BIO CONCRETE: A BACTERIA BASED SELF-HEALING CONCRETE

A PROJECT REPORT

submitted in partial fulfilment of the requirements for the award of the Degree of

BACHELOR OF TECHNOLOGY

IN

CIVIL ENGINEERING

Under the supervision of

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to

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May – 2019

STUDENTS' DECLARATION

We hereby declare that the work presented in the Project report entitled **"Bio Concrete: A Bacteria based Self-Healing Concrete"** submitted in partial fulfilment of the requirements for the degree of Bachelor of Technology in Civil Engineering at **Jaypee University of Information Technology, Waknaghat** is an authentic record of our work carried out under the supervision of **Mr. Abhilash Shukla.** This work has not been submitted elsewhere for the reward of any other degree/diploma. We are fully responsible for the contents of this project report.

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CERTIFICATE

This is to certify that the work which is being presented in the project report titled **"BIO CONCRETE: A BACTERIA BASED SELF-HEALING CONCRETE"** submitted in partial fulfilment of the requirements for the degree of Bachelor of Technology in Civil Engineering at **Jaypee University of Information Technology, Waknaghat** is an authentic record of work carried out by **Vinayak Sharma (151648) & Ayush Thakur (151660)** under the supervision of **Mr. Abhilash Shukla, Assistant Professor (Grade-II),** Department of Civil Engineering, Jaypee University of Information Technology, Waknaghat.

The above statement made is correct to the best of our knowledge.

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ABSTRACT

Concrete is the most extensively used building material. Cracks are responsible for the deterioration of concrete structures. The use of biological techniques and processes in the concrete has lead to the invention of a new building material, bio-concrete. The present study focuses on isolation and screening of spore forming, calcite mineral precipitating bacteria and analyzes the effect of potential bacterial strain embedded on the strength and crack healing characteristics of concrete. Here, a total of 15 soil samples were collected from the various locations of Himachal Pradesh, India. The bacterial strain Bacillus megaterium MTCC 1684, procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India was used as the standard culture to compare the results with selected isolates. The first phase of investigation focuses on the culturing of bacteria. For this, enrichment culture technique was performed in Luria Broth as a nutrient medium and further processed on Urea Broth. Then, 26 distinct bacterial isolates were further processed for screening to select potential bacterial isolates. Finally 2 isolates were selected based on urease assay and calcite precipitation results. M20 concrete specimens were prepared with water/cement ratio of 0.45 and the concentration of bacterial cells embedded in concrete was 10^7 to 10^8 cell/ml of mixing water. The cracks were induced in the concrete specimens by introducing a thin copper plate of thickness 0.3 mm to 0.5 mm in the fresh concrete paste up to a depth of 10 mm to 15 mm. The compressive strength test, splitting tensile strength test, flexural strength test and visual inspection of crack healing were performed on concrete specimens incorporated with and without bacteria. It was observed that concrete prepared with isolate 3 bacterial cells reported greatest increment in compressive strength (17.28%), split tensile strength (27.76%) and flexural strength (35.03%) as compared to the control concrete specimens. Also, the maximum healable crack width for isolate 3 concrete was 0.3 - 0.4 mm, for standard concrete: 0.2 - 0.3 mm and it was only 0.1 - 0.2 mm for control concrete specimens. Therefore, it was concluded that Isolate 3 concrete can be successfully used in the crack remediation of concrete structures.

Keywords: calcite precipitation, crack healing, biomineralization, bacillus, concrete.

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
BM	Bacillus megaterium
BOD	Biochemical Oxygen Demand
CSL	Corn Steep Liquor
CTM	Compression Testing Machine
DNA	Deoxyribonucleic Acid
EDS	Energy Dispersive Spectroscopy
FST	Final Setting Time
IS	Indian Standard
IST	Initial Setting Time
LB	Luria Broth
LPR	Linear Polarization Resistance
MICP	Microbiologically Induced Calcite Precipitation
MTCC	Microbial Type Cell Culture
RC	Reinforced Concrete
RNA	Ribonucleic Acid
SEM	Scanning Electron Microscopy
UA	Urea Agar
UB	Urea Broth
XRD	X-Ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 GENERAL

Concrete is the most extensively used building material in construction practices. However, the formation of cracks is common in the concrete structures. Cracks are one of the major factors that adversely affect the durability and strength of concrete. Cracks are responsible for the deterioration of the structures. Cracks make it easy for carbon dioxide, moisture, sulphates, other liquids and gases to enter the concrete structure more easily up to its core. In reinforced concrete structures, the initiation of corrosion occurs due to the ingress of moisture, carbon dioxide and chloride ions through the crack in concrete to the steel surface which increases the permeability of concrete-steel matrix [1]. Thus, it is suffice to say that the cracks are not at all desirable in concrete structures.

For crack repair, there are various techniques available such as cracks impregnation by cement or epoxy or other synthetic fillers, surface treatments with water repellents or pore blockers [2]. However, these treatments have some disadvantageous aspects such as variation in thermal expansion, degradation with age and the need for constant maintenance [3]. These repair techniques can be time consuming and expensive. Also, the epoxy treatment is harmful to the environment and health as toxic fumes and gases evolved may cause skin and breathing issues. Therefore, it is essential to suggest ecologically friendly methods for crack repair. In this context, bacterial induced calcium carbonate precipitation has been suggested as an alternate and sustainable, environment friendly technique for the repair of cracks [4-6]. A variety of studies have revealed that under certain situations, small cracks in concrete can heal themselves [7-11]. This process is described as "*Autogenous healing* "or "*Self healing"*. Calcium carbonate precipitation is considered as the most significant factor influencing the self-healing of concrete [8, 11].

Microbiologically Induced Calcite Precipitation (MICP) involves the calcite formation by some alkaliphilic bacterial species, *Bacillus,* provided with a calcium based nutrient and mixed in the concrete suspended in mixing water. This fills the cracks in concrete by

producing calcium carbonate. Biodeposition techniques have already been used for sand consolidation [12-15], limestone monuments repairing [16-18], and filling of pores and cracks in concrete [19-22]. The mechanism behind formation of $CaCO_3$ by the bacteria is based on the enzyme hydrolysis of urea into ammonia and carbon dioxide. These reactions cause an increase in pH value, thus forming carbonates and bicarbonate ions that undergo precipitation with the calcium ions and produce calcium carbonate [18, 23]. An integrated bacterium will therefore depicts an internal self-healing agent and can be used to increase the strength and durability of concrete structures.

When the cracks begin to appear in a concrete structure, then moisture (water) enters the cracks. After coming in contact with the oxygen and water, the spores of bacteria starts microbial activities. They undergo multiplication and germination in the calcium-based nutrient i.e calcium lactate. When they feed on the calcium lactate, these bacteria mix calcium with carbonate ions to produce the limestone thus sealing the crack [24].

The present study focuses on isolation and screening of spore forming, calcite precipitating bacteria to analyze the effect of potential bacterial strain embedded on the strength and durability characteristics of concrete.

1.2 BACTERIA

Bacteria are simple single-celled organisms that are capable of thriving in a variety of environments. These can endure life within soil, acidic hot springs, radioactive waste, ocean, and deep in the earth crust. There are approximate ten times as much bacterium cells as human cells in the human body. On the basis of relative complexness of their cells, all living organisms are generally classified as prokaryotes or eukaryotes. Bacterium are prokaryotes. The entire organism consists of one cell with a simpler internal structure. In contrast to eukaryotic polymer, that is neatly packed into a cellular compartment referred to as the nucleus, bacteria DNA float free, in a very twisted thread like mass known as the nucleoid. Bacteria cells also constitute separate, circular pieces of DNA known as plasmids. Bacteria don’t have membrane bound organelles, special cellular structures which are designed to perform a variety of cellular functions like producing energy or transporting proteins [24-26].

1.2.1 Classification of Bacteria

A. *Classification On the basis of Shapes:* Bacteria are classified into 5 groups according to their basic shapes as shown in Figure 1.1

a) Rod shaped (Bacilli)
b) Spherical (Cocci)
c) Spiral (Spirilla)
d) Corkscrew (Spirochaetes)
e) Comma (Vibrios)

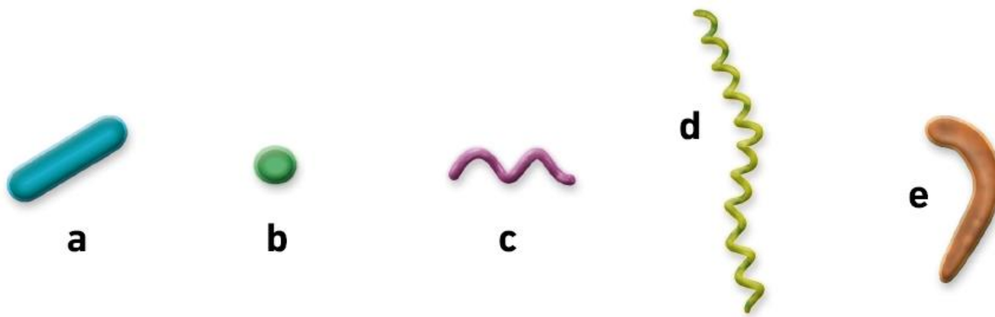

Figure 1.1 Classification of Bacteria on the basis of Shape (a-bacillus, b-coccus, c-spirillum, d-spirochaete, e-vibrios) [26]

B. *Classification On the basis of Gram Stain:* Bacteria are classified into 2 groups according to Gram Staining method as shown in Figure 1.2

a) Gram Positive (thick layer of peptidoglycan- 90% of cell wall) – Stains Purple.
b) Gram Negative (thin layer of peptidogycan- 10% of cell wall) – Stains Red/Pink.

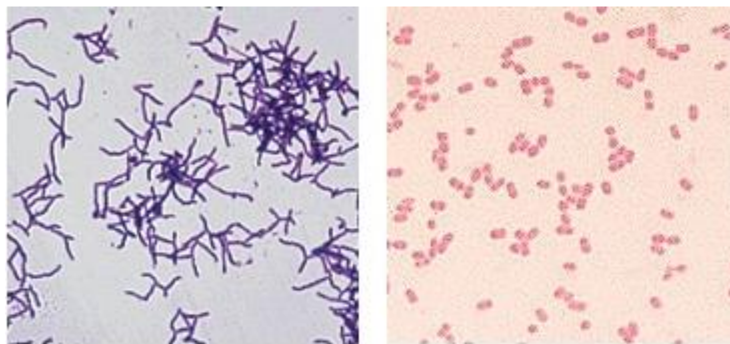

Figure 1.2 Classification of Bacteria on the basis of Gram Stain (a-gram positive, b-gram negative) [27]

C. *Classification On the basis of Oxygen Requirement:* Bacteria are classified into 2 groups according to their oxygen requirement for the survival of bacteria.

   a) Aerobic (uses molecular oxygen as terminal electron acceptor)
   b) Anaerobic (does not use molecular oxygen as terminal electron acceptor)

1.2.2 Growth and reproduction of Bacteria

Almost all bacteria multiply by the method of binary fission. A single bacterium cell, the "parent," makes a duplicate of its DNA and grows larger in size by increasing its cellular content by two times. The doubled contents are forwarded to each end of the cell. Then, a small opening emerge in the centre of the parent, ultimately splitting it into 2 similar "daughter" cells as shown in Figure 1.3. A few bacterium species like *cyanobacteria* and *firmicutes* multiply by budding. During budding, the daughter cell grow as an offspring of the parent. It starts off as a tiny nub, grows till it is the size of its parent and finally splits off [24,26].

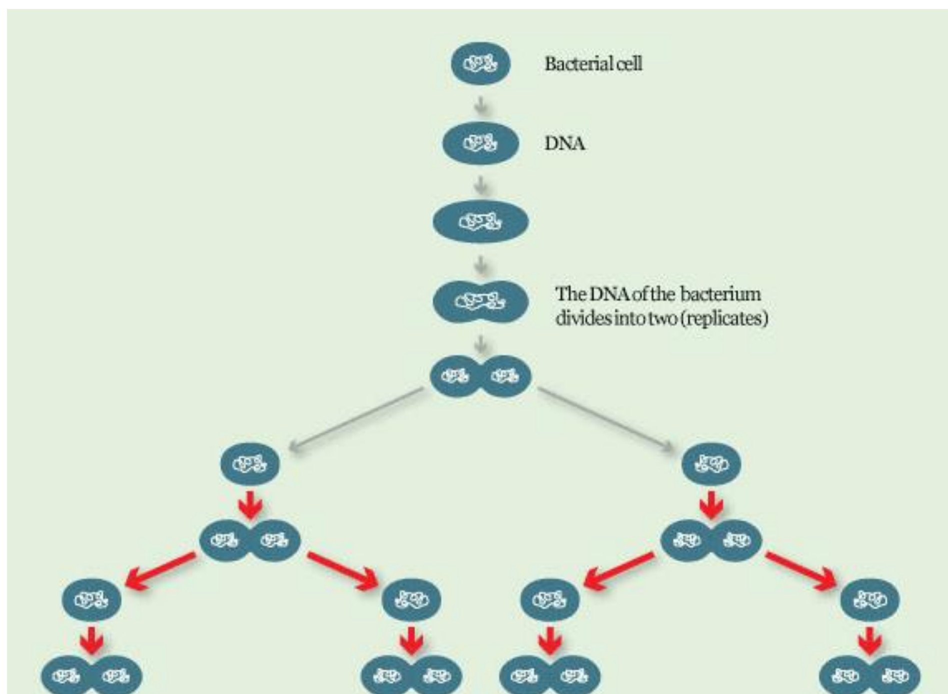

Figure 1.3 Bacteria Reproduction through Binary Fission [26]

1.2.3 Growth curve of Bacteria

It is a curve on a graph that depicts the variation in size of a bacteria population over time. The bacterium are cultured in a sterile nutrient medium and incubation is done at the optimum temperature. Specimens are removed at regular intervals and the quantity of living bacteria is measured [28-29]. A logarithmic growth curve is drawn, which shows 4 phases as shown in Figure 1.4

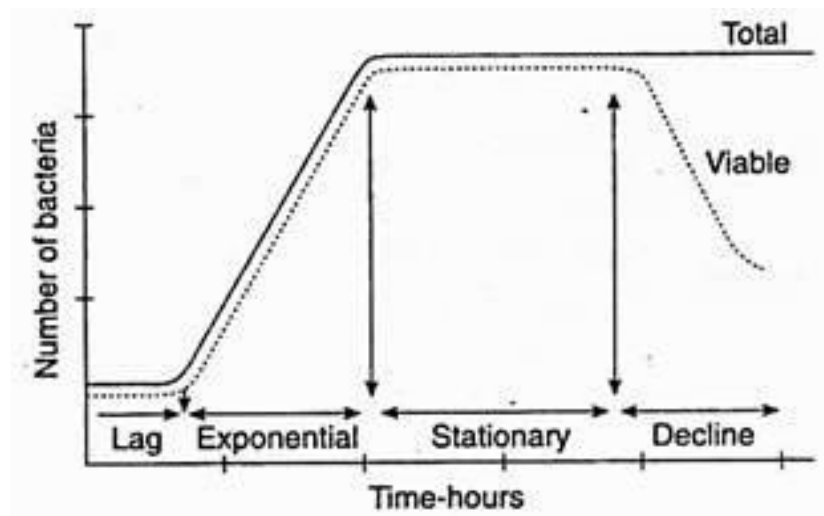

Figure 1.4 Bacteria Growth Curve [29]

a) Lag Phase - In this phase, only a little increase in quantity occurs as the bacteria absorb water, and synthesize ribosonal RNA and afterwards enzyme, in adapting to the new environment. The duration of this phase depend on which medium was used to grow the bacteria before the test and in which phase the bacteria are already present.

b) Log or Exponential Phase – With the decrease in the life span (generation time) of the cells, they enter the log phase, during which the cells attain a maximum reproducing rate and the quantity of bacteria increases proportionally with time, depicting a linear slope on a logarithmic scale (i.e. exponential growth). The rate of growth of bacterial cells can be predicted in this phase.

c) Stationary Phase - As time increases, the bacterial population grows, it enters the stationary phase, in which the nutrient and electron acceptor are depleted and the pH

falls as CO_2 and other poisonous waste accumulates. With the depletion in the cell's energy reserves, the rate of cell division drops.

d) Decline Phase - The decline phase happens when the rate at which the bacteria die rise above the rate at which they are reproduced; the population decreases as the nutrient level drops and toxin level increases.

1.2.4 Selection of Bacteria

The fresh concrete matrix is highly alkaline having pH 11-13, especially due to the production of calcium hydroxide. The common bacterium cannot survive in these environmental conditions [24]. Thus, the bacteria to be incorporated in the concrete mix not only need to withstand mechanical stresses of mixing but should also be able to tolerate a higher alkalinity for a prolonged duration. The bacterium additionally needs to be oxygen tolerant because the concrete matrix is toxic owing to ingress oxygen. Researchers have found that the aerobic alkaliphilic spore forming bacterium belonging to genus *bacillus* fulfil these criteria. Such spores have extraordinarily thick cell walls which facilitate them to remain intact for up to 200 years [30].

The bacteria belonging to the *Bacillus* species which can be used in the concrete are:-

a) Bacillus subtilis
b) Bacillus sphaericus
c) Bacillus pasteurii
d) Bacillus cohnii
e) Bacillus megaterium
f) Bacillus pseudofirmus
g) Bacillus halodurans
h) Bacillus alkalinitrilicus

1.3 OBJECTIVES OF THE PROJECT

The main aim of the present work is to study the strength, durability and crack healing characteristics of bio concrete as compared to the standard concrete. To achieve this aim, following objectives have been framed:-

a) To isolate the most efficient bacteria for crack remediation.

b) To characterize the calcite producing bacteria based on physiological characteristics.

c) To compare the compressive strength, split tensile strength and flexural strength of bacterial and non bacterial concrete.

d) To check the efficiency of potential bacterial strain towards crack remediation of concrete structure.

CHAPTER 2

LITERATURE REVIEW

The previous studies show that the bacteria can be used to enhance the performance and crack remediation of concrete structures. It has been successfully reported in most of the studies that the bacteria has tendency to precipitate the calcite and this precipitation forms a layer of crystals over the cracked area. The objective of this literature review is to provide an in-depth knowledge about the mechanism and various factors involved in the MICP. The following studies have promoted the concept of Bio Concrete as a Self-healing Concrete over the past few years.

2.1 Santhosh K. Ramachandran, V. Ramakrishnan and Sookle S. Bang (2001) [20]

This study proposed a novel biotechnology utilizing MICP for concrete remediation. Here, calcite precipitation produced by *Bacillus pasteurii* was investigated in 2 types of Portland cement (PC) mortar samples: one obtained from blending with micro-organisms, and second from artificial cracks filled with microbe mix. They conducted a durability investigation on concrete beams incorporated with bacteria, exposed to alkaline, sulfate and freeze-thaw conditions. It was observed that all the specimens with bacterial cells performed better than the control beams (i.e. without bacteria). As the bacterial concentration increases, the durability performance increased. MICP was evaluated by X-ray diffraction (XRD) analysis and visualisation was done by SEM. The importance of this study was to use micro-organisms that are found common in soil to be used for crack remediation of concrete. The important findings of this study were:

1. Chloride ions present in PC exhibit an adverse effect on the compressive strength of PC mortar specimens. Thus, before adding in the cement mix, the chloride ions already available in the microbial medium need to be cleaned thoroughly.
2. *Bacillus pasteurii,* at smaller concentrations, improves the compressive strength of PC mortar specimens. However, an increase in microbial biomass (lifeless forms particularly) decreased the strength of mortar samples.

iii. At pH above 12, neither *B. Pasteurii* nor *P. aeruginosa* cultivate actively. Thus, the increase in overall strength of cubes when incorporated with bacteria occurred due to the availability of sufficient quantity of organic matter in the matrix due to microbial biomass, and not from the calcite produced by microbial growth.

iv. Microbial remediation proves to be more productive in shallow cracks, not in deeper cracks. This is due to the fact that the bacteria develop more actively when the air is present.

Thus, it was concluded that *B. Pasteurii* is competent in crack remediation, but not in the strength improvement of the cement mortar specimens.

2.2 P. Ghosh, S. Mandal, B.D. Chattopadhyay and S. Pal (2005) [31]

This paper investigated a method of strength enhancement of cement-sand mortar via MICP. Here, an anaerobic bacterium associated with the *Shewanella* species was incorporated at various cell concentrations ranging from 10 - 10^7 per ml to the water mixed in mortar specimens. It was observed that:

i. The highest enhancement in compressive strength of mortar specimens was reported at a cell concentration of 10^5 cells per ml of mixing water.

ii. With the help of Mercury porosimetry, the alteration in pore size distribution was confirmed because of adding bacteria. It depicted that the cell concentration of 10^5 cells per ml of mixing water resulted in the highest decrease in porosity of specimens.

iii. By adding *E.coli,* no increase in the strength of specimens was seen.

2.3 Willem De Muynck, Kathelijn Cox, Nele De Belie and Willy Verstraete (2008) [21]

In this study, the durability of concrete and mortar specimens was studied due to biological calcium carbonate precipitation. Here, *Bacillus sphaericus* and ureolytic bacteria were compared with conventional surface treatments in crack remediation of concrete structures. The surface treatments applied included surface coatings and penetrating sealants. The morphology and mineralogy of the calcium carbonate crystals were visualized with SEM and XRD. The following observations were made:

i. The kind of micro-organism and the nutritional medium had a significant effect on crystal size and morphological behaviour of the specimens.
ii. Unlike the silicones, all other traditional methods caused a decrease in the quantity of water uptake.
iii. All the methods cause a reduction in the permeability toward oxygen. The greatest decrease in permeability were reported by surface coating and penetrating sealants.
iv. The calcite layer deposits on the surface of the samples caused a reduction in capilary suction and gas permeabillity.
v. Bacterial treatment caused a restricted variation in the chromatic features of mortar and concrete surface.
vi. Thus, it was concluded that the results of *Bacillus sphaericus* were comparable with the conventional treatments.

2.4 Henk M. Jonkers, Arjan Thijssen, Gerard Muyzer, Oguzhan Copuroglu and Erik Schlangen (2010) [23]

In this paper, the authors studied the bacterial potential as a self healing agent, i.e. their capability to remediate cracks in concrete structures. For this, a certain class of alkaliphilic spore forming micro-organism: *Bacillus pseudofirmus* and *Bacillus cohnii*, originally cultivated from alkaline soil specimens were selected. Cement-stone samples with and without bacterial cells were made to study the growth of mixed bacteria, pore-size distribution of samples, consequences of adding agents on strength and self healing capabilities. By estimating the quantity of living bacterium present in cement-stone samples, the surviving rate of mixed bacteria spore was measured by the most probable number (MPN) technique. Pore-size distribution of samples prepared with a water/cement ratio of 0.5 was evaluated by mercury intrusion porosimetry (MIP). It was obtained that:

i. The quantity of living cells significantly reduced as the sample age increased. Also, the viability of the most of the spores directly incorporated to the cement paste mix was less than four months.
ii. An adequate mineral precursor component is required to be added in the matrix to produce a self-governing repair process, because bacteria act as a catalyst.

iii. Bacteria and calcium lactate, forming a two component healing operator, allowed producing huge quantity of micro sized mineral like precipitates on cracked surface of cement-stone samples.
iv. The role of biological calcite precipitation of the mixed healing agent seems restricted to fresh concrete.

2.5 Kim Van Tittelboom, Nele De Belie, Willem De Muynck and Willy Verstraete (2010) [32]

The interest of this study was to compare the crack healing capability of bacteria and traditional repair methods using water permeability tests, ultrasound transmission test and by visualization. This study gives the chemical reaction for calcium carbonate precipitation as:

$$CO(NH_2)_2 + H_2O \longrightarrow NH_2COOH + NH_3 \quad (1)$$

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3 \quad (2)$$

$$H_2CO_3 \longleftrightarrow HCO_3^- + H^+ \quad (3)$$

$$2NH_3 + 2H_2O \longrightarrow 2NH_4^+ + 2OH^- \quad (4)$$

$$HCO_3^- + H^+ + 2NH^{4+} + 2OH^- \longleftrightarrow CO_3^{2-} + 2NH^{4+} + 2H_20 \quad (5)$$

$$Ca^{2+} + Cell \longrightarrow Cell\text{-}Ca^{2+} \quad (6)$$

$$Cell\text{-}Ca^{2+} + CO_3^{2-} \longrightarrow Cell\text{-}CaCO_3\downarrow \quad (7)$$

In this study, the cracks in concrete specimens were made in 2 ways. The first technique produced samples with standard crack and the second technique resulted in more realistic crack in specimens. The standard cracks were prepared in concrete specimens by introducing a thin copper plate of 0.3 mm thickness in the fresh concrete mix upto 10-20 mm depth. After 24 hours, the plates were detached during demoulding. The realistic cracks were prepared by doing splitting tests on concrete cylindrical specimens wrapped in fibre reinforced polymers (FRP).

The traditional repair techniques used in this study were a two-component epoxy resin and two-component cement bound mortar. *Bacillus sphaericus* strains, cultivated from calcareous sludge was also used. For few treatments, the bacterium was immobilized in silica gel to prevent the bacterial cells from the highly alkaline conditions in concrete mixture. To check the efficiency of different crack repair methods, various tests were performed like water permeability, ultrasonic measurements, visual inspection and thermogravimetric analysis. In water permeability test setup, because of the water flowing through the crack, the drop of water level in pipette was evaluated at regular time intervals, and water was restored every time to the starting level. To calculate the coefficient of water permeability (k), Darcy's law was used (Eq. 8).

$$k = \frac{aT}{At} ln\left(\frac{h_0}{h_f}\right) \tag{8}$$

here,

a	cross-sectional area of pipette (m2)
A	cross-sectional area of specimen (m2)
t	time (sec)
T	specimen thickness (m)
h_o	initial water head (cm)
h_f	final water head (cm)

The following observations were made:

i. The bacterial protection by silica gel matrix appeared to be working as the precipitation of calcium carbonate crystals occurred within the gel matrix and when only bacteria was used without immobilizing it in the silica gel, it wasn't effective.

ii. It was seen that crack healing by this bio mineral treatment caused a reduction in water permeability. But, the reduction in water permeability was also observed when autoclaved bacteria were used rather than viable bacteria. This concluded that the crack sealing by the silica gel matrix played a greater role in the reduction in water permeability.

iii. Thermogravimetric analysis on the crack repaired specimen depicted that the calcite crystals were present only in active bacteria.

iv. It was observed that the treatment of cracks by *Bacillus sphaericus*, incorporated in silica gel improved the ultrasonic pulse velocity which indicated that sealing of crack was successful. Also, by visually evaluating the crack, it was reported that this technique can be used in completely filling the cracks.

2.6 Virginie Wiktor and Henk M. Jonkers (2011) [33]

This research was performed to evaluate the crack healing potential of a two component bio chemical self healing agent (calcium lactate and bacterial spores) incorporated in porous expanded clay particle. The bacterium was isolated from alkaline lake soil which was later reported as *Bacillus alkalinitrilicus*.

For the oxygen consumption measurement of cement mortar samples, optical oxygen micro sensors were used. Oxygen uptake was evaluated by measuring the change in oxygen concentration using Fick's first law of diffusion [34] (Eq. 9).

$$J = -Do_2 \frac{dC(z)}{dz} \tag{9}$$

here, Do_2 diffusion coefficient of O_2 in water

$C(z)$ concentration of O_2 at depth z

For crack healing quantification, crack width measurements were done and the healing percentage was determined as (Eq. 10).

$$\text{Healing \%} = \frac{Cw_i - Cw_t}{Cw_i} \times 100 \tag{10}$$

here, Cw_i Initial crack width

Cw_t Width at time t

The critical findings of this study were:

i. Energy dispersive X-Ray analysis (EDAX) depicted the two major crystal morphologies: ‘‘deformed’’ lamellar rhombohedra and “needle like clusters”, possibly polymorphs of calcium carbonate, on cracked surface of bacterial samples. The observations from Fourier-Transform Infrared (FT-IR) spectra indicated that calcite and aragonite were present.
ii. It was observed that at longer duration, crack healing was sufficiently greater in bacterial samples. In both control and bacterial samples, a similar inverse relationship between crack width and healing percentage was reported: smaller the crack width, higher the healing percentage.
iii. The oxygen consumption was only seen in case of bacterial concrete, and not in control concrete.

2.7 Varenyam Achal, Xiangliang Pan and Nilüfer Özyurt (2011) [35]

This paper discusses the significance of microbiological calcium carbonate precipitation in improving the durability of fly ash concrete. Here, the role of *Bacillus megaterium* ATCC 14581 was studied on compressive strength, water absorption and water impermeability of mortar and concrete. For testing compressive strength of mortar specimens, fly ash was added by replacing the quantity of cement at the concentration of 10 - 40 %. For water absorption test, a sorptivity test according to RILEM 25 PEM (II-6) was conducted on mortar specimens. The sorptivity coefficient (k) was determined as (Eq. 11).

$$\frac{Q}{A} = k\sqrt{t} \tag{11}$$

here, Q quantity of water absorbed (cm^3)

A cross section of sample in contact with water (cm^2)

t time (sec)

The following observations were made in this study:

i. The quantity of live bacterial cells reduced as the age of mortar samples increased.

ii. The bacterial count was more in fly ash amended mortar or concrete specimens irrespective of their ages and higher the percentage of fly ash, more was survival rate.
iii. The compressive strength tests revealed that greater the amount of fly ash present, lesser was compressive strength, incorporated with or without bacterial cells. It was reported that the improvement in compressive strength is mainly due to the consolidation of $CaCO_3$ that was produced by bacteria in the pores of mortar cube specimens.
iv. There was a decline in water absorption and water permeability of the mortar samples due to the deposition of calcite layer on the surface and inside pores attributing the presence of bacteria.
v. Scanning Electron Microscope (SEM) analysis confirmed that the calcite induced by rod-shaped bacteria was present in the specimens.

2.8 Rafat Siddique and Navneet Kaur Chahal (2011) [24]

This is a critical review paper which discusses all the major factors influencing the calcite precipitation in bacterial concrete. This study has helped us in understanding the concept behind self healing mechanism of bio concrete. Here, the effects of bacteria on a variety of parameters in concrete like compressive strength, water permeability, water absorption, and chloride ingression are studied in detail. It also describes the applications of bacteria in concrete as: an alternative surface treatment; and water purifier. It was concluded that almost all of the studies reported an increase in compressive strength of mortar or concrete specimens filled with bacteria. The biodeposition treatments resulting in an improved resistance towards carbonation caused a reduction in gas permeability. Also, in water absorption tests, the bacterial absorption and of calcium carbonate crystals precipitation increased the weight of the mortar samples.

For measuring resistance against chloride ingression, accelerated migration test was conducted on biodeposition treated samples. The improvement in resistance toward the chloride migration of cubes treated with biodeposition proved to be effective in reducing the rate of reinforcement corrosion. Finally, it was concluded that MICP proves to be a promising technique for crack remediation.

2.9 Varenyam Achal, Abhijit Mukherjee and M. Sudhakara Reddy (2011)[36]

In this study, the authors studied the role of *Bacillus* sp. CT-5, isolated from cement, on the compressive strength and water absorption. This bacterial strain was grown in nutrient broth urea (NBU) medium. Both, compressive strength test and water absorption test were performed for control and *Bacillus* sp. CT-5 specimens.

It was obtained that:

i. The compressive strength of the mortar cube specimens incorporated with bacterial cells had considerably increased, as much as 36% in relative to control samples.
ii. For confirming microbial calcite precipitation, the cube specimens were visualized under SEM. The bacterial samples had shown calcite crystals and precipitated with rod shaped impressions (possibly *Bacillus*) having specific and sharpened edges, depicting a complete development of the crystals..
iii. Also, due to the bacterial cells present, there was a considerable reduction in the water uptake relative to control samples. The $CaCO_3$ crystals deposited on the surface caused a reduction in the sorptivity. Thus, it can be said that the incoming of destructive substances may be restricted.

2.10 Varenyam Achal, Abhijit Mukherjee, Shweta Goyal and M. Sudhakara Reddy (2011) [1]

This paper focussed on the capability of MICP in diminishing the corrosion of reinforcement. Here, reinforced concrete samples incorporated with bacterial cells were subjected to accelerated reinforced corrosion similar as in [37-38]. In this study, an industrial by-product, corn steep liquor (CSL), was used as a nutrient medium for the bacteria within the concrete. The corrosion rate in bacterial concrete specimens was evaluated by measuring corrosion current density and linear polarization resistance. Also, destructive tests like pullout strength and mass loss of the reinforcement were conducted.

The critical findings of this study were:

i. MICP caused a reduction in the current in reinforced concrete samples, as shown by Corrosion current density measurements.

ii. Linear polarization resistance measurements revealed that there was a significant variation in polar resistance in bacterial samples.
iii. There was an increase in pullout strength and reduction in the mass loss owing to calcite precipitation.

2.11 J.Y. Wang, H. Soens, W. Verstraete and N. De Belie (2014) [39]

This paper investigated the viability of encapsulated spores and the role of microcapsule on cement mortar samples. In this study, *Bacillus sphaericus* was used to make self-healing concrete. Self healing capability was determined using crack-healing ratio and water impermeability test.

The following observations were made:

i. Even after immobilizing into the microcapsules, the spores continued to live. This was concluded as the urea decomposed only after 3 days for broken bacterial microcapsules; and it was not the case for unbroken and broken microcapsules without bacterial spores.
ii. Due to microcapsules, both the tensile strength as well as the compressive strength of mortar specimens decreased, with latter having greater negative impacts.
iii. SEM images verified the breakage of microcapsules upon cracking under tensile force.
iv. It was observed that calcium-nitrate not only accelerated cement hydration but also had a beneficial impact on the hydration degree.
v. The crack healing ratio was much greater for bacterial samples (48 - 80%) as compared to the control specimens (18 - 50%).
vi. The maximum crack width sealed for bacterial samples was 0.97 mm, which was about four times wider as compared to non bacterial specimens (0.25 mm).
vii. The overall water permeability for bacterial specimens was about ten times lower as compared to non bacterial specimens.

2.12 Mian Luo, Chun-xiang Qian and Rui-yang Li (2015) [40]

In this research, a self healing concrete was prepared to depict microbiologically induced calcite precipitation. Water curing proved to be the most favourable method for curing

bacterial concrete. On the cracked surfaces of the mortar samples, the precipitates were visualized with SEM equipped with an EDAX and then examined by XRD. It was observed that with increasing average crack width, the crack was proving more troublesome to be healed. The effectiveness of microbial healing medium to heal cracks was restricted to 0.8 mm. After 60 days of cracking age, the crack healing ratio was considerably low; it decreased consequently with increasing cracking duration.

2.13 Yusuf Çağatay Erşan, Filipe Bravo Da Silva, Nico Boon, Willy Verstraete and Nele De Belie (2015) [41]

This paper discussed the effectiveness of some commercially provided protection methods on the setting time and compressive strength of mortar specimens when mixed with either *Bacillus sphaericus* or *Diaphorobacter nitroreducens*. In this investigation, initial setting time (IST), final setting time (FST) and compressive strength tests were conducted.

It was observed that:

i. When granular activated carbon, expanded clay or zeolite was mixed in the sample, the specimens showed a reduction up to 20 min in IST and an delay up to 30 min in FST.
ii. For mortar mixture having diatomaceous earth or metakaolin, the IST and FST reduced up to 100 min and 250 min, respectively.
iii. Adding nutrients for *B. sphaericus* caused a delay of 40 min in IST and 340 min in FST. But, when the medium sources for *D. nitroreducens* were mixed with cement paste, no considerable difference was reported in IST whereas the FST delayed by 40 min.
iv. CERUP increased IST by 40 min and ACDC resulted in the reduction by 20 min. Although, both CERUP and ACDC increased FST by 50 min.
v. Compressive strength test on different specimens did not yield a reliable result as it varied greatly for different healing agents.

2.14 S. Krishnapriya, D.L. Venkatesh Babu and Prince Arulraj G. (2015)[43]

The interest of this study was to determine the effectiveness of calcite precipitating bacteria for improving the strength of concrete specimens. The bacteria were isolated from the alkaline soil samples. For comparing the results, *Bacillus megaterium* MTCC 1684 was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Wheat bran was used as a substitute for growing bacterial cells. M25 concrete grade was prepared according to Indian standards. Control specimens were made without incorporating bacterial cells into them. To induce crack in the concrete beams, a thin copper plate 0.3 mm thick was introduced up to a depth of 10 mm in the young concrete. At the time of demoulding, the plates were removed to allow a clear visibility of cracks in the beam samples. Through SEM images, the precipitated calcite by bacterial cells in the micro cracks and pores in concrete specimens were visualized.

. The critical findings of this study were:

i. Every bacterial isolate was able to form endospores. However, *Bacillus flexus* could show only restricted amount of endospore formation as compared to the other isolates and standard culture.
ii. Each of the three bacterial isolate and the standard culture were able to precipitate calcite that would help in plugging the micro cracks and pores in concrete. Here also, *Bacillus flexus* produced the lowest amount of calcite and thus, was concluded to be least effective in improving compressive strength and crack healing of concrete specimens.
iii. The compressive strength of bacterial concrete specimens has increased as compared to control concrete specimens (without bacterial cells).
iv. The bacterial concrete specimens had shown distinct calcite crystals under SEM analysis.
v. Wheat bran can be used as an alternative substrate for growing bacteria that will result in reducing the cost of bacterial concrete.

2.15 E. Tziviloglou, V. Wiktor, H.M. Jonkers and E. Schlangen (2016) [45]

This research focussed on investigating the role of self-healing agent when mixed into light weight aggregates (LWA) with fresh cement paste. It evaluated the recovery of water tightness after cracking and under two different healing regimes by water permeability testing.

The important findings of this study were:

i. By replacing sand with light weight aggregates, a significant decrease in the bulk density and increase in the air content was observed, while it barely affected the consistency of the fresh mix. Moreover, the healing agent considerably affected the properties of the mortar mix at the duration of 3 days, which lead to a weakened material. But, after 7 days, the flexural strength of all 3 kinds of specimens followed a similar pattern.
ii. The compressive strength of samples with normal weight aggregates was significantly greater at all duration in comparison with the other mixes with light weight aggregates.
iii. SEM analysis showed that the major crystal shapes observed in the cracked mortar specimens were either cubic or clustered asymmetric rhombohedral, indicating the precipitation of calcite.
iv. FT-IR spectra for 3 different samples depicted strong calcite bands appearing at wave numbers 1400 cm^{-1}, 870 cm^{-1} and 712 cm^{-1}, which is generally the range of carbonate vibration in calcite.
v. Oxygen consumption was found only in bacterial specimens.
vi. The lightweight mortar with incorporated bacterial cells showed better crack healing, especially when exposed to wet dry cycles, rather than immersing in water continuously.

2.16 Kunal, Rafat Siddique, Anita Rajor and Malkit Singh (2016) [46]

In this paper, the authors treated Cement kiln dust, a bypass dust with bacterium *Bacillus halodurans.* They investigated the influence of bacterial treated cement kiln dust as a partial replacement for portland cement on various properties of concrete like compressive strength

and splitting tensile strength, water absorption, porosity, ultrasonic pulse velocity (UPV), and chloride permeability of concrete. After that, the concrete specimens were extracted from the inner core of the matrix and visualized by SEM and then by XRD.

The following observations were made in this study:

i. The compressive strength and splitting tensile strength enhanced by adding 10% bacterial treated cement kiln dust, by 26.6% and 25.6% respectively. However, for replacements greater than 10% cement, both the compressive strength and splitting tensile strength decreased significantly.
ii. By replacing cement by 10% bacterially treated CKD, the permeation characteristics reduced and above that, these characteristics increased for 28 and 91 days.
iii. The UPV test revealed that the sample with 10% cement kiln dust had higher UPV, which was an indication of denseness with lesser pores, thus allowing quicker wave propagation and higher strength.
iv. Also, SEM and XRD images indicated higher formation of calcium silicate hydrate gel that caused denser structure and lower permeability.
v. It was observed that at 10% CKD, the water absorption reduced by 64%, porosity by 53%, and chloride permeability by 22%.
vi. Thus, it can be said that the optimum concentration of bacterially treated cement kiln dust for replacing cement in concrete was 10%.

2.17 Ramin Andalib, Muhd Zaimi Abd Majid, Mohd Warid Hussin, Mohanadoss Ponraj, Ali Keyvanfar, Jahangir Mirza and Han-Seung Lee (2016) [47]

In this study, five bacterial concentrations of *Bacillus megaterium* (10 x 10^5 to 50 x 10^5 cfu/ml) were incorporated in concrete specimens to determine the optimum amount of bacteria to be added for structural concreting. The bacteria was isolated from the soil sample collected from a silty clayey location. MICP was quantified through XRD analysis, visualized by SEM and analysed by Energy dispersive spectrometer (EDS). To ascertain the optimum bacterial concentration for preparing concrete, the micro structural investigation was also conducted by compressive strength and flexural strength tests.

The important findings of this study were:

i. The microbial concentration of 30 x 10^5 cfu per ml depicted significant improvement in the compressive strength and flexural strength of concrete specimens..
ii. SEM, XRD, and EDS analysis confirmed that bacterial concentration of 10^5 cfu per ml and 30 x 10^5 cfu per ml proved to be best for improving the concrete properties.
iii. It was concluded that the optimum bacterial concentration had a profound impact on high strength concrete. Thus, the concrete specimens prepared with *Bacillus megaterium* can be proposed to be used as an ecological substance in the construction practices.

2.18 Rajneesh Vashisht, Sampan Attri, Deepak Sharma, Abhilash Shukla and Gunjan Goel (2018) [48]

This study investigated the influence of calcite precipitating bacteria isolated from alkaline soil samples in improving the strength and healing potential of concrete. For this, 16 samples comprising eight samples of alluvial soil and sewage samples each were obtained. Qualitative and quantitative urease assay tests were conducted on 14 different bacterial isolates. Then, calcium carbonate precipitation test was performed to finally select 5 potential isolates for crack remediation of concrete. For inducing cracks, hexa blade was used on concrete cubes. The precipitated calcite was examined using FE-SEM for *B. megaterium* MTCC 1684 and isolate I13 in concrete and then analyzed by EDS.

The crucial findings of this study were:

i. Only isolate I13 (*Lysinibacillus* sp.) was able to form endospore.
ii. Isolate I13 revealed highest conductance and greatest calcite precipitation among all the selected isolates.
iii. The FT-IR spectra of bacterial specimens showed single bonding present between carbon and oxygen, typical for carbonate group lying between wave number of 1000 – 1300 cm^{-1}.
iv. An improvement in compressive strength was revealed both in *Bacillus megaterium* MTCC 1684 (by 14.8%) and *Lysinibacillus* sp. I13 (by 34.6%) concrete sample as compared to the control concrete specimen.

v. The visual inspection revealed that the maximum crack healing was for the sample with *Lysinibacillus* sp. as compared to the standard culture (*B. Megaterium*) and control specimens.
vi. Thus, it was concluded that *Lysinibacillus* sp. can be used in self-healing concrete for crack remediation of concrete structure.

CHAPTER 3

MATERIALS & METHODS

The present investigation is divided into five phases:

A. Isolation and identification of calcite precipitating bacteria
B. Testing of construction materials
C. Preparation of concrete specimens
D. Testing of concrete samples
E. Quantification of crack healing

3.1 Isolation And Identification Of Calcite Precipitating Bacteria

This phase deals with the isolation of bacteria to get its pure colony. This section gives the methodology of culturing bacteria and screening it to select the desired strains of calcite precipitating bacteria. Various tests were performed for selecting the best isolates for calcite precipitatin. Moreover, a bacterial strain *Bacillus megaterium* MTCC 1684, procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India was used as the standard culture to compare the results with selected isolates. The stages below describe the process of isolation and identification of bacteria.

3.1.1 Sample Collection

A total of 15 samples were obtained from various locations of Himachal Pradesh, India. The selected samples are of alkaline nature and rich in lime and iron oxide. Few of the samples were obtained from Barmana of Bilaspur district, Himachal Pradesh (India) and remaining alkaline soil samples were randomly collected from Jaypee University of Information Technology campus, Waknaghat, District Solan, (H.P, India). All the samples were collected in sterile plastic bags and were kept at 4°C until their use. The storage is maintained at 4°C in microbiology experiments to slow down/almost stop the growth of bacterial culture. Also, 4°C slow down the degradation of purified DNA sample.

Table 3.1 gives detailed information about the sources of all the samples.

Table 3.1 Samples Collected

Sample No.	Type	Source
1	Alkaline Soil	ACC Cement Plant, Barmana, (District Bilaspur)
2	Crusher Soil	Ambuja Cement Plant, Darlaghat, (District Solan)
3	Natural Soil	Darlaghat roadside
4	Vegetative Soil	Koldam, Barmana (District Bilaspur)
5	Natural Soil	
6	Poultry Farm Soil	District Hamirpur
7	Limestone Sample	Darlaghat
8	Cement Sample	Concrete Technology Lab, JUIT, Waknaghat (District Solan)
9	Fine Sand	Structural Mechanics Lab, JUIT
10	Crushed Brick Sample	JUIT Campus
11	Natural Soil	Domeher roadside (District Solan)
12	Sandy Soil	JUIT Campus, Waknaghat (District Solan)
13	Natural Soil	
14	Vegetative Soil	
15	Sandy Soil	Domeher

3.1.2 Culturing of Bacteria

1) **Luria Broth Preparation -** For the enrichment of urease producing bacteria, Luria Broth (LB) (Casein enzymic hydrolysate 10.0 g/L; Yeast extract 5.0 g/L; NaCl 5.0 g/L, pH 7.0 ± 0.2) (HiMedia, India) was prepared (2% in distilled water). Thus, for 15 samples, 15g LB was mixed in 750 ml of distilled water and then distributed into 15 conical flasks (50 ml each). Then, the flasks were plugged tightly with the help of cotton plugs and covered with paper catalogues. Cotton plugs are used to keep fungal or bacterial infection from penetrating inside the flask while still allowing for proper aeration. Covering with paper catalogue is done so as to protect the sterilized flasks from being contaminated again after

picking them out from autoclaving. Table 3.2 gives the equipments and glassware used for the isolation of bacteria.

Table 3.2 Equipments used for isolation and identification of bacteria

S. No.	Equipment	Purpose
1	Analytical Balance	For weighing chemicals & samples
2	Spatula	For weighing & mixing materials
3	Conical Flask	For mixing nutrient media
4	Measuring Cylinder	To measure media & distilled water
5	Autoclave	To sterilize media & glassware
6	Inoculating Loop	For transferring & streaking cultures
7	Static Incubator (@37°C)	For growing bacteria
8	Shaker Incubator (@37°C, 150 rpm)	For cultivating & mixing bacteria
9	Laminar Air Flow	To work aseptic (in sterilized environment)
10	Micropipette	To transfer small quantity of liquid (in µl)
11	Eppendorf Microcentrifuge Tubes	To conduct serial dilution
12	Cell Spreader (L shaped)	To spread bacteria on petri plate
13	pH Meter	To determine pH
14	Water bath	To melt agar
15	Hot Plate	
16	Microwave Oven	
17	Petri plate	For culturing bacteria
18	Test tube	To grow bacteria & make slants
19	Glass Slide	To perform staining
20	Light Microscope	For viewing bacterial stains

2) **Autoclave** – It is an apparatus which is used to sterilize equipments, media and supplies by subjecting them to pressurized saturated steam at 121°C (249°F) for around 15–20 minutes depending on the size of the load and the contents. The basic concept of an autoclave is to have each item sterilized -whether it is a liquid, plastic ware, or glassware, coming in direct contact with steam at a specific temperature and pressure for a specific amount of time. Time, steam, temperature, and pressure are the

four main parameters required for a successful sterilization using an autoclave. In this study, we autoclaved our nutrient media at 121°C for 15 minutes at 15 psi pressure. Figure 3.1 shows the autoclave used in this study.

Figure 3.1 Autoclave

3) **Inoculation of Samples into LB flasks** – After autoclaving the media, one gram of each sample was inoculated into respective flask by sterilized spatula under Laminar Air Flow and then incubated at 37°C, 150 rpm for 3-5 days in shaker incubator.

4) **Urea Broth Preparation** – In the meantime, urea broth (UB) and urea agar (UA) were prepared separately as nutrient media in a similar process like that of Luria Broth and adjusted to pH 8.0 using a few drops of Sodium Hydroxide. The flasks were then autoclaved. The composition of media chemicals are given in the Appendix A of this report.

5) **Inoculation of LB into UB** – After incubation of sample inoculated LB flasks for 3 days, from each LB flask, 1 ml was transferred to respective 50 ml UB flask by micropipette under Laminar Air Flow. Then, the UB flasks were subjected to incubation at 37°C, 150 rpm for 3-5 days in shaker incubator.

6) **Pouring UA in petri plates** – Subsequently, frozen autoclaved urea agar medium was subjected to melting under water bath or hot plate. Then, the molten urea agar was poured into 45 (15 x 3) petri plates inside Laminar air flow.

7) **Serial Dilution** – A serial dilution is a step-by-step process used for reducing a dense concentration of bacterial cells to a more convenient concentration. Each dilution, thus reduces the bacterial concentration by a certain quantity. This process was performed under laminar airflow using micropipette. Here, 0.9 ml saline (0.89% NaCl in distilled water) was firstly added into each eppendorf tube (6 tubes per sample, for dilutions: 10^{-1} to 10^{-6}). For first sample, 0.1 ml of incubated UB was added to tube having 10^{-1} dilution and mixed thoroughly. Then, 0.1 ml of this tube was transferred to 10^{-2} dilution tube and mixed. This process is continued up to 10^{-6} dilution. And, this procedure was repeated for all the remaining samples (14 in this case). Figure 3.2 shows the serial dilution process under laminar air flow.

Figure 3.2 Serial Dilution

8) **Spreading of dilutions on Petri plates** – After serially diluting bacteria into eppendorf tubes, for each sample, 3 concentrations were selected for spreading on petri plates, namely: 10^{-2}, 10^{-4}, 10^{-6}. For this, 1 ml of dilution was transferred on petri plate and spread evenly by cell spreader. This process was performed for all samples

(i.e. total 45 spreadings). The plates were then incubated @ 37°C for 5 days in Static Incubator. Figure 3.3 shows the spreading on petri plates under laminar air flow.

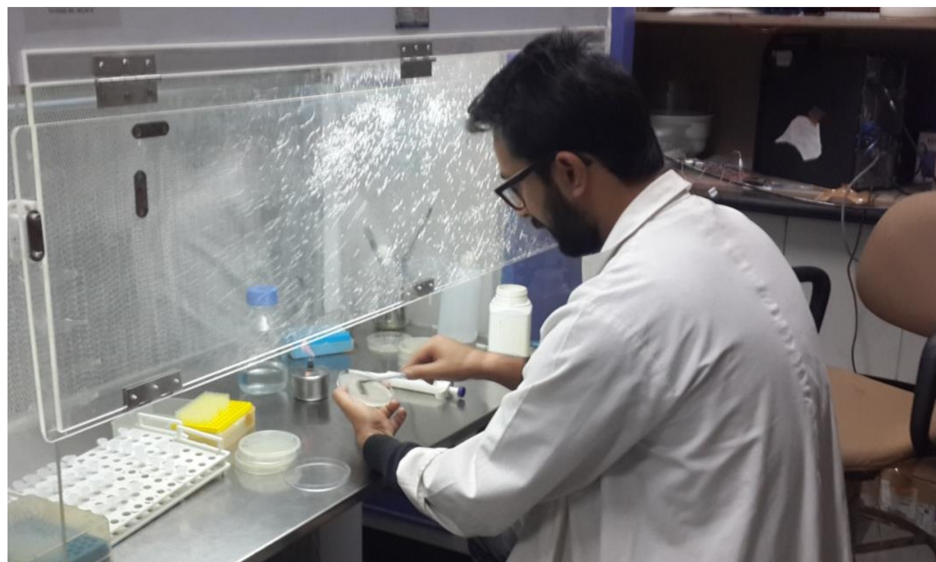

Figure 3.3 Spreading on Petri plates

9) **Streaking of colonies on UA plates** – The individual visible colonies were streaked on urea agar petri plates and incubated at 37°C for 5 days in Static Incubator.

10) **Sub Culturing of colonies -** The grown bacterial colonies from the petri plates were sub cultured two more times on the same medium from where they were picked. This was done so as to get the pure colonies of bacteria.

11) **Inoculation on UB tubes** – 5 ml of urea broth tubes were prepared for each distinct colony and autoclaved. Then, single colony from petri plate was inoculated in the respective UB tubes and incubation was done at 37°C for 3-4 days in Static Incubator.

12) **Streaking of colonies on UA plates** – Then, streaking of the above colonies was done on urea agar plates and left for incubation at 37°C for 5 days in Static Incubator. From this, 26 distinct bacterial colonies were obtained and were further processed for physiological characterization. The appearance of bacterial colonies is shown in the Appendix B of this report.

3.1.3 Physiological characterization of Bacterial isolates

Various morphological and biochemical studies were performed on the selected 26 bacterial isolates to characterize them. These were:

1) **Gram Staining –** Gram staining is used to characterize bacteria in two groups, gram positive bacteria or gram negative bacteria**.** Gram positive bacteria generally exhibit greater affinity for crystal violet as compared to gram negative bacteria when gram's iodine is applied [49]. Table 3.3 gives the chemicals used in gram staining of bacteria.

Table 3.3 Chemicals used in gram staining of bacteria

S. No.	Chemical	Acts as
1	Crystal Violet	Primary Stain
2	Gram's Iodine	Mordant
3	Ethanol (95%)	Decolourizer
4	Safranin	Counter Strain
5	Immersion oil	To increase resolving power of microscope

Firstly, UB tubes were prepared, autoclaved and then inoculated with respective bacterial isolate. Then, the tubes were incubated @ 37°C for 1 day in Static Incubator. After that, glass slides were washed and cleaned with water and later, wiped with spirit or ethanol and left for drying. Under laminar airflow, bacterial smear from UB tubes were spread on glass slide and heat fixed. Then, the heat fixed smear was gently flooded with crystal violet and left for 60 sec. The slide was then rinsed with wash bottle or in a water beaker. Now, the slide was flooded with Gram's iodine and left undisturbed for 60 sec and washed briefly in water. The smear was decolourized with 95% ethanol until the alcohol runs almost clear and then immediately washed in water. After that, slide was gently flooded with safranin and washed with water after 45 sec. Finally, the smear was visualized under light microscope using oil immersion.

2) **Endospore Staining –** Endospore staining is performed to determine whether the bacterium can form endospore or not. *Bacillus* species are known to be spore forming bacteria [50]. Table 3.4 gives the chemicals used in gram staining of bacteria.

Table 3.4 Chemicals used in endospore staining of bacteria

S. No.	Chemical	Acts as
1	Malachite Green	Primary Stain
2	Tap/Distilled water	Decolourizer
3	Safranin	Counter Strain
4	Immersion oil	To increase resolving power of microscope

In this study, Schaeffer-Fulton`s method is used. The primary stain applied here was malachite green, which stains both vegetative cells and endospores. The heat was applied to help the primary stain penetrate the endospore. The bacterial cells were then decolorized with water to remove the malachite green from the vegetative cell but not the endospore. Then, Safranin was applied to counter stain any cells which had been decolorized. On visualizing the slides under light microscope, vegetative cells appear pink, and endospores green.

3) **Calcite Precipitation –** This test was performed to determine the calcite precipitation potential by all the 26 bacterial isolates. For this, bacterial isolates were inoculated in the respective 5 ml Urea broth test tubes and incubated @37°C for 7 days. The calcite precipitation was determined in broth state as per the study [48]. The precipitated calcite was filtered through Whatman Filter Paper and dried in the oven @60°C for 3 hours and then weighed. The weight of precipitated calcite was determined (Eq. 12).

$$W_c = W_2 - W_1 \quad (12)$$

here, W_c Weight of precipitated calcite

W_1 Weight of empty filter paper

W_2 Weight of filter paper containing calcite

4) **Urease Assay –** Qualitative urease assay test was performed on all the 26 bacterial isolates to check their urease activity. For this, few drops of phenol red was added to each Urea Agar test tube and autoclaved. Then, the tubes were kept in a slanted position for the solidification of media. Later, the cultures were streaked on respective slant tubes. This is known as slant streaking. The tubes were then incubated @37°C

and monitored daily to check change in colour from pink to yellow which indicated positive urease activity.

5) **Growth Curve** – This test is done to determine the growth parameters of the selected bacterial isolates 3 and 26 (standard culture). The cells were cultured in Urea Broth medium and the Optical Density of isolates was determined using Spectrophotometer at 600 nm from 0 to 64 hours. This provided the growth rate curve and also the phase of maximum growth of isolates.

3.2 Testing of construction materials

This phase deals with the testing of construction materials (cement, fine aggregate and coarse aggregate). All of these tests are performed according to the Bureau of Indian Standards. The 43 grade Ordinary Portland Cement used in this study must conform to IS 8112: 1989 [53]. These tests are performed so as to get the data required for preparing mix design and to also confirm the quality of construction materials. Table 3.5 shows the equipments and apparatus used in the testing of construction materials.

Table 3.5 Apparatus/Equipment used for testing of construction materials

S. No.	Apparatus/Equipment	Test(s)
1	Vicat's Apparatus	Consistency, IST and FST of cement
2	Le Chatelier Mould	Soundness of cement
3	Le Chatelier Flask	Specific Gravity of cement
4	90 micron sieve	Fineness of cement
5	Wire basket	Specific Gravity of coarse aggregate
6	Pycnometer Bottle	Specific Gravity of fine aggregate

Table 3.6 describes the equipments used in the preparation of cement or concrete specimens.

Table 3.6 Equipments used in the preparation of cement or concrete specimens

S. No.	Equipment	Purpose
1	Weighing Balance	For weighing materials
2	Measuring Cylinder	For measuring water
3	Trowel	To perform gauging
4	Stop Watch	To note time
5	Tamping Rod	For compaction
6	Vibration Machine	For compacting concrete

3.2.1 Testing of Cement

1) **Consistency Test –** This test is performed according to IS 4031 (Part 4): 1988 [54]. For a cement paste, standard consistency is that consistency at which Vicat's plunger of 10 mm dia and 50 mm length can penetrate up to a depth of 7 - 5 mm from bottom of the mould. This test is useful in determining the water content for other tests such as IST & FST, and soundness test.

2) **Initial and Final Setting Time Test –** It follows IS 4031 (PART 5): 1988 [55]. Initial setting time is the duration from when the water is added to cement to time at which the cement paste starts losing its plasticity. Final setting time is the duration from when the water is added to cement up to the time when the cement paste has lost all of its plasticity. The water to be added in this test need to be "0.85 P" by weight of cement, where "P" is the normal consistency of cement obtained from consistency test.

3) **Soundness Test –** This test is performed according to IS 4031 (Part 3): 1988 [56]. Soundness of cement means its ability to resist volume expansion. Because of unburnt lime, dead burnt magnesia and calcium sulphate, unsoundness occurs in cement. Only the presence of unburnt lime (CaO) can be determined by Le-chatelier method. The water added in this test must be "0.78 P" by weight of cement, where "P" is the standard consistency of cement.

4) **Fineness Test –** It follows IS 4031 (PART 1): 1988 [57]. This test was performed using 90 micron sieve. 100 g of cement was sieved for 15 minutes and the retained cement percentage gives the fineness of cement. For fineness of cement greater than 10%, there is a risk of drying shrinkage of concrete which allows cracking in structure.

5) **Specific Gravity Test –** This test is done as per IS 4031 (Part 11): 1988 [58]. Specific gravity is described as the ratio between the weight of a particular volume of cement and weight of an equal volume of water at 4°C. This test is performed using Le Chatelier flask, by using kerosene. The portland cement must have a specific gravity of value around 3.15.

3.2.2 Testing of Fine Aggregate

1) **Fineness Modulus Test –** Fineness modulus of fine aggregate is determined using sieve analysis. This test gives the gradation of sand zone. Fineness modulus is a factor obtained by adding the cumulative percentages of aggregate retained on each of the standard sieves ranging from 10 mm to 150 μ and dividing this sum by 100.

2) **Specific Gravity Test –** This test is performed according to IS 2720 (Part 3): 1980 [59] using pycnometer bottle.

3.2.3 Testing of Coarse Aggregate

1) **Specific Gravity Test –** It is performed according to IS 2386 (Part 3): 1963 [60] using wire basket. This test is done to measure the strength or quality of the material. The aggregates with low specific gravity are usually reported to be weaker as compared to the ones having higher specific gravity value.

3.3 Preparation of concrete specimens

Three different types of concrete samples were prepared, as given in Table 3.7:

Table 3.7 Types of Concrete Specimen prepared

S. No.	Specimen	Description
1	Control Concrete	Without bacterial cells
2	Standard Concrete	Incorporated with standard culture (*Bacillus megaterium*)
3	I3 Concrete	Incorporated with isolate 3 bacterial cells

Bacterial cells were incorporated in concrete with 10^7 to 10^8 cells/ml of mixing water to the design mix using water/cement ratio of 0.45. These cell concentrations were determined using Optical Density of 0.7 to 1.0. Using 43 grade OPC cement, zone II gradation sand, 20 mm coarse aggregates with water/cement ratio of 0.45, the concrete specimens were prepared. The mix design was prepared as per IS 10262: 2009 [52] and the calculations are given as:

1. **Target Strength for mix proportioning:**
   Target Mean Compressive Strength = 20 + 1.65 (4) = 26.6 MPa

2. **Selection of water-cement ratio:**
   Assume water-cement ratio as 0.45

3. **Selection of water content:**
   For 20 mm coarse aggregate, max water content = 186 litre (From Table 2 of IS 10262:2009)

4. **Calculating cement content:**
   Cement content = $\frac{186}{0.45}$ = 413 kg/m^3

5. **Proportion of Volume of aggregate:**
   For Zone II fine aggregate and 20 mm coarse aggregate,
   Volume coefficient of coarse aggregate = 0.62 + 0.01 = 0.63 (From Table 3 of IS 10262:2009)

Volume coefficient of fine aggregate = 0.37

6. **Mix Calculations:**

Volume of Concrete = 1 m^3

Volume of cement = $\frac{413}{3.15}$ x $\frac{1}{1000}$ = 0.13 m^3

Volume of water = 0.186 m^3

Volume of aggregate = 1 – 0.13 – 0.186 = 0.684 m^3

Mass of coarse aggregate = 0.684 x 0.63 x 2.73 x 1000 = 1176 kg

Mass of fine aggregate = 0.682 x 0.37 x 2.62 x 1000 = 663 kg

Table 3.8 presents the Design Mix quantity of M20 concrete used in the study.

Table 3.8 M20 Design Mix Quantity

S. No.	Material	Quantity (kg/m^3)
1	Cement	413
2	Water	186
3	Fine Aggregate	663
4	Coarse Aggregate	1176

3.4 Testing of concrete samples

This phase focuses on compressive strength test, split tensile strength test and flexural strength test of concrete specimens, with and without incorporated bacterial cells. Compression testing machine (CTM) and Flexural testing machine are used in these tests. The tests are performed to compare the strength of bacterial and non bacterial concrete.

1) **Compressive Strength Test –** This test is conducted as per IS 516: 1959 [61]. Compressive strength is the capability of material to carry the loads on its surface without any crack or deflection. The compression test was performed on concrete cubes of dimension 150 mm x 150 mm x 150 mm. The specimens are tested under CTM after 7 days, 14 days and 28 days curing. The load was applied gradually at the rate of 140 kg/cm^2/minute (0.22 MPa/s) till the sample fails. Figure 3.4 shows the compressive strength test setup performed on concrete cube samples.

Figure 3.4 Compressive strength test setup under CTM

The compressive strength is calculated using the following formula (Eq. 13):

$$\text{Compressive Strength} = \frac{P}{A} \qquad (13)$$

here, P Failure load (N)

A Area of specimen (mm^2)

2) **Split Tensile Strength Test –** It follows IS 5816: 1999 [62]. The concrete is very weak in tension due to its brittle nature, so, it develops cracks when tensile forces exceed its tensile strength. Thus, it is essential to determine the tensile strength of concrete to determine the load at which the concrete members may crack. The test is performed under CTM on concrete cylinder of 100 mm diameter and 200 mm height. The load is applied at a constant rate of 1.2 to 2.4 MPa/min until the sample fails. Figure 3.5 shows the split tensile strength test performed on concrete cylinder sample.

Figure 3.5 Failure of concrete cylinder under CTM

The split tensile strength is calculated using the below mentioned formula (Eq. 14):

$$\text{Split Tensile Strength} = \frac{2\,P}{\pi\,L\,D} \tag{14}$$

here, P Failure load (N)

L Cylinder length (mm)

D Cylinder diameter (mm)

3) **Flexural Strength Test –** This test is done according to IS 516: 1959 [61]. Flexural strength test determines the tensile strength of concrete indirectly. The test was conducted on beams of size 100 mm x 100 mm x 500 mm (as the size of aggregate was less than 19 mm). Flexural test was conducted by applying four point loading using flexural testing machine. The load was applied at a rate of loading of 400 kg/min. Figure 3.6 shows the flexural strength test performed on beam specimen.

Figure 3.6 Flexural strength test setup under flexural testing machine

The flexural strength is calculated using the following formula (Eq. 15, 16):

Flexural Strength, $f = \frac{P\,L}{B\,D^2}$: when $a \geq 133$ mm (15)

Also, $f = \frac{3\,P\,a}{B\,D^2}$: when 110 mm $< a \leq$ 133 mm (16)

here,

P	Failure load (N)
L	Span length (mm)
B	Beam width (mm)
D	Beam depth (mm)
a	Distance between line of fracture and nearer support (mm)\

3.5 Quantification of crack healing

In this phase, crack healing by bacteria is to be quantified during and after curing. The cracks are induced in the concrete specimens by introducing a thin copper plate of thickness 0.3 mm to 0.5 mm in the fresh concrete paste up to a depth of 10 mm to 15 mm. The plates are removed after 24 hours, after demoulding. A similar method of introducing cracks was done by the researchers [32,43]. For quantifying the crack healing process, visual inspection of concrete specimens, at regular intervals is done. Figure 3.7 shows the crack induced in fresh concrete cube specimen.

Figure 3.7 Concrete cube specimens with cracks induced (left) and without cracks (middle and right)

CHAPTER 4

RESULTS & DISCUSSION

This chapter discusses the results obtained from various experiments performed.

4.1 Isolation And Identification Of Calcite Precipitating Bacteria Results

4.1.1 Isolation of Bacteria Results

The colonies appeared for most of the samples after cultivating bacteria in nutrient medium. Table 4.1 describes the colonies size and time taken for appearance of colonies.

Table 4.1 Colonies size of samples and the time taken for appearance of colonies

Sample No.	Colonies Size	Time taken to obtain colonies
1	Small	3 days
2	Medium	4 days
3	Small	4 days
4	Very Small	4 days
5	Medium – Large	4 days
6	Very Small	5 days
7	Small	3 days
8	Small	5 days
9	No colony	—
10	Medium	5 days
11	No colony	—
12	No colony	—
13	Very Small	5 days
14	Medium	4 days
15	No colony	—

From these results, colonies were selected for sub culturing them to get the best bacterial isolates.

26 distinct bacterial isolates, including *Bacillus megaterium* MTCC 1684 (BM 1684), were obtained on petri plates after growing them from urea broth tubes. Table 4.2 represents the morphology of these selected isolates.

Table 4.2 Morphology of Selected Isolates

Isolate No.	Sample No.	Colony Size	Shape	Colour
1	1	Small	Round	Greyish white
2	1	Small	Perfect Round	Greyish white
3	1	Small	Round	White
4	1	Very Small	Round	White
5	1	Small	Round	Pale White
6	1	Small	Irregular Round	Pale White
7	1	Small	Round	White
8	1	Small	Round	Pale White
9	1	Very Small	Perfect Round	Greyish White
10	1	Very Small	Round	White turned safranin
11	1	Small	Irregular Round	White turned green
12	2	Large	Irregular Round	White
13	2	Large	Irregular Round	Faded White
14	2	Medium	Irregular Round	Faded Yellow
15	7	Small – Medium	Round	Greyish White
16	5	Medium	Perfect Round	White
17	5	Medium – Large	Irregular Round	Greyish White
18	5	Medium	Perfect Round	White
19	5	Small	Round	White
20	6	Very Small	Round	Yellow
21	10	Medium	Irregular Round	Greyish White
22	14	Medium	Irregular Round	Greyish White
23	14	Medium	Irregular Round	Greyish White
24	14	Small	Round	Pale White
25	14	Small	Round	Pale White
26	BM 1684	Medium - Large	Perfectly Round	White

4.1.2 Gram Staining Results

The gram staining results have shown that most of the isolates are of the genus *Bacillus,* which are known to be calcite precipitating bacteria. The images of gram staining results are provided in Appendix C. Table 4.3 presents the gram staining results of isolates.

Table 4.3 Gram Staining Results of Isolates

Isolate No.	Gram	Shape
1	+	Bacilli
2	+	Streptococci
3	+	Bacilli
4	+	Bacilli
5	+	Bacilli
6	+	Bacilli
7	+	Bacilli
8	+	Cocci
9	Not found	—
10	+	Bacilli
11	+	Bacilli
12	+	Bacilli
13	+	Bacilli
14	+	Cocci
15	+	Bacilli
16	+	Bacilli
17	+	Bacilli
18	+	Bacilli
19	+	Bacilli
20	+	Bacilli
21	+	Bacilli
22	+	Bacilli
23	+	Streptobacilli
24	+	Cocci
25	+	Cocci
26	+	Bacilli

4.1.3 Calcite Precipitation Results

This test depicts that the isolates 1, 3, 4, 6, 12, 13, 19, 24, 25 and 26 are heavy calcite precipitating bacteria. The difference of weight of dry filter paper containing calcite and that of empty filter paper for all isolates are shown in Table 4.4.

Table 4.4 Results of Calcite Precipitation

Isolate No.	Weight of Calcite Precipitated (mg)
1	79.6
2	68.9
3	117.5
4	86.4
5	68.9
6	92
7	45.1
8	39
9	16
10	47.6
11	51.1
12	116.8
13	112.1
14	41.3
15	64.4
16	74.5
17	62
18	76.2
19	97.3
20	56.5
21	53.8
22	22.6
23	19.8
24	117.9
25	96.6
26	109.2

4.1.4 Urease Assay Results

It is observed that only isolates 21, 24 and 25 are showing negative urease activity (no colour change). The images of Urease Assay results are shown in Appendix D. Table 4.5 presents the results of urease activity.

Table 4.5 Results of Urease Activity

Isolate No.	Colour Change	Urease Activity
1	Yes	+
2	Yes	+
3	Yes	+
4	Yes	+
5	Yes	+
6	Yes	+
7	Yes	+
8	Yes	+
9	Yes	+
10	Yes	+
11	Yes	+
12	Yes	+
13	Yes	+
14	Yes	+
15	Yes	+
16	Yes	+
17	Yes	+
18	Yes	+
19	Yes	+
20	Yes	+
21	No	-
22	Yes	+
23	Yes	+
24	No	-
25	No	-
26	Yes	+

4.1.5 Growth Curve Results

The growth curve of selected isolates (3 and 26) was done for 64 hours. These cultures were selected on the basis of calcite precipitation and urease assay results. Figure 4.1 and 4.2 shows the growth curve of isolate 3 and isolate 26 (standard culture) respectively. It can be seen from the curves that Isolate 3 exhibits higher and quicker growth as compared to the standard culture (Isolate 26).

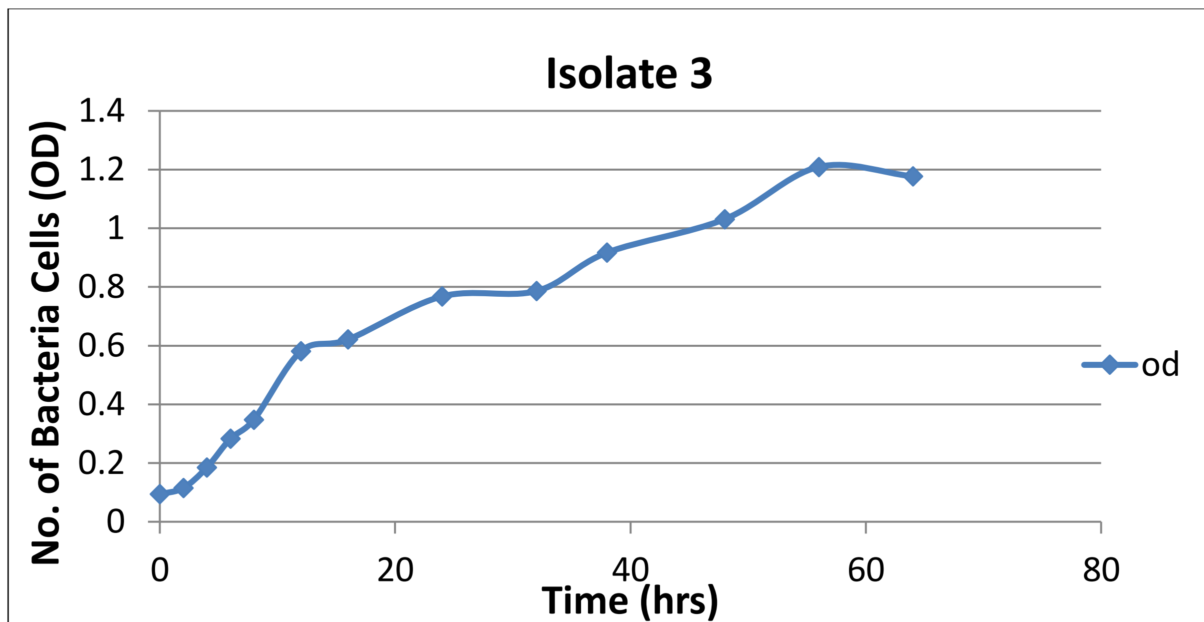

Figure 4.1 Growth curve of Isolate 3

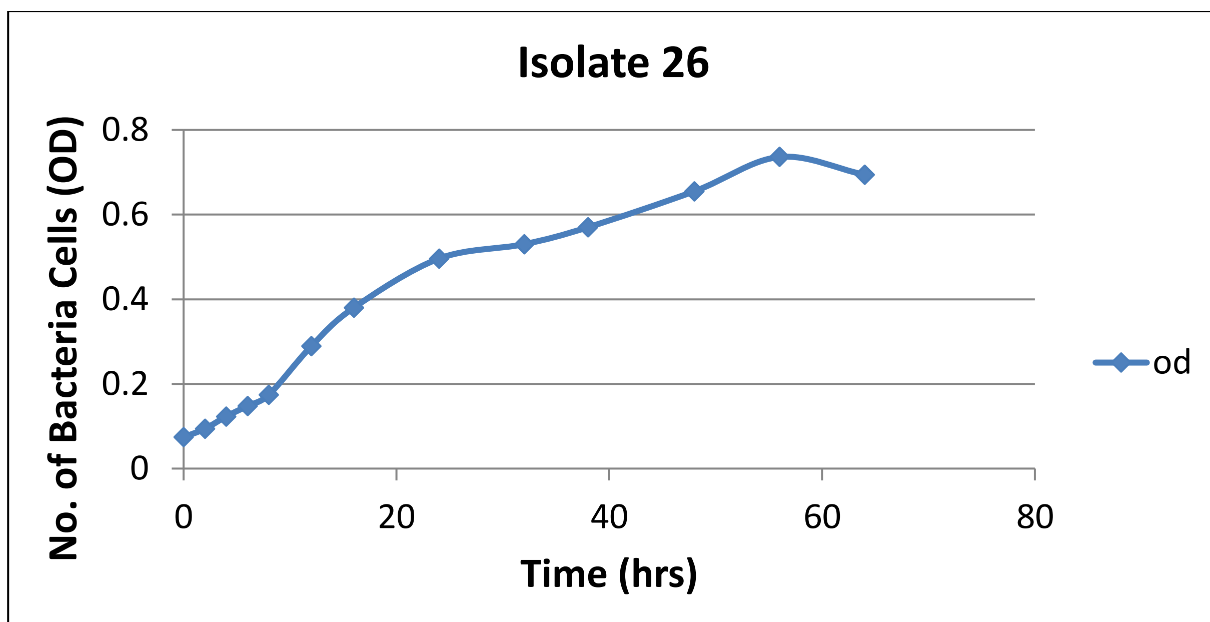

Figure 4.2 Growth curve of Isolate 26 (standard culture)

4.2 Construction Materials Testing Results

The results obtained after testing the materials (cement, fine aggregate and coarse aggregate) were compared with their respective specifications given by Indian Standards. It was found that all the materials used were of adequate quality and there was no need to replace any material. Table 4.6 describes the results of tests performed on construction materials.

Table 4.6 Results of Material Testing

S. No.	Test performed	Value Obtained
1	Standard consistency of cement	30%
2	Initial Setting Time of cement	40 min
3	Final Setting Time of cement	230 min
4	Soundness of cement	2.43 mm
5	Fineness of cement	2.18%
6	Specific Gravity of cement	3.15
7	Fineness Modulus of Fine Aggregate	2.75
8	Specific Gravity of Fine Aggregate	2.62
9	Specific Gravity of Coarse Aggregate	2.73

As the fineness modulus of sand comes out to be 2.75, the average size of fine aggregate is in between the second sieve (0.3 mm) and third sieve (0.6 mm). According to IS 383 - 1970, the given fine aggregate is of Zone II and as per the fineness modulus, the sand can be considered as Medium sand.

4.3 Concrete Specimens Testing Results

4.3.1 Compressive Strength Test Results

The results of compressive strength tests of concrete specimens performed under compression testing machine at 7, 14 and 28 days curing are provided in Table 4.7, Table 4.8 and Table 4.9, respectively.

Table 4.7 Compressive Strength Test Results at 7 days curing

Concrete Specimen	Compressive Strength (MPa)				
	Sample 1	Sample 2	Sample 3	Average	% Increment in Strength
Control Concrete	17.72	18.24	18.19	18.05	-
Standard Concrete	20.22	20.75	19.47	20.15	11.63%
I3 Concrete	22.42	23.91	24.75	23.69	31.25%

Table 4.8 Compressive Strength Test Results at 14 days curing

Concrete Specimen	Compressive Strength (MPa)				
	Sample 1	Sample 2	Sample 3	Average	% Increment in Strength
Control Concrete	22.23	22.45	23.04	22.57	-
Standard Concrete	24.49	25.11	24.73	24.78	9.80%
I3 Concrete	25.44	25.80	26.14	25.79	14.27%

Table 4.9 Compressive Strength Test Results at 28 days curing

Concrete Specimen	Compressive Strength (MPa)				
	Sample 1	Sample 2	Sample 3	Average	% Increment in Strength
Control Concrete	23.74	24.15	25.38	24.42	-
Standard Concrete	26.70	26.88	27.32	26.97	10.44%
I3 Concrete	28.55	28.43	28.94	28.64	17.28%

As it can be seen from the results above, I3 concrete depicted greater increment (17.28%) in compressive strength than standard concrete (10.44%), as compared to control specimen. This improvement in the compressive strength of bacterial concrete specimens was due to the calcium carbonate deposition on the surface of cell and inside the pores of concrete matrix [15,20,31,36]. When the curing of bacterial concrete cubes started, the bacterial cells obtained adequate nourishment due to the cubes being porous at younger age. As the fresh concrete is highly alkaline, the microbial cells adapt to a new environment during the curing duration. When the bacteria start growing inside the cube, calcite begins to precipitate on the cell surface and within the pores of matrix, making concrete less porous [36]. This causes an improvement in the compressive strength of microbiologically treated concrete samples. With increase in incubation duration, the improvement in compressive strength of specimens is expected due to the extra cellular growth generated by bacterial cells. The significant difference between standard concrete and I3 concrete can be attributed to the results obtained from the calcite precipitation results (see section 4.1.3). Isolate 3 gave higher precipitated calcite as compared to the standard culture. Figure 4.3 shows the comparison in compressive strength of control, standard and Isolate 3 concrete cube specimens.

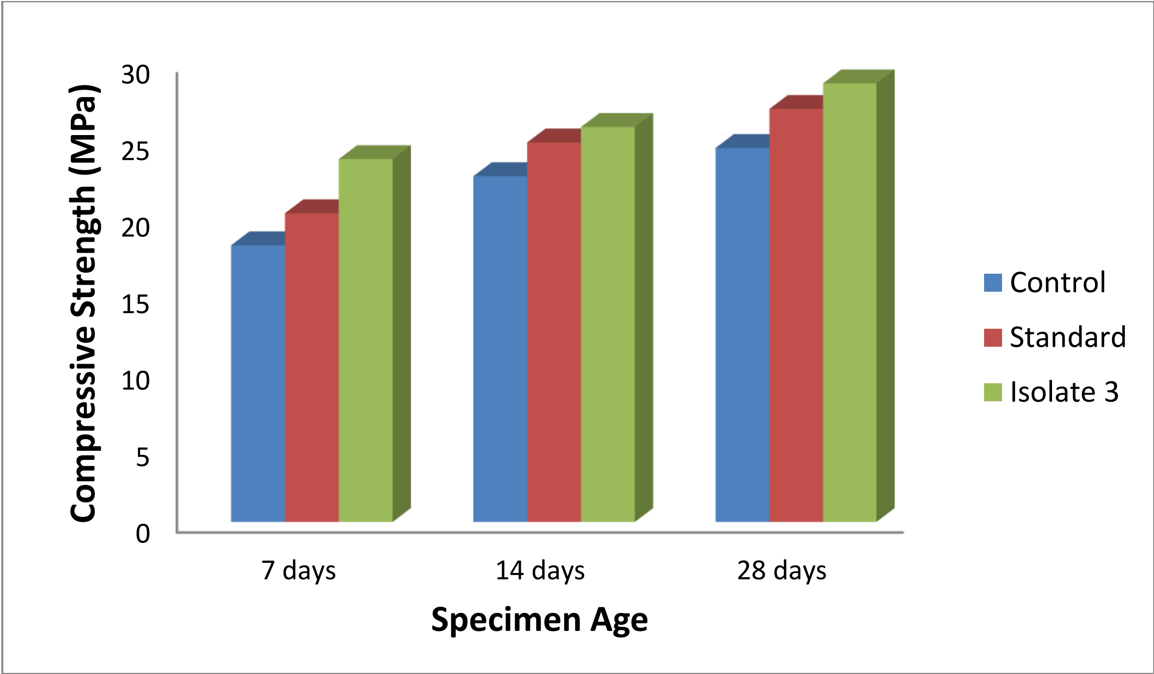

Figure 4.3 Comparison in Compressive strength of Concrete Specimens

4.3.2 Split Tensile Strength Test Results

The results of split tensile strength tests of cylindrical concrete specimens performed under compression testing machine at 28 days are given in Table 4.10.

Table 4.10 Split Tensile Strength Test Results at 28 days curing

Concrete Specimen	Split Tensile Strength (MPa)				
	Sample 1	Sample 2	Sample 3	Average	% Increment in Strength
Control Concrete	2.33	2.49	2.52	2.45	-
Standard Concrete	2.88	2.74	2.97	2.86	16.73%
I3 Concrete	2.93	3.11	3.34	3.13	27.76%

It is evident from these results that the tensile strength of bacterial concrete is significantly greater than that of control concrete samples. Maximum increase (27.76%) is seen in case of concrete prepared with Isolate 3 bacteria. This increase in strength is due to the formation of additional calcium silicate hydrate and reduction in the pore space of concrete matrix. Figure 4.4 shows the comparison in split tensile strength of control, standard and Isolate 3 concrete cube specimens.

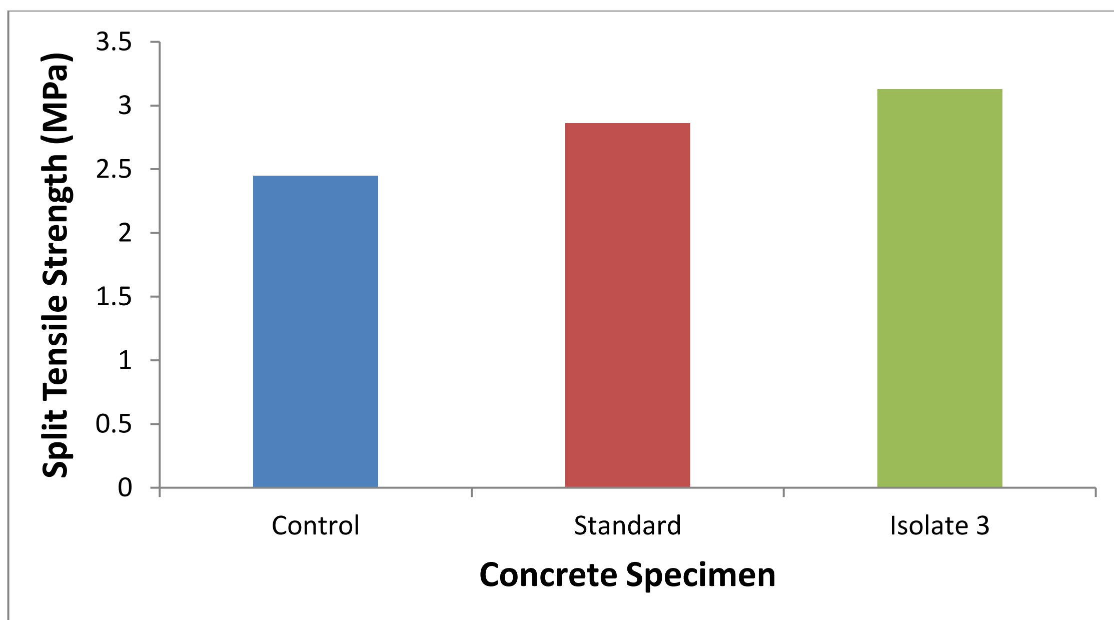

Figure 4.4 Comparison in Split Tensile strength of Concrete Specimens

4.3.3 Flexural Strength Test Results

Table 4.11 shows the results of flexural strength tests of concrete beam specimens performed under flexural testing machine at 28 days.

Table 4.11 Flexural Strength Test Results at 28 days curing

Concrete Specimen	Flexural Strength (MPa)				
	Sample 1	Sample 2	Sample 3	Average	% Increment in Strength
Control Concrete	3.03	3.18	3.21	3.14	-
Standard Concrete	3.58	3.77	3.82	3.72	18.47%
I3 Concrete	3.96	4.48	4.29	4.24	35.03%

The above results prove that the increase in flexural strength for I3 concrete (35.03%) is much greater than that of standard concrete (18.47%) relative to control concrete specimen. The comparison in flexural strength of control, standard and Isolate 3 concrete cube specimens is depicted in Figure 4.5.

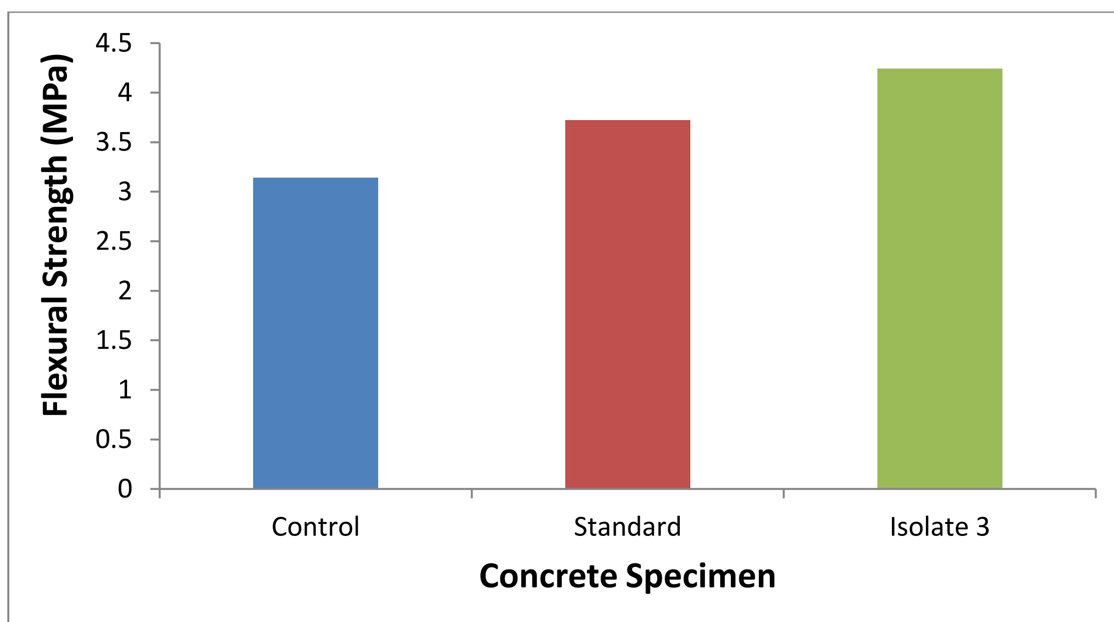

Figure 4.5 Comparison in Flexural strength of Concrete Specimens

4.3.4 Crack healing Quantification Results

This section illustrates the self-healing concept in both bacterial and non-bacterial concrete specimens. The results of crack healing quantification for control concrete specimen are given in Figure 4.6. It is clearly seen from the images that the self-healing phenomenon (without bacterial cells) started after 7 days of curing. After 14 days, the 0.7 mm wide crack reduced to 0.5 mm and 0.5 mm to 0.4 mm. Even at 28 days of curing, this crack width did not reduce. This is in accordance with various studies that the Autogenous healing without microbial cells only occurs by 0.1 - 0.2 mm width [7-11].

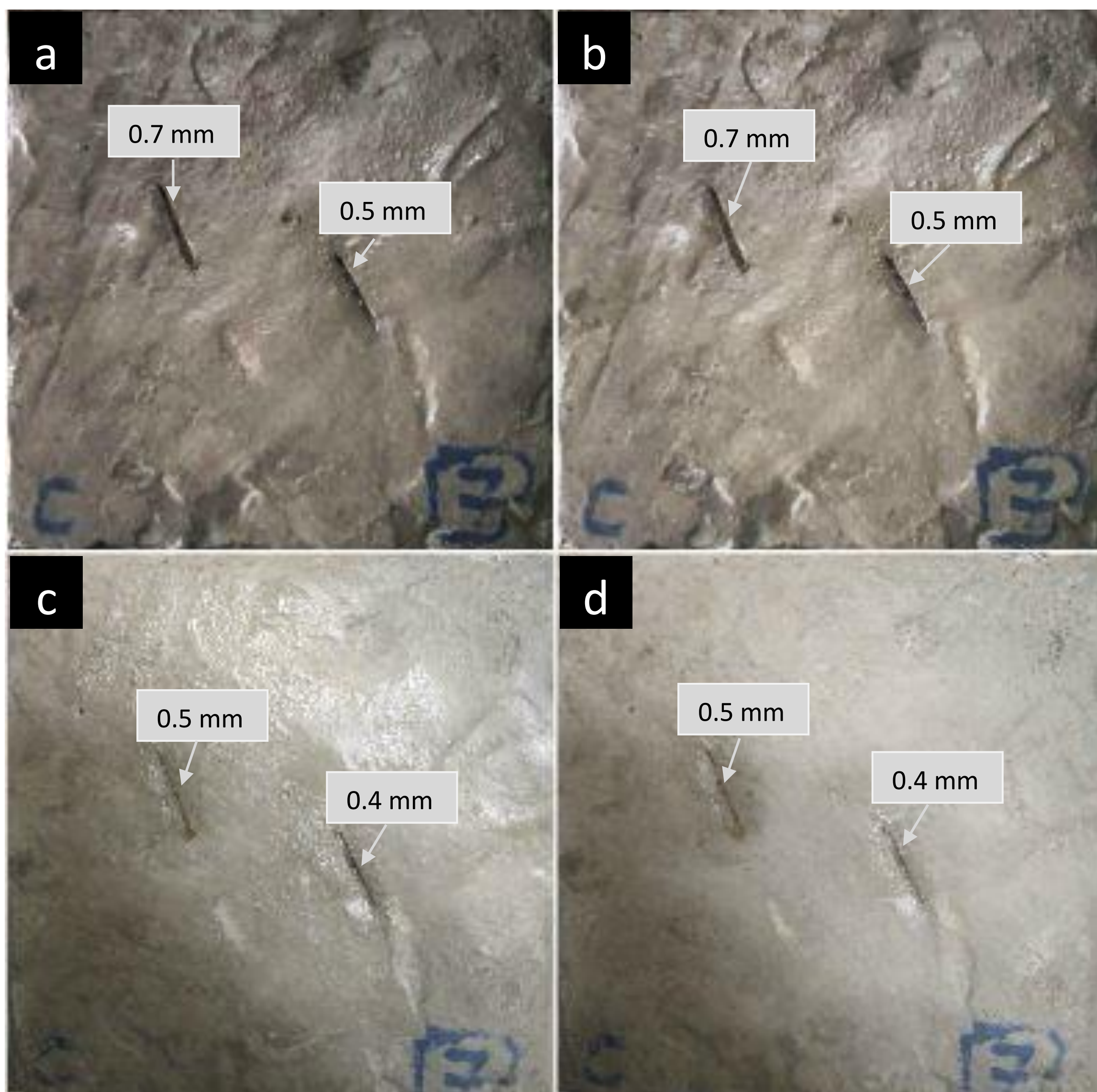

Figure 4.6 Crack healing images of Control concrete specimens (a- 0 day cured, b- 7 days cured, c- 14 days cured, d- 28 days cured)

The crack healing images of standard culture concrete are shown in Figure 4.7. It can be observed from these illustrations that healing in standard concrete specimen started after curing for a few days itself. At 7 days, the 0.6 mm and 0.5 mm crack healed by 0.1 mm each and finally after 28 days, the crack width reduced to 0.3 mm and 0.4 mm. Although, the crack healing took place for only 0.2 - 0.3 mm but the calcite precipitation was clearly visible (whitish-yellow substance) near the crack edges. This layer of calcite deposited on the crack surfaces was a result of hydrolysis of urea into ammonia and carbonate, thus forming calcium carbonate.

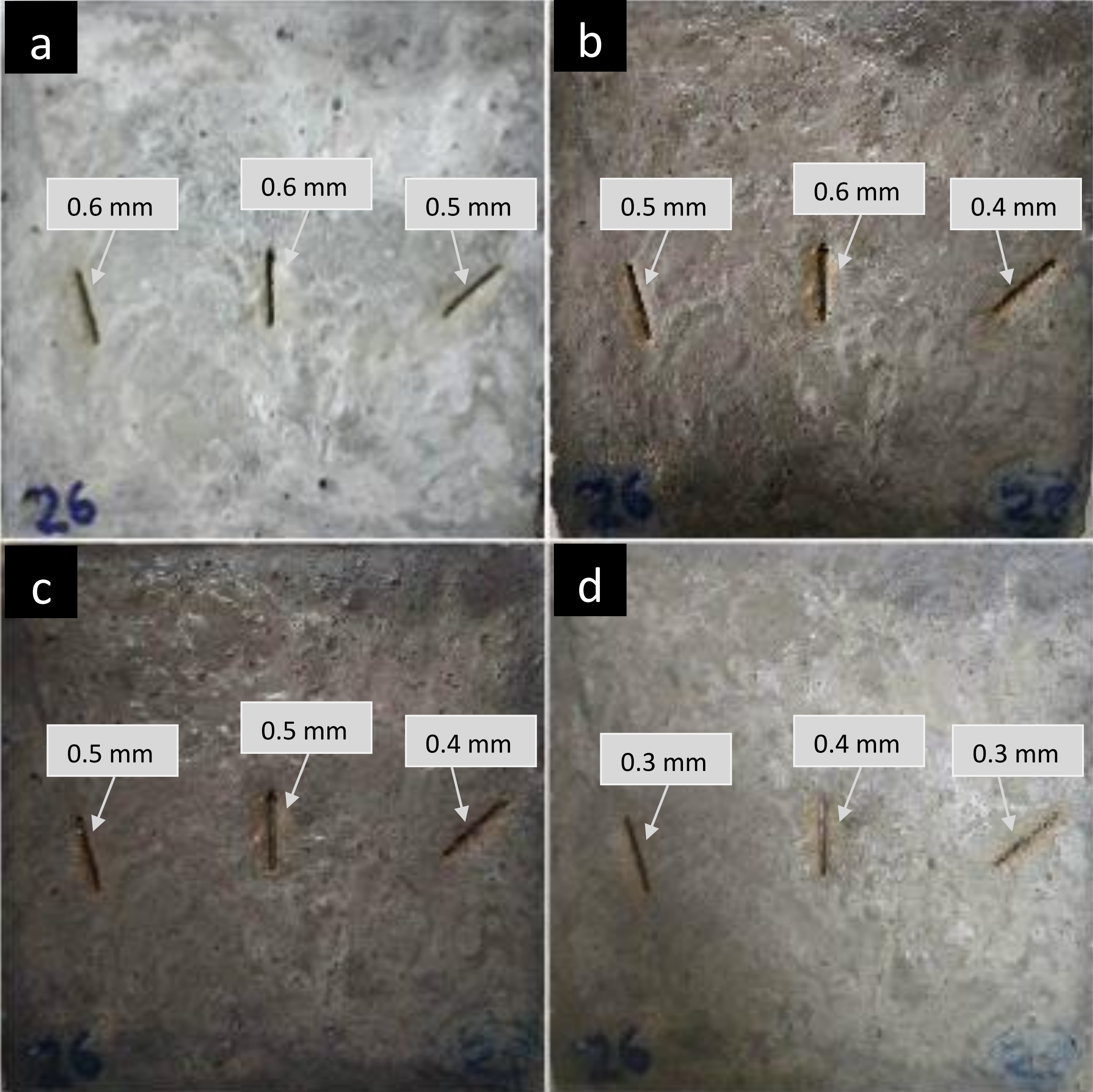

Figure 4.7 Crack healing images of Standard concrete specimens (a- 0 day cured, b- 7 days cured, c- 14 days cured, d- 28 days cured)

Figure 4.8 shows the self-healing phenomenon for Isolate 3 concrete. By looking at these images, we can confidently say that Isolate 3 is far better than the standard culture in remediating cracks in concrete. The healing width is between 0.3 – 0.4 mm for I3 concrete specimen which is significantly greater than that for standard concrete. Moreover, there are deposits of calcite near the edges of cracks as well as on the surface of specimen. It can be said that with prolonged duration, this culture may be able to completely fill the cracks induced.

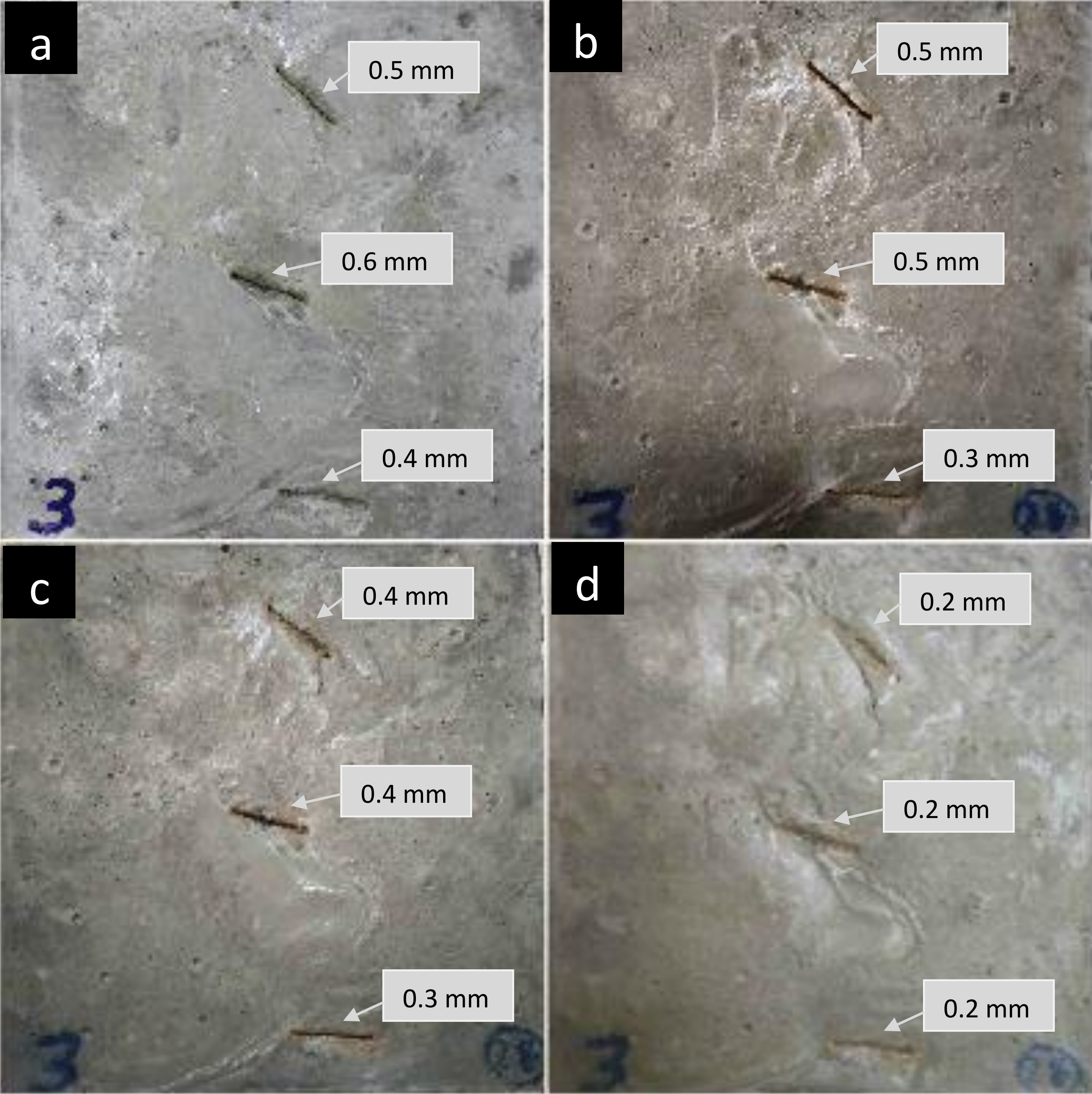

Figure 4.8 Crack healing images of I3 concrete specimens (a- 0 day cured, b- 7 days cured, c- 14 days cured, d- 28 days cured)

CHAPTER 5

CONCLUSION

Based on the results obtained from this study, the use of microbial cells embedded in concrete can be successfully implemented for healing macro cracks in concrete structures. From isolation stage, 26 potential bacterial isolates were obtained and as the screening process continued, this number reduced to only 2. Not every bacterium is able to survive in the harsh environment within the concrete matrix, so the only isolates viable after undergoing thorough characterization process were used in preparing concrete specimens. The strength studies performed on concrete samples depicted that the concrete made from bacterial cells was superior to the control concrete sample. This is due to the calcium carbonate deposition by bacteria on the surface of cell and inside the pores of concrete matrix. The microbial cells fill the pores inside the concrete and when. The visual investigation performed on bacterial and non bacterial concrete specimens at regular time intervals revealed that after 7 days of curing, the bacterial cubes started producing whitish-yellow coloured precipitates near the cracks induced. But this layer was not observed in case of the control concrete specimens. As the curing duration increased, the cracks started to fill, both in control as well as bacterial specimens. However, after 28 days, the maximum healable crack width increases by 2 times for I3 concrete as compared to the concrete prepared without bacterial cells. Thus, following conclusions can be drawn from this research:

i. For isolation of calcite precipitating bacteria from soil samples, alkaline soil rich in lime and iron oxide should be used, as done in the present study.
ii. Enrichment culture technique is much better choice than direct plate count method for cultivating bacterial cells. This narrows down the potential bacteria from all the possible bacteria to urease producing bacteria to finally calcite precipitating bacteria.
iii. Only bacterial isolates showing positive urease assay and endospore formation should be considered for crack remediation in concrete samples. This is because the bacteria unable to form endospore cannot survive in highly alkaline environment of fresh concrete.

iv. The concrete prepared with Isolate 3 bacterial cells depicted highest increase in compressive strength (17.28%), split tensile strength (27.76%) and flexural strength (35.03%) as compared to the control concrete specimens. Therefore, this isolate can be used for further investigations including the durability of self-healing concrete.

v. The maximum healable crack width for isolate 3 concrete was 0.3 to 0.4 mm, for standard concrete: 0.2 to 0.3 mm and it was only 0.1-0.2 mm for control concrete specimens. Thus, Isolate 3 concrete can be successfully used in the crack remediation of concrete structures. However, for cracks greater than 0.5 mm, the bacterial cells will be of little use as per various studies.

vi. As the microbial cells have been proven to consume oxygen, future scope of these self-healing bacteria can be seen as an oxygen diffusion barrier preventing the corrosion in reinforced concrete.

vii. While this research focussed on the strength studies and quantification of the crack healing process of bacteria, several other properties like long term durability, suitability in marine environment, and cost efficiency of bacterial concrete need to be investigated before using it in construction practices.

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APPENDIX A

Composition of Urea Broth & Urea Agar

For the culturing of bacteria, urea broth and urea agar were prepared as the nutrient media. To make urea agar, the agar is added in the urea broth media itself. The only purpose of agar is solidification of nutrient media. Table A.1 describes the composition of urea broth & urea agar.

Table A.1 Composition of Urea Broth & Urea Agar

S. No.	Chemical	Composition (gm/L)
1	Urea (CH_4N_2O)	20
2	Ammonium Chloride (NH_4Cl)	10
3	Sodium Bicarbonate ($NaHCO_3$)	2.12
4	Calcium Chloride two Hydrate ($CaCl_2.2H_2O$)	25
5	Nutrient Broth	3
6	Agar ($C_{14}H_{24}O_9$) (in Urea Agar only)	20

Nutrient Broth: Peptone 5.0 g/L; NaCl 5.0 g/L; Yeast extract 1.5 g/L; Meat extract 1.5 g/L, pH 7.4 ± 0.2 (HiMedia, India).

APPENDIX B

Appearance of Bacterial Colonies

Figure B.1 presents the appearance of bacterial colonies after spreading selected serial dilution concentration on petri plate.

Figure B.1 Appearance of Bacterial Colonies after Spreading

Figure B.2 presents the appearance of bacterial colonies after streaking individual colonies on petri plate.

Figure B.2 Appearance of Bacterial Colonies after Streaking Individual Colonies

Figure B.3 presents the appearance of bacterial isolates after streaking selected colonies on petri plate.

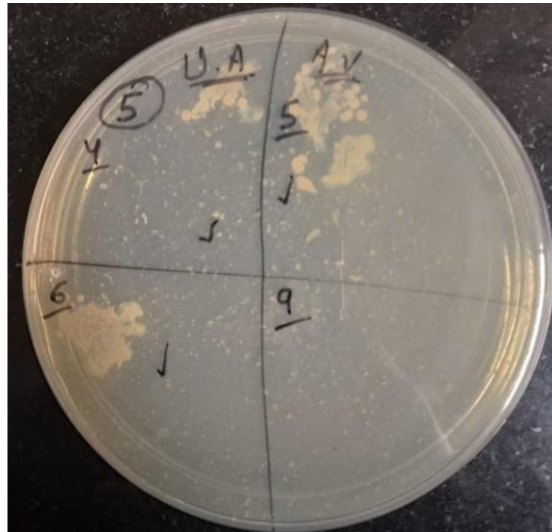

Figure B.3 Appearance of Bacterial Isolates after Streaking Selected Colonies

APPENDIX C

Microscopic Images of Gram Staining Results

Figure C.1 shows the microscopic images of gram staining results at magnification 100X. The rod shaped structures in the images represents the genus *Bacillus* and the spherical shaped structures represents *Coccus* bacteria.

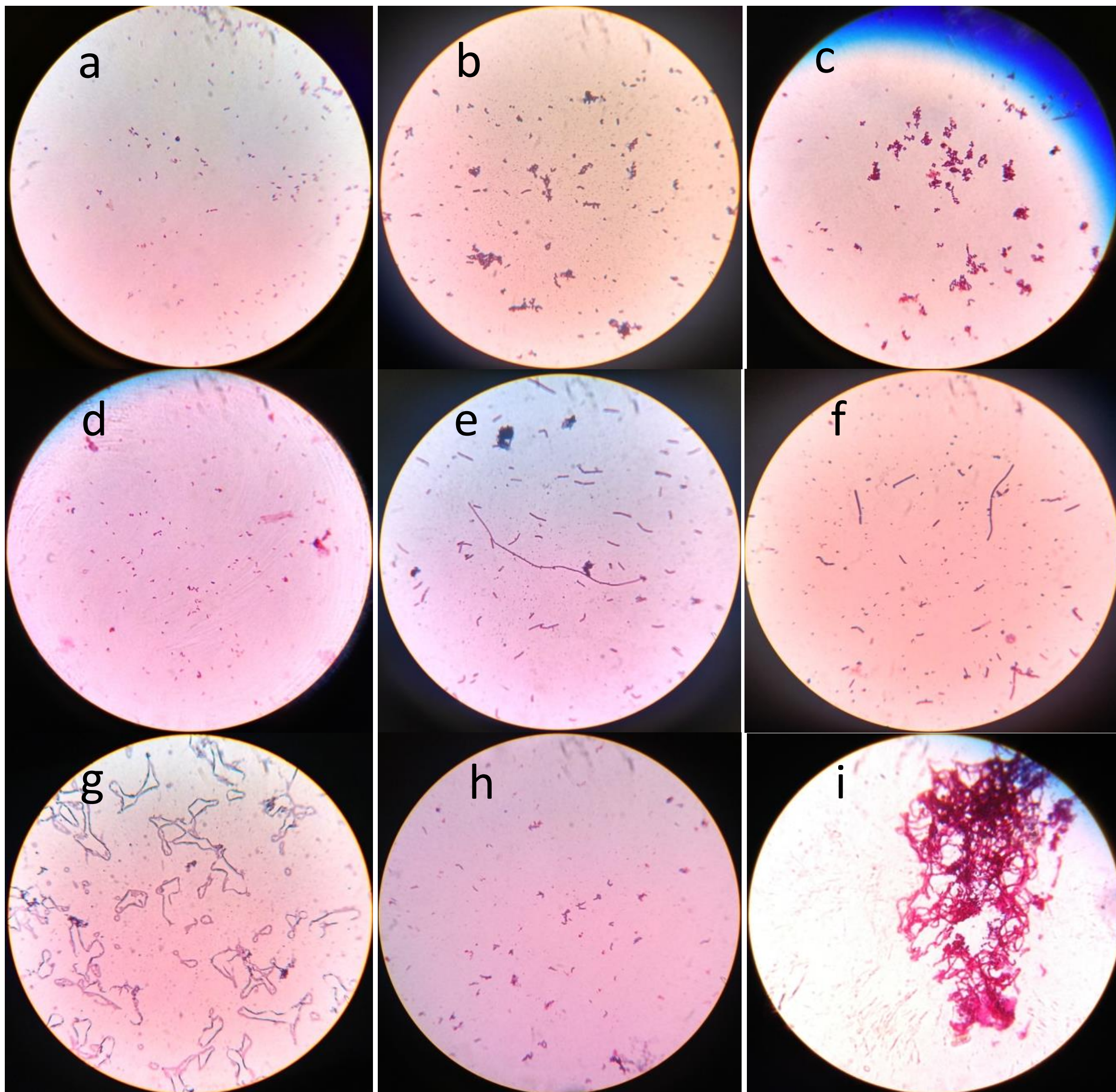

Figure C.1 Microscopic images of Gram Staining results of Isolates (a-Isolate 1, b-Isolate 3, c-Isolate 4, d-Isolate 6, e-Isolate 12, f-Isolate 13, g-Isolate 18, h-Isolate 24, i-Isolate 26)

APPENDIX D

Urease Assay Results

Figure E.1 presents the slants depicting urease activity. The yellow colour slants (left) shows positive urease activity and the pink colour slants (right) shows negative urease activity.

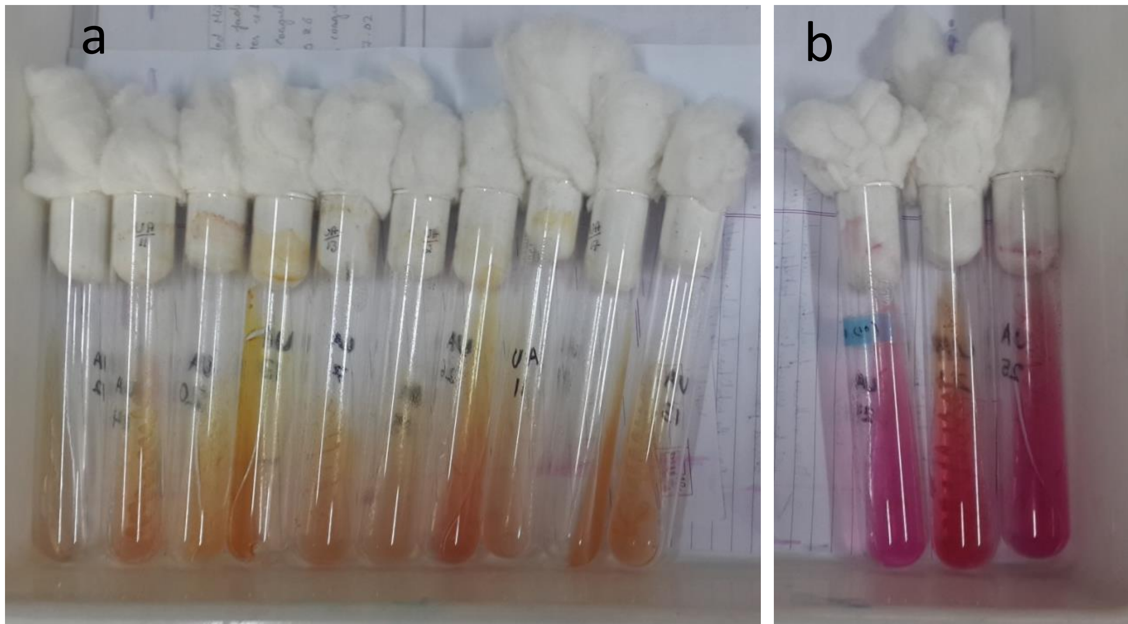

Figure D.1 Urease activity on Slants {a-Urease positive (yellow) and b-Urease negative (pink)}