55 |
| Calcium Gluconate pH 10 | 19.58 | 38.97 | 23.18 |
| Calcium Gluconate pH 12 | 16.91 | 27.39 | 24.10 |

## 4.7 Sieve analysis of fine aggregates

In order to confirm the particle size distribution of sand, sieve analysis was performed. This test was performed according to ASTM standard C136/C136M. After performing this test, Fineness Modulus of sand was 2.352071.

Table 4. 2 Sieve analysis flow sheet of fine aggregates

| Sieve Size | wt Retained (kg) | wt Retained (gm) | Retained (%) | Commulative % Retained | % Passing |
|---|---|---|---|---|---|
| 3/8in | 0 | 0.00 | 0% | 0% | 100% |
| #4 | 0 | 0.00 | 0% | 0% | 100% |
| #8 | 0.008 | 8.00 | 1% | 1% | 99% |
| #16 | 0.036 | 36.00 | 4% | 4% | 96% |
| #30 | 0.377 | 377.00 | 37% | 42% | 58% |
| #50 | 0.495 | 495.00 | 49% | 90% | 10% |
| #100 | 0.08 | 80.00 | 8% | 98% | 2% |
| #200 | 0.018 | 18.00 | 2% | 100% | 0% |

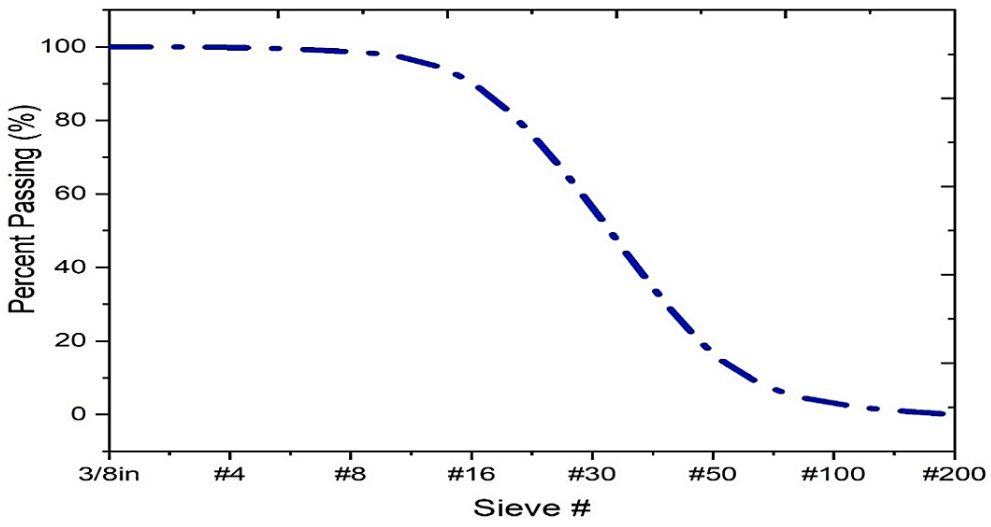



Figure 4. 15 Sieve analysis of fine aggregates was performed. In this test fine aggregates were passed through series of sieves, according to ASTM standard C136/C136M

## 4.8 Compressive and Flexural strength test

In order to identify the characteristics of concrete, compressive test was performed and to evaluate the tensile strength of concrete, flexural test was performed. To perform this test, three specimens were casted including calcium source and bacteria for each formulation, tested at $7^{th}$ and $15^{th}$ day for compressive and flexural strength investigations. Higher compressive strength of calcium hydroxide and bacteria was observed after 15 days of curing. In case of flexural strength only control formulation showed increase in flexural strength. On exposure of mortar to atmosphere calcium hydroxide reacts with $CO_2$ forming $CaCO_3$ and hence strengthens the construction, whereas calcium gluconate retards hydration of all phases of the cement.

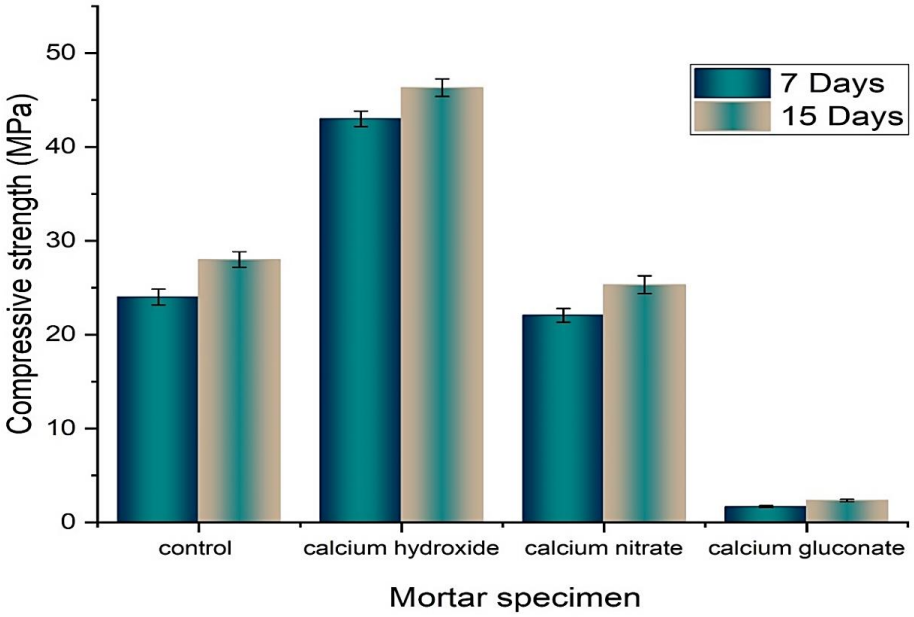



Figure 4. 16 Compressive strength results of *B.safensis* and calcium sources after 7 and 15 days of interval. Higher compressive strength of calcium hydroxide and bacteria was observed after 15 days of curing.

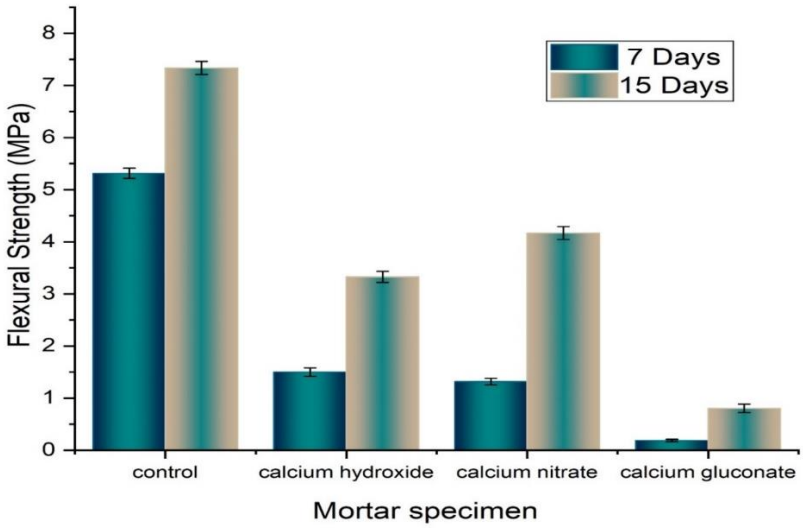



Figure 4 17 Flexural strength results of B.safensis and calcium sources after 7 and 15 days of interval. In case of flexural strength only control formulation showed increase in flexural strength.

## 4.9 Self-healing test:

Self-healing was observed in concrete. Cracks were generated on mortar cubes and observed at day 1 and day 7 to check healing of cracks. Self-healing of cracks in concrete was achieved at interval of 7 days of curing. Cracks were measured under crack measuring microscope. Control samples was generated with width of 0.66mm crack. Whereas hydroxide formulation sample was generated with width of 0.7 mm crack.


Fig. 4.19 (a) Control formulation crack day 1

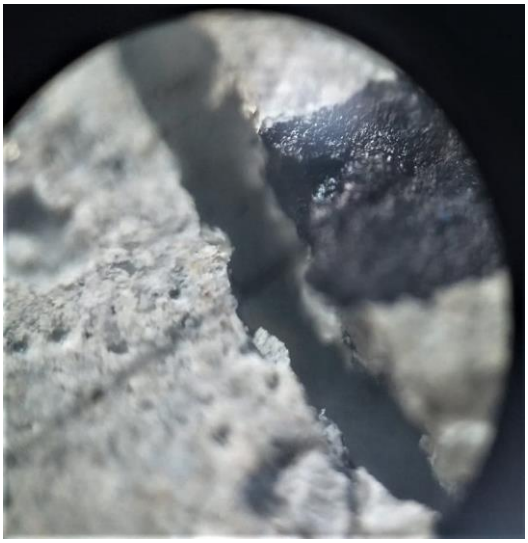


Fig. 4.19 (b) Control formulation crack day 7

Figure 4. 18 Control formulation is presented with no addition of bacteria and calcium source. Crack was generated at width of 0.66mm. No healing was observed in control formulation after 7 days of interval.

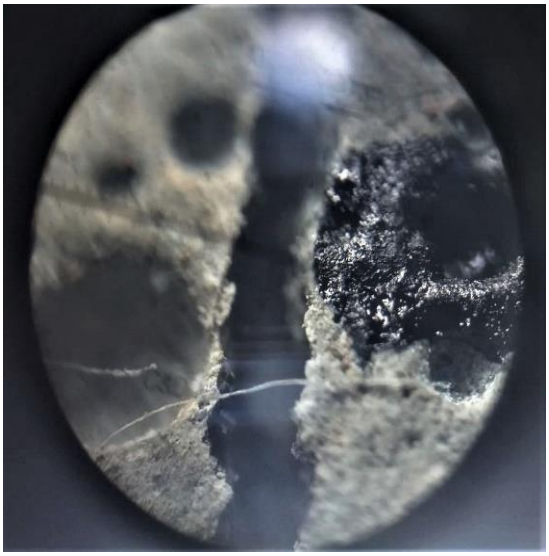


Fig. 4.20 (a) Hydroxide formulation crack day 1

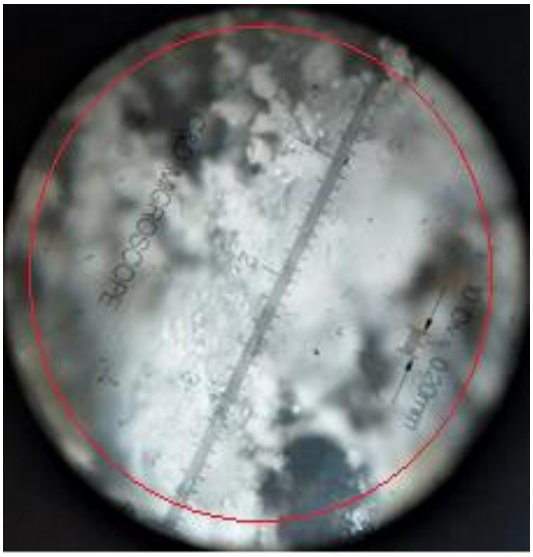


Fig. 4.20 (b) Hydroxide formulation crack day 7

Figure 4. 19 Hydroxide formulation is presented with addition of *B.safensis* and calcium hydroxide. Crack was generated at width of 0.7mm. Healing was observed in concrete after 7 days of interval.

## 4.13 Confirmation of calcifying genes

Study was performed to identify and confirm the presence of *lcfA, etfA,* and *etfB* in *Bacillus safensis* and *Bacillus subtilis.* The sequences of the Bacillus subtilis lcfA, etfA, and etfB genes were acquired from NCBI. Basic local alignment was performed of obtained sequences against the genomes of strains involved in calcite production. Alignment of the gene sequences (lcfA, etfA, etfB) to Bacillus safensis showed 70.18% identity to lcfA, 69.24% for etfA, and 70.22% for etfB. Alignment of the gene sequences (lcfA, etfA, etfB) to Bacillus australimaris showed 69.54% percentage identity for *lcfA*, 68.55% for *etfA* and 69.94% for *etfB*. Gel electrophoresis confirmed the presence of calcifying genes in *B. subtilis and B. safensis*.

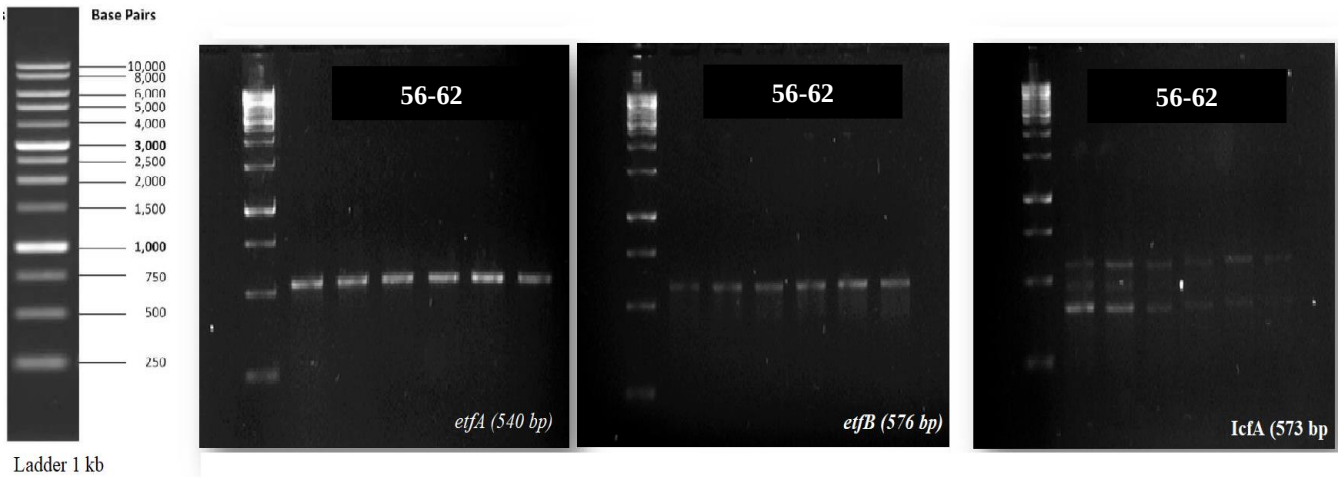



Figure 4. 20 PCR results of all three genes of B. subtilis (B.S), temperature ranges 56, 58, 59, 60, 61 and 62

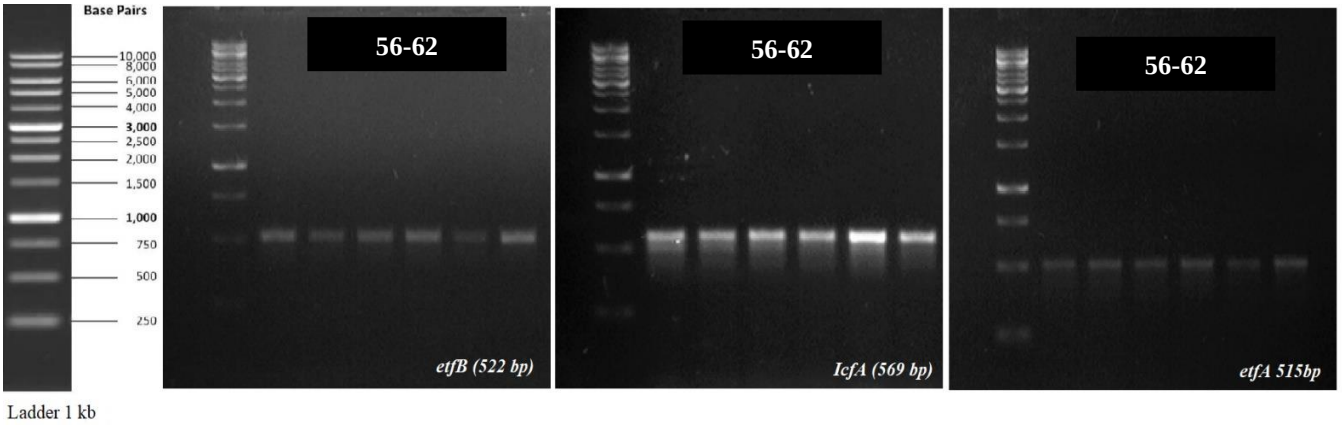



Figure 4. 21  PCR results of all three genes of B. safensis (1G), temperature ranges 56, 58, 59, 60, 61, and 62

# Chapter 5

# Discussion

Concrete is one of the commonly used construction material due to its exclusive properties. Concrete has so many advantages comprising high compressive strength, flexibility, accessibility, economical, outstanding thermal mass, and can be casted in any desired shape (Makul, 2020). Regardless of these advantages, formation of cracks is a major problem in concrete structures. Cracks usually appear due to low tensile strength and stresses. There are two different types of cracks, structural and non-structural cracks. Structural cracks are caused by improper design, imperfect construction, or overloading whereas non-structural cracks are due to internal forces generated in the material due to humidity fluctuations. Exposure to temperature changes, hairline cracks, gases, liquids, etc (Nama, Jain, Srivastava, & Bhatia, 2015). There are many remedies which has been previously used to heal cracks in concrete. But many of them are not suitable as they are not economical, requires skilled labour, not environmental friendly and also reduces the useful life of concrete (Thagunna, 2014). To address all these problems a process named as MICP is used to heal these cracks. This process consists of microorganism and nutrients added in the concrete during preparation. When a crack forms in concrete, healing agent activates and form calcium carbonate ($CaCO_3$) which then heals the crack (Kulkarni, Nemade, & Wagh, 2020).

The main purpose of this research was to maximize the production of $CaCO_3$ while using different calcium sources and bacterial strains, presence of calcifying genes in bacterial strains and to investigate the mechanical effect of these bacteria and calcium sources in concrete. Soil samples were collected from six different locations from highly alkaline cementitious environment. Ten

calcite producing bacteria were isolated and 16s rRNA sequencing was performed. This analysis was performed using MEGA X. Phylogenetic tree shows the genetic diversity of mineralizing strains isolated from local soils. Individual phylogenies are represented in the form of rootless trees with two overlays. Among strains Bacillus species were common and other species were Arthrobacter , Chryseomicrobium, Planococcus, Glutamisibacter and Corynebacterium. Bacillus, Arthrobacter and Planococcus classes are known for their calcite production (Mykytczuk, Lawrence, Omelon, Southam, & Whyte, 2016) (Jonkers, 2021) (Park, Park, & Ghim, 2013). *Corynebacterium strain* has also been stated as calcifying bacterial strain (Cacchio & Del Gallo, 2019).

Quantification of calcite precipitation was performed using five types of calcium sources (calcium formate, calcium nitrate, calcium gluconate, calcium hydroxide and calcium chloride) along with ten selected calcifying bacterial strains. All bacterial strains were gram-positive. This experiment was performed at three different pH values (pH 7, pH 10 and pH 12). Each calcium source in combination with bacteria acted differently. After 15 days of incubation, calcite was successfully formed with all five calcium sources. Greater amount of calcite was achieved at pH 12. Calcium hydroxide precipitated 1420 mg of calcite, calcium gluconate 460 mg, calcium formate 260mg, calcium chloride 142 and calcium nitrate130mg. all bacterial strains exhibited higher calcite precipitation at greater pH. Higher pH increases rate of bacterial calcite precipitation (De Belie, 2016). Calcite is more formed at high pH because the actual pH of cementitious system is high (Lors, Ducasse-Lapeyrusse, Gagné, & Damidot, 2017).

Calcium hydroxide was the highest producer of calcite among all other calcium sources and formed pure crystals of calcite. Calcium hydroxide showed great affinity with bacterial strains in formation of calcite. All bacterial strains showed more calcite precipitation with calcium hydroxide

at pH 10 and pH 12. Calcium chloride is an optimal calcium supplement that is useful for generating calcium carbonate therefore more calcite was formed after 15 days. After 7 days of incubation higher calcite precipitation was observed at pH 10 and pH 12. After 15 days of incubation higher calcite precipitation was observed at pH 7 and pH 12. Calcium chloride is a good source of calcium for the MICCP procedure as it delivers higher urease activity and calcite production (Achal & Pan, 2014). Calcite formation using calcium nitrate was successful, after performing calcite precipitation under calcium nitrate, higher calcite precipitation was observed at PH 7 AND B. safensis and B.pumilus possessed high calcite potential at ph 12. Calcium nitrate has been used previously as a source of calcium in MICP process (AKOĞUZ, ÇELİK, & BARIŞ, 2019). Incase of calcium formate only few bacterial strains were actively participating. *B. pumilus, B. safensis ,B. australimaris and Glutamicibacter mysorens* possessed higher calcite formation potential at pH 10 and pH 12. Calcium gluconate was the second highest producer of calcite among all sources. Higher calcite precipitation was observed at interval of 15 days as compared to 7 days of incubation.Calcium formate prevents the growth of bacteria in growth medium, as it is said to be inhibiting growth of bacteria like *E.coli, pseudomonas aeruginosa* and *staphylococcus aureus* (Qiu, Dong, Ashour, & Han, 2020). SEM and EDX was performed for the confirmation of calcite production with help of these calcium sources and bacterial strains. These tests were conducted to observe the crystal morphology of these crystals and to check the elemental composition of the calcite produced. EDX and SEM endorsed the precipitation of $CaCO_3$, bacterial type and calcium source influence crystal morphology. Sharp peaks of EDX represented good crystallinity of particles. Presence of Ca, C and O confirmed the formation of CaCO3. SEM micrograph was well matched with literature (Shaheen, Jalil, Adnan, & Arsalan Khushnood, 2021). Calcium source and bacterial strain greatly affected the crystal morphology (Xu, Du, Jiang, & She, 2015). In case of

calcium gluconate Spherical crystals were formed which are known as vaterite while rhombohedral crystals are called calcite. Whereas calcium hydroxide formed aragonite crystals. There are three types of crystal naturally present known as Calcite, vaterite and aragonite (Seifan, Khajeh Samani, Hewitt, & Berenjian, 2017). Furthermore, mechanical evaluation of cementitious system was done. The effect of bacterial inoculation and three different calcium sources in cementitious system was investigated through determination of compressive and flexural stresses. In this test, three formulations were casted consist of Calcium hydroxide, calcium gluconate and calcium nitrate along with *B.safensis.* Higher compressive strength of calcium hydroxide and bacteria was observed after 15 days of curing. In case of flexural strength only control formulation showed increase in flexural strength. Addition of bacteria showed increase in concrete compressive strength, this improvement is because of adding calcifying bacterial strains in mixture. The increase in compressive strength is due to self-healing mechanism (Sierra-Beltran & Jonkers, 2012). On exposure of mortar to atmosphere calcium hydroxide reacts with $CO_2$ forming $CaCO_3$ and hence strengthens the construction. (Marangu, Thiong'o, & Wachira, 2019). Whereas Calcium Gluconate which acted as a retarder in cement mortar, show no compatibility with cement and quickly dissolved in water during water (Singh, 1976). Calcium gluconate retards hydration of all phases of the cement and is also known as the most efficient retarders (Ramachandran & Lowery, 1992). These formulations consisting calcium hydroxide and *B.safensis* were also tested for their self- healing abilities in concrete. Mortar specimens were pre-cracked with a width of 0.7mm and after interval of 7 days self-healing of cracks were achieved. Production of CaCO3 in large amount was observed in these cracks. Presence of CaCO3 was responsible for the healing of cracks. Conversion of calcium source along with bacterial solution into calcium carbonate was also observed (Jonkers, Thijssen, Muyzer, Copuroglu, & Schlangen, 2010). Calcium hydroxide

successfully healed the concrete after 7 days of curing. Calcium hydroxide is one of the products formed when Portland cement is hydrated with water. Numerical simulations shows that the dissolution of calcium hydroxide supplies to the significant increase in porosity of hydrated cement pastes (Marchand, Bentz, Samson, & Maltais, 2001). After that genes were identified at molecular level which are responsible for calcite precipitation. For idenetification of genes icfA operon was used which is composed of five genes named as *etfA, etfB* and *IcfA.* This *icfA* Operon is said to be involved in calcite precipitation in *Bacillus subtilis.* This cluster of genes are used in fatty acid metabolism. It is also suggested that it could be participating in calcium carbonate precipitation. Moreover other studies have also confirmed that there is a link between calcium carbonate and fatty acid metabolism (Barabesi et al., 2007). Therefore three of genes from *lcfA* operon were selected for *B.safensis* and *B.australimaris* followed by primer designing ,DNA was extracted and PCR was performed which resulted in proving presence of calcifying genes in *B.subtilis* and *B.safensis.*

# Chapter 6

# Conclusion

The main purpose of this research was to maximize the production of $CaCO_3$ for MICP while using different calcium sources and bacterial strains. After 16S rRNA sequencing Bacillus sp. were most common in identified strains and also gave higher calcite precipitation than rest of the species. Among all calcium sources, calcium hydroxide possessed higher calcite secretion potential and comprises higher mechanical strength. Calcium hydroxide also proved to be the effective source for self-healing in concrete and also a reliable source for increasing compressive strength of concrete. Presence of *etfA*, *etfB* and *icfA* genes are confirmed in *B. safensis* same like in *B. subtilis.*

# Chapter 7

# Future Prospects

The role of these genes in calcite precipitation can be further confirmed through gene knockout experiments. To enhance compressive and flexural stress more tests can be performed with extended curing time. Self-healing test can be performed furthermore in presence of more calcifying bacterial strains and calcium sources.

# Chapter 8

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