"EXPERIMENTAL INVESTIGATION OF BIO-CONCRETE"

A Thesis

Submitted in partial fulfilment of the requirements for the award of the degree of

MASTER OF TECHNOLOGY

IN

CIVIL ENGINEERING

With specialization in

STRUCTURAL ENGINEERING

Under the supervision of

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To

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CERTIFICATE

This is to certify that the work which is being presented in the thesis titled **"Experimental Investigation of Bio-Concrete"** in partial fulfilment of the requirements for the award of the degree of Master of Technology in Civil Engineering with specialization in "**Structural Engineering**" and submitted to the Department of Civil Engineering, Jaypee University of Information Technology, Waknaghat is an authentic record of work carried out by **Rajneesh Vashisht (152654)** during a period from July 2016 to May 2017 under the supervision of **Mr. Abhilash Shukla** Assistant Professor, 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

The present study focussed on the screening and identification of calcite precipitating bacteria with a goal to check the suitability of the potential strain in concrete to advance its strength and durability. For this, a total of sixteen samples comprising eight alluvial soil (alkaline in nature and rich in Iron oxide and lime) samples and eight sewage samples were collected from the different locations of district Solan (H.P). For isolation, enrichment culture technique was used to enrich calcite precipitating strains in Urea broth. After enrichment, fourteen distinct bacterial colonies were obtained on Urea agar. All the strains were gram-positive rods or coccus in shape. All the fourteen bacterial cultures were screened for qualitative urease assay and quantitative electric conductivity test. The calcite precipitation was also determined by taking dry weight of the precipitates in urea broth supplemented with calcium chloride. Based on the screening results, five isolates were selected possessing high calcite formation along with higher urease activities. All the results were compared with standard strain of *Bacillus megaterium* MTCC 1684, already reported for high calcite formation. The compressive strength and quantification of crack healing observed and the positive results shown by two bacterial species. The DNA extraction of these species done and the sequencing was performed.

CHAPTER 1

1.1 **INTRODUCTION**

As we know, concrete play vibrant role in present construction practices. Concrete is the second most used material on this Earth after water. The massive manufacturing of concrete leads to negative environmental effects. The key constituents of concrete are cement and aggregates, which needs to be produced. The making of cement only leads to 7% CO_2 emission by human's activities. This happens due to heating limestone and clays @ 1500°C. Upon every ton of concrete production, 100 Kg CO_2 emitted (Wout Knoben, 2011). By knowing these facts, we cannot say that concrete is an ecological material. To overcome these and make concrete eco-friendly we replace cement partially with greener alternatives like fly ash, slag, blast furnace or rice husk ash which are by products of iron, coal and agricultural materials or industries and other way is to increase the life stretch of the concrete by using any external agent, which can repair the concrete properties. Cracks are the major shortcoming in concrete structures, both Micro cracks and Macro cracks are accountable for the deterioration of concrete. The major impact over durability and facility life of concrete made by these cracks. Any kind of crack helping outside medium to contact with concrete's microstructure. Cracks makes it easy for moisture, Carbon dioxide (CO_2), Sulphate, other liquids and gases to trans-pass the concrete structure easily up to its core and reinforcement which results in decay of reinforcement and decline the strength and durability of concrete. Therefore, cracks are not desirable in concrete structure. The micro cracks can rehabilitated by concrete itself. This process of healing known as *"Autogenic healing"* which is also known as *"Self-healing"*.

However, cracks can healed by mixing specific healing mediator in the concrete matrix. In present study, we are going to study the consequences of bacteria as a healing agent. The concrete is extremely alkaline in nature due to the creation of calcium hydroxide; its pH is about 11 to 13. Therefore, we need bacteria, which should be alkaliphilic (alkali-resistant) and withstand against high mechanical stresses during mixing.

Previously bacteria based healing process was done externally. In that, a bacteria solution applied manually to the crack, which appeared to the naked eye. This was a method of surface treatment of concrete and required regular observations and regular application of bacterial solution, which was time-consuming process. Henk M. Jonkers took first initiative to mix bacteria in a concrete matrix. He studied different parameters and bring concrete a new life.

The concept behind using bio-concrete is that some alkaliphilic *Bacillus* species helps to fill cracks in concrete by producing calcite ($CaCO_3$). The calcite formation mechanism by these bacteria based on enzymatic hydrolysis of urea to ammonia and then carbon dioxide. These reactions causing increase in pH value, which forms carbonates and bicarbonate ions, which are going to precipitate with the present calcium ions. Those leads to the formation of calcium carbonate.

This is the most efficient method to save environment and improve durability of concrete. This study based on the bacteria based self-healing and effect on concrete by immobilizing bacterial species in it.

1.2 BACTERIA

Bacteria are the single cell microbes. There is no nucleus and any other membrane in them therefore, they have simple cell structure. Their generic information contains in the single loop of DNA, all this present in the control centre of the bacteria. Some have different group of genetic material called plasmid. It contains genes, which give advantages to bacterium over bacteria.

1.2.1 Classifications of Bacteria:

Classification based on shapes: According to their basic shapes, bacteria can be classified into 5 groups.

i) *Spherical (Cocci);*
ii) *Rod (Bacilli);*
iii) *Spiral (spirilla);*
iv) *Comma (vibrios) &*
v) *Corkscrew (spirochaetes).*

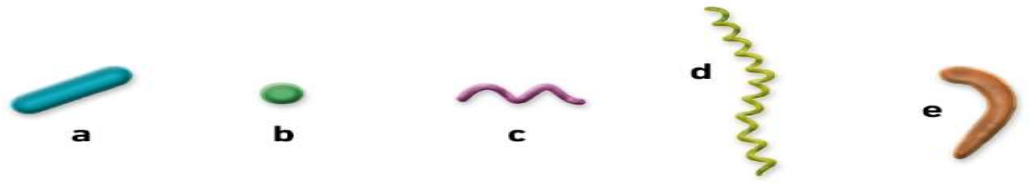


Figure 1. Classification of bacteria based on shapes. (*a. Bacillus; b. Coccus; c. Spirillum; d. Spirochaete & e. vibrios*) "Source: microbiologyonline.org"

Classification based on Gram strain: According to gram strain, bacteria can be classified into 2 groups.

i) Gram Positive (gives positive results in gram strain test) &
ii) Gram Negative (gives negative results in gram strain test).

Classification based on Oxygen requirement: According to oxygen, requirement bacteria can be classified into 2 groups.

i) Aerobic (required molecular oxygen as terminal electron acceptor) &
ii) Anaerobic (do not use molecular oxygen as terminal electron acceptor).

1.3 GROWTH OF BACTERIA

Growth of bacteria done by asexual reproduction. Single bacteria cell divided in two daughter cells under the process of binary fission. These cells further divided into many more cells to grow population. The growth of bacteria can be of two levels one by increase in number of bacteria cells and other by increase in size of bacteria. Growth can be count quantifiably by number of viable bacteria cells count. Total number of bacteria gives bacteria cells count for both living and dead cells while viable count consider only living cells, which are capable of multiplication.

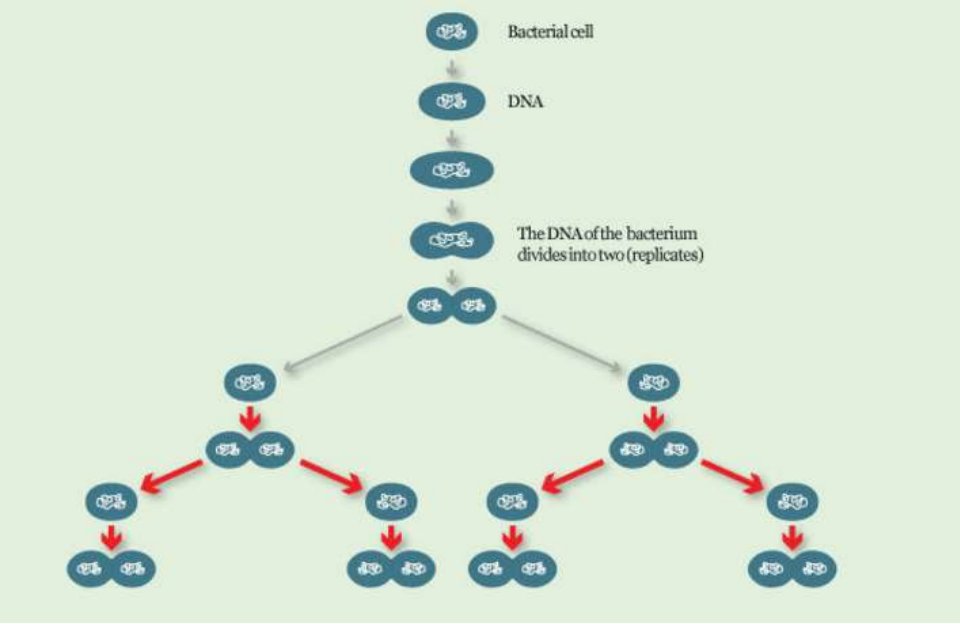


Figure 2. Binary Fission "Source: microbiologyonline.org"

1.4 BACTERIA GROWTH CURVE

The growth of bacteria characterized by growth curve of bacteria, which follows following phases:

i) *Lag Phase:* The first phase in the growth of bacteria, which flinches instantaneously after the inoculation, this phase considers as the new environment recognition time for the bacteria. This period varies with species, nature of culture medium and temperature.

ii) *Log or Exponential Phase:* In this phase, the cells leads to replicate and the number of cells amplified exponentially with respect to time.

iii) *Stationary Phase:* After the exponential growth, phase the cell separation stops due to the consumption of nutrient. Therefore, the viable count leftover stationary as a balance in the dying cells and freshly formed cells.

iv) *Decline Phase:* In this phase, the inhabitants of cells decreased due to the cell death.

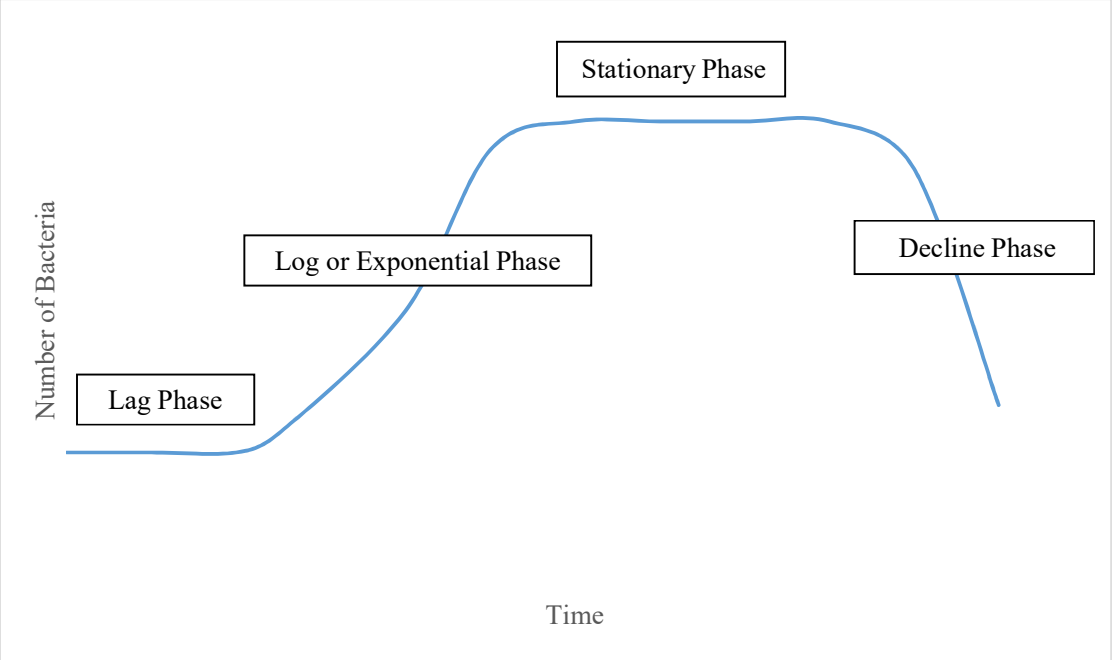


Figure 3. The growth curve of bacteria.

1.5 BACTERIA USED IN CONCRETE

Concrete is extremely alkaline, its pH is about 11 to 13 and it mixed under high mechanical stresses. Therefore, immobilized bacteria must be alkaliphilic (alkali-resistant) and must have propensity to endure against the mechanical stresses. The key point against crack repairing is

that the bacteria must precipitate with calcite ($CaCO_3$) to form crystalline layer over cracked surface. Bacillus spores show this kind of properties. The crack-filling phenomenon is due to the urease activities due to the alakliphilic bacteria, which form calcite.

The following *Bacillus* species, which used in the bio-concrete, are:

i) *Bacillus pasteurii.*

ii) *Bacillus subtilis.*

iii) *Bacillus megaterium.*

iv) *Bacillus cohnii.*

v) *Bacillus halodurans.*

vi) *Bacillus pseudofirmus.*

And other similar species.

CHAPTER 2

2.1 LITERATURE REVIEW

List of previous studies tell us that bacteria can play repairing characteristics in concrete. The following studies cleared that bacteria can be arrested in the concrete matrix and bacteria has tendency to precipitate the calcite ($CaCO_3$), the precipitation of calcite form a layer of crystals over cracked area. These crystals can fill the cracked area. The following studies leads to important results, which helped to accept bacteria as self-healing agent for concrete mix.

2.1.1 Henk M. Jonkers and Erik Schlangen (2007)

The interest of this study was to inspecting self-healing capacity of bacteria in concrete. Formerly the bacteria treatment applied on the cracks externally, Henk M. Jonkers tried to get results by bacteria mixing with constituents of concrete. Flexural tensile and compressive strength tests were accomplished in this study to get the consequence of bacteria on concrete possessions. The tests were performed over three different samples (bacterial concrete, organic compound concrete and control concrete). Alkaliphilic (alkali-resistant) spore forming bacteria was used in this study. The culture of bacteria was acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. These were cultured in yeast extract medium. In this study *Sporosarcina pasteurii, Bacillus cohnii, Bacillus halodurans* and *Bacillus pseudofirmus* bacteria were used. The growth and sporulation was tested in mineral medium with organic carbon sources of 6g Na-citric acid or 5g peptone in accumulation to 3g yeast extract per litre. The pH maintained about 9.2 and the growth was checked by using Burger-Turk counting chamber a microscopy method instrument. Three concrete bars (16 x 4 x 4 cm) of bacterial, organic and control concrete were prepared using Ordinary Portland cement (ENCI CEMI 32.5R). The tests were done according to Netherlands standards and results were obtained, from the results it was cleared that there were no great changes in the tensile and compressive strength of the bacterial concrete while in case of organic concrete a huge loss in strength is obtained even Na-gluconate and Na-ascorbate gives zero tensile and compressive strength. By these results, it was assured that there is no issue in bacteria mix concrete. This kind of concrete can be used as important structural construction material. The ESEM (Environmental Scanning Electron Microscope) analysis was observed

on every sample so that bacterial spore's reaction and mineral production can be seen. It was observed that control concrete does not show any crystal formation while in case of bacterial concrete a huge amount of crystal formation takes place. In this study, it was detected that bacteria can be used as incorporated organism and it does not show any loss in strength of concrete mix while crystallization in concrete matrix is high.

2.1.2 Henk M. Jonkers, Arjan Thijssen, Gerard Muyzer, Oguzhan Copuroglu and Erik Schlangen (2008)

In this study Henk M. Jonkers and team trying to identify suitable bacteria for bacterial concrete. The main target was to get bacteria, which not only survive concrete incorporation for long time, but also can act as better self-healing agent. As we know that concrete is highly alkaline (pH values in between 11 and 13) the alkliphilic bacteria is most suitable for such conditions. The concrete matrix is also oxic because of ingression of oxygen (diffusion through matrix capillaries) so, that the bacteria must be oxygen resistant. Therefore, aerobic alkaliphilic spore forming bacteria is required for self-healing concrete. This type of bacteria occored in genus *Bacillus*. Few bacillus bacteria tested for compatibility. Two species, which were cultivated from alkaline soil samples Bacillus pseudofirmus and Bacillus cohnii, were acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. These were cultured in liquid medium with 5g pepton, 3g meat extract, 0.42g $NaHCO_3$ and 0.53g Na_2CO_3 per litre distilled water and in alkaline mineral supplemented with manganese for better sporulation. The pH of the solution was about to 10. To investigate viability of assimilated bacteria sample with and without incorporated bacteria (mixture of vegetative cells and bacteria cells) were prepared. Ordinary Portland cement (CEM I 32.5R, ENCI, The Netherlands) was used to prepare the samples. Water cement ratio of 0.4 or 0.5 and bacterial specimens of 1-10 x 10^8 spore's cm^{-3} cement stone were used. Testing moulds of 4 cm x 4 cm x 4 cm were used. Viability test of cement stone-incorporated bacteria has been done by Most Probable Number (MPN) technique. The bacteria spore's endurance rate was determined by valuation of number of viable bacteria existing in cement stone specimens at the age of 9, 22, 42 and 153 days. The MPN numbers were calculated by computer program. For this technique, bacteria should be freed from cement stone matrix and must be brought in a single cell suspension. In this study, they crushed and pulverised the sample using high mechanical force and suspended by ultrasonic treatment. It was obtained that according to age the number of bacteria spores decreased. It was found that the 9 days cured sample had 1.8 x

10^6 of the assimilated 2.4 x 10^8 spore's cm^{-3} cement stone was retrieved. The number of viable bacteria cells decreased with increase in age. The number of viable cells from 135 days cured sample were below the MPN detection limit (i.e., < 0.5 x 10^3 cm^{-3}). The mineral producing capacity was analysed by ESEM analysis (Philips XL30 Series ESEM). The specimen was dipped in distilled water and ESEM analysis has been concluded. It was seen that in case of control sample cured for 7 & 28 days 1-5 µm sized particles on crack surface. While in case of bacteria treated concrete Copious amount of 20-80 µm sized mineral like particles were seen on the crack surface of 7 days but not on 28 days cured sample. This is due to viability of bacteria cells decreases with age. It was observed that alkali–resistant spore forming bacteria related to genus *Bacillus* showed very good results as self-healing agent. It was observed from this study that alkaliphilic *Bacillus* spore forming bacteria play important role in self-healing of concrete, precipitation by such bacteria is high for early age and it could be used as self-healing agent.

2.1.3 Kim Van Tittelboom, Nele De Belie, Willem De Muynck and Willy Verstraete (2009)

This study was entitled as use of bacteria to repair cracks in concrete. Kim Van Tittelboom gives chemical reaction of precipitation of $CaCO_3$. According to this study $CaCO_3$ precipitation depends upon few factors which are dissolved inorganic carbon concentration, pH value, calcium ion concentration and nucleation site. The inorganic carbon concentration, pH value and calcium ion deliberation provided by bacterial metabolism while nucleation site was provided by cell wall of the bacteria. The bacteria used in this study produce urease which catalyses the hydrolysis of urea [$CO(NH_2)_2$] into carbonate (CO_3^{2-}) and ammonium (NH^{4+}). The reactions given below (Van Tittelboom, Kim et al. 2010):

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

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

$$H_2CO_3 \rightarrow HCO^{3-} + H^+ \quad (3)$$

$$2NH_3 + 2H_2O \rightarrow 2NH^{4+} + 2OH^- \quad \text{(pH increase)} \quad (4)$$

$HCO^{3-} + H^{+} + 2NH^{4+} + 2OH^{-} \rightarrow CO_3^{2-} + 2NH^{4+} + 2H_2O$ (5)

$CO_3^{2-} + Ca^{2+} \rightarrow CaCO_3$ (carbonate precipitation) (6)

$CO(NH_2)_2 + 2H_2O + Ca^{2+} \rightarrow 2NH^{4+} + CaCO_3$ (overall reaction) (7)

Eq. (1) one mole of urea hydrolysed intracellularly to 1mol of ammonia and 1mol of carbonate. Eq. (2) which further hydrolysed to form an additional 1mol of ammonia and carbonic acid. Eq. (3) & (4) they form bicarbonate. 2mol of ammonium and 2mol of hydroxide ions when equilibrate with water. Eq. (5) the rise in pH move to the bicarbonate equilibrium, resulting in the formation of carbonate ions. Eq. (6) which precipitate $CaCO_3$ due to the presence of soluble calcium ion (Ca^{2+}). Eq. (7) shows the overall reaction of urea and calcium to form ammonium and calcium carbonate production. The bacterial cell wall negatively charged therefore; bacteria can attract cations to deposit on their cell surface from environment including Ca^{2+}. The reactivity between Ca^{2+} ions and CO_3^{2-} ions causing the precipitation of $CaCO_3$ at the cell surface as nucleation site. Eq. (8) & (9).

$Ca^{2+} + Cell \rightarrow Cell\text{-}Ca^{2+}$ (8)

$Cell\text{-}Ca^{2-} + CO_3^{2-} \rightarrow Cell\text{-}CaCO_3 \downarrow$ (9)

For testing Ordinary Portland cement CEM I 52.5 N was used in this study. The strength tests were performed over 150 mm x 150 mm x 150 mm, 150 mm x 150 mm x 600 mm and 160 mm x 160 mm x 70 mm moulds. The $CaCO_3$ precipitation bacteria (*B. sphaericus*) were collected from BCCM culture collection in Ghent, Belgium. Two more samples of epoxy resin (Sikadur 52) and cement-bound mortar (SikaTop 111) used for crack repairing and compared with $CaCO_3$ precipitation technique by bacteria. The crack creation was done manually. The bacteria immobilized in silica gel to overcome strong alkaline environment in the concrete. The crack repair were evaluated by water permeability, ultrasonic measurements and by visual evaluation. These crack repair techniques are reliable and can be done easily. For water permeability test, a setup was prepared and "Darcy's Law" calculated the results. The coefficient of water permeability k was obtained from Darcy's law i.e.,

$$k = \frac{aT}{At} ln \frac{h_o}{h_f}$$

a = cross-sectional area of pipette (m^2)

A = cross-sectional area of specimen (m^2)

T = specimen thickness (m)

t = time (s)

h_0 and h_f = initial and final water heads (cm)

It was observed that the waster permeability decreases with increase in age of concrete. This depends upon the precipitation rate and size of crack. It was also observed that the concrete mix with bacteria show better results than epoxy resin and cement-bound mortar. The ultrasonic measurements were also done over same sample. Ultrasonic waves travel much easily in hardened concrete (4000-5000 m/s) as compare to water (1480 m/s) and air (350 m/s). By using such technique, they conclude that the there is a decrease in the transmission time in cracked sample of bacterial concrete as compared to others. Different repairing technique lead to different results, but this method of testing helps to get the repairing rate of different healing agents. They also done visual inspection, in that they inspect micro cracks regularly and note the repairing rate. High definition pictures were taken and compared to obtain the results. It was observed that different crack healing samples had different rates of repairing. The better results were obtained by *Bacillus sphaericus* mixes compared to others. This study observed that *Bacillus sphaericus* bacteria immobilized with silica gel resulted in better results and it was highly acceptable because mineral precipitation technique does not pollute the environment.

2.1.4 Virginie Wiktor and Henk M. Jonkers (2011)

In this study, the microstructural analysis by ESEM and X-Ray diffraction was studied along with the oxygen consumption of bacteria-based specimen. As we know the aerobic bacteria required oxygen for its viability. Oxygen helps in precipitation. Therefore, collecting information about oxygen consumption helps in the bacterial mix. The basic objective of this study was to analyse the self-healing phenomenon by using bacteria in concrete matrix and getting the X-Ray diffraction of the concrete matrix. They used calcium lactate and bacterial spores mixed with reinforced mortar made up of Ordinary Portland Cement (CEM I 42.5 N,

ENCI, The Netherlands), fine aggregates and lightweight aggregates (LWA). A prism of 4 x 4 x 16 cm was cast for testing purpose. Tensile loading was applied to get cracks over prismatic samples. The precipitation was analysed by Environmental Scanning Electron Microscope (ESEM, Philips XL 30 Series) equipped with an Energy Dispersive X-Ray (EDAX) element analysing system and at last it was examined by Fourier-Transformed Infrared (FT-IR) spectrometry. They observed that the mineral precipitation was based up on calcium carbonate ($CaCO_3$). ESEM analysis results in active precipitation by bacteria at high rate. The oxygen consumption was only seen in case of bacterial concrete; while in case of control concrete, no oxygen consumption was seen. By using Fick's law of diffusion, it was calculated that O_2 concentration was about 4.14×10^{-2} 1mol m^{-2} s^{-1}. The quantification of crack healing was given by this study is based on following formulae;

$$Healing\ \% = \frac{C_{wi} - C_{wt}}{C_{wi}}\ X\ 100$$

Where, Cwi is the initial crack width, Cwt the width measured at time t.

Number of calculations was done at different time for both control concrete and bacterial concrete sample. It was found that very few cracks were 100% healed in case of bacterial concrete and mostly cracks were healed up to appropriate level of healing while there was not any kind of healing in case of control concrete. It was observed from this study that the bacterial concrete could be used for construction purposes.

2.1.5 J.Y. Wang, H. Soens, W.Verstraete and N. De Belie (2013)

The main objective of this study was to getting self-healing concrete by the use of microencapsulated bacterial spores. Microcapsules used to encapsulate bacteria spores for self-healing concrete. This encapsulation technique used to increase the bacterial life in concrete. The viability of encapsulated bacteria was investigated. This was a great research by author, which helped to attain more life for incorporated bacteria. Bacteria spores were encapsulated in a size of 5 μm. The capsule contains inert substances to protect the bacterial spores. The concentration of bacterial spores in the microcapsule was about 10^9 cells/g microcapsule (dry weight). The microcapsules were broken down under high tensile force (crack generates). These broken capsules liberates bacteria in the concrete matrix and precipitation takes place. Number of tests were performed for different concentration. The viability of the spores can be calculated by using the amount of decomposed urea. The spores in the capsules only germinate when the capsules were broken. Then they reached the nutrients. They need time to transform

from dormant state to active state. It was found that spores remained viable after immobilization into the microcapsules. The micro capsulation does not affect the volume of the sample but this process show decrease in tensile and compressive strength. In addition to higher than 3% microcapsules dosage there was a significant loss in tensile strength while in case of 1% to 5% microcapsules dosage there was a huge loss of about 15% to 34% in compressive strength was recorded by testing specimens. In case of water absorption there was a lower water absorption in case of nutrients and microencapsulated bacteria. The pore size distribution and porosity was obtained by the Mercury intrusion porosimetry (MIP) test. It was observed that the microencapsulated technique increased the viability of the bacterial spores but it certainly decreases the mechanical properties of the concrete like decrease in tensile and compressive strength. This type of technique could be useful in further scopes of bacterial concrete.

2.1.6 LUO Mian, QIAN Chunxiang, LI Ruiyang and RONG Hui (2014)

The main objective of this study was to get efficiency of concrete crack healing based on biological carbonation precipitation. In this study, the author observed different aspects of crack healing by bacteria. The microstructural analysis was done by using SEM images and SEM observation of carbonation precipitation and by X-Ray diffraction (XRD). The compressive strength and water permeability along with visual inspection were also observed. The tests specimens and testing methods were used according to the Chinese standards. They observed slight increase in the compressive strength of concrete. The water permeability of bacterial concrete was relatively decreased by 84% and 96% for 7 and 28 days' immersion in water, which was greater than that of control concrete. The microstructural analysis by (SEM) Scanning Electron Microscope shown a complete precipitation and mineral formation, visually the healing capacity of bacteria was observed clearly. The crack with width of 0.48 mm was completely healed after 80 days. This study concludes that using biological healing agent helps in proper crack repairing and give eco-friendly concrete, which helps in green construction.

2.1.7 S. Krishnapriya, D.L. Venkatesh Babu and G. Prince Arulraj (2015)

The main objective of this study was isolation and identification of bacteria to improve the strength of concrete. It was India based research. The Bacteria culture (*Bacillus megaterium*) collected from Microbial Type Culture Collection (MTCC) and Gene Bank, Chandigarh, India. An alternate substrate for growth of bacteria wheat bran was used in this study for maintaining

economical sporulation. Ordinary Portland cement of grade 53 was used according to India standards. Concrete grade of M25 was prepared according to the Indian standards. The compressive strength was obtained over the specimen prepared. It was observed that the compressive strength of bacteria incorporated concrete was greater than that of control concrete specimen. The microstructural analysis was done using SEM micrographs using Jeol JSM – 6390 apparatus. The SEM analysis shown positive results towards precipitation of calcium carbonate ($CaCO_3$) by bacteria and visual inspection also shown crack healing capacity of bacteria. It was observed that the use of Bacillus genus spore forming bacteria as self-healing agent is possible and it could help in increasing life of structural concrete.

2.1.8 Farzaneh Nosouhian, Davood Mostofinejad and Hasti Hasheminejad (2015)

The main objective of this study was to getting durability results of bacterial concrete against sulfate environment. This study was conducted on two bacteria spores used for self-healing purpose. a) *Bacillus subtilis* b) *Sporosarcina pasteurii*. The behaviour of these two bacteria was checked by mixing them in concrete mixtures designed according to ACI-211 to have a compressive strength of 25 MPa after 28 days. Different cell concentration was used to know whether cell concentration affects the results or not. Rapid chloride permeability test (RCPT) was performed according to ASTM C 1202 standards. It was observed that the inclusion of bacteria enhances the resistance of concrete against chloride penetration. In Case of sulfate environment, other observations like mass variation, volume variation, density, water absorption, porosity and compressive strength were also considered. It was observed that the specimen submerged in sulfate solution shown reduction of mass while in case of volume variations they shown increased volume. It was concluded that in sulfate environment the mass of concrete gets decreases while volume gets increases. The bulk density of concrete found to be increased and lowering in the voids seen. It was seen that the compressive strength of bacterial concrete in sulfate environment gets increased by 29.3% at 270 days. By all above results, it was observed that the bacterial concrete provides good durability to the structure and it can show great results in sulfate environment.

2.1.9 Yusuf Çağatay Erşan, Filipe Bravo Da Silva, Nico Boon, Willy Verstraete and Nele De Belie (2015)

The main objective of this study was to check the protection materials compatibility with concrete. The protection material used was bacteria. The initial and final setting time along

with the compressive strength was concluded in this study. *B. sphaericus* and *D. nitroreducens* two bacteria were tested along with them two non-axenic bacteria Cyclic EnRiched Ureolytic Powder (CERUP) and Activated Compact Denitrifying Core (ACDC) were tested. *B. sphaericus* and CERUP helped for microbial crack repair by ureolysis while *D. nitroreducens* and ACDC helped in microbial crack repair by denitrification. The mortar used was prepared by using CEM I 52.5N. The bacteria were incorporated in the design mix. The final and initial setting time were determined by using ASTM C807-13 standards using Vicat needle. It was observed that there is a decrease up to 20 minutes in initial setting time while increase up to 30 minutes in the final setting time. The initial and final setting time get varied according to the concentration and incorporated nutrients but an increase in setting time by using any incorporated bacteria was observed. In case of compressive strength, no loss but slight gain in compressive strength was observed in samples with different incorporated healing agent.

2.1.10 Kunal, Rafat Siddique, Anita Rajor and Malkit Singh (2016)

The main objective of this study was to used major by-product of cement manufacturing plan in structural concrete i.e., Cement Kiln Dust (CKD). The tests were performed to check the tensile, compressive strengths along with ultrasonic pulse velocity and rapid chloride permeability test. Microstructural analysis was done by SEM and XRD. It was observed that the compressive strength and splitting tensile strength increased with the addition of 10% bacteria-treated CKD, for the replacement of more than 10% cement. There were no qualitative measures were found by using CKD by treated with bacteria while quantitative changes were observed with respect to Portlandite phase percentage. The optimum used of bacteria-treated CKD as a cement replacement was found to be 10% and this approach was a great approach towards green construction and green engineering.

2.1.11 Mian Luo and Chun Xiang Qian (2016)

The main objective of this study was to get the performance of two bacteria-based additives used for self-healing concrete. Two types of bacteria-based additives were used, (1) calcium lactate and bacteria spores powder and (2) calcium formate and bacteria spores powder. Cement paste specimen with dimensions of 40 x 40 x 160 mm was casted using Ordinary Portland cement along with bacteria-based additives. The self-healing characterizations were done by using area repair rate method and microstructural analysis. The microstructural

analysis was done by using SEM images and XRD method. The setting time was also observed by using Vicat needle according to Chinese standards (GB/T 1346-2011). It was observed that the setting retardation was caused by calcium lactate while setting acceleration was caused by calcium formate furthermore; the bacteria spores' powder had slight retardation in setting. It was concluded that both bacteria-based additives could be used in construction concrete as the good healing agents. The incorporation of Type 1 additive and Type 2 additives leads to certain decrease in the compressive strength of the concrete specimen but in case of Type 2 additive increase in compressive strength after 28 days curing was observed. The carbonation depth was calculated after 3 days of accelerated carbonation in control, Type 1 and Type 2 samples. It was observed that the carbonation depth in control, Type 1 and Type 2 was 6.6 mm, 7.0 mm and 6.5 mm respectively. The SEM and XRD analysis shown the complete precipitation of calcium carbonate. It was observed that both additives could be used as healing agents.

2.1.12 E. Tziviloglou, V. Wiktor, H.M. Jonkers and E. Schlangen (2016)

The main objective of this study was to use bacteria-based self-healing concrete to increase liquid tightness of cracks. In this study, recovery of water tightness (RWT) was concluded along with different tests performed against water ingression. The healing agent was used were alkaliphilic bacteria of *Bacillus* genus and organic mineral compounds. The healing agent incorporated in OPC (CEM I 42.5 N, ENCI, The Netherlands) along with normal weight aggregates and lightweight aggregates (LWA) were mixed to get desirable mortar mix. The sealing efficiency of the bacteria-based agent was investigated by using stereomicroscopic images. The test was performed and it was observed that lightweight mortar with incorporated bacteria-based healing agent gives improved crack sealing than other samples. The better liquid tightness was also observed in this sample. It was observed that oxygen was only consumed by the bacteria-based healing agent samples. This study concludes that the lightweight mortar shows better liquid sealing along with better crack sealing. The lightweight aggregates could perform better role in self-healing bacterial concrete.

CHAPTER 3

3.1 MATERIALS & CHEMICALS

The material and chemicals required for the isolation of bacteria, samples taken for isolation of bacteria and material required for the casting of concrete cubes discussed in this section. Table 1 summarizes the culture media for the microbes of interest.

Table 1. Materials required for the nutrient media of bacteria growth.

Sr. No.	Material & Chemical Name	Purpose
1.	Urea (CH_4N_2O)	Nutrient Media
2.	Sodium Bicarbonate ($NaHCO_3$)	Nutrient Media
3.	Ammonia Chloride (NH_4Cl)	Nutrient Media
4.	Nutrient Broth	Nutrient Media
5.	Calcium Chloride two Hydrate ($CaCl_2.2H_2O$)	Nutrient Media
6.	Agar ($C_{14}H_{24}O_9$)	Solidification of Nutrient Media

The above table consists the materials required for the nutrient media composition, the purpose of each material mentioned along with the material. These materials used to grow the alkaliphilic bacteria. Table 2 consists of various samples taken for the isolation of bacteria. These alkaliphilic bacteria mostly isolated from the alkaline soils, sewage and from water. For this, a total of sixteen samples comprising eight alluvial soil (alkaline in nature and rich in Iron oxide and lime) samples and eight sewage samples were composed from the different locations of district Solan (H.P).

Table 2. Samples taken for the isolation of bacteria.

Sr. No.	Sample Type	Location
1.	Cement Sample	Concrete Technology Lab. JUIT, Waknaghat
2.	Sandy Soil	Building Construction Site Near Meet & Treat PG For Boys, Waknaghat
3.	Crusher Soil	Thank You Gate JUIT, Waknaghat
4.	Natural Soil	Kaithlighat
5.	Crusher Soil	Building Construction Site Near Thank You Gate JUIT, Waknaghat
6.	Vegetative Soil	Kandaghat
7.	Tap Water	JUIT, Waknaghat
8.	Pure Water Jogni Falls Water Sample	Environmental Engineering Lab. Civil Department JUIT, Waknaghat
9.	Waste Water Sample (Unknown)	Environmental Engineering Lab. Civil Department JUIT, Waknaghat
10.	Waste Water Sample (Unknown)	Environmental Engineering Lab. Civil Department JUIT, Waknaghat
11.	Waste Water Sample (Unknown)	Environmental Engineering Lab. Civil Department JUIT, Waknaghat
12.	Waste Water Sample (Unknown)	Environmental Engineering Lab. Civil Department JUIT, Waknaghat
13.	Waste Water Sample (Unknown)	Environmental Engineering Lab. Civil Department JUIT, Waknaghat

14.	Sandy Soil	Civil Engineering Block JUIT, Waknaghat
15.	Crusher Soil	Building Construction Site on the way to JUIT, Waknaghat
16.	Sandy Soil	Ashwani Khad, Solan

The material required for the cement and concrete testing are as follow:

Table 3. Material required for cement and concrete testing.

Sr. No.	Material
1.	Cement (PPC-Fly Ash Based)
2.	Fine Aggregate
3.	Coarse Aggregate
4.	Kerosene or Benzene

The chemicals used for the urease activity tests given in Table 4.

Table 4. Chemicals used for urease activity tests.

Sr. No.	Material
1.	Phenol Red ($C_{19}H_{14}O_5S$)
2.	Urea (CH_4N_2O)
3.	Deionised Water

Table 5 comprised of different chemicals used for the gram straining and sporulation.

Table 5. Chemicals used for gram straining, sporulation and endospore straining.

Sr. No.	Material
1.	Safranin (Straining)
2.	Malachite Green (Endospore Straining)
3.	Manganous Sulphate (Sporulation)
4.	Dextrose (Sporulation)

3.2 EQUIPMENTS USED

The isolation of bacteria required equipment shown in Table 6:

Table 6. Equipment used for the isolation and growth of bacteria.

SR. NO.	EQUIPMENT	PURPOSE
1.	Digital Weighing Balance (1-220 gm)	Weighing Materials
2.	Inoculating Loop	For Inoculating Bacteria
3.	Laminar Airflow	Provides Sterilised Environment
4.	Autoclave	To Sterilised Media & Glass Plates
5.	BOD Incubator (@ 37°C)	For Growth of Bacteria
6.	Freezer (@ 4°C)	Prevent culture against over growth and contamination
7.	pH Meter	To measure pH
8.	Conductivity Meter	To measure bacterial activities
9.	Microwave Oven	For melting Agar

Table 7 shown different glassware and plastic ware used for the isolation of bacteria.

Table 7. Glassware used for the preparation of bacteria culture.

SR. NO.	EQUIPMENT	PURPOSE
2.	Petri Dish (90 X 15 mm)	Culturing Bacteria
2.	Conical Flask	Mixing Media
3.	Test Tube	For making Slants & Growing Bacteria
4.	Measuring Cylinder	Measuring Media & Distilled Water
5.	Centrifuge Tubes (2 ml, 15 ml and 50 ml)	For Centrifuge Cells.

Preparation of cement and concrete samples required following equipment:

Table 8. Equipment used for the preparation of Cement and Concrete samples.

SR. NO.	EQUIPMENT	PURPOSE
1.	Trowel	Gauging
2.	Tamping Rod	Compaction
3.	Measuring Cylinder	Measuring Water
4.	Sieve	Gradation of Material according to their particle size
5.	Weighing Balance	Weighing Materials
6.	Stop Watch	Note Down Time
7.	Vibration Machine	Compaction of Mortar & Concrete

Table 9 comprises of equipment and apparatus used for the testing of cement and concrete samples along with their specification according to Indian Standards of apparatus and testing in civil engineering.

Table 9. Equipment and apparatus used for testing of Cement and Concrete samples.

SR. NO.	EQUIPMENT/APPARATUS	PURPOSE
1.	Vicat Apparatus "IS:4031(4)-1988" & "IS:4031(5)-1988"	Consistency, Initial and Final Setting Time of Cement
2.	90 micron Sieve "IS:4031(1)-1996"	Fineness of Cement
3.	Le-Chatelier Mould "IS:4031(3)-1988"	Soundness of Cement
4.	Le-Chatelier Flask "IS:4031(11)-1988"	Specific Gravity of Cement
5.	Pycnometer Bottle	Specific Gravity of Fine Aggregate
6.	Wire Basket	Specific Gravity of Coarse Aggregate

3.3 METHDOLOGY

This study consists of three phase: phase 1, phase 2, phase 3 and phase 4. These phases described below:

1) Phase 1: Isolation and screening of bacteria.
2) Phase 2: Preparation Concrete testing samples.
3) Phase 3: Testing of samples.
4) Phase 4: DNA Extraction, PCR Amplification and DNA Sequencing.

3.3.1 Phase 1: Isolation and screening of bacteria

The methodology of culturing bacteria explained stepwise step in this section. The first stage in Phase 1 of this study is to isolate the bacteria of purpose and to get its pure colony. The process of culturing consists of preparing nutrient media for the growth of bacteria to getting pure colonies. This process of isolation and culturing shown as in Figure 4. Different tests were performed for selecting the best isolates this whole process termed as the screening of the bacteria. A standard bacterial species *Bacillus megaterium* MTCC 1684 was collected from MTCC, India. This culture was further taken as the standard culture. It was already reported for high electric conductivity and calcite formation (S. Krishnapriya et. al. 2015). All the screenings results compared with this culture. The stages of isolation and screening procedure explained below:

Sample Collection: A total of sixteen samples comprising eight alluvial soil (alkaline in nature and rich in Iron oxide and lime) samples and eight sewage samples were collected from the different locations of district Solan (H.P). The soil samples were diluted in the distilled water for the purpose of getting isolates. These samples were mentioned along with their location in Table 2.

Preparation of Nutrient Media: The chemicals used in the preparation of urea broth already discussed in Table 1. 200 ml of urea broth was prepared and autoclaved. The composition of media chemicals given in the Appendix of this report. Agar powder was used to solidify the media to prepare urea broth plates. Media was prepared in the conical flask and plugged tightly with the help of cotton plugs. Sterilization of the media was done with the help of autoclave.

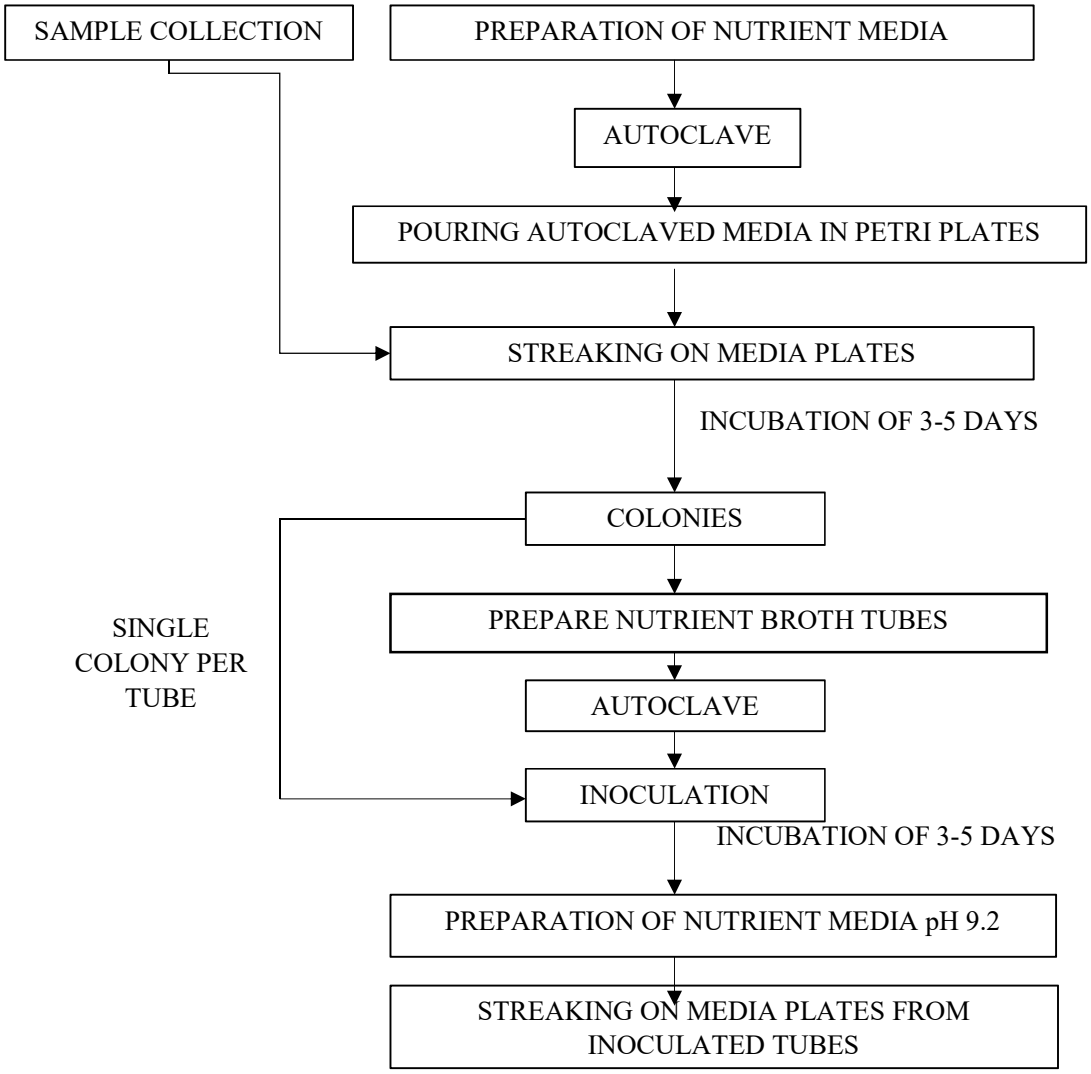


Figure 4. Flowchart of the isolation of Bacteria.

Autoclave: An autoclave is used for the sterilization of media, glass wares and plastic wares (tools and materials). Autoclaving effectively destroys potential viral or bacterial contamination through exposure to extreme heat i.e. 121°C for 15 minutes at 15 psi pressure. It works under the principle of steam under pressure.

Figure 5. Autoclave.

Pouring: The autoclaved media was then poured in the Petri plates inside the Laminar Airflow and incubated at 37°C.

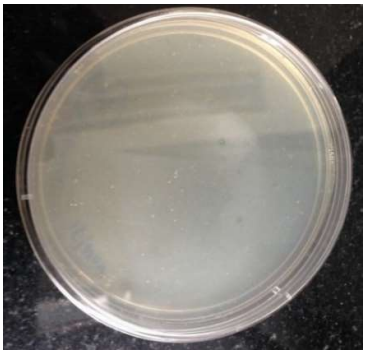


Figure 6. Petri Plate after Pouring.

Streaking: Soil and sewage samples were diluted in distilled water and then streaked.

Incubation: Streaked plates were incubated at 37°C for 3 to 4 days.

Appearance of Colonies:

Figure 7. Mixed colonies on nutrient media (precipitated and non-precipitated).

Preparation of Nutrient Broth Tubes: 5ml of nutrient broth tubes were prepared and then autoclaved.

Inoculation: Single colony was taken from the petri plate and then inoculated in the nutrient broth tubes. Tubes were incubated at 37°C for 2-3 days.

Figure 8. Inoculation Loop.

Streaking: Streaking was done on the plates having media of pH 9.2 from the tubes and then incubated at 37°C for 3-4 days.

3.3.1.1 Tests for urease assay:

To check the urease activities two tests were performed,

1) Qualitative Test (Phenol Red Test).
2) Quantitative Test (Electrical Conductivity Test).

The methodology of these tests is explained below.

1) Qualitative Test (Phenol Red Test): In this, we can measure the activity rate of bacteria precipitation and urease assay. Using phenol red as the indicator, which turned to yellow colour if such activities takes place. First, the media was prepared by adding indicator phenol red in it then the media composition was microwaved for the melting of agar powder. After microwaving, the media was poured to the test tubes. These test tubes then autoclaved. After autoclaving these test tubes were placed in slanted positions so that media solidifies in slanted position. This procedure is known as slants making. The colour of these slants must be pink. Further, the slants were streaked using isolated bacterial strains. After streaking the test tubes were incubated for 3-4 days to get results. The slants which turned to yellow colour were considered to be urease positive (+) and those remained pink are considered to be urease negative (-).

Figure 9. Slant (Indicator: Phenol Red)

2) *Quantitative Test (Electrical Conductivity Test):* For getting measurable, extant of isolated bacterial species activities, the electrical conductivity test was performed. A solution of 1M Urea along with deionised water was prepared. 2 ml of culture was inoculating to this solution by adjusting the culture OD (Optical Density) up-to 1. The conductivity was measured by using conductivity meter.

Figure 10. Conductivity Meter.

The conductivity meter first calibrated with distilled water. Distilled water was used as the cell constant. After calibration the media of urea was checked for its conductivity. By using this method, the rate of activity can be measured. Its unit is Mhos/cm.

3.3.1.2 $CaCO_3$ Precipitation test:

This test was performed to check the efficiency of isolates towards calcite precipitation. This test was performed by making solution of nutrient broth and calcium chloride two hydrates. The solution was prepared in the centrifuged tubes of 15 ml. The incubation of these tubes after

inoculating isolated bacterial strains was done for 4 days at 37°C. The centrifuged tubes then centrifuged @ 7000 rpm for 10 – 15 minutes to get the cell pellet. The supernatant was discarded and the wet and dry pellet weight was compared. The isolates that shown greater results then further selected for other screening processes.

3.3.1.3 FT-IR Spectroscopy:

FT-IR (Fourier Transform Infrared Spectroscopy) was performed by using Agilent Technologies Cary 630 FTIR instrument. A dry sample of $CaCO_3$ precipitated pellet was used for the performance of this test. The results obtained in the form of graphical peaks compared to the standard wavelengths of the different compounds groups and the confirmation of the compound group was done.

Figure 11. Agilent Technologies Cary 630 FT-IR.

3.3.1.4 Gram Straining:

The cells were strained on the microscopic glass slide with crystal violet dye and the gram iodine solution was added to form a complex between crystal violet and iodine. A decolourizer such as ethyl alcohol or acetone was added to the sample, which dehydrates the peptidoglycan layer, shrinking and tightening it. A counter strain of weak water soluble safranin was added. Since, safranin is lighter than crystal violet dye; it does not disrupt the purple coloration in gram-positive cells.

3.3.1.5 Growth Curve:

This experiment was performed to obtain the growth parameters of the bacteria. The selected isolates along with standard culture was grown at the same time with same inoculum. The OD (Optical Density) of the sample cells was checked by using Spectrophotometer at 600 nm from 0 hour to 48 hours. The graphs were obtained by getting the results. These results helped to form a mathematical model of the growth rate of the bacteria. Using growth curve we observed the phase of maximum growth of cultures.

3.3.1.6 Endospore Straining:

This test was performed to obtain the results of isolated bacteria, whether they form spores or not. The spore forming bacteria are generally *Bacillus* species. The spore forming bacteria can survive in worst conditions. Spores are resistant to heat, desiccation, chemicals and radiations. This technique works on Schaeffer-Fulton method, which used to distinguish between vegetative cells and the endospores. A primary strain "malachite green" is used to strain the endospores. The malachite green permeate the spore wall by heating. In this technique heating acts as a mordant. There is no need of using decolourizer in this spore straining. Only water is sufficient for the decolourizer. Vegetative cells have been disrupted by heat. As the endospores are resistant to straining, the endospores are equally resistant to de-straining and will retain the primary dye while vegetative cells will lose the strain. Safranin was used as the counter strain. The vegetative cells should appear pink/red in colour. The vegetative cells that contain endospore should strain pink while the spore should be seen as green ellipses. While free endospores should not be associated with the vegetative bacteria and should be seen as green ellipses.

3.3.2 Phase 2: Preparation of Cement and Concrete testing samples

In this study PPC Fly Ash based cement was used therefore, basic cement properties checks were done. To do so basic hydraulic cement tests were performed. Vicat's mould was filled with cement water paste to get consistency and initial & final setting time. For soundness Le-Chatelier mould was prepared and Le-Chatelier flask was used to obtain the specific gravity of the cement. The specific gravity of fine and coarse aggregate was find out along with the fineness modulus of sand and zone of sand. Further Design mix for concrete testing was designed of M20. The immobilization of standard culture, isolate 2 and isolate 13 with 1.21 x

10^{10}, 1.21 x 10^{10} and 5.2 x 10^{8} CFU/ml to the design mix along. The composition of this design mix given in appendix.

3.3.3 Phase 3: Testing of construction materials

The basic hydraulic cement tests were performed to get the specification of cement used. These tests include consistency, initial & final setting time, fineness, soundness, specific gravity, compressive strength of cement. These tests were performed according to the Bureau of Indian Standards. A stepwise test performance shown in Figure 12. PPC Part1 must satisfy the specifications according to IS 1489-1: 1991.

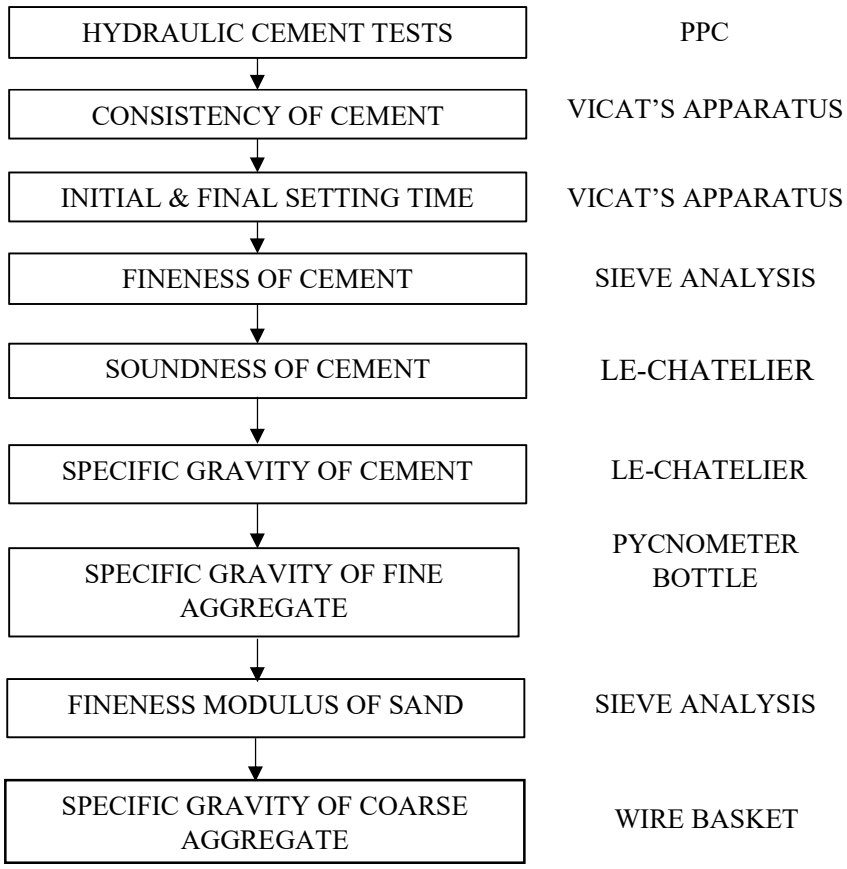


Figure 12. Flowchart of materials testing.

Consistency of Cement: The consistency of cement test follows the IS 4031 (4) – 1988. The test was performed using Vicat apparatus and consistency plunger. Cement water paste was prepared and filled in the vicat mould. The water cement ratio for preparation of paste was started from 25% of cement weight than it was increased up to 35%. Paste which gives penetration value in between 7 to 5 from bottom was taken as consistent and that water

percentage is considered as the consistency of the cement. Consistency of cement helps to get the water percentage for initial & final setting time, soundness and compressive strength tests.

Initial & Final Setting Time: Initial and final setting time follows IS 4031 (5) – 1988. This test was done using the vicat apparatus and setting time needles. The water added must be "0.85P" by weight of cement, where "P" is the standard consistency. The initial setting time was measured using 1 mm penetration needle failed to penetrate at 5 mm – 7 mm from bottom of the mould. In addition, final setting time is that time when 1 mm penetration needle makes an impression on the mould but 5 mm assembly failed to make any impression.

Fineness of Cement: It was done according to the IS 4031 (1) – 1988. The fineness of cement was done using sieve analysis. 90-micron sieve was taken and 10g of cement was sieved for 2 minutes. The percentage retaining was calculated and it must not exceed 10% by weight of cement.

Soundness of Cement: This test was done according to the IS 4031 (3) – 1988. The soundness means ability to resist volume expansion. The expansion in cement is due the presence of free lime in it. The water used in this test is about 0.78P, where "P" is the standard consistency of cement. The testing sample was placed in the Le-Chatelier mould and it was placed for 1-day curing in water and after that 3 hours Le-Chatelier water bath treatment than the expansion was noted. The expansion must not exceed 10 mm.

Specific Gravity of Cement: This test was done according to the IS 4031 (11) – 1988. The specific gravity was performed by using Le-Chatelier flask. The specific gravity of must be as 3.15 and it must be done by using kerosene or benzene.

Specific Gravity of Fine Aggregate: This test was performed using Pycnometer bottle, under the consideration of IS 2720(3): 1980.

Fineness Modulus of Fine Aggregate: Test was performed over the sand used in the design mix of concrete samples. It was done to get the gradation of sand zone. It was done by sieve analysis.

Specific Gravity of Coarse Aggregate: This test was done according to the IS 2386(3): 1963. The specific gravity was performed by using wire basket.

Compressive Strength of Concrete: Compressive strength of concrete blocks can be checked by compressive testing machine after 28 days curing. The concrete cubes of 100 mm x 100 mm x 100 mm were prepared. Total four number of samples were prepared one of control concrete sample and other of bacteria immobilized concrete.

Quantification of crack healing by bacteria: Crack healing was quantify by visual inspection of the concrete cubes during curing and after curing. The regular visual inspection was done, time-to-time reports were collected, and the pictures of the healed cracks and voids were taken.

3.3.4 Phase 4: DNA Extraction, PCR Amplification and DNA Sequencing

The genomic DNA extraction was carried out using overnight grown cultures of bacterial isolates. The cell pellets from 1ml culture was mixed with 1ml of extraction buffer [1 M Tris-HCL (pH 8), 5 M NaCl and 0.5 M EDTA] and DNA was extracted with some modifications (Sharma *et al*., 2017). The isolates were identified using 16 rRNA gene sequencing. The PCR reaction was carried with total volume of 25µl containing 1 µl of DNA, 2.5 µl of 10× PCR buffer, 0.5 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTP (Promega) mix, 1 µl each of 10 pmol, 0.25 µl of 5 U Taq DNA polymerase (Intron) using Total Bacteria primers Bact27f (5′-GTTTGATCCTGGCTCAG-3′) and 1492r (5′- CGG CTA CCT TGT TAC GAC-3′) (Felsk, 2001). Using reaction cycle with initial denaturation step of 7 minutes at 95°C with an amplification of 35 cycles with denaturation step of 1 minute at 94°C then annealing for 40 seconds at 52°C with an extension of 1 minute at 72°C and final extension of 10 minutes at 72°C. After the complete reaction, the PCR amplified DNA fragments were analysed followed by visualisation in a UV trans-illuminator. The amplified 16s RNA gene from different bacterial were sequenced and the sequence data was analysed by using BLAST and clustal omega (http://www.ncbi.nlm.nih.gov/). 16s rRNA gene sequences have been deposited in gene bank database.

CHAPTER 4

4.1 RESULTS

The results, which were obtained by different experiments, are considered in this chapter. The result part consists of each test concluded on the bacterial isolates and materials, which used in concrete sample preparation.

4.1.1 Isolation and Screening of Bacteria Results:

Mixed colonies were obtained from the samples. Description of appearance of colonies and time taken given in Table 10.

Table 10. Samples with colonies size and time taken to obtain colonies.

SR. NO.	SAMPLE NO.	SIZE OF COLONIES	TIME TAKEN
1.	1	Small Colonies	3 Days
2.	2	Bigger Colonies	3 Days
3.	3	Bigger Colonies	5 Days
4.	4	Medium Size Colonies	4 Days
5.	5	No Colony	-
6.	6	Bigger Size Colonies	4 Days
7.	7	No Colony	-
8.	8	No Colony	-
9.	9	No Colony	-
10.	10	Medium Size Colonies	4 Days
11.	11	No Colony	-
12.	12	No Colony	-
13.	13	No Colony	-
14.	14	Medium Size Colonies	4 Days
15.	15	No Colony	-
16.	16	Small Size Colonies	5 Days

Single colonies further taken from the petri dish and inoculated to the Nutrient Broth. The morphology of each colony is given the table 11 below along with colony number and sample number.

Table 11. Selected Colonies and their description.

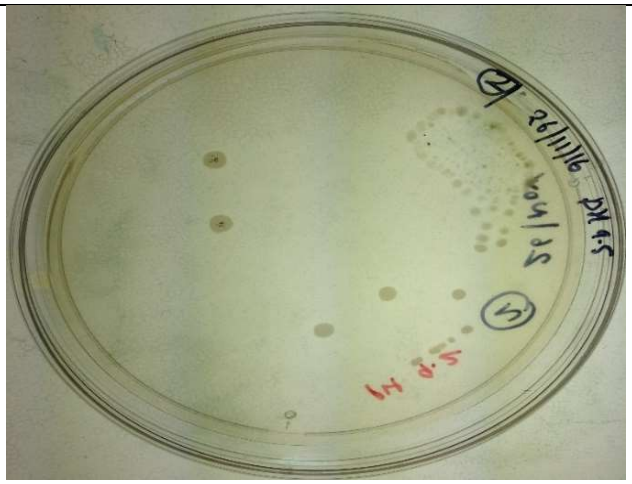
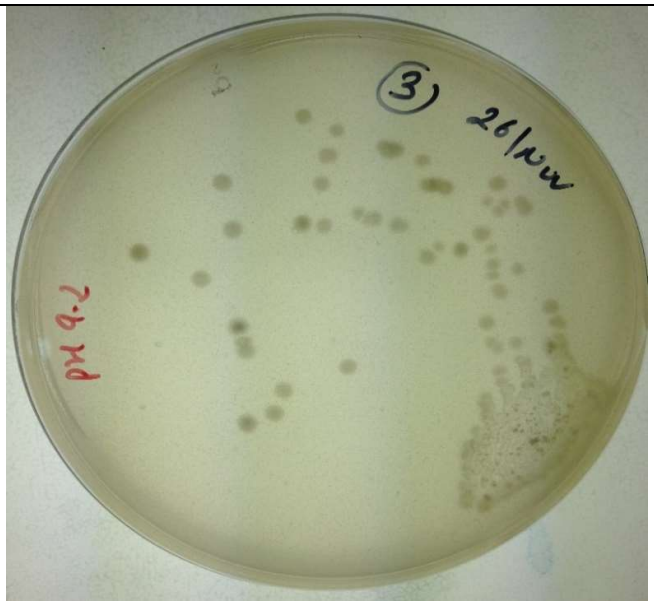
SR. NO.	SAMPLE NO.	COLONY NO.	DESCRIPTION/MORPHOLOGY
1.	1	1	I) Perfectly Round Shaped. II) Pale in Colour. III) Medium sized colony.
2.	2	2	I) Irregular Round Shaped. II) Grey in Colour. III) Bigger sized Colony.
3.	2	3	I) Round Shaped. II) Grey in Colour. III) Bigger sized Colony
4.	2	4	I) Perfectly Round Shaped. II) Brown in Colour. III) Small sized Colony.
5.	3	5	I) Round Shaped. II) Pale and Grey in Colour. III) Bigger sized Colony.
6.	4	6	I) Irregular Round Shaped. II) Black dot in centre and Faded-Grey in Colour. III) Medium sized Colony.
7.	4	7	I) Round Shaped. II) Faded-Grey in Colour. III) Small sized Colony.
8.	6	8	I) Irregular Round Shaped. II) Pale in Colour. III) Medium sized Colony.

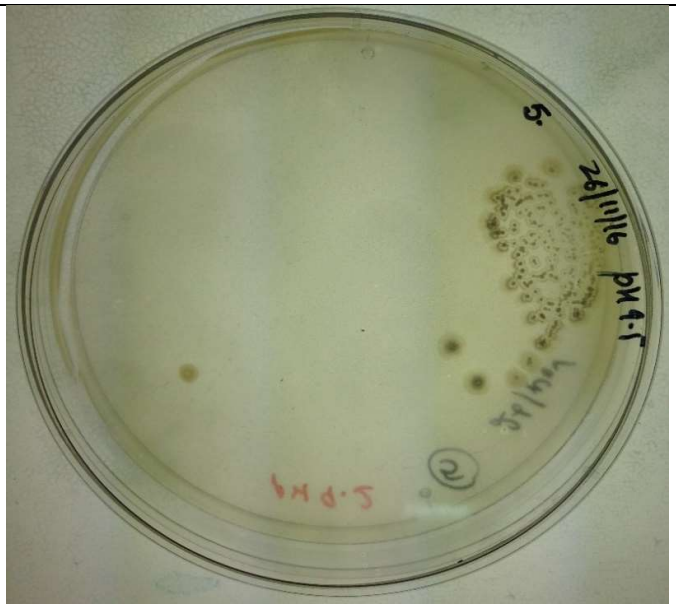
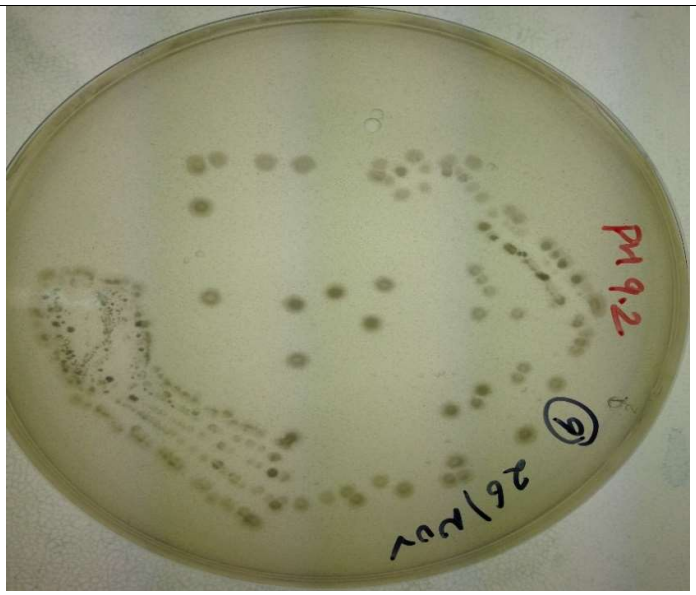
9.	6	9	I) Irregular Round Shaped. II) Black in Colour. III) Bigger sized Colony.
10.	6	10	I) Irregular Round Shaped. II) Faded-Yellow in Colour. III) Bigger sized Colony.
11.	10	11	I) Irregular Round Shaped. II) Faded-Brown in Colour. III) Medium sized Colony.
12.	10	12	I) Round Shaped. II) Faded-Yellow with Black dot in centre. III) Bigger sized Colony.
13.	14	13	I) Round Shaped. II) Brown in Colour. III) Medium sized Colony.
14.	16	14	I) Perfectly Round. II) Faded-Brown with Dark centre. III) Small sized Colony.

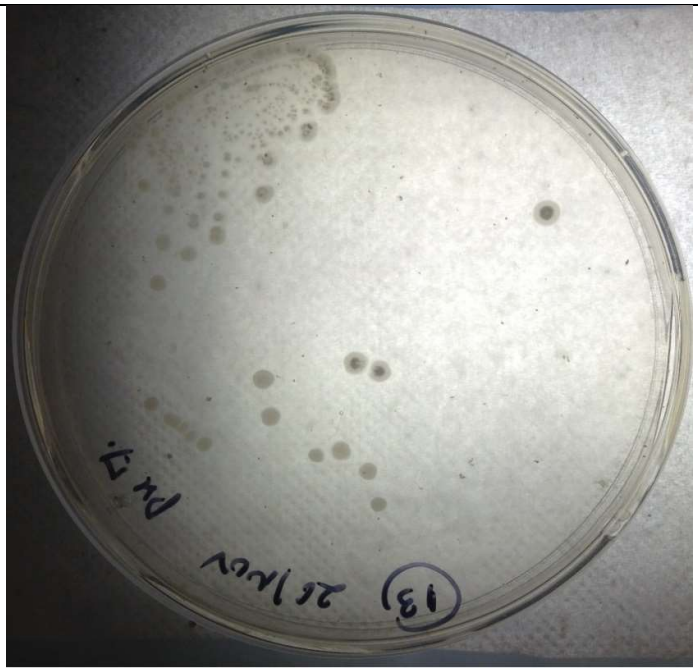
These 14 colonies were taken and inoculated to the Nutrient Broth media test tubes. These 14 single colonies were streaked over new nutrient media plates and pure colonies were obtained after 3-5 days.

The isolates were selected finally getting the precipitation over 9.2 pH nutrient media (Urea broth) petri plates. The selected plates were as following:

Table 12. Selected colonies (Isolates).

SR. NO.	COLONY NO.	IMAGE OF PERTI DISH AFTER INOCULATION OF SINGLE COLONY
1.	2	 **Figure 13. Pure colonies by colony no. 2.**
2.	3	 **Figure 14. Pure colonies by colony no. 3.**

3.	5	 **Figure 15. Pure colonies by colony no. 5.**
4.	9	 **Figure 16. Pure colonies by colony no. 9.**

5.	13	 **Figure 17. Pure colonies by colony no. 13.**

The precipitated and non-precipitated colonies shown in the appendix with description. These 5 isolates were further tested to advance screening stages.

4.1.2 Urease Assay Tests Results:

The results of urease assay test by phenol red test given in Table 13. This test was the qualitative result of urease assay.

Table 13. Results of phenol red test.

SR. NO.	COLONY NO.	CHANGE IN COLOUR	RESULT	PRECIPITATION
1.	2	Yes “Yellow”	+	Yes “Little”
2.	3	No “Faded Pink”	-	Yes “Heavy”
3.	5	Yes “Yellow”	+	Yes “Heavy”
4.	9	No “Pink”	-	Yes “Heavy”
5.	13	No “Pink”	-	Yes “Heavy”

a) **b)**

a) Picture showing negative urease (Left) and positive urease (Right).

b) Picture results negative and positive urease for different colonies.

Figure 18. Slants showing urease activity.

The conductivity test was performed to gain the quantitative results of urease assay. The following results were obtained by conductivity test. The conductivity of deionised water was recorded as 0.006 mMhos/cm and the conductivity of 1M urea solution was recorded as 0.019 mMhos/cm. The conductivity measured in µMhos/cm. The detailed conductivity results shown in table 14.

Table 14. Conductivity results for different isolates. (Units - µMhos/cm)

TIME (Min.)	STANDARD CULTURE	ISOLATE 2	ISOLATE 3	ISOLATE 5	ISOLATE 9	ISOLATE 13
0	58	63	29	28	29	35
5	67	65	34	31	34	38
10	69	68	34	31	34	39
15	72	73	36	32	37	41
20	74	75	36	33	38	42
25	75	76	37	33	39	42
30	76	77	37	34	39	42
35	76	77	37	33	39	42
40	75	76	38	34	41	43
45	74	76	38	34	42	43
50	73	75	38	34	42	43
55	74	76	37	34	42	42
60	72	74	38	33	42	41
65	70	72	37	33	43	41
70	70	71	38	42	43	42
75	69	70	41	34	43	42
80	69	69	39	34	43	41
85	68	71	41	35	44	42
90	67	71	41	35	44	43

4.1.3 $CaCO_3$ Precipitation Results:

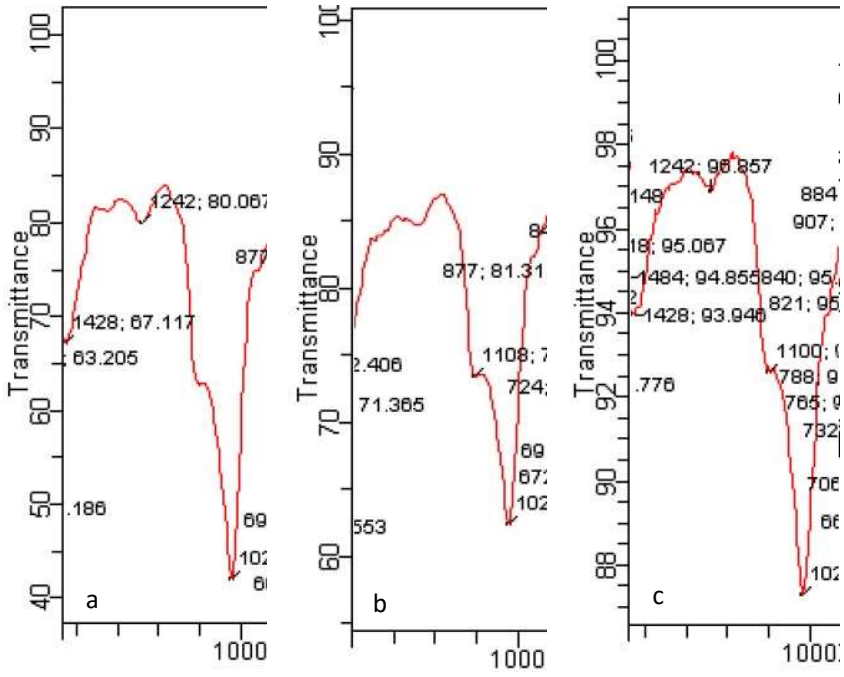
The weight comparison was done between the dry and wet weight of the precipitated isolated and recorded as in table 15.

Table 15. $CaCO_3$ Precipitation by different cultures.

Sr. No.	Culture	Dry Weight of Precipitation (mg)
1.	Standard Culture	9
2.	Isolate 2	12
3.	Isolate 3	20
4.	Isolate 5	15
5.	Isolate 9	14
6.	Isolate 13	21

4.1.4 FT-IR Spectroscopy Results:

The IR (Infrared) wavelength for C - O compound of alcohols, esters, ethers, carboxylic acids, anhydrides is 1000 to 1300 cm^{-1}. The results obtained are as in following images under the required wavelength.



Wavenumber (cm^{-1})

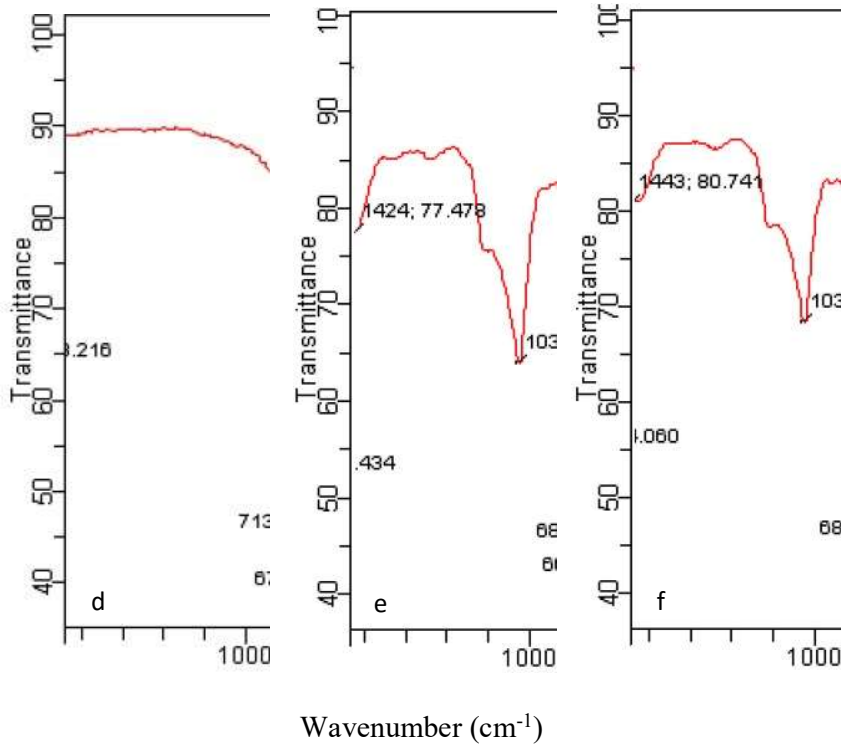
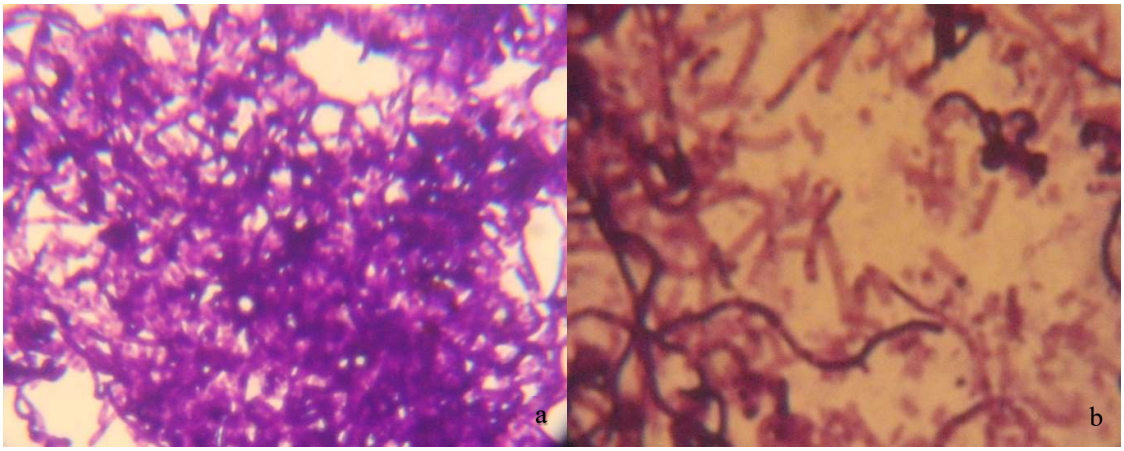


Figure 19. FT-IR transmittance value for IR wavenumber from 1000 to 1300 cm^{-1} for a) standard culture, b) isolate 2, c) isolate 3, d) isolate 5, e) isolate 9 and f) isolate 13.

The figure 19 shown peaks in between the desired wavenumber (1000-1300 cm^{-1}). Therefore, we can assure the precipitated product as Calcite.

4.1.5 Gram Straining Results:

The gram straining was done and all the isolates were found to be gram positive. The gram straining results was optimised using microscopic images. These microscopy analysis results are shown for each isolate as in figure 20.



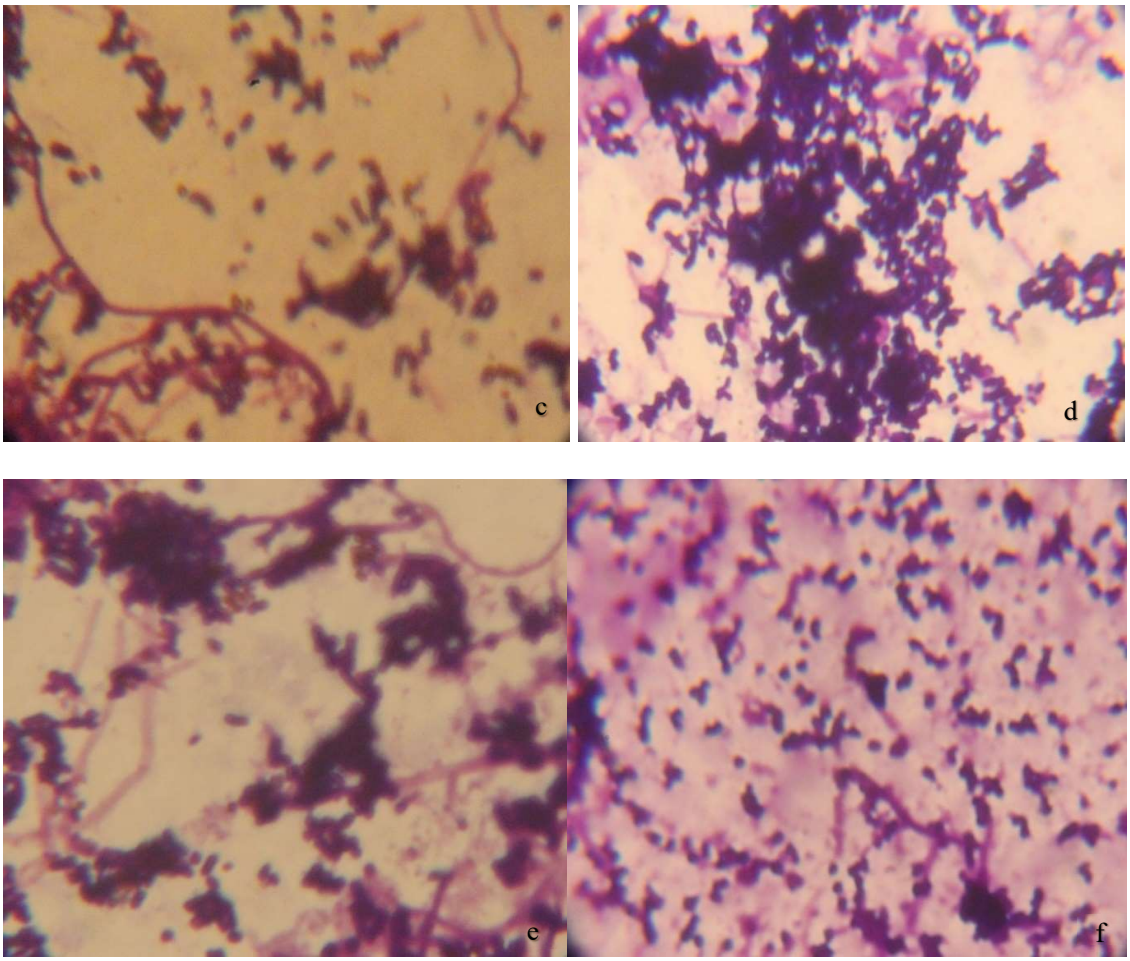


Figure 20. Microscopy images of gram straining a) Standard culture, b) Isolate 2, c) Isolate 3, d) Isolate 5, e) Isolate 9 and f) Isolate 13.

4.1.6 Growth Curve of Selected Isolates:

Growth curve of select two isolates (2 and 13) based on the urease assay along with standard culture were done for 48 hours. These three cultures were selected based on urease assay test and gram straining. The growth curve of these species shown in figure 21, figure 22 and figure 23.

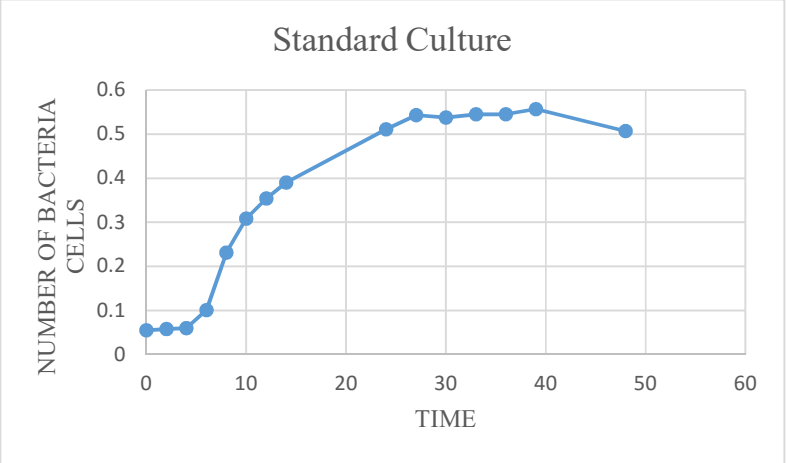


Figure 21. Growth curve of standard culture.

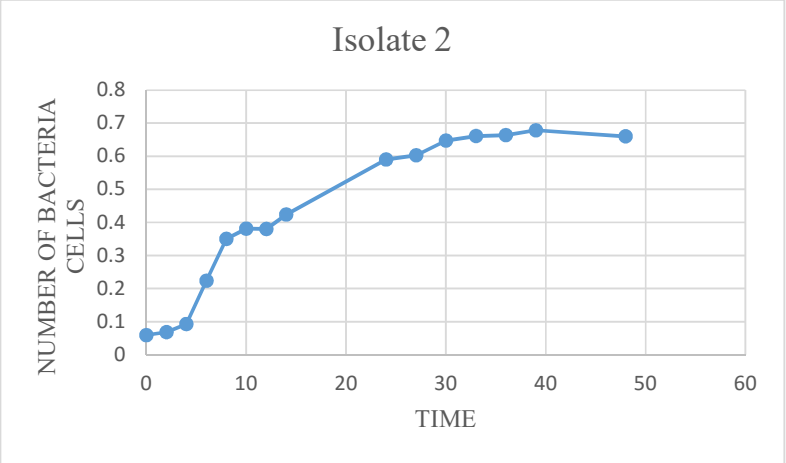


Figure 22. Growth curve of isolate 2.

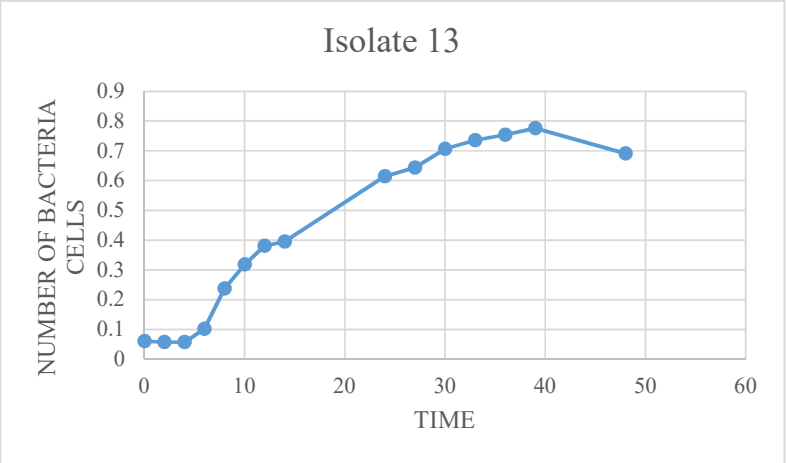


Figure 23. Growth curve of isolate 13.

4.1.7 Endospores of Selected Isolates:

The endospore formation of three bacterial cultures were analysed by microscopy. The microscopy images shown in figure 24.

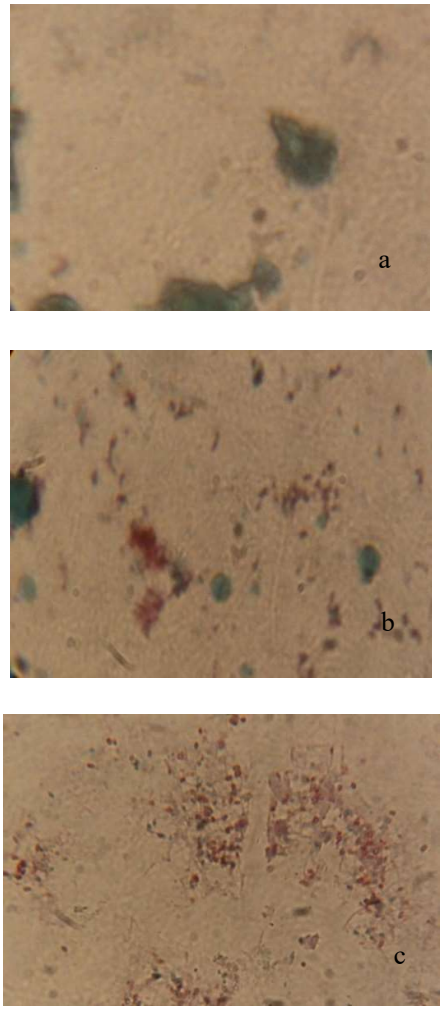


Figure 24. Microscopy images of endospores a) Standard culture, b) Isolate 2 and c) Isolate 13.

4.1.8 Material Testing Results:

Testing PPC Part 1 obtained following results by performing different tests and these values were compared with the specifications of PPC Type 1 along with the coarse and fine aggregate testing. These results are included in following table.

Table 16. Experimental values of materials used.

Sr. No.	Experiment	Experimental Values
1.	Consistency of Cement	32%
2.	Initial setting time of Cement	55 min.
3.	Final setting time of Cement	245 min.
4.	Fineness of Cement	3.8%
5.	Soundness of Cement	5 mm
6.	Specific Gravity of Cement	3.15
7.	Specific Gravity of Fine Aggregate	2.56
8.	Specific Gravity of Coarse Aggregate	2.88
9.	Fineness Modulus of Fine Aggregate	2.81
10.	Zone of Fine Aggregate	II

Fineness modulus of fine aggregate was done by sieve analysis and the results of the sieve analysis was concluded as bellow:

Table 17. Fineness Modulus of Fine Aggregate.

Sr. No.	Sieve Size	Weight retained (gm)	Cumulative weight retained (gm)	Cumulative percentage weight retained (%)
1.	4.75 mm	8.1	8.1	.81
2.	2.36 mm	13.5	21.6	2.16
3.	1.18 mm	20.4	42	4.2
4.	0.6 mm	40.3	82.3	8.23
5.	0.3 mm	621.6	703.9	70.39
6.	0.15 mm	255.1	929	92.9
7.	Pan	35.2	964.2	96.42
8.	Total			275.11

Therefore, fineness modulus of sand is 2.75.

Type of sand = Medium soil.

Zone of sand = 2.

4.1.9 Compressive Strength Results:

The compressive strength of the samples were performed by using compression-testing machine after 28 days. The results for different samples were as following:

Table 18. Compressive Strength of samples.

Sample	28 Days Compressive Strength (MPa)
Control Concrete	21
Standard Culture Concrete	22
Isolate 2 Concrete	22
Isolate 13 Concrete	23

4.1.10 Quantification of crack healing by bacteria:

The visual inspection was done for obtaining the quantification of crack healing. It was observed that after 7 days of curing of concrete cubes the white colour layer starts appeared on the concrete cubes. The macro voids on the surface of concrete cube stars filling autogenously. The pictures were taken at regular interval of 7 days. The results are as shown in Figure 25.

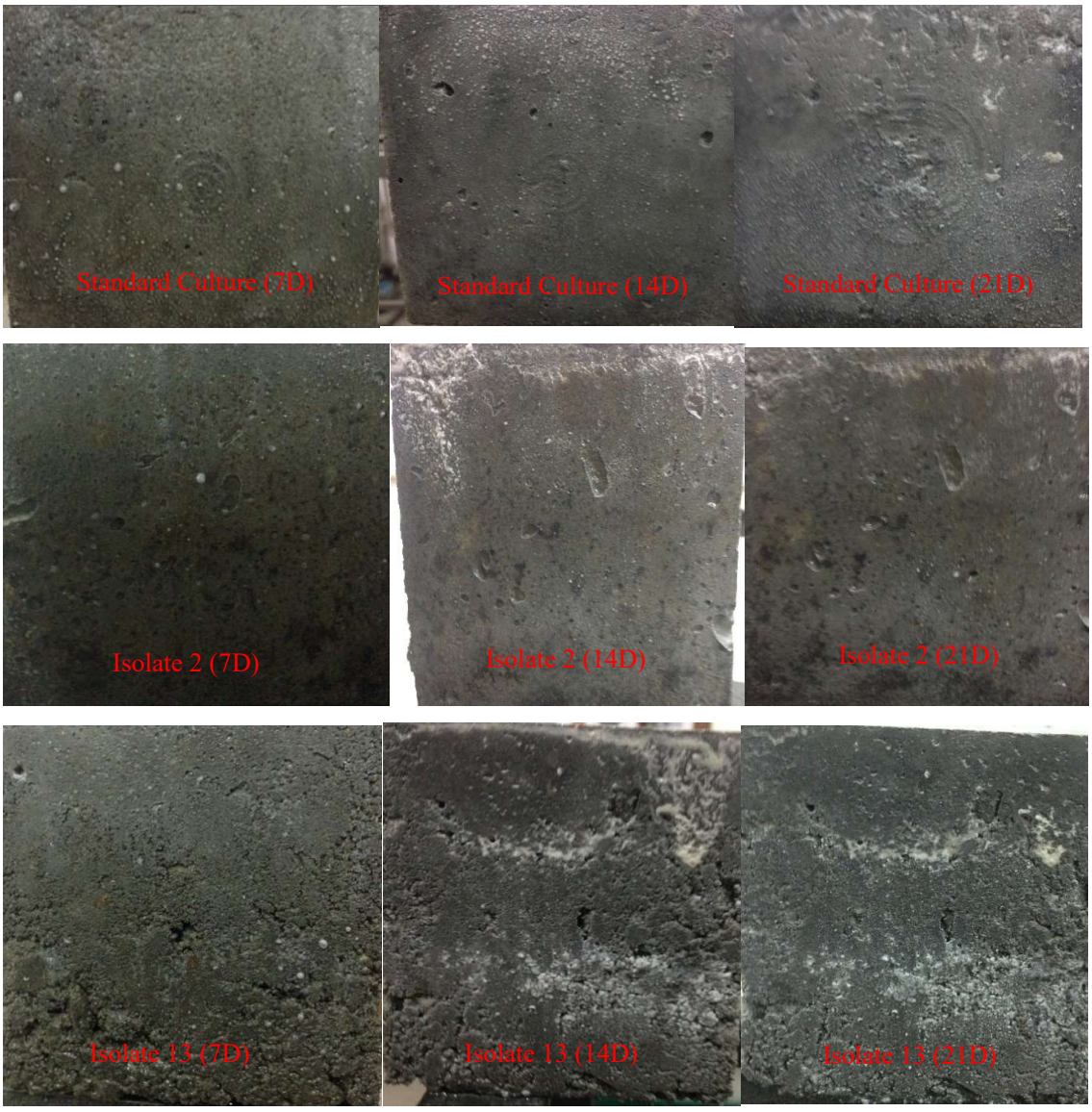


Figure 25. Images of quantification of crack healing control, standard culture, isolate 2 and isolate 13 concrete samples after 7, 14, 21 days of casting.

The above images shown potential healing properties in standard culture, isolate 2 and isolate 13 concrete samples. While the control concrete samples remained the same in both of the cases. Detailed pictorial representation of healing by bacteria shown in the appendix.

4.1.11 DNA Extraction, PCR Amplification and DNA Sequencing Results:

DNA was extracted using modified protocol defined by Sharma et. al., (2017). DNA bands were visualised using UV trans-illuminator and image was captured using Alpha imagerEP gel imaging system.

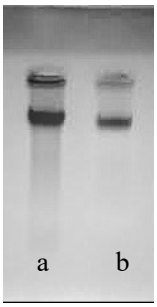


Figure 26. DNA bands of a) isolate 2 and b) isolate 13.

16s RNA gene for bacterial species was amplified using T100 Thermal Cycler (Bio-Rad) using total bacteria primers as mentioned in material and methodology. Product size of bands was 1400 Basepair.

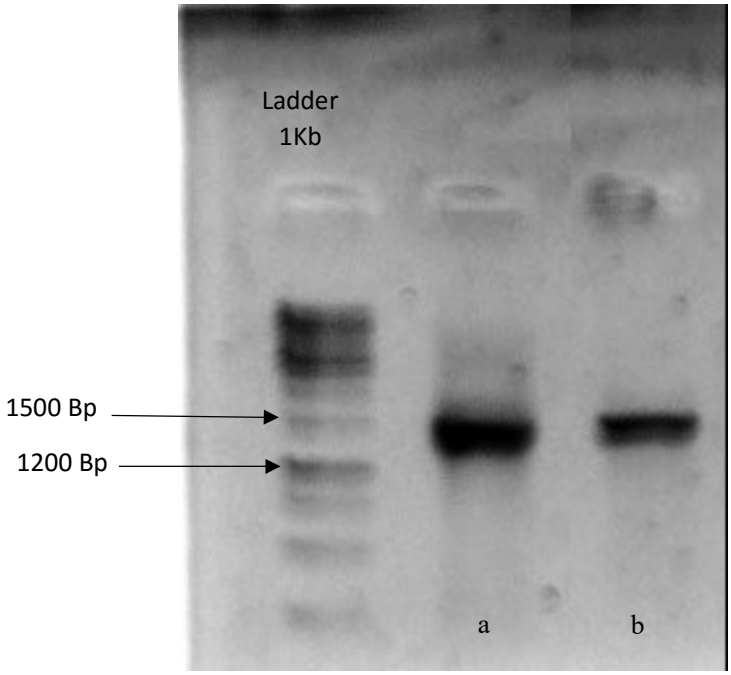


Figure 27. PCR amplification using total bacteria primer a) isolate 2 and b) isolate 13.

The bacterial species were identified by sequence analysis of 16S rRNA gene. Comparative analysis of sequences, within the NCBI database revealed *Enterococcus* sp. and *Lysinibacillus* sp.

4.2 CONCLUSION

By analysing above results, it was concluded that the bacteria culture showing positive urease results and heavy precipitation. These bacterial isolates shown great calcite formation. Due to high conductivity and potential spore forming behaviour, these bacterial cultures survive in the concrete and can heal crack properly. The screening of these bacterial isolates done by comparing the results of these isolates with the standard culture *Bacillus megaterium* MTCC 1684. The calcite formation was confirmed by the FT-IR spectroscopy. The quantification of crack repairing can be seen by visual inspection. It was recorded that the potential calcite formation seen after 7 days. Great impact of calcite formation seen after 21 days of curing of concrete cubes. The compressive strength shows similar results in all the concrete samples.

APPENDIX – 1

A-1.1 COMPOSITION OF NUTRIENT MEDIA

The nutrient media (Urea Broth and Urea Agar) prepared for the culturing of bacteria given in Table A.1.1.

Table A.1.1. Nutrient media composition per litre.

SR. NO.	CHEMICAL	COMPOSITION (gm/l)
1.	Urea	20
2.	Sodium Bicarbonate	2.12
3.	Ammonia Chloride	10
4.	Nutrient Broth	3
5.	Calcium Chloride two hydrate	25
6.	Agar	17

A-1.2 INCREASING pH OF NUTRIENT MEDIA COMPOSITION

The pH of nutrient media was increased by adding 4 pellets of Sodium Hydroxide.

A-1.3 PHENOL RED ADDITION FOR MAKING SLANTS

Phenol red was used as indicator for getting urease activities. 0.018gm/l phenol red was added to the nutrient media composition.

APPENDIX – 2

A-2.1 IMAGES OF PRECIPITATED AND NON-PRECIPITATED COLONIES

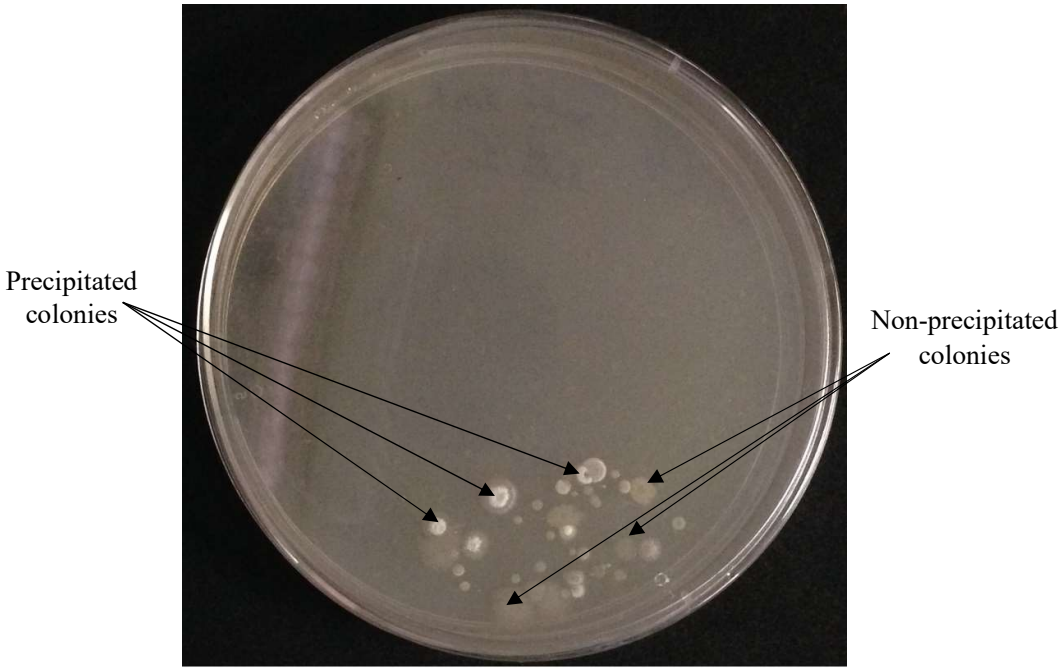


Figure A.2.1. Mixed colonies precipitated and non-precipitated.

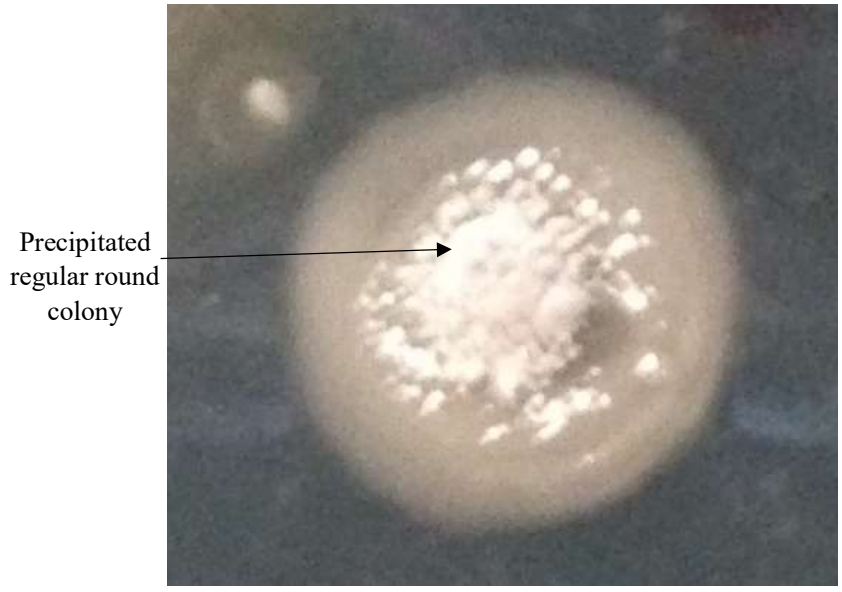


Figure A.2.2. Precipitated colony.

A-2.2 IMAGES OF PURE COLONIES

Figure A.2.3. Pure colonies.

A-2.3 IMAGES OF SLANTS FOR UREASE ASSAY

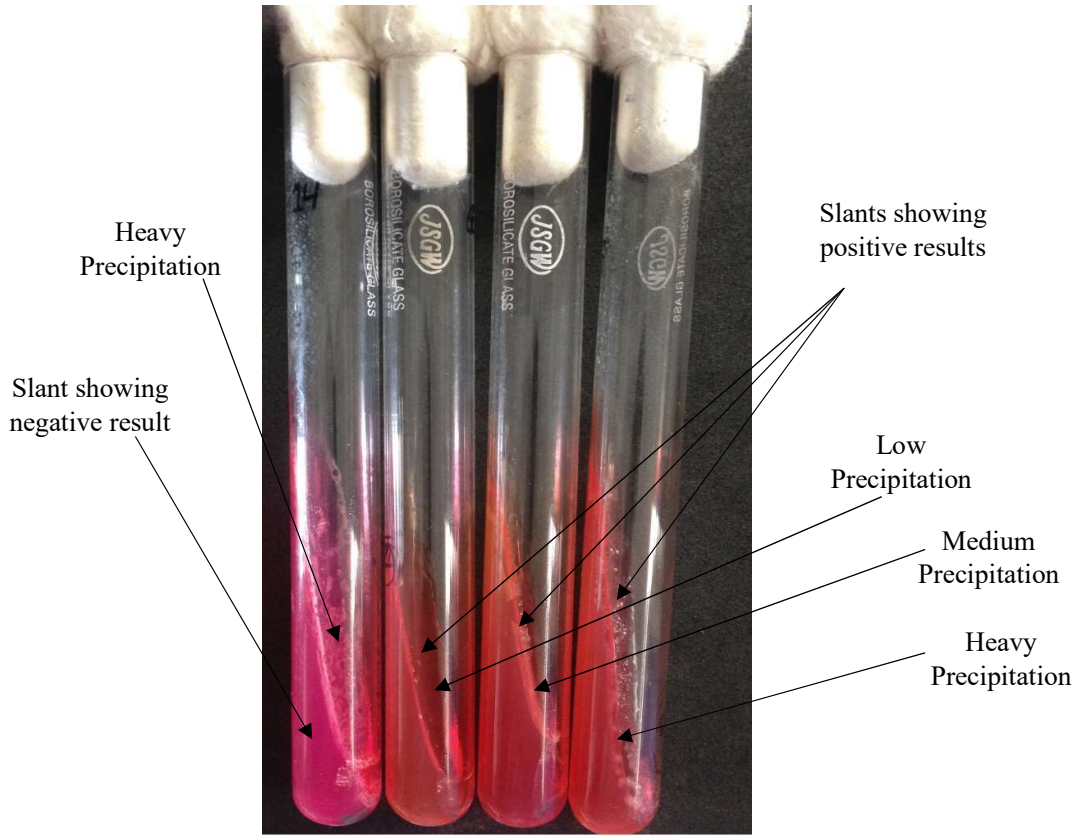


Figure A.2.4. Slants.

APPENDIX – 3

A-3.1 DESIGN MIX QUANTITY PER CUBIC METER OF CONCRETE

The quantity of each material for the preparation of design mix for zone II gradation sand, 10 mm coarse aggregate along with 0.45 water to cement ratio (design of M20 grade of concrete) described in the Table A.3.1.

Table A.3.1. M20 grade of concrete design mix.

Sr. No.	Type	Quantity kg/m^3
1.	Cement	462
2.	Water	208
3.	Fine Aggregate	896
4.	Coarse Aggregate	852

APPENDIX – 4

A-4.1 IMAGES OF CRACK HEALING BY BACTERIA

The visual inspection of crack healing by bacteria shown in the following images and the comparison made with control concrete sample.

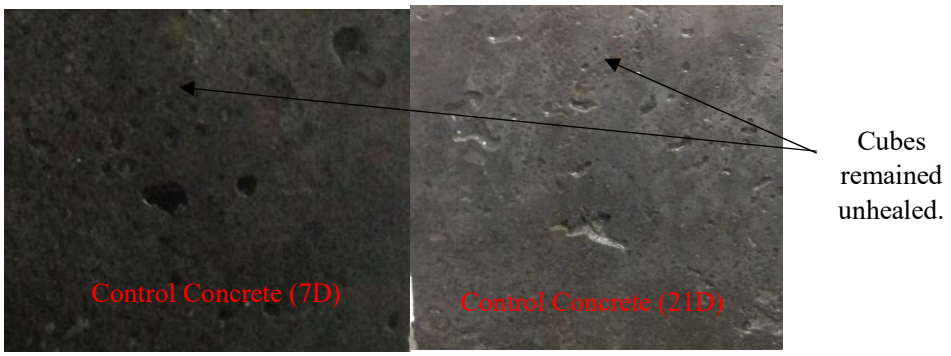


Figure A.4.1. Control concrete sample after 7 and 21 days of concrete cubes curing.

Figure A.4.2. Potential of crack healing by standard culture seen after 7 days and 21 days of concrete cubes curing.

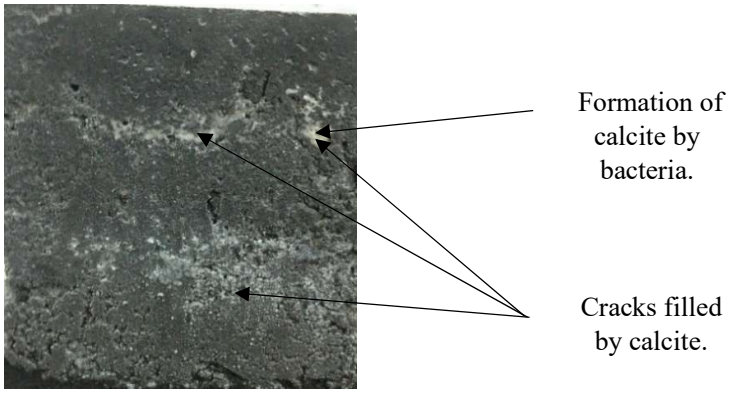


Figure A.4.3. Heavy calcite formation in concrete by isolate 13 after 21 days of concrete cubes curing.

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PUBLICATIONS

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