# Identification of Genes in Bacterial Strains (*Bacillus australimaris* and *Bacillus safensis*) and Prediction of the Pathway Involved in Microbial Induced Calcite Precipitation (MICP) for Concrete Self-healing


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# Thesis Acceptance Certificate

It is certified that the contents and form of thesis entitled "Identification of Genes in Bacterial Strains (*Bacillus australimaris* and *Bacillus safensis*) and Prediction of the Pathway Involved in Microbial Induced Calcite Precipitation (MICP) for Concrete Self-healing" submitted by Aisha Mehmood, Mahad Ahmad, Sameen Ahmed Malik, and Urooj Fatima has been found satisfactory for the requirement of the BS degree.

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We certify that this research work titled "Identification of Genes in Bacterial Strains (*Bacillus australimaris* and *Bacillus safensis*) and Prediction of the Pathway Involved in Microbial Induced Calcite Precipitation for Concrete Self-healing" is our 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.

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Aisha Mehmood: 

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***This thesis is dedicated to our beloved parents and teachers who have supported us in every step of the way and our family and friends whose council and guidance has been unmatched.***

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# ABSTRACT

Microbially Induced Calcite Precipitation (MICP), secreted through bacterial metabolic activity has assisted in providing restorative measures within the construction industry and reduced the negative impact on the economy as well as the environment. The bacterial strains precipitate calcite through active and passive pathways. The passive pathway includes urea hydrolysis, amino acid ammonification, denitrification, and dissimilatory sulphate reduction, whereas the active pathway involves oxidation of organic matter. The process of $CaCO_3$ precipitation also enhances the mechanical and flexural strength of cement matrix. However, the repairing efficiency of MICP is limited by its adverse effects, such as accumulation of new products owing to the chemical reactions between the bacterial metabolic by-products and the cement minerals, and formation of stained patches due to fungal growth as a consequence of nutrient availability, in cement matrix. Therefore, in the present study calcite precipitating genes in the already isolated bacterial strains were identified to investigate the mechanism at molecular and genetic level. For this purpose, 16S rRNA sequencing was performed to identify the isolates capable of $CaCO_3$ production, followed by phylogenetic analysis through MEGA X. The bacterial strains were identified as species of *Bacillus, Arthrobacter, Planococcus, Chryseomicrobium and, Corynebacterium*. Furthermore, *lcfA* operon (*lcfA, ysiA, ysiB, etfB, and etfA*) in *Bacillus subtilis* was reported to be involved in $CaCO_3$ precipitation. Local alignment was performed between the calcifying gene sequences of *Bacillus subtilis* and other strains of calcite precipitating bacteria. Consequently, gene sequences of bacterial strains (*Bacillus australimaris* and *Bacillus safensis*) with highest sequence homology were retrieved from NCBI, followed by primer designing (using Primer3) and PCR. Moreover, for the confirmation of genes, gradient PCR and Gel electrophoresis were performed. The results indicated the presence of calcifying genes. Besides, the gene sequences were translated to amino acid sequences that were used for the modelling of protein structure via Swiss-Model Web Tool. In addition, the non-ureolytic pathway was also first time predicted that is likely to be involved in the precipitation of calcium carbonate by these bacterial strains. A link between the fatty acid metabolism and calcite precipitation was reported as the genes responsible for β-oxidation of fatty acids were similar to the genes of calcite precipitation. Moreover, *etfA* encodes a membrane associated flavoprotein that is involved in the exchange of ions across the cell that are essential for the formation of calcium carbonate, outside the cell.

# CHAPTER 1

## INTRODUCTION

In the recent years, urbanization has led to a surge in the development of infrastructure. Until now, cement is the most widely used construction material due to its high strength, durability, and resistance, but with the passage of time, cracks appear which can lead to the loss of strength and also the ingress of water in the cement matrix leads to the corrosion of steel. This occurrence of cracks requires recurrent maintenance as they cannot be avoided in these structures. Various kind of healing mechanisms are being implemented to overcome the problem of external repairs. Recently, a self-healing concrete system known as Microbially Induced Calcite Precipitation (MICP) has been of more importance as it involves bacteria that dwell nearly everywhere on earth and can survive in harsh conditions. In the past few years, revolution in science and technology has introduced novel dimensions to the cement industry. Researchers have employed MICP in numerous engineering techniques such as in, remediation of soil and water that have been contaminated by heavy metals, metalloids and other cations, bio-sequestration of CO2, bio-consolidation of soil and in bio-concrete (Dioxide et al., 2018). Bio-concrete has proved its unique potential in the field of construction. This material can repair itself from cracks, thus enabling builders to complete projects timely and at a faster pace. Bio-concrete adds to the structural integrity and resilience of the cement matrix.

In addition, Microbially Induced Calcite Precipitation is influenced by various parameters such as the type of bacterial strains, temperature, pH, pathway for precipitation, availability of nutrients, relative humidity, and dissolved inorganic carbon (Cho et al, 2015). The MICP process is mostly carried out by species of heterotrophic alkaline soil bacteria such as *Bacillus*, *Pseudomonas*, *Sporosarcina*, *Pantoea* and few others (Daskalakis et al, 2013). These bacteria not only induce precipitation of calcium carbonate, but also provide nucleation sites for the stratification of calcite (De Muynck et al, 2010).

The process of biomineralization occurs through bacterial activity when a range of cations such as $Ca^{2+}$, $Mg^{2+}$, Fe and MnO2 react with oxides, phosphates, sulphides, and carbonates. Biomineralization is categorized into three classes: Biologically Controlled Mineralization (BCM), Biologically Induced Mineralization (BIM), and Biologically Mediated Mineralization

The bacterial strains precipitate calcite through active and passive pathways. The passive pathway includes urea hydrolysis, amino acid ammonification, denitrification, and dissimilatory sulphate reduction. The limitation of nitrogen and sulphur cycle is that it produces ammonia and elemental sulphur products which are harmful to the climate as well as to concrete (Vijay et al., 2017). In our previous research, an alternative method known as the active pathway was adopted. It involves the oxidation of organic matter and is more environment- friendly and safe as compared to conventional methods that includes the nitrogen and sulphur reduction methods to harvest calcite precipitation (Shaheen et al., 2021). This pathway also has an added advantage of eliminating the sulphide formations and toxic ammonia.

Regardless of the pathways involved, the repairing efficiency by MICP can also have adverse effects such as: accumulation of new products as a result of chemical reactions between the cement minerals and by-products of bacterial metabolism; growth of fungus, due to organic nutrients, (needed for bacterial growth) can lead to the formation of stained patches (Mastromei, 2000).

To overcome the drawbacks of MICP, in our research, we isolated 10 strains of alkaliphilic bacteria from soil that are higher calcite producers. The strains were identified through 16S rRNA sequencing, followed by identification of genes involved in the process of mineral formation in bacterial strains namely *Bacillus safensis* and *Bacillus australimaris,* to have a better control over the conditions required for precipitation of calcite in cementitious system. This study will enable the isolation of bacterial strains that precipitate calcite faster, and therefore speed up the process of crack healing and thus minimize the limitations of MICP.
In addition, we predicted the non-ureolytic pathway that is likely to be involved in the precipitation of calcium carbonate by bacteria as this area was poorly understood. A study suggested that the process of biomineralization is genetically controlled. A gene cluster known as *lcfA* operon (*lcfA, ysiA, ysiB, etfB, etfA*) has a role in calcite precipitation, as well as in β-oxidation of fatty acids.

## 1.1 Rationale of the study

### 1.1.1 Problem Statement

The occurrence of cracks in the cementitious system requires recurrent repair and is an expensive procedure. The process of Microbially Induced Calcite Precipitation (MICP) is being employed to overcome this problem. However, this approach has its own drawbacks, such as accumulation of new products and formation of stained patches on the cement matrix, which limits the efficiency of the repairing mechanism.

### 1.1.2 Hypothesis

To overcome this problem, we will identify the genes involved in calcite precipitation by bacteria that could possibly improve the conditions required for this process in cement matrix. This will further help us to identify bacterial strains that might have better capability of calcite precipitation process, and therefore speed up the process of crack healing.

## 1.2 Objectives

- Identification of the calcifying bacterial strains and phylogenetic analysis via16SrRNA sequencing.
- Identification of genes in bacterial strains (*Bacillus australimaris* and *Bacillus safensis*) that are involved in calcite precipitation.
- Prediction of non-ureolytic pathway for calcite precipitation in bacteria.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 General Introduction

Concrete is one of the most important and widely used material in construction, because of its high strength, resistance, ability to with-stand huge compressive stress, durability, and cost effectiveness. At a universal level, about 10 billion tons of concrete is used annually, and it has been estimated that the demand for concrete is expected to increase and reach about 16 billion tons till 2050 (Achal et al., 2011). Despite of its large usage, there are adverse effects on the global climate and economy, due to the technology utilized by the manufacturing industry. About 2-3% of global energy demand is consumed in the industrial processes that are involved in the production of concrete from its precursor, lime. As a result of these processes, about 0.73-0.99t $CO_2$/t of cement is generated, which is responsible for 8-10% of the universal greenhouse gas emissions of $CO_2$ and total $CO_2$ emissions of about 3.4% (Castro-Alonso et al., 2019).

Due to the vulnerability of infrastructures to physical and chemical damage, the consumption of concrete is constantly increasing. The damage is usually because of natural disasters, varying temperatures, exposure to destructive gases and radioactive materials, and also due to certain level of microbial activity (Jroundi et al., 2010). These physical, chemical, and biological factors result in the formation of microcracks, which have a negative impact on the durability, permeability, compressive, flexural, and mechanical strength of concrete. Consequently, the beneficial life of concrete is reduced, and the repair and maintenance cost of infrastructures is increased (Achal & Mukherjee, 2015).

Recently, alternative methods to decrease the environmental impact and costs have been developed, of which a metabolic process of bacteria recognized as Microbially Induced Calcite Precipitation (MICP) is of more importance and extensively studied (Basaran Bundur et al., 2015). This process helps in self- healing and further enhances the mechanical properties of cementitious system.

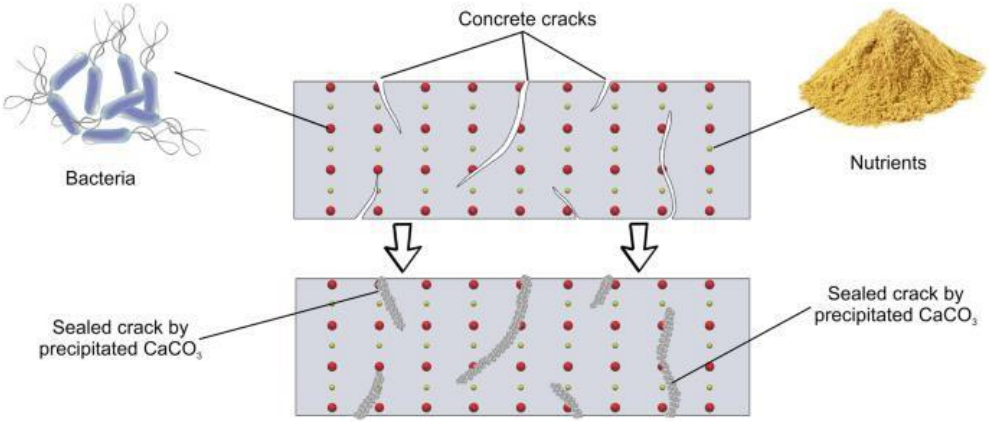



*Figure 1. Crack healing through calcium carbonate precipitation by bacteria (Achal et al., 2011).*

## 2.2 Biomineralization

Generally, this process involves the biosynthesis of minerals by microbes. The microorganisms involved in the biomineralization process belong to the diverse taxonomies with different metabolic pathways in a variety of environment. The formation of biominerals takes place through microbial activity when a wide range of cations such as $Ca^{2+}$, $Mg^{2+}$, Fe, and $MnO_2$ reacts with phosphates, carbonates, sulphates, hydroxides, or oxides (Ghosh et al., 2009).

Depending upon their mechanism of action, biomineralization process is classified into three categories.

- **Biologically Controlled Mineralization (BCM)**

  This process takes place when composition, crystal morphology, nucleation, and location of biominerals are directly and completely controlled by the metabolic activity of the microbes and it involves the regulation of several genes. The involvement of organic macromolecule such as exopolysaccharides (EPS), in this phenomenon further categorizes this process into extracellular (BCMe), intracellular (BCMin) and intercellular (BCMint) (Castro-Alonso et al., 2019). In BCM, mineral deposition is generally inside the cells within special vesicles which demonstrated that cell has complete control over the morphology and composition of minerals (Dupraz et al., 2009).

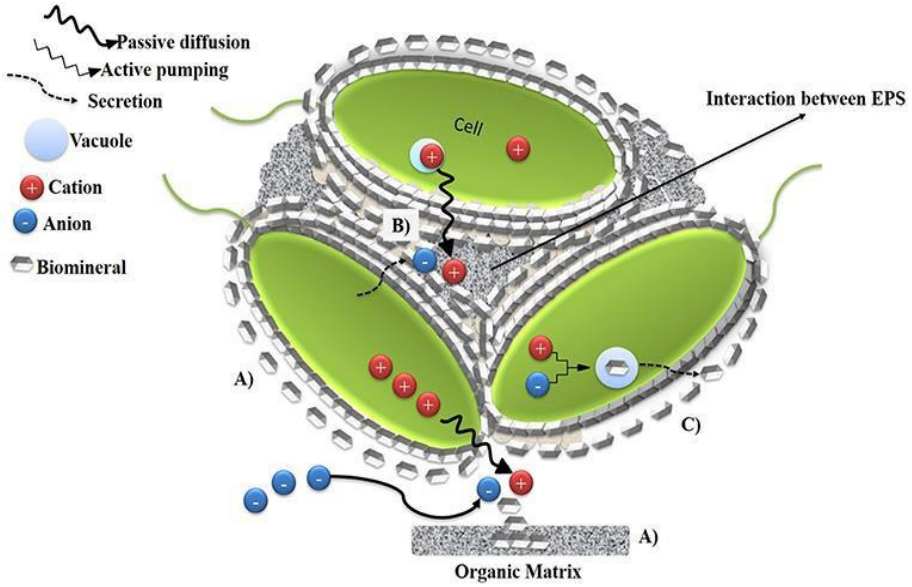



*Figure 2. The diagrammatic representation of Biologically Controlled Mineralization (Castro-Alonso et al., 2019).*

- **Biologically Induced Mineralization (BIM)**

  In this type of biomineralization, indirect precipitation of minerals occurs which is the result of the reaction between the ions present in the environment and metabolic by-products of microbes (Fu et al., 2005). However, in BIM there is the limited involvement of microbial cells in the crystal morphology, growth, and localization of minerals (Erşan et al., 2015). Mineral formation by this process involves the extracellular growth and nucleation. In BIM, mineral deposition may be the result of uncontrolled interactions of metabolic by-products of the microbial cell with the ions in nature which causes the poor specificity and crystallinity as well as broad distribution of particle size of minerals (Castro-Alonso et al., 2019).

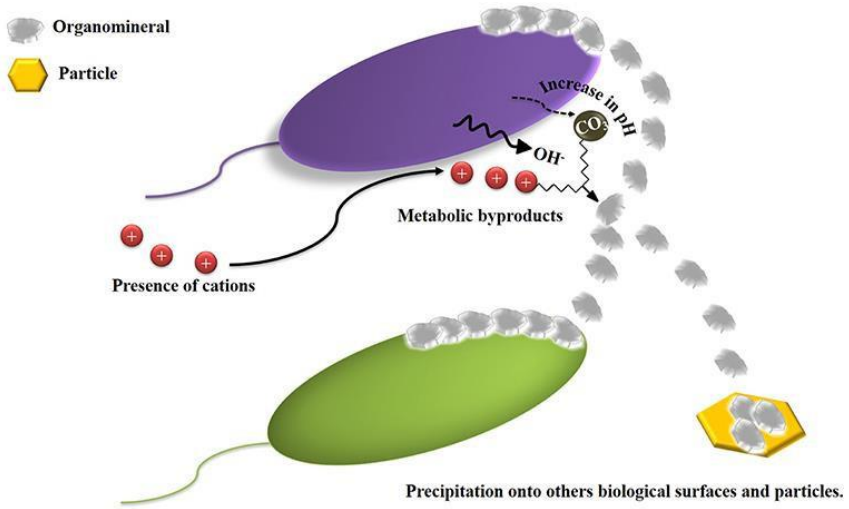



*Figure 3. The diagrammatic representation of Biologically Induced Mineralization (De Muynck et al., 2008).*

- **Biologically Mediated Mineralization (BMM)**

  This process of biomineralization is independent of intracellular or extracellular biological activity of the bacterial cells and formation of minerals in BMM is the consequence of interactions of organic matrix with the inorganic or organic compounds (Fu et al., 2005).

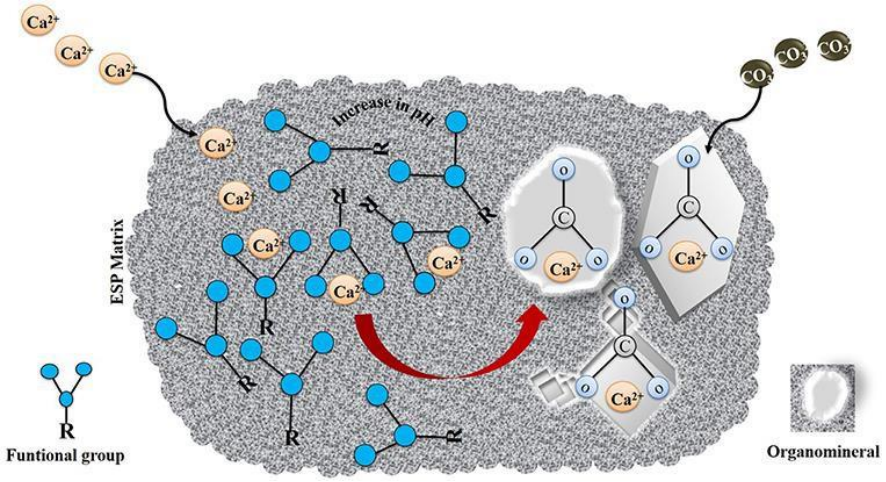



Figure 1. The diagrammatic representation of Biologically Mediated Mineralization (Basaran Bundur et al., 2015).

## 2.3 Microbially Induced Calcite Precipitation (MICP)

In this process, calcium carbonate is precipitated due to interaction between different by-products of metabolic pathways and ions present in the immediate micro-environment, namely calcium ions (Salman et al., 2016). Although, MICP takes place principally via extracellular processes, a number of research have shown intracellular means of calcium carbonate precipitation, primarily in cyanobacteria (Cam et al., 2015). It was identified that *Achromatium oxaliferum* has adapted to intracellular mode of calcium carbonate precipitation with 70% of total cell volume being dominated by crystals of calcium carbonate, encircled by membranes without the formation of vesicles (Head et al., 2000). The process of MICP involved in crack healing, is affected by a number of factors such as bacterial strain, pH, temperature, pathway of calcite precipitation, humidity, nutrients availability and dissolved inorganic carbon (Shaheen et al., 2021).

### 2.3.1 Applications of MICP

MICP has a number of applications in biotechnology, such as soil and water remediation that has heavy metal, cations and metalloids contamination, in other processes such as $CO_2$ bio-sequestration, bio-consolidation of soil, and in bio-concrete (Achal & Mukherjee, 2015).

## 2.4 Mechanism of Action

To restore the strength and the longevity of concrete, the mechanism of action of MICP can be categorised into two ways. Depending upon the type of bacteria, mechanism of action varies. For ureolytic bacteria and non ureolytic bacteria, calcite precipitation is primarily based on the hydrolysis of urea and respiration, respectively. In case of urea hydrolysis, the urease enzyme acts on the urea and produce a notable amount of carbonate ions in presence of ammonium (Dioxide et al., 2018). In presence of calcium source, these precipitated $CO_3^{2-}$ ions lead to the formation of calcium carbonate. This causes an increase in the surrounding pH which is responsible for the self-healing of cracks in the cement, owing to the accumulation of calcium carbonate in the cracks. The two types of bacteria that are largely used in hydrolysis of urea in several investigations include *Sporosarcina pasteurii* and *Bacillus Sphaericus* (Shivangi Mathur, 2018).

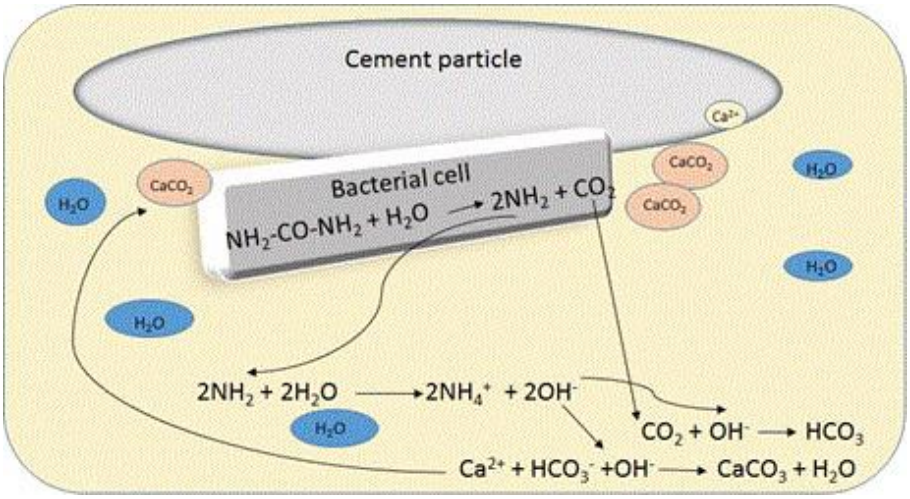



*Figure 5. Microbial interaction with the cement matrix (Castro-Alonso et al., 2019).*

However, the calcite precipitation by this method results in the environmental pollution by ammonia which limits the efficiency of ureolysis. To overcome these problems, non-ureolytic bacteria are used for obtaining promising and environment-friendly outcomes. These include *Bacillus subtilis, Bacillus cohnii* and *Bacillus alkalinitrilicus* (Shivangi Mathur, 2018). To initiate calcium carbonate precipitation, non-ureolytic bacteria use organic compounds such as lactate and acetate as carbon source in place of urea. The carbon dioxide released from the carbon source reacts with calcium hydroxide in the cement matrix and result in the precipitation of calcite in place of crack (Achal & Mukherjee, 2015).

## 2.5 Bio-concrete

Bio-concrete is one of the most climate-friendly, cost-effective, and economic technologies, as it minimizes the emissions of the greenhouse gas $CO_2$ and further reduces the cost of repair and maintenance (Vijay et al., 2017). It has gained particular attention due to its self-healing nature and enhancement of mechanical properties of cement structures. Bio-concrete is basically made by the addition of calcium carbonate precipitating bacteria to concrete mixture and helps in sealing any cracks that might appear. This is known as a self-healing property. The process is promoted through the intrusion of $H_2O$, carbon dioxide and certain other substances such as $NO_3^-$ and $SO_4^{2-}$ (Basaran Bundur et al., 2015).

The major components of Bio-concrete include:

- MICP capable bacteria
- Nutrients
- Calcium ions that are needed to form the cementitious product.

Bacteria that have been identified in the self-healing of cracks mainly include, *Bacillus pseudofirmus, Bacillus alkalinitrilicus, Pseudomonas aeruginosa, Bacillus cohnii, Diaphorobacter nitroreducens* (Erşan et al., 2015).

Various experiments have been conducted to restore historic infrastructures by the process of MICP, such as Angera Cathedral, Thouars church tower, and Alcazar de Guadalajara (Páramo Aguilera et al., 2015).

## 2.6 Major Bacterial Metabolic Processes Involved in MICP

These bacterial processes include:

- The Nitrogen Cycle:
    - Urea hydrolysis
    - Amino acid Ammonification
    - Denitrification
- The Sulphur Cycle:
    - Dissimilatory Sulphate Reduction
- Photosynthesis
- Methane Oxidation
- Fatty Acid Catabolism

Among the above-mentioned pathways, hydrolysis of urea is less complex, and strains involved in urea hydrolysis have shown higher calcium carbonate precipitation as compared to strains that carry out other metabolic pathways. As a result, the most studied MICP process is urea hydrolysis (Wang et al., 2012).

## 2.6.1 The Nitrogen Cycle

**Urea Hydrolysis**

The enzymes that drive the complex reactions in hydrolysis of urea are known as Urease (UE) and Carbonic Anhydrase (CA). Firstly, Urease (UE) hydrolyses one mole of urea to one mole of carbamate and ammonia, followed by spontaneous hydrolysis of carbamate to one mole of carbonic acid and ammonia (De Muynck et al., 2008). The enzyme Carbonic Anhydrase (CA) further converts the carbonic acid to bicarbonate along with the formation of two moles of ammonia and hydroxide as a result of hydrolysis of ammonia (Akada et al., 2000).

Therefore, the pH around the cell is increased and when soluble $Ca^{2+}$ ions are present, calcium carbonate precipitation is induced. Furthermore, when conditions are unfavourable, the cell can survive because it allows the entrance and accumulation of $Ca^{2+}$ ions, which further results in removal of protons excessively (Bachmeier et al., 2002). Therefore, calcium is actively exported by the cells to counteract the proton loss. Thus, for carbonate ions secretion, a high $Ca^{2+}$ ion concentration and low proton concentration is required in the immediate microenvironment, carbonate ion supersaturation stimulates calcium carbonate precipitation on the cell surface (Achal et al., 2011).

Moreover, nucleation sites for these reactions can be provided by exopolymers, inactive spores and biofilms.

| Reaction Steps | Reaction Equation |
|---|---|
| **Step 1** | $CO(NH_2)_2 + H_2 \rightarrow NH_2COOH + NH_3$ |
| **Step 2** | $NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3$ |
| **Step 3** | $H_2CO_3 \rightarrow HCO_3^- + H^+$ |
| **Step 4** | $NH_3 + 2H_2O \rightarrow 2NH_4^+ + 2OH^-$ |
| **Step 5** | $HCO_3^- + H^+ + 2NH_4^+ + 2OH^- \rightarrow CO_2^{3-} + 2NH_4^+ + 2H_2O$ |
| **Step 6** | $Ca^{2+} + Cell \rightarrow Cell - Ca^{2+}$ |
| **Step 7** | $Cell - Ca^{2+} + CO_2^{3-} \rightarrow Cell - Ca^{2+} + CaCO_3$ |

*Table 1. Urea hydrolysis chemical reaction steps (Castro-Alonso et al., 2019).*

A broad range of kinetic parameters for Carbonic Anhydrase (CA) and Urease (UE) have been reported. These include pH; that has an influence on the affinity of Urease to its substrate and also effects the activity of *Sporosarcina pasteurii* enzymes (Van Vliet et al., 2004). It was found that Urease activity decreases at a pH of 7 and increases when the pH is increased to about 8.3-9.0 (Wang et al., 2012). In an experiment, the UE activity of immobilized and free recombinant *E. coli* (HB101) was compared. The results showed that higher concentrations of substrate are required by immobilized UE as compared to free cells. These results suggest that because of inadequate concentrations of substrate, bacteria will be under stress in bio-concrete conditions. (Bachmeier et al., 2002)

Furthermore, UE and CA from bacillus megaterium were characterized, and the function and effect of CA on Urease activity was confirmed and the significance of certain factors such as pH, temperature, calcium ions concentration, and nucleation sites availability was also highlighted (Anbu et al., 2016).

Besides, the durability of the formed crystals is also influenced by their morphology. Some of the biomineral polymorphs include aragonite, calcite and vaterite, that have distinct crystallization types namely, orthorhombic, rhombohedral, and hexagonal (Van Tittelboom et al., 2010).

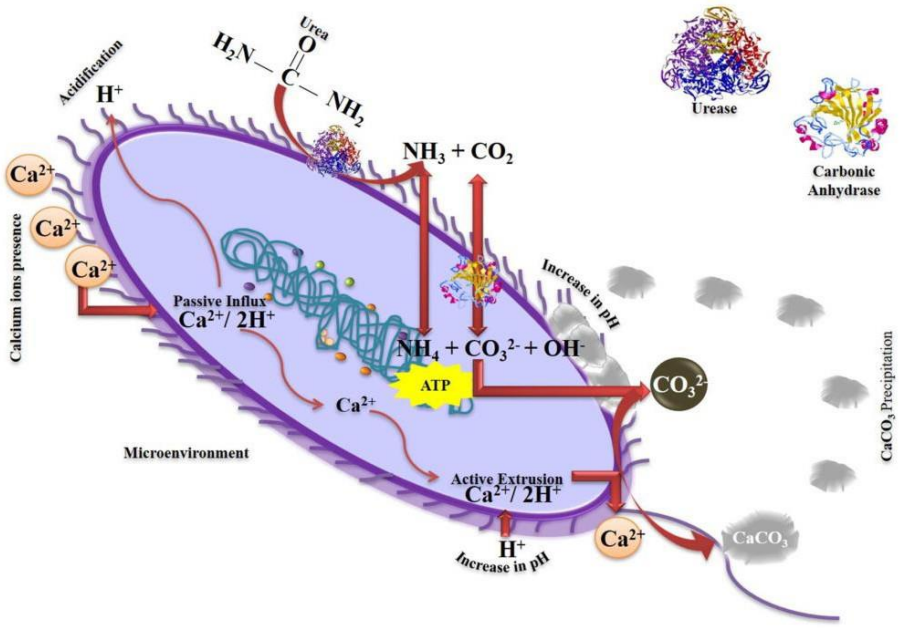



Figure 2. The schematic representation of hydrolysis of urea (Mastromei, 2000).

**Amino acid ammonification**

Amino acid ammonification is another bacterial mechanism involved in MICP. In this process, amino acids metabolism is facilitated by microbial activity which produces ammonia and CO2. Ammonia hydrolysis results in the production of $OH^-$ and $NH_4^+$ ions which accumulate in the surroundings of cell. This causes the supersaturation of ions which ultimately results in the calcite participation (formation of $CaCO_3$) (Zhu & Dittrich, 2016).

*Table 2. Amino acid ammonification chemical reaction steps (Rodriguez-Navarro et al., 2003).*

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

A bacterium named *Myxococcus xanthus* during its growth has been found using this process in both solid and liquid mediums. Moreover, this bacterium is responsible for the precipitation of uranium as meta-autunite, which causes the protection of concrete structures when exposed to radioactive material (Pflock et al., 2005).

**De-nitrification**

This process involves the use of $NO^{3-}$ as final acceptor of electron which oxides the organic matter and consequently favours the precipitation of calcium carbonate (MICP). In the presence of soluble $Ca^{2+}$ ions, bacteria produce an alkaline environment by the absorption of $H^+$ ions and by formation of CO2, NO2 and $OH^{-\cdot}$ (Ruan et al., 2019).

*Table 3. De-nitrification chemical reaction (Achal & Mukherjee, 2015).*

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

It has been studied that addition of denitrified clay particles caused the immobilization of bacterial strains such as *Diaphorobacter nitroreducens* or *Pseudomonas aeruginosa*

which in turn reduces the permeability of cracks by 47 and 42%, respectively and closure of microcracks of size range from 200 to 250 micrometre (Sarayu et al., 2014). Incorporation of special denitrifying particulates known as "Activated compact denitrifying core (ACDC)" results in an increased cracks healing (greater than 70%) of size more than 400 micrometre. However, it has been found that the efficiency of ureolysis in calcite precipitation is more than the denitrification mechanism as the accumulation of by products such as nitrous oxide and nitrite appears to be toxic for the latter process (Sarkar et al., 2019).

## 2.6.2 The Sulphur Cycle

**Dissimilatory Sulphate reduction**

Sulphate reducing bacteria (SRB) in presence of calcium induces the production of calcium carbonate minerals in anaerobic environments (rich in organic matter) by dissimilatory sulphate reduction process (Schreier et al., 1989). Removal of sulphates from gypsum in *Desulphovibrio sp.* is responsible for calcite precipitation through the three different mechanisms named as; diffusion, dissolution, and calcium carbonate precipitation (Seifan et al., 2018). Owing to the removal of sulphide in alkaline environment, a sulphate reduction reaction takes place between $Ca^{2+}$ ions released from the gypsum and CO2, which results in precipitation of CaCO2 (Seifan et al., 2018).

*Table 4. Dissimilatory Sulphate Reduction chemical reaction (Achal et al., 2011).*

| Reaction Steps | Reaction Equation |
|---|---|
| **Step 1** | $6CaSO_4 + 4H_2O + 6CO_2 \rightarrow CaCO_3 + 4H_2S + 2S + 11O_2$ |

A decrease in permeability of water by 8.5% and increase in the concrete' compressive strength has been reported upon incorporation of sulphate reducing bacteria (SRB) in concrete matrix (Golovkina et al., n.d.). Moreover, the addition of SRB which is isolated from the domestic acidic water has demonstrated an increase in the flexural strength by 52.30% and compressive strength by 60.78% (Golovkina et al., n.d.).

However, this mechanism for MICP is limited because of the production of H2S which corrodes the structure of concrete. Partially oxidized sulphur or elemental sulphur species

which are recognized as corrosion product of concrete structure are formed as a result of interaction between H2S and oxygen (Wen et al., 2019). Sulphate reducing bacteria have shown advancement in calcite formation by its interaction with cyanobacteria. Cyanobacteria has appeared to improve the SRB precipitation kinetics since it produces the extracellular polymeric substances (EPS) as its nucleation sites which facilitates the mobility of $Ca2^{+}$ ions and also affects the diffusion barrier (Mitchell et al., 2018). This microbial consortium of cyanobacteria and sulphate reducing bacteria has promising applications in bio-concrete industry, however, more research is required in this regard to combine the anaerobic SRB with oxygen producing cyanobacteria.

### 2.6.3 Photosynthesis

The possibility of MICP by processes that are autotrophic, namely methane oxidation and photosynthesis, have also been identified. In an aquatic environment, the major microorganisms that are involved in MICP are the photosynthetic Cyanobacteria and Micro-algae (Rodriguez-Navarro et al., 2003). Precipitation in these micro-organisms takes place due to exchange of HCO-3HCO3- and CO2-3CO32- exchange. First, through the cell membrane, HCO-3HCO3- diffuses and once inside the cytosol, it dissociates into $OH^-$ and CO2, Carbonic Anhydrase catalyses this reaction (Qabany et al., 2012). This results in pH increase because OH- is generated, this, in reaction with $Ca^{2+}$ ions that are found in the microenvironment stimulates MICP.

*Table 5. Photosynthesis chemical reaction steps (Achal et al., 2011).*

| 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** | $2HCO_2^- \leftrightarrow CO_2 + CO_2^{3-} + H_2O$ |

However, for photosynthetic microorganisms to be used in bio-concrete, exposure to $CO_2$ and sunlight must be present as they are the key factors for photosynthesis.

## 2.6.4 Methane oxidation

The methane oxidizing bacteria are largely responsible for maintaining the concentration of carbon dioxide under anoxic as well as aerobic conditions, in freshwater and marine sediments. When the conditions are aerobic, i.e., when oxygen is present, the enzyme methano mono- oxygenase converts methane to methanol (Anbu et al., 2016). In the cell periplasm, numerous enzymatic processes convert methanol to formate, furthermore, when formate and formic acid are in equilibrium, formic acid is oxidized to $CO_2$ by the activity of formate dehydrogenase (Seifan et al., 2018). The carbon dioxide formed converts to $CO_3^{2-}$ and therefore, precipitation of calcium carbonate occurs around cells in the presence of $Ca^{2+}$ ions.

*Table 6. Methane Oxidation chemical reaction steps (Achal et al., 2011).*

| 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** | $Ca^{2+} + CO_2 + 2OH^- \leftrightarrow CaCO_3 + H_2O$ |

Besides, anaerobic methanotrophic microorganisms also form bicarbonate when methane anaerobic oxidation takes place. In this process, sulphate is the final electron acceptor, and this process occurs in the presence of $Ca^{2+}$ions (Sarayu et al., 2014).

## 2.6.5 Catabolism of Fatty acids

To mediate the bacterial induced calcite mineralization (BICM) in *B. subtilis*, the process has been categorized into three mechanisms depending on the presence and absence of urea. One process takes place through urea hydrolysis whereas the other two occur in urea-deprived growth medium either through interaction with fatty acid metabolism or by the production of techoic and dipicolinic acids (Zhang et al., 2015).

It has been found that the importance of participation of several operons (containing *etf, yus and yko* genes) in cacite precipitation has been highlighted as a result of certain mutants in urea-free growth media. In this scenario, *lcfA* operon which is composed of five genes named as *lcfA*, *fadB*, *fadR,etfA* and *etfB* has gained more attention of the researchers. A putative flavoprotein encoded by *etfA* showed its involvement in the calcium carbonate precipitation in urea-deprived media as well as in the fatty acid metabolism. This depicts the association of fatty acid metabolism and calcite precipitation as *Bacillus Subtilis* contains most of the genes that are involved in fatty acid metabolism (Perito et al., 2014).

It has been investigated that in *etfA* mutants, there is a comparatively greater release of hydrogen ions outside the cells and not being used by fatty acid metabolism which limits the calcite precipitation as well in mutant strains as compared to the wild type strains (Mitchell et al., 2018). *etfA* has also been found to have role in the membrane composition of phospholipids as mutation in its gene changes the membrane composition as well as limits the calcium carbonate precipitation (Seifan et al., 2018). Depending on the nature of culture conditions, it is evident that *Bacillus Subtilis* has been involved in inducing calcite precipitation through different mechanisms (Perito et al., 2014).

## 2.7 Isolation of alkaliphilic calcifying bacteria and their feasibility for enhanced $CaCO_3$ precipitation in bio-based cementitious composites

The Passive pathway of calcite precipitation consists of the following:

- Nitrogen cycle (amino acid ammonification, dissimilatory nitrate reduction, uric acid or urea degradation)
- Sulfur cycle (dissimilatory sulfate reduction) and,

The active pathway of calcite precipitation involves:

- Organic matter oxidation when calcium and dissolved or gaseous oxygen is present.

As compared to the passive pathway of calcite precipitation, the active pathway is seldom investigated. The limitation of nitrogen cycle is that it produces ammonia which is harmful to the climate as well as to concrete (Sarayu et al., 2014).

In our previous research, an alternative method was adopted to get rid of sulfide formations and toxic ammonia. An active pathway of calcite precipitation, known as organic matter

oxidation, was implemented to screen the calcite precipitating microbes that are particularly alkaliphilic (Anbu et al., 2016).

In the first step, screening for calcifying bacteria was carried out, and about 24 strains of bacteria secreted calcite on Calcite Precipitation Media (CPM) plate and also in another method known as stationery falcon method. Halos were produced around the inoculation of bacteria on CPM agar plate, by bacterial strains that were calcifying along with the formation of crystals as shown below. These crystals were further dried and their chemical verification was performed by adding acetic acid which resulted in effervescence, thus the presence of $CaCO_3$ was confirmed (Shaheen et al., 2021). Moreover, the dried powder was further confirmed for the presence of calcite by dissolving in hydrochloric acid (HCl). From the bottom of the falcon tubes, the precipitated crystals were filtered out, then dried and the chemical detection confirmed that they were precipitates of calcite.


Figure 3. The stepwise identification of calcifying bacteria (Shaheen et al., 2021).

Then, using different calcium sources, calcite precipitation for 24 bacterial strains was quantified at a pH of 10. Calcium sources that were used include calcium acetate and calcium lactate. As compared to 7 days, calcite precipitation was higher at a period of 14 days (Shaheen et al., 2021). In addition, the rate of calcite precipitation via the active pathway is

much slower as compared to urea hydrolysis. With calcium lactate, greater calcium precipitation was observed as compared to calcium acetate (Shaheen et al., 2021). Finally, based on the amount of calcite precipitation, 10 strains that possessed the highest calcite secretion potential were chosen for further research.

The quantification protocol of Calcium carbonate was formed to screen out the high strength calcifying microbes at different pH. Furthermore, results of gram-staining showed that all of the 10 bacterial strains were gram-positive, with three being spore formers and the remaining were non-spore formers (Shivangi Mathur, 2018). Spore-forming bacteria can survive in harsh environments. Moreover, most of the strains did not show any hydrolysis of urea and were able to precipitate calcite through the active pathway.

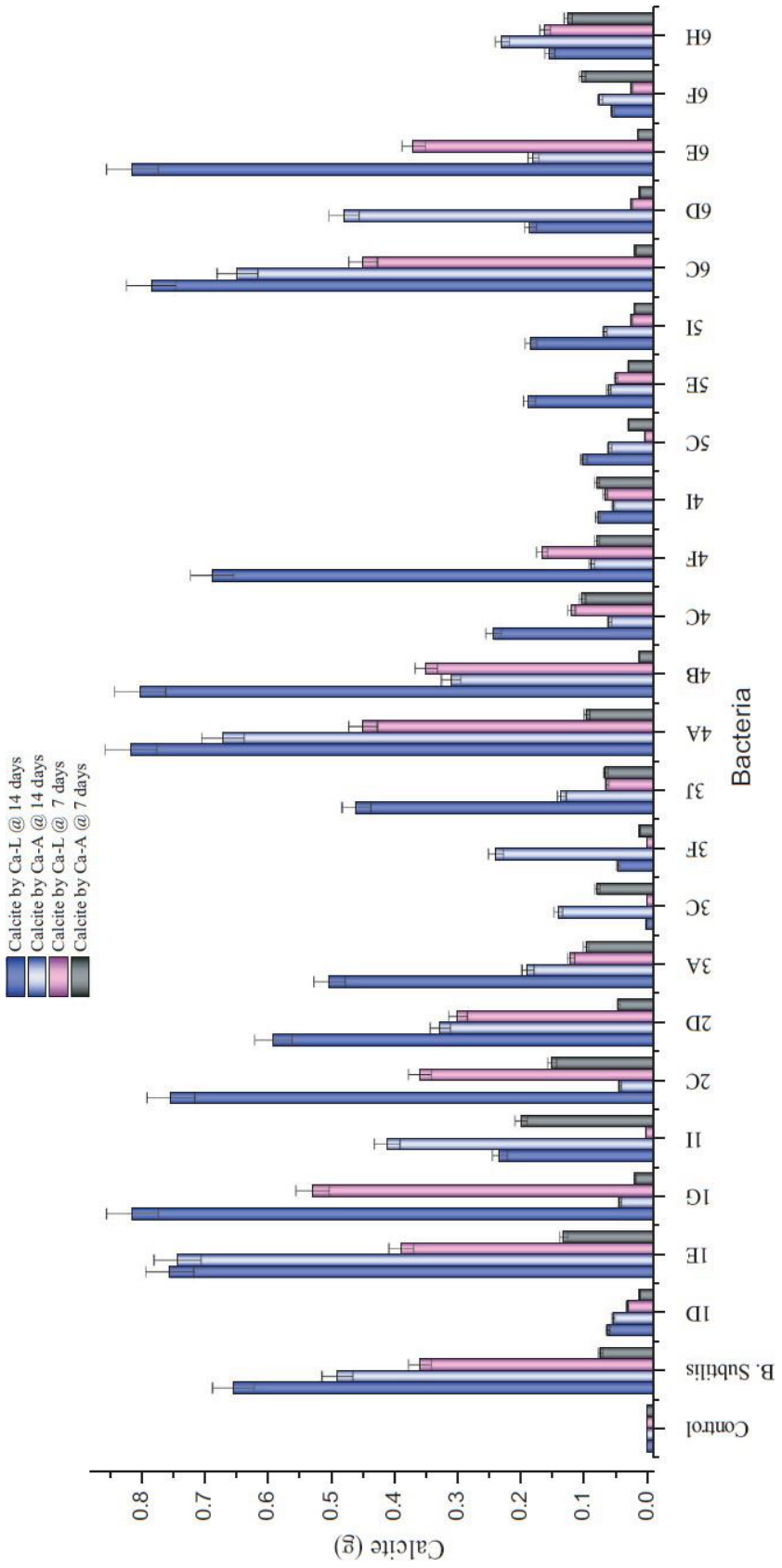



*Figure 8. Calcite precipitation quantification by using different calcium sources (calcium lactate and calcium acetate) at an interval of 7 and 14 days (Shaheen et al., 2021).*

The predominant strains were identified as bacillus species. At a pH of 10, calcite precipitation was increased to about 24.32% in *Bacillus safensis* whereas in *Arthrobacter luteus*, calcite precipitation increased to about 58.2% at pH 7 (Shaheen et al., 2021). In addition, during the process of precipitation, the pH of the system increased due to the calcifying strains.

It should be noted that, species of *Arthrobacter, Planococcus and, Bacillus* have already been identified as producers of calcium carbonate whereas chryseomicrobium and Glutamicibacter were identified for the first time as precipitators of $CaCO_3$ in the research being mentioned (Páramo Aguilera et al., 2015). The strains are capable of enhancing the strength of cementitious material through climate-friendly pathway (Zhang et al., 2015). It was recommended in the mentioned research that pH change should be monitored continuously during the process of precipitation.

In order to characterize the precipitated calcite powder Scanning electron microscope (SEM), X-ray diffraction (XRD) and Thermogravimetric analysis (TG) were performed which results in the classification of calcium carbonate polymorphs as calcite and vaterite (Perito et al., 2014). SEM was complimented with EDS and it was quite evident from the result that varying nature of calcium sources affects the crystal morphology of calcium carbonate precipitates (Jroundi et al., 2010). $CaCO_3$ crystals are of rhombohedral form in case of calcite and when calcium source is of calcium acetate while of spherical form in case of calcium lactate as source.

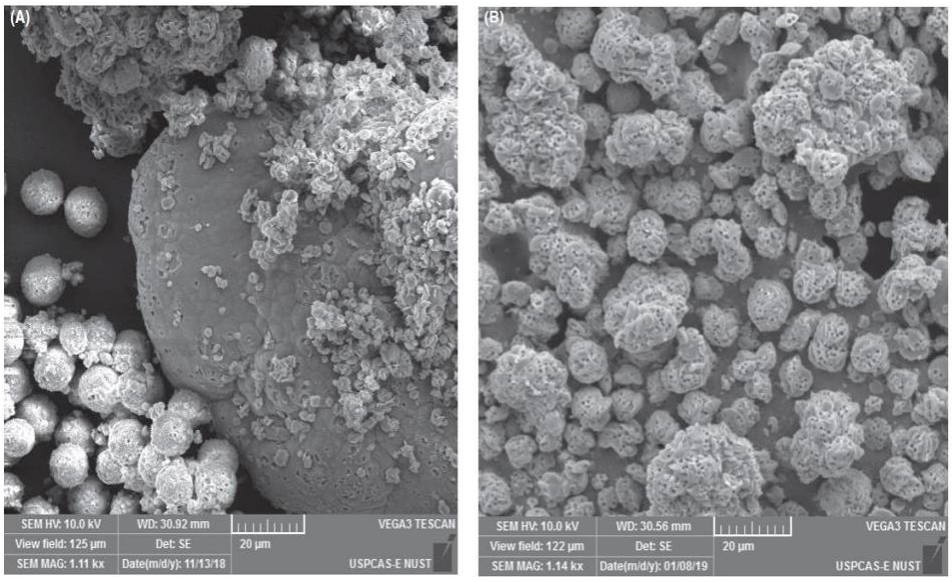



*Figure 9. Representation of $CaCO_3$ crystal morphology through SEM by using different calcium sources (A: Calcium Lactate, B: Calcium Acetate (Shaheen et al., 2021)*

It was also depicted from the SEM analysis that size is also affected by varying the source of calcium as the size of calcium lactate precipitates was smaller than that of calcium acetate ones (Shaheen et al., 2021). Different bacterial strains also affect the nature and morphology of $CaCO_3$ precipitates.

For further confirmation, XRD was performed to identify the structure of precipitates. Vaterite, with time transforms into calcite as it is one of the unstable forms of calcium carbonate (Achal & Mukherjee, 2015). After 14 days of incubation, conversion of vaterite to calcite was reported.

TG analysis of the calcite precipitates as a result of bacterial activity demonstrates that at temperature range of 600-850°C, there occurs a weight loss of 38.97% in the healing compound (CaCO3) (Shaheen et al., 2021). This certifies that the chemical analysis of the crystals comprised of calcium carbonate as it decomposes into calcium hydroxide and carbon dioxide at temperature of 600 to 850° C.

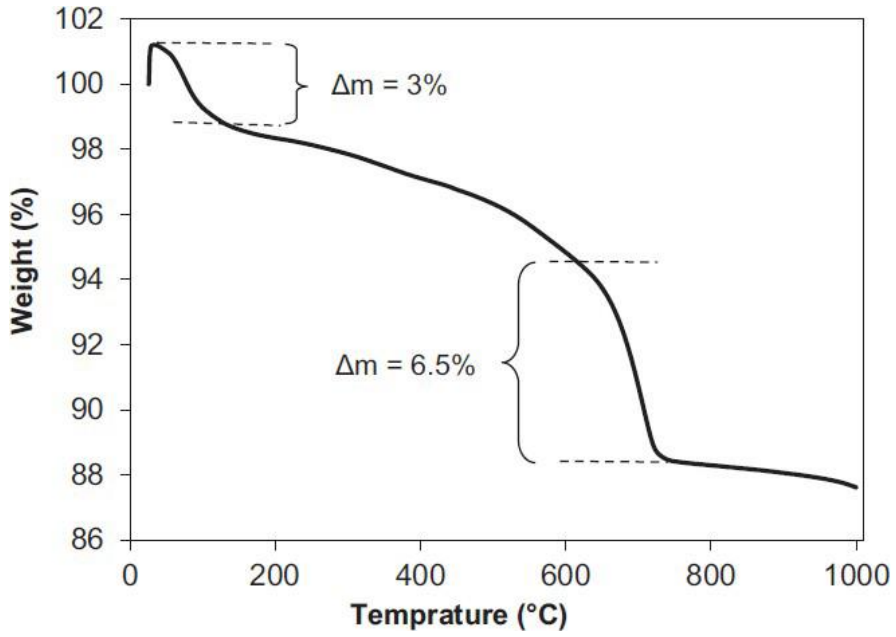



Figure 4. Thermal decomposition curve of $CaCO_3$ precipitates at a temperature range of 600-800 degree Celsius (Shaheen et al., 2021).

Furthermore, a total of 10 bacterial strains were tested for mechanical evaluation in cement matrix. All bacterial strains showed high mechanical strength. It is evident that both flexural strength and compressive strength were increased by 96 and 36%, respectively (Zakari et al., 2016). The attribution of increase in the above-mentioned strengths of cementitious matrix should be given to the deposition of bio-calcite. This improvement in strength is due to the refinement in pore formation of cement matrix. Among all, Arthrobacter and Bacillus species demonstrated higher mechanical strength (Shaheen et al., 2021).

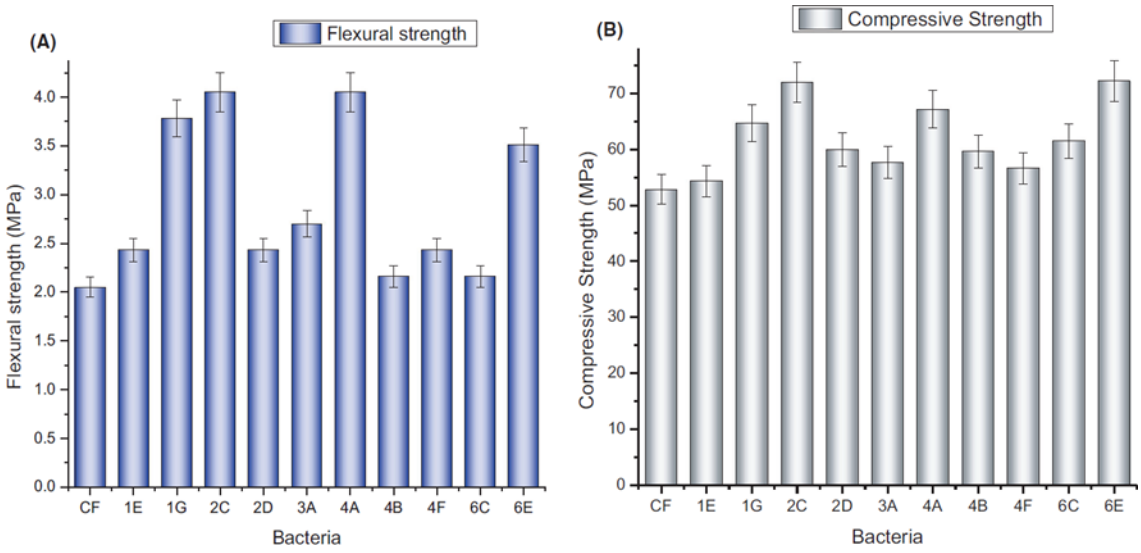
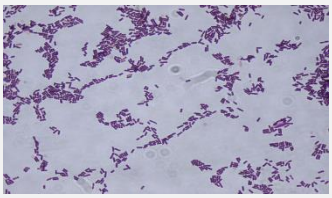
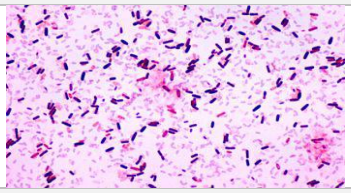
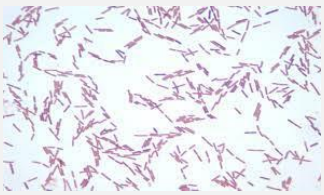
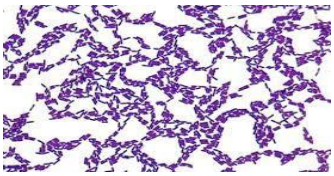
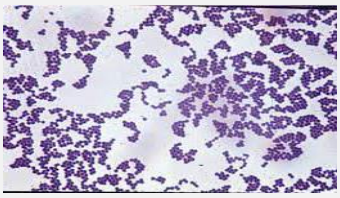
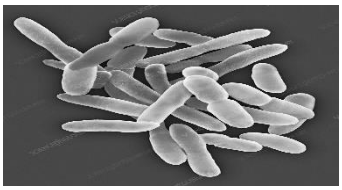


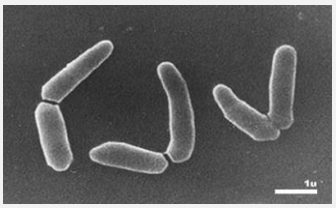
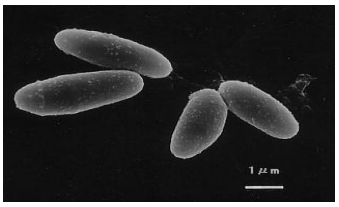
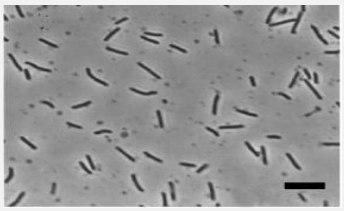

*Figure 11. Mechanical evaluation of cement matrix (A: Flexural Strength, B: Compressive Strength) (Shaheen et al., 2021*

## 2.8 Major Calcite precipitating bacteria and their characteristics.

The calcium carbonate precipitating bacteria along with their characteristics include:

*Table 7. Characteristics of calcifying bacterial strains.*

| Bacteria | Characteristics | |
|---|---|---|
| ***Bacillus Subtilis*** | These are rod-shaped, Gram-positive, spore-forming, flagellated, heterotrophic bacteria, with a circular chromosome located in the nucleoid region. |  |
| ***Bacillus pumilus*** | These are rod-shaped, Gram-positive, endospore forming, aerobic bacteria that are mostly present in the soil. |  |
| ***Bacillus australimaris*** | These are rod-shaped, Gram-positive, spore-forming, obligate aerobes. These are motile and form colonies that are circular. |  |
| ***Bacillus safensis*** | These are rod-shaped, Gram-positive, spore-forming bacteria. They are also aerobic chemoheterotroph. |  |
| ***Planococcus plakortidis*** | These are coccus shaped, Gram-positive, mesophilic, aerobic bacteria, that are motile and were first isolated from Plakortis simplex, a marine sponge. |  |
| ***Arthrobacter koreensis*** | These are rod-shaped, Gram-positive, catalase-positive, aerobic and coryneform bacteria. |  |

| | | |
|---|---|---|
| ***Arthrobacter luteolus*** | These are rod-shaped, catalase-positive bacteria that have an oxidative metabolism. |  |
| ***Corynebacterium efficiens*** | These are Gram-positive, glutamic acid producing bacteria that are non-motile. |  |
| ***Chryseomicrobium amylolyticum*** | These are Gram-positive, rod-shaped bacteria that are non-motile, and have been identified in semi-arid soil in India. |  |

*B. subtilis* is a model bacterium which is most often used in the laboratory, it is able to form precipitates of calcite on media provided with a suitable calcium source (Dupraz et al., 2009).

## 2.9 Bacterial Gene Cluster Involved in Calcium Carbonate Biomineralization

In a research, calcium carbonate formation by *B. subtilis* was studied, and the genes that are involved in the process of biomineralization were identified. In order to investigate the genes involved in calcite precipitation, mutants of *B. subtilis* were formed by a technique named as UV mutagenesis using pMUTIN plasmid (Páramo Aguilera et al., 2015). 1190 UV mutants of

*B. subtilis* were retrieved from the European consortium of *B.* subtilis Functional Analysis (BFA) which in turn facilitated the isolation and identification of strains mutated in the precipitation phenotype.

A cluster of five genes such as *lcfA, ysiA, ysiB, etfB,* and *etfA* which has been recognized as *lcfA* operon was appeared to be involved in the precipitation of calcium carbonate crystals (Perito

et al., 2014). *B. subtilis* strains containing the *lcfA* operon i.e, each of the above mentioned five genes were altered by UV mutagenesis.

Upon setting up of experiment, it was demonstrated that all of the strains carrying the altered *lcfA* operon were unable to produce the $CaCO_3$ crystals, with the exclusion of the non-mutated ones (Tojo et al., 2011). It has been reported that the most significant role performed by the *lcfA* operon is by its last gene identified as *etfA*.

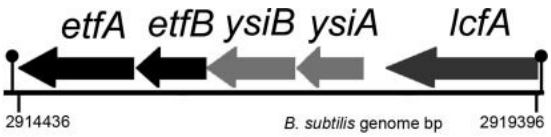



Figure 5. LcfA operon gene cluster (Perito et al., 2014).

Owing to the putative nature of the genes of *lcfA* operon, result of the research indicates an association between the calcite precipitation by bacteria and fatty acid metabolism (Bachmeier et al., 2002).

The *B. subtilis* primers used for carrying out PCR in the mentioned research are listed as follows:

*Table 8. Primers for genes of LcfA operon in B. subtilis (Perito et al., 2014).*

| 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.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Phylogenetic Analysis (16S rRNA sequencing)

Our group extracted out 10 strains of calcite producing bacteria and performed 16S rRNA sequencing of the PCR product of these bacteria and checked homology in their sequences via BLAST. Among the strains, *Bacillus* species were the most common and other common species were *Arthrobacter* and *Chryseomicrobium*. Species left belongs to *Planococcus, Glutamicibacter, Corynebacterium.*

*Table 9. Following table shows the homology between sequences performed via BLAST.*

| ***Strain*** | ***Bacteria*** | ***Similarity*** | ***Accession Number*** |
|---|---|---|---|
| *1G* | *Bacillus safensis* | 98.92% | MN865802 |
| *4A* | *Bacillus pumilus* | 99.44% | MN865840 |
| *4B* | *Bacillus australimaris* | 99.63% | MN865842 |
| *2C* | *Glutamicibacter mysorens* | 97.99% | MN865826 |
| *6C* | *Arthrobacter koreensis* | 99.29% | MN865864 |
| *2D* | *Planococcus plakortidis* | 97.78% | MN955850 |
| *1E* | *Corynebacterium efficiens* | 98.18% | MN865795 |
| *6E* | *Arthrobacter luteolus* | 99.29% | MN865845 |
| *3A* | *Chryseomicrobium amylolyticum* | 98.09% | MN865977 |
| *4F* | *Chryseomicrobium imtechense* | 97.27% | MN867026 |

## 3.2 Basic Local Alignment Tool

One of the most important tools in bioinformatics is BLAST also known as Basic Local Alignment Search Tool. By using this tool, we compare the biological sequence information for example we can compare nucleic acid sequences, protein sequences. There is a library of database of sequence available at blast and that can be compared with the protein or nucleotide sequence that is also called a query. For instance, there is an unknown gene in a mouse, whose sequence is compared with that of human gene sequence to see if humans have that gene. If the similarity is found, we can say that the function performed in human genome by that gene can be like that in mouse. This all is based on the similarity found in the sequences.

### 3.2.1 Process

BLAST finds similarity in sequences by using heuristic as a method. It basically identifies the short matching sequences in the two sequences. This whole process of finding same sequences is known as seeding. Words are very important in this case; these are the sets of common letters.

We took the gene sequences of *lcfA, etfA, etfB* of *B. subtilis* and performed blast against the whole genome sequences of *B. safensis* and *B. australimaris*. The sequence showing the similarity was downloaded for primer designing.

## 3.3 Primer3 (a web interface)

We use primers in a lot of biological research especially the polymerase chain reaction aka PCR. It is very crucial for a primer to bind the exact target sequence of the gene because primers are the target where DNA replication starts. There are several factors which play a vital role in the binding of primers to sequences, and these are the melting temperature denoted as Tm, GC content of the primer that how much guanine and cytosine is present and also the self-complementarity. Several online programs are available to design primers. Among them we used

We put the Fasta, sequences of our genes, primer3 designed primers for us and also told us about the melting temperature and GC content of our primers.

Primers for bacillus subtilis were taken from a research paper published.

*Table 10. Primers for B. subtilis, B. safensis, B. australimaris*

| Sr. No. | Bacteria | Genes | Primers | | Amplicon size | Nucleotides |
|---|---|---|---|---|---|---|
| 1. | *Bacillus subtilis* | *lcfA* | F | CCGCTTGGATGTATGACG | 573 | 18 |
| | | | R | CTCTCAGCACTGCGGCGG | | 18 |
| | | *etfA* | F | TGAGAGGTGTTATCGAGC | 540 | 18 |
| | | | R | GAGTAACGACTTTCCCGG | | 18 |
| | | *etfB* | F | GATTCAGGATGACGGAGC | 576 | 18 |
| | | | R | TCAGCATCCTCTTCGTCC | | 18 |
| 2. | *Bacillus safensis* | *lcfA* | F | ATTTGACGCAGCTGATACCC | 569 | 20 |
| | | | R | GGGCTTCTTCGACCTCTCTT | | 20 |
| | | *etfA* | F | TCTGGAAAAGCCTTCGAAAA | 515 | 20 |
| | | | R | AACAAGTCACCGACGATTCC | | 20 |
| | | *etfB* | F | GAAGAAGCCATTCAGCTTCG | 522 | 20 |
| | | | R | TGGTACATCGTCTTCGTCCA | | 20 |
| 3. | *Bacillus australimaris* | *lcfA* | F | CTTTTCGATGTTCGGGTGAT | 541 | 20 |
| | | | R | GACAAGCATGGAGCACTTGA | | 20 |
| | | *etfA* | F | TGTTGATGGCCACAATGACT | 562 | 20 |
| | | | R | GACTTGTCGCCGAAAATAGC | | 20 |
| | | *etfB* | F | ATGGATAGCGAGGCTCATTG | 535 | 20 |
| | | | R | CGTACGTTTGACACGGAAGA | | 20 |

## 3.4 *In silico* PCR

*In silico PCR* is based on the theoretical reaction of polymerase chain reaction using computational tools present online. This is used to check the amplification of the primers for the desired DNA. There are basically two factors involved in the optimization of primers that

are selectivity and efficiency. Efficiency is based on factors such as the GC content, how the primer binds to the DNA, the structure, and the melting and annealing temperature. This is used to predict the PCR product using the primers. *In silico* PCR also gives some other information about the length of amplicons, location of the primers etc.

## Wet lab Analysis

For wet lab, we started with the revival of bacteria to make sure that our new culture contains the same DNA, which was stored in glycerol. To recover the bacteria, we opened the tube and with the help of a sterile loop, we took some of the frozen bacteria and streak it on the agar plate. We used three bacterial strains that are *B.safensis, B. australimaris*, and *B.subtilis*. we revive the bacteria for 15 minutes on ice. After reviving the bacteria, we vortexed the bacteria samples for equal mixing.

## 3.5 Streak Plate Method

We used streak plate method to obtain the pure culture of our desired strains from a mixed population. We streaked the plate in such a way that our bacteria thinned out and the resultant bacterial cells are separated.

### 3.5.1 Principle

The main principle of streak plate method is to obtain isolated colonies. By streaking, the original sample is diluted to such an extent that there is only one bacterial cell spaced at a difference from the other and when that bacterial cell divides and produce enormous daughter cells, a new isolated colony form. By using that isolated colony, we can obtain our pure cultures.

### 3.5.2 Materials

1. Ethanol
2. Bacterial culture
3. Sterile loop
4. Spirit lamp
5. N-agar plates

### 3.5.3 Procedure

1. We sprayed ethanol to avoid contamination. In the laminar flow hood, we sterilize our loop by heating it until it is red hot.
2. After cooling the loop, we pick our bacteria from the stock culture and streak it over the N-agar plate in such a way that it does not gouge the agar plate.
3. After streaking the plates, we incubate our plates at 37°C for overnight growth.

### 3.5.4 Results

The colonies obtained should have the same appearance. If they do not have the same general appearance, then the colonies should be streaked again to get the pure culture.

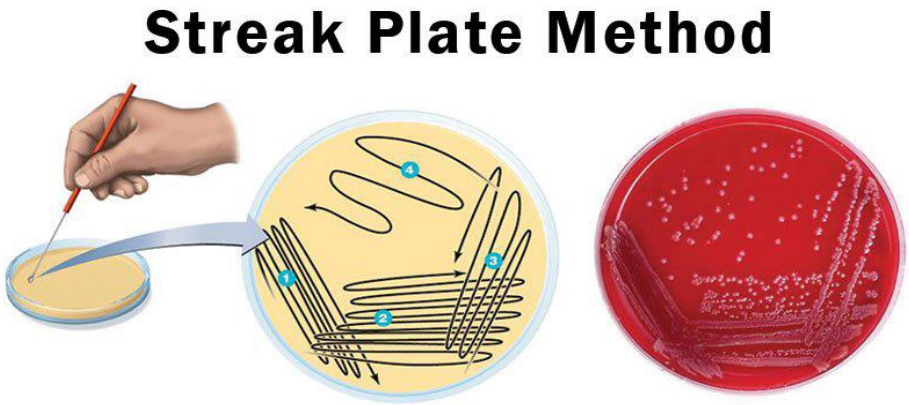



*Figure 13. Streak Plate Method.*

## 3.6 Gram Staining

Gram staining is the technique which we use to differentiate between two largest groups of bacteria that gram positive and Gram-negative bacteria by the help of dyes that colours the cell walls of bacteria either pink or purple. Gram-positive bacteria are stained purple which is attributed to the hefty layers of peptidoglycan which are present in their cell wall. While the pink colour of gram-negative bacteria is because of the presence of thin layer of peptidoglycan.

In staining three processes are involved basically. First process is the staining with crystal violet, fixing with mordant, decolorizing, and counterstaining.

Crystal violet gives the characteristic purple to the Gram-positive bacteria, after decolorizing. While after treating with primary dye and after decolorizing, Gram-negative bacteria lose their primary dye and take the secondary dye.

When the cells are stained with crystal following fixing with gram iodine which fixes the dye with cell walls and is mordant. There is the formation of a complex molecule having both crystal violet and iodine. After that decolourizer dehydrates the layer of peptidoglycan. Because of the thick layer crystal violet-iodine complex does not get a chance to escape the cell wall and get trapped in it. However, Gram-negative bacteria have a very low amount of peptidoglycan, so the complex formed between iodine and crystal violet can easily go out of the cell upon decolorizing and after counterstaining retain the counter stain which is safranin.

## 3.6.1 Materials

1. Crystal violet acting as the primary dye colours the cell purple.
2. Gram iodine which fixes the crystal violet to the cell and is mordant.
3. Decolorizing Agent for example Ethanol or Acetone.
4. Counter stain that is Safranin.
5. Water for washing purposes.
6. Droppers.
7. Bacterial culture
8. Slides

## 3.6.2 Procedure

1. Firstly, we made a smear on a slide of our bacterial culture. We heat fixed the sample onto the slide with the help of a spirit lamp.
2. After that, we add crystal violet and let it sit for 1 minute. After that washed the slide with water to let go the unbound stain.
3. Then we added Gram Iodine for 1 minute, so the crystal violet is fixed with the bacterial cell wall. Then washed the slide with water.

4. Then ethanol is added to wash the slide for 5 seconds following the washing with water.
5. After that counter stain safranin is added. And the slide is washed with water after 30 seconds.
6. To check the results that whether the bacteria are Gram-positive or Gram-negative, we performed microscopy.

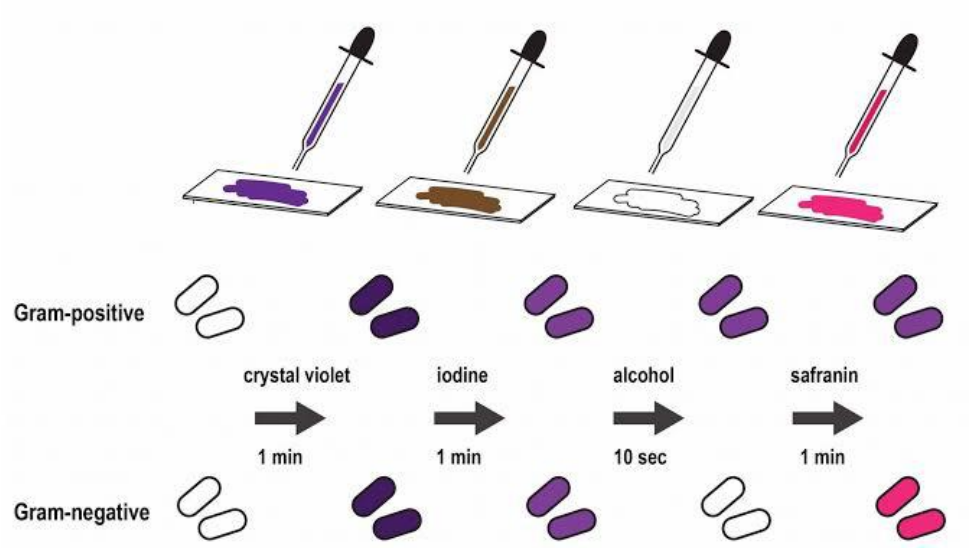



*Figure 14. Gram Staining*

## 3.7 Microscopy

Microscopy is a technique used to visualize the microorganisms because they cannot be seen with naked eye. In light microscopy, light is used as a source of visualization, and it is transmitted from a source that is opposite to the slide carrying the sample. Firstly, the light is passed from 1 of 2 lenses that is condenser lens to focus the light on the sample so that we can have maximum brightness. After passing through the condenser lens, light passed through the objective lens so that the image can be magnified and then go through the oculars, where we can view the magnified image produced.

### 3.7.1 How to Use a Microscope

1. First, we prepared a slide of our sample. We put immersion oil on the smear present on the slide and cover it with cover slip.

2. Then we put the slide on the stage and adjust the stage so that the light is falling onto the sample.
3. By turning the revolving turret, we set the magnification power to 100X.
4. We looked through the objective lens and then eyepiece and turned the focus knob so that the image can come into the focus.
5. For increasing the amount of light, we adjusted the condenser lens and light intensity.

When a clear image of the sample is obtained, we investigated them.

6. The images ensured us that the bacterial strains used are Gram-positive and rod shaped.
7. We took the pictures and removed the slide.

### 3.7.2 Cultural Characteristics of Strains (Colony Morphology)

When the microorganisms are grown on media, they exhibit difference in their microscopic appearance of the growth. These differences are generally called as cultural characteristics and these characteristics are used as a basis for separating the microorganisms into different taxonomic groups. The characteristics that are observed included Shape (circular, irregular, rhizoid), margins (entire, undulate, lobate, serrate, filamentous), elevations (flat, raised, convex, umbonate), pigmentation, optical property (opaque, translucent, transparent), and consistency. In order to observe the cultural characteristics of strains, the strains were first streaked on the agar plates in the manner that well-isolated colonies were appeared and then those isolated colonies of each strain were thoroughly observed for determining the colony morphology.

## 3.8 Primer Dilution

We prepared the stock solutions of primers, we ordered. We received the lyophilized primers. Firstly, master stock of the primer is produced which is then diluted to the working solution. This reduces the number of times we must thaw the primer stock and it is a great way to avoid contamination in the original primer stock.

### 3.8.1 Materials

a. Primers

b. Nuclease free water

### 3.8.2 Procedure

a. Firstly, we vortexed our tubes containing the primers.
b. Then we added 300uL nuclease free water to the primers to make the stock solution

### 3.8.3 Working solution for Primers

a. We spun the stock solutions produced.
b. We prepared the working solution of the primers by taking 10uL primer solution from the stock and mixing it in 90uL nuclease free water.

## 3.9 Colony PCR

We performed Colony PCR to confirm the presence of our desired genes in the bacterial strains.

### 3.9.1 Materials

a. PCR tubes
b. Nuclease free water
c. Bacterial colonies
d. PCR water
e. Master Mix
f. Forward and reverse primers.

### 3.9.2 Procedure

1. We took 20uL nuclease free water in PCR tubes. Then we carefully picked the bacterial colonies with the help of pipette tip from the plate.
2. We mixed them until the solution got turbid.
3. After that, we spun the tubes in a mini spin.
4. Then we prepared the PCR mix with the reaction volume of 25uL. In the PCR mix, 12.5uL is the dreamTAQ master mix, 1uL froward primer, 1uL reverse primer, 2uL

of PCR water having bacterial colonies and nuclease free water was added to complete the volume.

5. Again, we spun the tubes. After spinning, PCR tubes were placed in the thermocycler for 10 minutes at 95°C.
6. Refrigerated centrifugation was carried out at 4°C, 6000 rpm for 3 minutes.
7. Then the PCR reaction was carried out by setting the annealing temperature at 55°C.

## 3.10 Genomic DNA Extraction/Purification

DNA is present in the chromosomes which are present in the cell. The removal of DNA from the cell is basically known as DNA extraction or DNA purification. DNA extraction is used for diagnosing certain genetic diseases and for detecting bacteria and viruses present in the environment. Basic outline of DNA extraction is as follows:

Firstly, we break open the cells containing our DNA of interest via centrifugation or sonication. To break the protein components of the cell, vortexing is done with phenol. Detergent like SDS or triton X 100 are used to break the lipid membranes of the cell. Proteins can also be degraded by the help of a protease. After vortexing with phenol, protein components of the cell come in the organic phase and are easily separated out. And usually, the DNA is present between the interface of two phases that are the organic and inorganic phase. Then DNA is mixed with alcohol, it is insoluble in alcohol and will be separated out of the solution. DNA is washed and then is resuspended in TAE buffer. After that gel electrophoresis s performed to confirm the presence of DNA.

**DNA extraction using Thermofisher DNA purification kit:**

We used Thermofisher kit to separate the DNA of our strains and followed the protocol for DNA extraction of gram-positive bacteria.

Before starting, we prepared the working solution for the **Lysis Buffer** including our detergent Triton X100, Tris HCl, and EDTA.

We required 20mM Tris HCl, 2mM EDTA at pH 8, and 1.2% Triton X100. And after preparing the working solution we will add 20mg/mL lysozyme immediately before use.

### 3.10.1 Calculation for Tris HCl

1M Tris HCl (100mL)

Mass= $\frac{molarity * molar\ mass * volume}{1000}$

Mass= $\frac{1*121.14*100}{1000}$ =12.1g.

We took 12.11g Tris HCl and dissolved it in 80mL distilled water. After dissolving, we added enough water to make the volume 100mL. we autoclaved the solution.

To make the working solution we took the volume 50mL. Our required concentration was 0.02M.

C1V1= C2V2

1M * V1 = 0.02 * 50mL

V1= 1mL

We took 1mL of Tris HCl and then add distilled water to make the volume 50mL.

## 3.10.2 Calculation for EDTA

We already have 0.5M Tris HCl present and we must make 2mM EDTA from that concentration. So, our required concentration is 0.002 M., and our desired volume was 100mL.

C1V1 = C2V2

0.5 *V1 = 0.002M * 100

V1 = 0.4mL

We took 0.4mL EDTA and dissolve in 80mL 99.6mL distilled water.

## 3.10.3 Calculation for 1.2% Triton X100

We took 1.2mL Triton X100 and dissolve in 98.8mL distilled water.

## 3.10.4 Procedure

1. We harvested our bacterial cells in a microcentrifuge tube by centrifugating the sample at 5000g x g for nearly 10 minutes. The supernatant obtained was discarded.
2. We took 180µL of the lysis buffer and resuspend the pellet in it. The solution was incubated for 30 minutes at 37°C.

3. We took 20µL proteinase K and add 200uL of lysis solution prepared in step 2. We mixed both by vortexing.
4. We incubated our sample at 56°C for nearly 30 minutes and used a shaking incubator to the point that our cells are perfectly lysed.
5. 20uL of RNase A solution was added to the previous it, vortexed the solution then incubate at room temperature for 10 minutes.
6. Then we added 400µL of 50% ethanol and mix the solution by vortexing.
7. Lysate has been prepared. We took the collection tube for purification which was present in a purification column. It was centrifuged for 1 minute at 6000 x g. We then discarded the tubed containing the flow through solution. We took the purification column and inserted it into a new 2mL collection tube.
8. After putting the insertion column into a new tube, wash buffer 2 around 500µL in which ethanol was pre-added, added into the DNA. After doing this step, solution was centrifuged at 8000 x g for 1 minute. Again, we throw away the flow through solution produced in the process and add the column into a new tube.
9. Again, 500µL of the wash buffer 2 present in the kit was added to the column and the column was then centrifuged for 3 minutes at 12000 x g.
10. Then we added 200µL elution buffer in the purification column to elute our desired DNA. Solution was incubated at room temperature followed by centrifugation at 8000 x g for 1 minutes.
11. Purification column was discarded. DNA was utilized immediately for gradient PCR procedure.

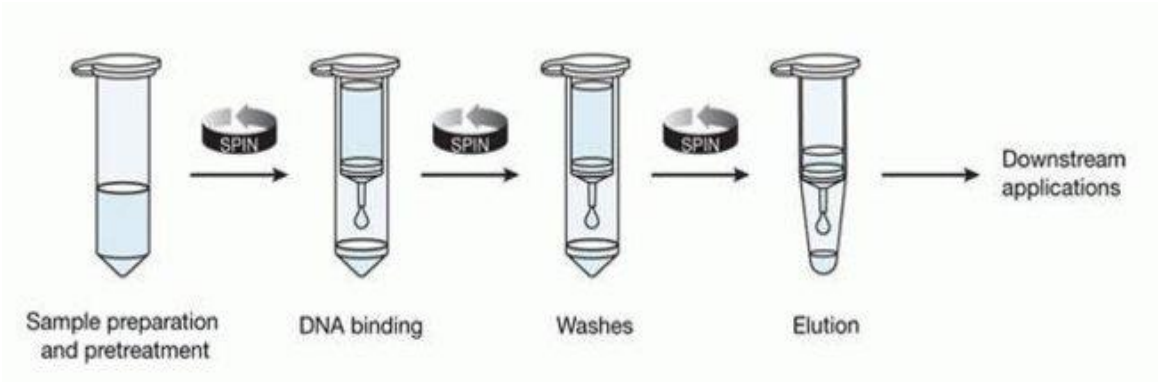



*Figure 15. DNA extraction protocol*

## 3.11 Gradient PCR

PCR is performed to amplify a specific region of DNA with a specific set of primers in a thermocycler. Each primer has its own specific melting temperature, the temperature at which half of the DNA gets denatured. If the primers have similar melting temperature, then it is very easy to perform simple PCR. But we had primers with different melting temperatures, and we wanted to perform PCR for all of the primers at the same time. So, we performed gradient PCR in which different temperatures were set in a gradient along with rows or columns.

a. For this purpose, we set the temperature range of 55-65°C under the section of annealing temperature.

b. Then we adjusted the extension temperature to 72°C.

c. We performed 30 cycles and the total time was 1 hour and 30 minutes.

d. After performing PCR, we performed gel electrophoresis to check the results.

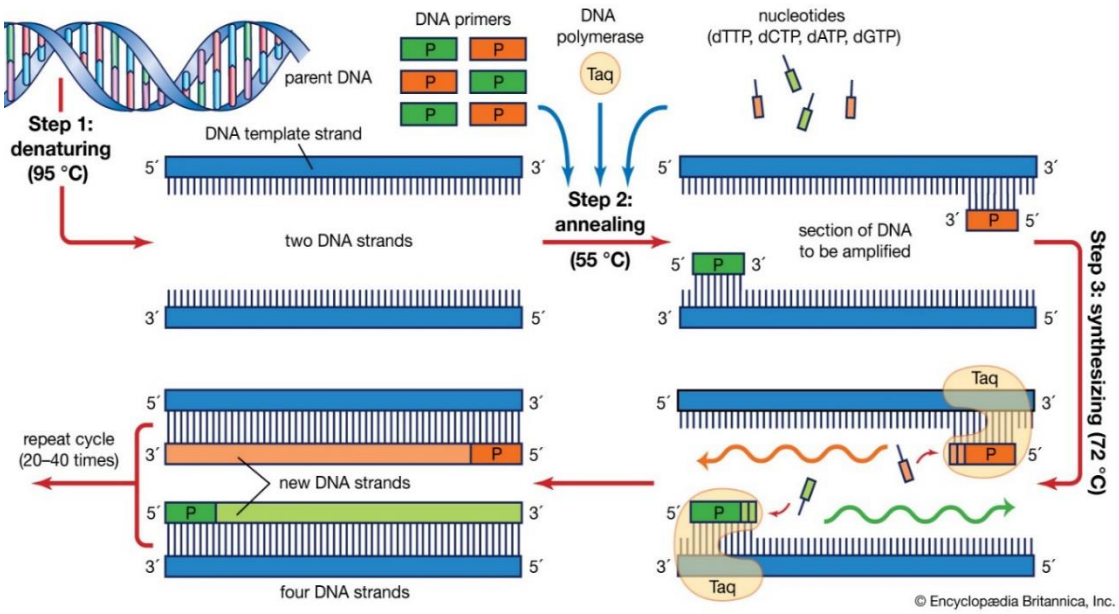



*Figure 16. Gradient PCR*

## 3.12 Gel Electrophoresis

Gel electrophoresis is one of the most common technique used in molecular biology. This is used to separate the DNA based on its size to view the DNA. Gel electrophoresis relies on an electrical field so that the DNA carrying the negative charge can move towards a positively charged electrode through the gel matrix. DNA fragments those are shorter are length can move quickly as compared to longer fragments. By using this we can determine the length of DNA fragments by running them along with DNA ladder.

### 3.12.1 Preparation of Agarose Gel

Agarose gels are the most important component of electrophoresis used to analyse DNA. Agarose is composed of Agar which is a natural product obtained from seaweed. Agarose allows for the diffusion of DNA because it forms a matrix that is porous. When the DNA is loaded in the gel and the voltage is given, negatively charged DNA moves towards the positively charged electrode.

### 3.12.2 Materials

a. Agarose Powder

b. A beaker or any flask.

c. TAE buffer

d. Gel casting tray

e. Oven

## 3.12.3 Procedure

a. To prepare 1% agarose gel, take 0.5g multipurpose agarose.

b. Take 50mL TAE buffer in a beaker and add agarose in it.

c. Heat the solution in the oven until there are no bubbles.

d. Let the solution cool down on its own.

e. Pour the gel in the plate slowly to avoid any bubble formation.

f. After cooling, add ethidium bromide in the gel.

g. Pour the solution in the gel casting tray and adjust the combs.

## 3.12.4 Electrophoresis

### 3.12.4.1 Materials:

a. Casting tray

b. Well combs

c. Current source

d. UV transilluminator

e. TAE

f. Ethidium Bromide.

### 3.12.4.2 Loading Samples and Running an Agarose Gel:

1. Firstly, we place our gel in the gel box.
2. We filled the gel box with TAE buffer.
3. Firstly, we load a DNA ladder in the first well of our gel.
4. Then we carefully loaded our samples into the wells.
5. Then we covered the gel unit. And let the gel run at 80volts for 60 minutes.
6. After running of the gel, we turned off the power and visualize the gel under UV transilluminator.

### 3.12.4.3 Analysing the gel:

We use DNA ladder to determine the size of our bands. After analysing we purify our samples from the gel.

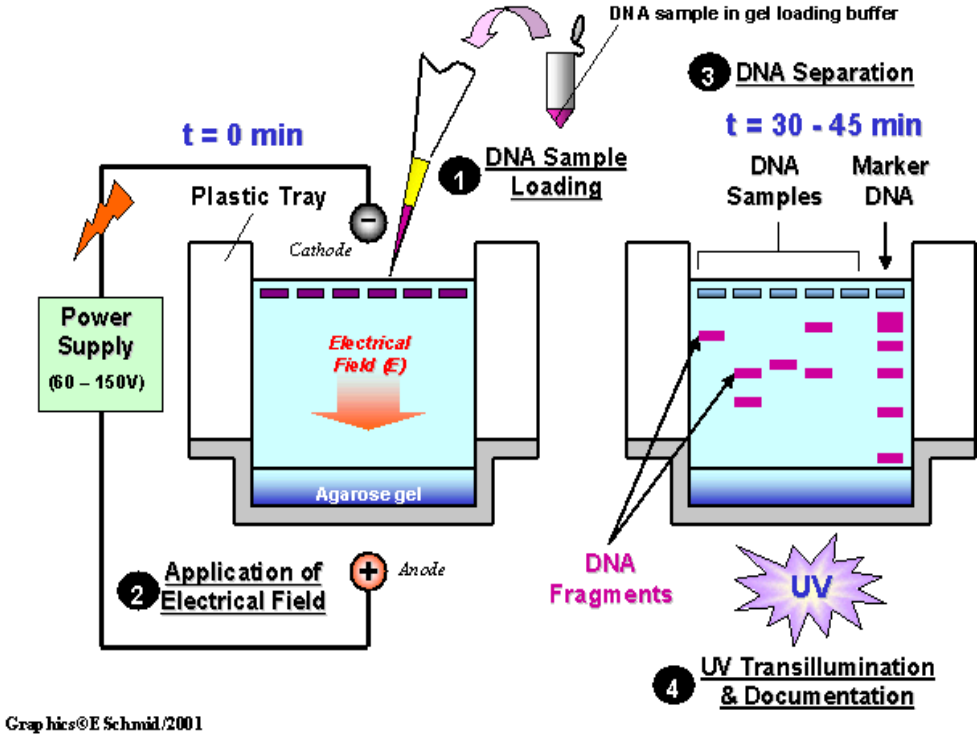



*Figure 17. Gel Electrophoresis*

## 3.13 Biochemical Activities of Isolated Strains

For understanding the biochemical activities isolated strains, two tests were performed: catalase test and oxidase test.

### 3.13.1 Catalase Test

This test is used to differentiate the bacteria that produce catalase enzyme from the non-catalase producing bacteria. Catalase acts as a catalyst in the breakdown of hydrogen peroxide. A strain is tested for catalase production by bringing it in contact with $H_2O_2$. Bubbles of oxygen are released if the strain produces catalase, but the culture should not be older than 24 hours.

To perform the test, glass slide was taken and few drops of catalase $H_2O_2$ (Table: 2.9) were added on the slide. Then a single isolated colony of the strain was picked and emulsified on the

slide containing reagent and observed for the bubble formation. The process was performed for all the strains.

### 3.13.2 Oxidase Test

The oxidase test is used in the identification of those strains that produce the enzyme cytochrome oxidase. Cytochrome oxidase catalyzes the oxidation of a reduced cytochrome by $O_2$ resulting in the formation of water or hydrogen peroxide. The ability of bacteria to produce cytochrome oxidase is determined by the addition of test reagent that serves as an artificial substrate, donates electrons, and thus becomes oxidized to a blackish compound in the presence of oxidase.

To perform the test, a filter paper was taken and properly labeled and then few drops of ρ-amino dimethylaniline (Table: 2.8) was added on the filter paper. Then the colony of the strain was picked with the help of inoculating loop and emulsify on the filter paper and the result was noted. The experiment was repeated for all strains.

### 3.13.3 Urease Test

Urease test is performed to differentiate between organism who produce the enzyme urease. It is an enzyme that destroys the bonds present between ammonia and carbon mainly in urea.

We can detect the presence of urea when the model organisms are grown in the urea broth. Urease if produced, breaks the nitrogen compound, and produces ammonia. Presence of ammonia makes the pH indicator go from red to deep pink. This is the result of a positive test.

To perform Urease test, we inoculate our desired organisms grown in urea broth, into their separate tube by using sterile loop. His also contain pH indicator phenol. Then we will incubate our cultures for up to 48 hours at 37°C.

# CHAPTER 4

# RESULTS

## 4.1 Phylogenetic analysis of the bacterial strains

Our team isolated ten unknown bacterial strains from the soil samples which were collected from the surrounding of cement factories. We conducted the evolutionary analysis of the 16S rRNA gene sequences of these 10 strains in MEGA X. The separated phylogeny is represented in the form of unrooted tree having two super clads as shown in Figure 20. Among the strains, *Bacillus* species were the most common and other common species were *Arthrobacter* and *Chryseomicrobium*. The other species belong to *Planococcus, Glutamicibacter, Corynebacterium.*

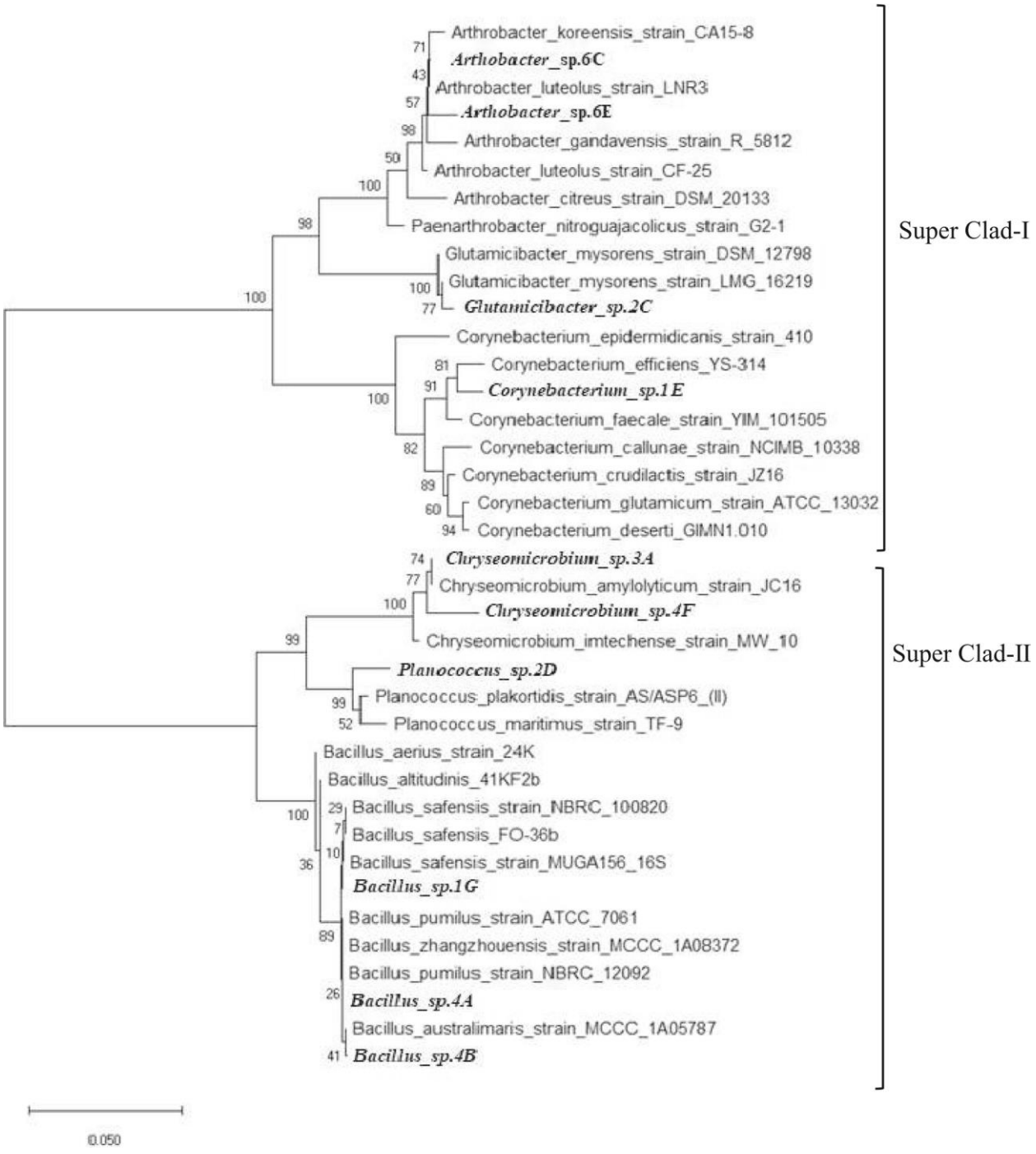



*Figure 18. Phylogenetic Tree of the Isolated Bacterial Strains*

Each super clad is comprised of 3 sub clads. The first super clad is comprised of *Corynebacterium*, *Glutamicibacter* and *Arthrobacter*. The second super clad is comprised of *Chryseomicrobium*, *Planococcus* and *Bacillus.* This study shows the diversity among strains involved in calcite production isolated from soil.

## 4.2 Identification of genes involved in calcite precipitation

We conducted the study to identify the genes involved in calcite production in novel bacteria. The gene cluster, comprised of *lcfA, ysiA, ysiB, etfB and etfA*, identified by (Cho et al, 2015). is involved in calcite precipitation. The bacteria responsible for calcite precipitation in their study was *Bacillus subtilis*.

The sequences of *lcfA, etfA* and *etfB* genes for *Bacillus subtilis* were retrieved from NCBI database. We performed the basic local alignment of acquired sequences against the genomes of eight novel strains involved in calcite production identified by our group. The purpose was to identify and confirm the presence of *lcfA, etfA,* and *etfB* in *Bacillus safensis, Bacillus australimaris, Bacillus pumilus, Arthrobacter koreensis, Arthrobacter luteolus, Planococcus plakortidis, Corynebacterium efficiens,* and *Glutamicibacter mysorens*.

### 4.2.1 Alignment result for *B. pumilus*

The alignment of the acquired gene sequences of *Bacillus subtilis* with our bacterial genomes displayed various percentage identity. The alignment result of the gene sequences (*lcfA, etfA, etfB*) against *Bacillus pumilus* shows 70.84% percentage identity for *lcfA*, 69.24% percentage identity for *etfA* and 68.52% percentage identity for *etfB.*

### 4.2.2 Alignment result for *B. safensis*

The alignment result of the gene sequences (*lcfA, etfA, etfB*) against *Bacillus safensis* shows 70.18% percentage identity for *lcfA*, 69.24% percentage identity for *etfA* and 70.22% percentage identity for *etfB.*

### 4.2.3 Alignment result for *B. australimaris*

The alignment result of the gene sequences (*lcfA, etfA, etfB*) against *Bacillus australimaris* shows 69.54% percentage identity for *lcfA*, 68.55% percentage identity for *etfA* and 69.94% percentage identity for *etfB*.

### 4.2.4 Alignment result for *P. plakortidis*

The alignment result of the gene sequences (*lcfA, etfA, etfB*) against *Planococcus plakortidis* shows 65.54% percentage identity for *lcfA*, 76.32% percentage identity for *etfA* and 69.33 percentage identity for *etfB.*

### 4.2.5 Alignment result for *A. koreensis*

The alignment result of the gene sequences (*lcfA*, *etfA*, *etfB*) against *Arthrobacter koreensis* shows 74.47% percentage identity for *lcfA*, 68.52% percentage identity for *etfA* and 75.86% percentage identity for *etfB*.

### 4.2.6 Alignment result for *A. luteolus*

The alignment result of the gene sequences (*lcfA*, *etfA*, *etfB*) against *Arthrobacter luteolus* shows 73.40% percentage identity for *lcfA*, 73.02% percentage identity for *etfA* and 79.63% percentage identity for *etfB*.

### 4.2.7 Alignment result for *C. efficiens and G. mysorens*

When the genomes of *Corynebacterium efficiens* and *Glutamicibacter mysorens* were aligned against gene sequences, both genomes show no similar regions for each gene (*lcfA*, *etfA* and *etfB*).

*Table 11. The Following table shows the alignment results for all the bacterial strains.*

| Strains | Putative Genes | Max Score | Query Cover | Percent identity | Accession Number |
|---|---|---|---|---|---|
| Bacillus pumilus | Putative *lcfA* | 587 | 77% | 70.84% | NZ_CP011007.1 |
| | Putative *etfA* | 383 | 100% | 69.24% | NZ_CP011007.1 |
| | Putative *etfB* | 269 | 92% | 68.52% | NZ_CP011007.1 |
| Bacillus australimaris | Putative *lcfA* | 589 | 86% | 69.54% | NZ_LGYN01000001.1 |
| | Putative *etfA* | 361 | 100% | 68.55% | NZ_LGYN01000001.1 |
| | Putative *etfB* | 307 | 89% | 69.94% | NZ_LGYN01000001.1 |
| Bacillus safensis | Putative *lcfA* | 625 | 86% | 70.18% | CP015611.1 |
| | Putative *etfA* | 397 | 100% | 69.24% | CP018100.1 |
| | Putative *etfB* | 309 | 89% | 70.22% | CP010405.1 |
| Arthrobacter koreensis | Putative *lcfA* | 62.6 | 14% | 74.47% | NZ_WACG01000001.1 |
| | Putative *etfA* | 94.2 | 27% | 68.52% | NZ_WACG01000001.1 |
| | Putative *etfB* | 35.6 | 7% | 75.86% | NZ_WACG01000001.1 |
| Arthrobacter lutelus | Putative *lcfA* | 58.1 | 5% | 73.40% | NZ_BCQM01000001.1 |
| | Putative *etfA* | 68.0 | 12% | 73.02% | NZ_BCQM01000001.1 |
| | Putative *etfB* | 41.9 | 6% | 79.63% | NZ_BCQM01000001.1 |
| Planococcus plakortidis | Putative *lcfA* | 224 | 70% | 65.64% | CP016539.2 |
| | Putative *etfA* | 280 | 81% | 76.32% | CP016539.2 |
| | Putative *etfB* | 270 | 89% | 69.33% | CP016539.2 |

## 4.3 Primer designing in the conserved region

The aligned sequences of *Bacillus safensis* and *Bacillus australimaris* were downloaded for primer designing. Then the primers were designed for *Bacillus safensis* and *Bacillus australimaris* for the confirmation of gene through PCR.

The primers were synthesized through Primer3 (A web-based application).

### 4.3.1 primers designed for *B. safensis*

For *Bacillus safensis*, both forward(F) and reverse(R) primers are of 20 nucleotides each. The amplicon size is 569 bp for *lcfA* primers, 515 bp for *etfA* primers and 522 bp for *etfB* primers.

**Primer3 Output**

```
PRIMER PICKING RESULTS FOR NZ_CP043404.1:2747768-2749227 Bacillus safensis strain PgKB20 chromosome, complete genome

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%   any   3'  seq
LEFT PRIMER        649   20   60.10   50.00  6.00  0.00 ATTTGACGCAGCTGATACCC
RIGHT PRIMER      1217   20   59.96   55.00  4.00  0.00 GGGCTTCTTCGACCTCTCTT
SEQUENCE SIZE: 1460
INCLUDED REGION SIZE: 1460

PRODUCT SIZE: 569, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00
```

*Figure 19. Primer3 result for lcfA in B. safensis*

**Primer3 Output**

```
PRIMER PICKING RESULTS FOR NZ_CP043404.1:2751688-2752665 Bacillus safensis strain PgKB20 chromosome, complete genome

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%   any   3'  seq
LEFT PRIMER        409   20   59.93   40.00  6.00  2.00 TCTGGAAAAGCCTTCGAAAA
RIGHT PRIMER       923   20   59.97   50.00  3.00  1.00 AACAAGTCACCGACGATTCC
SEQUENCE SIZE: 978
INCLUDED REGION SIZE: 978

PRODUCT SIZE: 515, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00
```

*Figure 20. Primer3 result for etfA in B. safensis*

**Primer3 Output**

```
PRIMER PICKING RESULTS FOR NZ_CP043404.1:2750871-2751559 Bacillus safensis strain PgK820 chromosome, complete genome

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'  seq
LEFT PRIMER        124   20   60.10   50.00  5.00  3.00 GAAGAAGCCATTCAGCTTCG
RIGHT PRIMER       645   20   60.11   50.00  4.00  1.00 TGGTACATCGTCTTCGTCCA
SEQUENCE SIZE: 689
INCLUDED REGION SIZE: 689

PRODUCT SIZE: 522, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 1.00
```

*Figure 21. Primer3 result for etfB in B. safensis*

## 4.3.2 Primers designed for *B. australimaris*

For *Bacillus australimaris*, both forward(F) and reverse(R) primers are of 20 nucleotides each. The amplicon size is 541 bp for *lcfA* primers, 562 bp for *etfA* primers and 535 bp for *etfB* primers.

**Primer3 Output**

```
PRIMER PICKING RESULTS FOR NZ_LGYN01000001.1:648962-650416 Bacillus australimaris strain NH7I_1 contig1, whole genome shotgun sequence

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'  seq
LEFT PRIMER        704   20   59.93   45.00  4.00  2.00 CTTTTCGATGTTCGGGTGAT
RIGHT PRIMER      1244   20   59.99   50.00  4.00  1.00 GACAAGCATGGAGCACTTGA
SEQUENCE SIZE: 1455
INCLUDED REGION SIZE: 1455

PRODUCT SIZE: 541, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00
```

*Figure 22. Primer3 result for lcfA in B. australimaris*

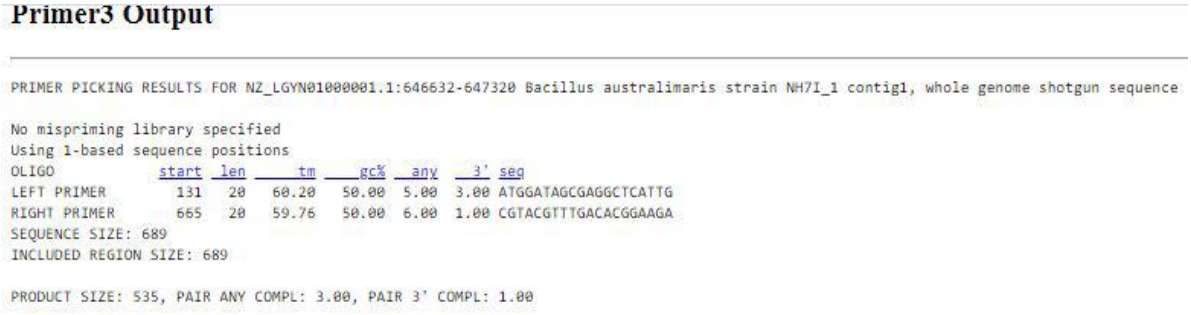
**Primer3 Output**

```
PRIMER PICKING RESULTS FOR NZ_LGYN01000001.1:645526-646503 Bacillus australimaris strain NH7I_1 contig1, whole genome shotgun sequence

No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm     gc%   any   3'  seq
LEFT PRIMER        114   20   59.97   45.00  8.00  1.00 TGTTGATGGCCACAATGACT
RIGHT PRIMER       675   20   59.85   50.00  7.00  2.00 GACTTGTCGCCGAAAATAGC
SEQUENCE SIZE: 978
INCLUDED REGION SIZE: 978

PRODUCT SIZE: 562, PAIR ANY COMPL: 6.00, PAIR 3' COMPL: 1.00
```

*Figure 23. Primer3 result for etfA in B. australimaris*



**Primer3 Output**

PRIMER PICKING RESULTS FOR NZ_LGYN01000001.1:646632-647320 Bacillus australimaris strain NH7I_1 contig1, whole genome shotgun sequence

No mispriming library specified
Using 1-based sequence positions

| OLIGO | start | len | tm | gc% | any | 3' | seq |
|---|---|---|---|---|---|---|---|
| LEFT PRIMER | 131 | 20 | 60.20 | 50.00 | 5.00 | 3.00 | ATGGATAGCGAGGCTCATTG |
| RIGHT PRIMER | 665 | 20 | 59.76 | 50.00 | 6.00 | 1.00 | CGTACGTTTGACACGGAAGA |

SEQUENCE SIZE: 689
INCLUDED REGION SIZE: 689

PRODUCT SIZE: 535, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

*Figure 24. Primer3 result for etfB in B. australimaris*

### 4.3.3 Primers for *B. subtilis*

The primers designed by Primer 3 for *Bacillus safensis* and *Bacillus australimaris* and the primers for *Bacillus subtilis* were taken from the literature to perform *in silico* PCR.

## 4.4 *In silico* PCR for the amplification of genes

We performed *in silico* PCR for the amplification of genes (*lcfA, etfA* and *etfB*) in *Bacillus Subtilis, Bacillus safensis* and *Bacillus australimaris*. This is used to predict the PCR product using the primers. *In silico* PCR also gives some other information about the length of amplicons, location of the primers etc.

The results *of in-silico* PCR of *lcfA, etfA* and *etfB* in *Bacillus subtilis, Bacillus safensis* and *Bacillus australimaris* shows perfect amplification.

## 4.5 Protein Structure Modelling

We predicted the tertiary structure of *lcfA, etfA* and *etfB* in Bacillus safensis through Swiss-Model web tool. The target sequence was compared with the template sequence and based on homology results the structure was predicted. The tertiary structure of *lcfA* was predicted in comparison with luciferin 4-monooxygenase from Japanese firefly with range 1-475. The sequence identity and similarity for the structure was 31.25% and 0.35 respectively (shown in Figure 25a). The tertiary structure of *etfA* was predicted in comparison with electron transfer flavoprotein alpha subunit from *Acidaminococcus fermentans* with range 1-323. The sequence identity and similarity for the structure was 37.77% and 0.39 respectively (shown in Figure 25b). The *etfB* gene structure was predicted in comparison with electron transfer flavoprotein from *Paracoccus denitrificans* having range 1-229. The sequence identity and similarity for the structure was 36% and 0.38 respectively (shown in Figure 25c).

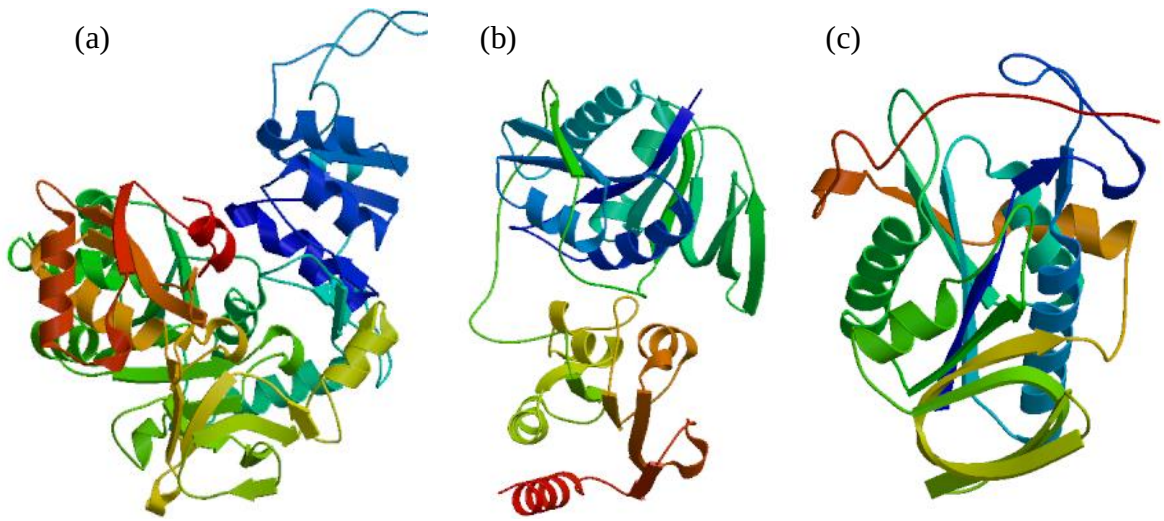



*Figure 25. (a) Tertiary Structure of lcfA, (b) Tertiary Structure of etfA, and (c) Tertiary Structure of etfB*

# Wet lab Analysis

## 4.5 Revival of Bacterial Strains

We revived *B. subtilis*, *B. safensis* and *B. australimaris*. We streaked and cultured the strains for pure bacterial colonies.


*Figure 26. Culturing bacterial strains on agar plates (streak plate method). (1G) B. safensis, (4B) B. australimaris, (B.S) B. subtilis*

## 4.7 Gram Staining to Check Bacterial Group (Gram-positive or Gram-negative)

Then gram staining was performed for the identification and confirmation of strains. The results shows that all the strains are showing purple colour and are gram positive. All the strains are rod shaped.

## 4.8 Result of PCR and Gel Electrophoresis

We extracted out DNA to check for the presence of our desired genes. We performed Gel electrophoresis to visualize our PCR product (bands) that confirm the presence of calcite producing genes.

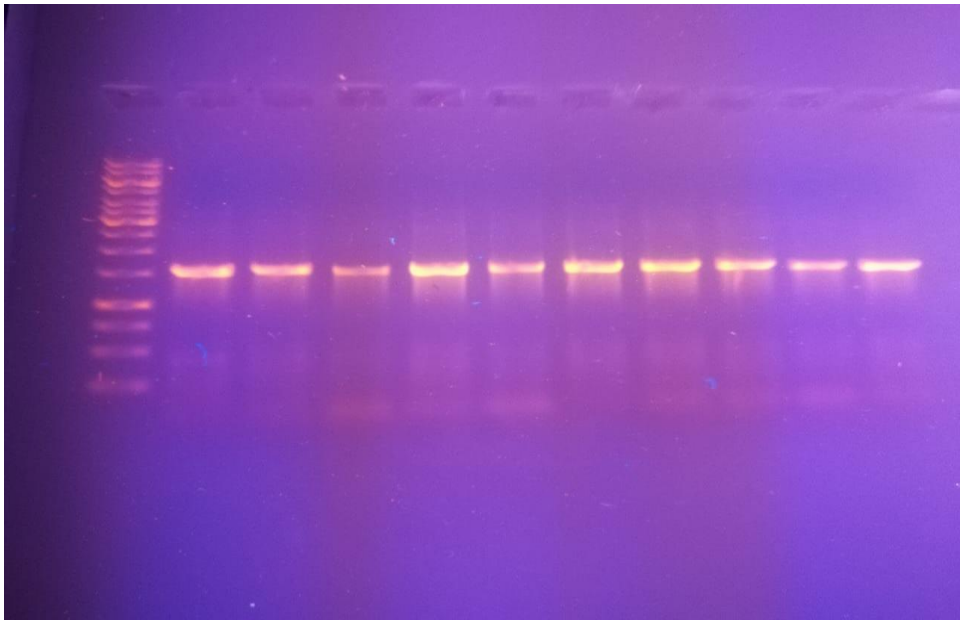


*Figure 27. Result of Gel Electrophoresis*

## 4.9 Biochemical Tests performed (oxidase and catalase tests)

*Bacillus safensis* and *Bacillus australimaris* were also tested for oxidase, catalase, and spore forming activities. Both the strains are oxidase and catalase positive as well as positive for spore formation while both the strains are urease negative.

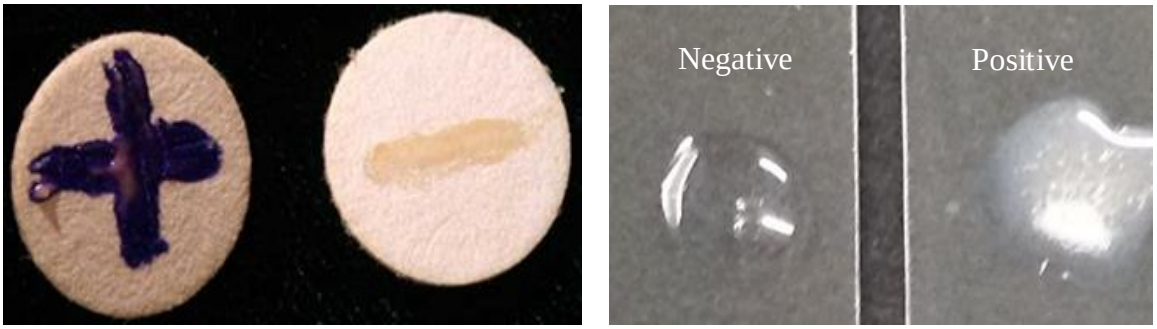



*Figure 28. Oxidase (Left) and Catalase (Right) test results*

## 4.10 Pathway prediction for calcite precipitation in non-ureolytic bacteria

We predicted a non-ueolytic pathway for microbial induced calcite precipitation (MICP) involved in the self-healing of concrete in the cement matrix as limited research was done in this regard. Certain pathways were available in the literature such as urea hydrolysis, denitrification, amino acids ammonification and dissimilatory sulphate reduction, but little was known about the active pathway.

From the literature, we found a link between calcite precipitation and fatty acid metabolism in calcifying bacterial strains (*B. subtilis) (*Basaran Bundur et al., 2015). The genes involved in calcite precipitation such as *lcfA, ysiA, ysiB, etfB, and etfA* are like few of the genes responsible for the β-oxidation of fatty acids (*lcfA, lcfB, fadB, fadA, fadE, etfA and etfB*).

Furthermore, *etfA* encodes a membrane associated flavoprotein that has a role in calcium carbonate precipitation (in urea-deprived media), as well as in fatty acid metabolism. This also validates the association of fatty acid metabolism and calcite precipitation.

Moreover, *lcfA* operon is involved in the synthesis of a lipid intermediate (2,3,4- saturated acyl-CoA intermediate) which is directly involved in the biomineralization process.

At neutral pH, the bacterial cell membrane is negatively charged due to the presence of highly electronegative groups, which attracts the positively charged metal ions such as $Ca^{2+}$ ions. This is followed by the entrance and accumulation of calcium ions into the cell, which results in excessive removal of protons. As a result, calcium is actively exported by the cells to counteract the proton loss. This exchange of ions is favoured by the membrane associated flavoproteins. Due to bacterial respiration, carbon dioxide is released by the bacterial cell. At the site of crack, carbon dioxide reacts with $H_2O$, resulting in the formation of carbon acid ($H_2CO_3$), which dissociate to form carbonate ions. Carbonate ions supersaturation in the microenvironment stimulates calcium carbonate precipitation around the bacterial cell. Figure 29 illustrates the predicted pathway involved in calcite precipitation.

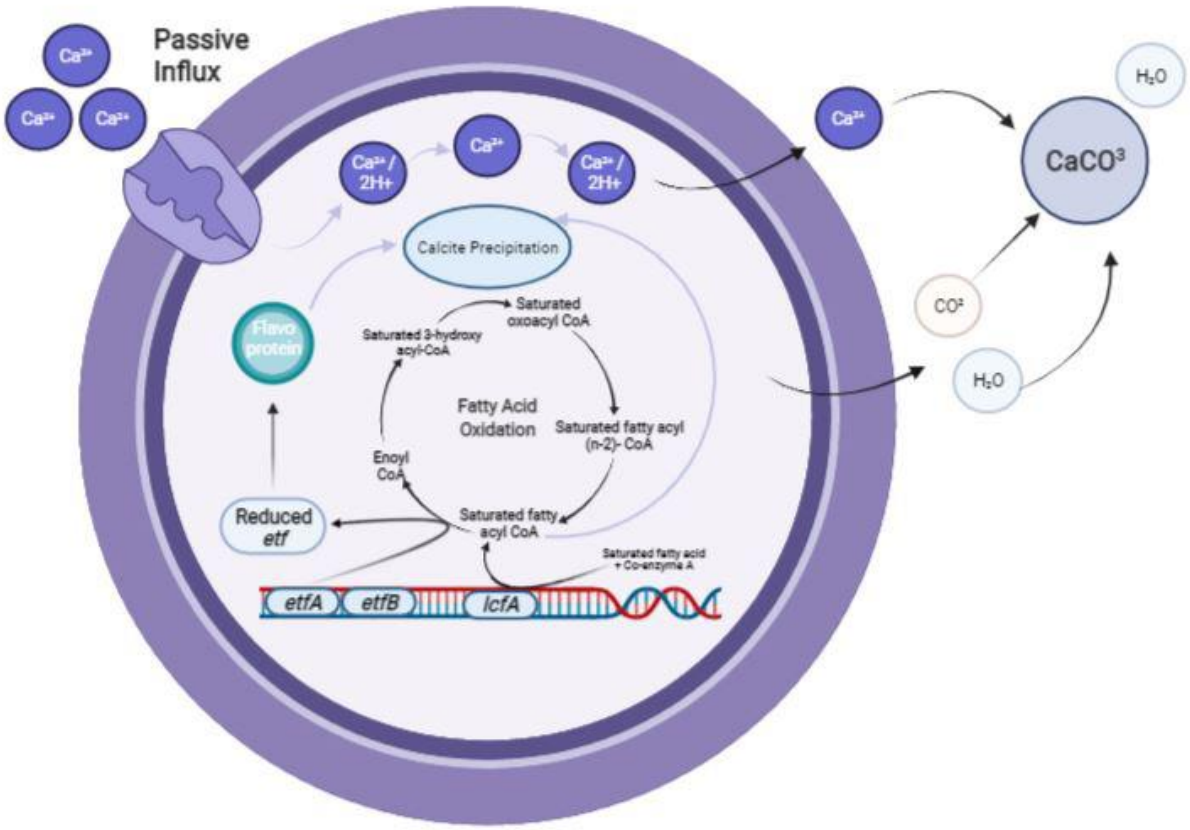



*Figure 29. Predicted Calcite Precipitation Pathway. (A) etfA encodes flavoprotein which is involved in the exchange of $H^+$ and $Ca^{2+}$ions across the cell. (B) 1. Negative charge of the cell attracts $Ca^{2+}$ ion, 2. The $Ca^{2+}$ ions accumulate inside the cell, 3. Removal of protons, 4. $Ca^{2+}$ is actively exported. (C) Bacterial respiration results in $CO_2$ formation which is released outside the cell and forms $CO_3^{2-}$ions. (D) Calcium carbonate precipitation. (E) lcfA forms Acyl-CoA intermediate which is directly involved in calcite precipitation.*

# CHAPTER 5

## DISCUSSION

This study conducted describes the identification of calcifying genes in *B. safensis* and *B. australimaris,* based on their sequence homology with the genes of *B. subtilis.* In our previous work, our group isolated 10 alkaliphilic calcifying strains. An alternative method was adopted to eliminate the drawbacks of the passive pathway as it leads to the formation of sulfide and toxic ammonia. Active pathway of calcite precipitation involved in organic matter oxidation was implemented for this purpose (Achal & Mukherjee, 2015).

The process of MICP has adverse effects such as accumulation of new products and growth of fungus which leads to the formation of stained patches. To avoid these problems and obtain the optimum conditions, we identified the bacterial genes responsible for calcite precipitation. (Basaran Bundur et al., 2015). We performed BLAST to obtain strains that have high sequence homology with the calcifying genes of *B. subtilis,* consequently we obtained two strains (*B. safensis* and *B. australimaris*) that show greater identity and are therefore categorized as high calcite producing strains. This was followed by primer designing using a web interface primer3 and *in silico* PCR. Primer3 provided the optimum conditions for primers such as melting temperature, and GC content.

To check the cultural characteristics of our strains, we performed colony morphology. The strains were first streaked on the agar plates in the manner that well-isolated colonies appeared and then those isolated colonies of each strain were thoroughly observed for determining the colony morphology. We performed Colony PCR to confirm the presence of our desired genes in the bacterial strains and obtained bands that were visualized under UV trans-illuminator, and this validated the presence of calcifying genes.

Furthermore, several biochemical tests like oxidase, catalase, and urease were performed. Our strains were oxidase and catalase positive. After performing these experiments, DNA extraction followed by gradient PCR and gel electrophoresis, was carried out to confirm the predicted amplicon size by *in silico* PCR. We further predicted the tertiary structure of proteins translated by the calcifying genes via Swiss-Model web tool. We also predicted the pathway that our bacterial strains use to precipitate calcite in the cracks. We predicted a link between calcite precipitation and fatty acid metabolism in calcifying bacterial strains as in *Bacillus subtilis* (Basaran Bundur et al., 2015). We found that the genes involved in calcite precipitation

such as *lcfA, ysiA, ysiB, etfB, and etfA* are similar to few of the genes responsible for the β-oxidation of fatty acids *(lcfA, lcfB, fadB, fadA, fadE, etfA and etfB*) (Cam et al., 2015).

# CHAPTER 6

## CONCLUSION

Microbially Induced Calcite Precipitation (MICP) is being implemented for the self-healing of cracks. In the present study, calcite precipitating genes in the bacterial strains were identified to optimize the conditions for this process.16S rRNA sequencing and phylogenetic analysis enabled us to identify 10 calcite producing strains. The BLAST results demonstrated that out of 10 strains, two strains named as *B. safensis* and *B*. *australimaris* had the highest sequence homology with calcite precipitating genes of *B. subtilis.* Furthermore, the results of gradient PCR and Gel electrophoresis confirmed the presence of calcifying genes in *B. safensis* and *B*. *australimaris*. The calcifying gene sequences were translated to amino acid sequences, and it resulted in the tertiary modelling of protein structure via Swiss-Model Web Tool. In addition, we predicted the non-ureolytic pathway that is likely to be involved in the precipitation of calcium carbonate by bacteria. It was found that the genes (*lcfA, ysiA, ysiB, etfB, and etfA*) are involved in calcite precipitation as well as in fatty acid metabolism. Moreover, *etfA* encodes a membrane associated flavoprotein which is likely to have a role in the exchange of ions across the cell membrane that facilitates the precipitation of calcite on the cell surface.

Finally, further research must be done in this regard in order to scale-up the process. The ultimate goal of this part of the research study was to identify the calcifying genes and prediction of the non-ureolytic pathway to determine the preliminary optimum conditions for the MICP process, making this technique one step closer to *in situ* application.

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