BIO MINERALISATION OF CALCIUM CARBONATE BY DIFFERENT BACTERIAL STRAINS AND THEIR APPLICATION IN CONCRETE CRACK REMEDIATION

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ABSTRACT

Concrete crack under sustained loading and on exposure to environmental agents. Cracks can lead to damage of the mineral matrix and corrosion of steel. Research has indicated that a material with low permeation properties lasts longer. Microbially Induced Calcite Precipitation (MICP) is a biochemical process in which specific organisms produce extracellular calcium carbonate which is capable of crack healing. In the present investigation bacterial strains were isolated from concrete environment, isolates were characterised till species level and their activity were compared with that of Bacillus pasteurii and Bacillus sphaericus. Bacillus flexus the isolated species was found to perform better when compared to that of Bacillus pasteurii and Bacillus sphaericus. MICP was quantified by X-Ray Diffraction (XRD) analysis and visualized by Scanning Electron Microscopy (SEM). The present investigation demonstrates that Bacillus flexus have better potential of calcite production than other species; hence this species could be effectively used in MICP.

KEYWORDS: *Bio mineralisation, Bacillus pasteurii, Bacillus sphaericus, Bacillus flexus, XRD, SEM.*

I. INTRODUCTION

Concrete is the most used and relatively cheap construction material for infrastructure, but most concrete structures are prone to cracking with time and with different exposure conditions. Micro cracks on the surface of the concrete make the whole structure vulnerable because water and other environmental agents seeps in through the cracks to degrade the concrete and corrode the steel reinforcement, greatly reducing the lifespan of a structure. This durability related problems impact a great economical loss. Methods currently used for crack remediation often use synthetic polymers that need to be applied repeatedly, which requires continuous monitoring and recurring expenses. Because of these disadvantages of conventional surface treatments, attention has been drawn to alternative techniques for the improvement of the durability of concrete and also environmentally friendly. Therefore a novel technique for remediating cracked structural elements has been developed by employing a selective microbial plugging process in which microbial metabolic activities promote calcium carbonate precipitation [1, 2]. Specially selected types of the genus Bacillus, along with a calcium based nutrient and nitrogen and phosphorus in presence of oxygen, the soluble calcium source is converted to insoluble calcium carbonate by ureolytic activity. The calcium carbonate solidifies on the cracked surface, thereby sealing it up. It mimics the process by which bone fractures in the human body are naturally healed by osteoblast cells that mineralize to reform the bone.

MICP occurs via far more complicated processes than chemically induced precipitation. The bacterial cell surface with a variety of ions can non-specifically induce mineral deposition by providing a nucleation site. Ca^{2+} is not likely utilized by microbial metabolic processes; rather it accumulates

outside the cell. In medium, it is possible that individual microorganisms produce ammonia as a result of enzymatic urea hydrolysis to create an alkaline micro environment around the cell. The high pH of these localized areas, without an initial increase in pH in the entire medium, commences the growth of $CaCO_3$ crystals around the cell. Possible biochemical reactions (vide Eq. 1 2 & 3) in Urea-$CaCl_2$ medium to precipitate $CaCO_3$ at the cell surface can be summarized as follows [1]:

$$Ca^{2+} + Cell \rightarrow Cell\text{-}Ca^{2+} \quad (1)$$

$$Cl^- + HCO_3^- + NH_3 \rightarrow NH_4Cl + CO_3^{2-} \quad (2)$$

$$Cell\text{-}Ca^{2+} + CO_3^{2-} \rightarrow Cell\text{-}CaCO_3\downarrow \quad (3)$$

A novel approach of MICP has been reported as a long-term remediation tool which has exhibited high potential for crack cementation of various structural formations such as granite and concrete [3, 1, 4]. They used *Bacillus pasteurii* to induce $CaCO_3$ precipitation. Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD) analysis has shown the direct involvement of microorganisms in calcium carbonate precipitation [5]. The presence of calcite was, however, limited to the surface areas of the crack. The authors attributed this to the fact that *Bacillus pasteurii* grows more actively in the presence of oxygen. Still, the highly alkaline pH (12–13) of concrete was a major hindering factor to the growth of the moderate alkaliphile *Bacillus pasteurii*, whose growth optimum is around a pH of 9. To retain high metabolic activities of bacterial cells at such a high pH, immobilization technology (where microbial cells are encapsulated in polymers) can be applied in order to protect the cells from the high pH. Day et al. [6] investigated the effect of different filler materials on the effectiveness of the crack remediation. Beams treated with bacteria and polyurethane showed a higher improvement in stiffness compared to filler materials such as lime, silica, fly ash and sand. According to the authors, the porous nature of the polyurethane minimizes transfer limitations to substrates and supports the growth of bacteria more efficiently than other filling materials, enabling an accumulation of calcite in deeper areas of the crack. No differences could be observed between the overall performances of free or polyurethane immobilized cells in the precipitation of carbonate [4, 7]. De Belie and De Muynck [8] further investigated the use of MICP for the repair of cracks in concrete by using *Bacillus sphaericus*. Al-thawad et al [9] studied calcium carbonate formation from isolated bacteria from Australian soil and sludge they genetically examined three isolated and found them to be closely related to bacillus species. Among them they used highest calcium carbonate forming bacillus species they have used urea and calcium chloride. The type, size and shape of crystals were characterized by using light microscope and scanning electron microscope and they found vaterit and calcite were precipitated at the surface of sand granules indicating the possibility of using these method to consolidate loose sand. Arunachalam et al [10] have examined biosealent properties of Bacillus sphaericus which were isolated from soil, they used bio mineralization on concrete cubes, and Studies showed that the bacterial treatment of the drilled cube has increased the strength to about 34%, when compared to the drilled, non-remediated cube. Varenyam achal et al., [11] studied the effects of Bacillus sphaericus and CT-5 isolated from cement on compressive strength and water-absorption tests. The results showed a 36% increase in compressive strength of cement mortar with the addition of bacterial cells. Treated cubes absorbed six times less water than control cubes as a result of microbial calcite deposition.

The main objective of the present investigation was to isolate more efficient bacteria for concrete crack healing and characterize them based on morphological, physiological and molecular characteristics, apply it for concrete crack remediation and also to compare with that of Bacillus pasteurii and Bacillus sphaericus.

In this paper crack remediation of concrete has been studied by isolating bacterial strain which has shown better crack healing ability than the reported bacterial species. The paper is divided into 8 sections, section 1 is the Introduction; section 2 is the materials and method used in the experimentation; section 3 is the characterization of bacteria and concrete casting; section 4 is the concrete crack remediation by bacterial mineralisation; section 5 is the discussion; section 6 is the conclusion; section 7 is the scope for future work and section 8 is the acknowledgement.

II. MATERIAL AND METHODS

In order to remediate concrete crack, it is necessary to either isolate calcium carbonate precipitating bacteria or procure such bacteria from bacterial banks. The bacteria obtained by above sources are to characterised for calcium carbonate precipitation by serious of tests as explained in the subsections.

2.1. Isolation of Calcium carbonate precipitating bacteria

Samples were collected from a concrete curing tank at Research laboratory of M.S. Ramaiah Institute of Technology, Bangalore using sterile container. The samples were suspended in a sterile saline solution (0.85%, NaCl), serially diluted and inoculated by pour plate technique on Precipitation Agar containing urea (20 g/l), $NaHCO_3$ (2.12 g/l), NH_4Cl (10 g/l), Nutrient broth (3 g/l), $CaCl_2.2H_2O$ (25 g/l). pH was maintained alkaline in the range of (7.5-8.0). All the inoculated plates were incubated at room temperature. Colonies were observed every 5 days with a stereo microscope at regular intervals until the crystal formation around the colonies. Such colonies were sub cultured and tested for urease activity, their morphology and gram reaction was observed. Presence and absence of endospore and the position of the endospore were also noted by staining the endospores.

2.2. Morphological biochemical studies of bacterial isolates

To characterize all the bacterial isolates conventional physiological and biochemical characterization tests were carried out as described in Bergey's Manual of Systematic Bacteriology [12].

2.2.1. Gram staining

Bacterial smear was prepared, on a glass slide and heat fixed. Smear was flooded with crystal violet for 60 sec. and then washed gently in water to remove excess crystal violet. Later it was flooded with Gram's iodine for 10 sec. and washed gently in water. Smear was decolourised with ethanol for 10 sec. and washed immediately in tap water. Counterstaining was done with safranin for 15 sec. and washed with water to remove the excessive stain. Finally samples were visualized under microscope at different magnification and observed for the Gram reaction and morphology of the bacterial cells.

2.2.2. Endospore staining

Bacterial smear was prepared on a clean glass slide and was heat fixed. The slide was placed over a water bath with some sort of porous paper over it, so that the slide is steamed. Malachite green (0.5%) is flooded over the slide, which can penetrate the tough walls of the endospores, staining them green. After 5 minutes, the slide is removed from the steam, and the paper towel is removed. After cooling, the slide is rinsed with water for thirty seconds. The slide is then counter stained with diluted safranin for 30 seconds, which stains most other micro organic bodies red or pink. The slide is then rinsed again, and blotted dry with bibulous paper. After drying, the smear was visualized under microscope at different magnification for the presence or absence of endospore, position and shape of endospore. Photo micrography was also carried out using labomed trinocular microscope CXL-PLUS.

2.2.3. Urease test

For preparation of Urea agar medium, following ingredients were used Peptone 1.0 g/lt , Sodium Chloride 5.0g/lt , Potassium di Hydrogen Phosphate 2.0g/lt, Agar 20.0g/lt and Distilled Water 1000ml. All the above ingredients were dissolved and the pH was adjusted to 6.8 and autoclaved at 121^0C for 15 minutes and cooled later 1g of glucose and 6ml of 0.2% phenol red was added and steamed for one hour, finally 20% aqueous 100ml of urea was added and sterilized by filtration and poured into the test tube and slants were prepared. The organisms isolated were streaked on the surface of the media and incubated at 37^0C and observed for the change of the colour of the media from yellow to pink.

2.2.4. Molecular characterisation of bacterial isolates

The pure cultures of bacterial isolate-1 were used for molecular identification. The extraction of DNA from the pure cultures was performed by Cetyl Tri methyl Ammonium Bromide (CTAB) method [13]. Agarose gel electrophoresis was performed in a horizontal submarine apparatus (Genei, Bangalore, India) as outlined by [14]. 10 µl of Gene Ruler 1kb DNA Ladder (Chro- mous Catalogue No. LAD03) was loaded into one well as a standard molecular weight marker. Electrophoresis was carried out at 60V for 40–60 min. The gel was viewed under UV transilluminator (352 nm). DNA band obtained was removed from the gel aseptically and Polymerized Chain Reaction (PCR) was performed in a Thermocycler (PTC-100TM programmable thermal controller, USA) to produce multi copies of a specified DNA using following PCR condition.

1. Initial Denaturation 94°C for 5 min.

2. Denaturation 94°C for 30 sec.

3. Annealing 55^0C for 30 sec

4. Extension 72^0C for 1 min.

5. Final extension 72^0C for 15 min.

6. Stop at 4°C for 1 h.

Universal primers for 16s rRNA specific primer 16s Forward Primer 5'-AGAGTRTGATCMTYGCTWAC-3', 16s Reverse Primer 5'-CGYTAMCTTWTTACGRCT-3' reverse primers were used. These primers were obtained from Chromous Biotech Pvt. Ltd. Bangalore, India. ITS region of rDNA was visualised by UV trans-illumination (352 nm) and the expected DNA band was excised from the gel using a sterile scalpel and placed into a 1.5 ml micro tube. This DNA was purified using gel extraction kit (Chromous Biotech Pvt. Ltd. Bangalore, India) according to the manufacturer's specifications. The purified PCR product was sequenced at Chromous Biotech Pvt. Ltd. Bangalore, India. Sequences were determined by the chain termination method using an ABI 3130 Genetic Analyser. Sequencing was done in the forward and reverse direction. The sequence was generated using data analysis software (Seq Analysis_ v 5.2).

2.2.5. Sequence data analysis

Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well-designed queries and alignments. Basic Local Alignment Search Tool (BLAST) provides a method for rapid searching of nucleotide and protein database. The rDNA gene sequence was used to carry out BLAST with the data base of NCBI gene bank.

2.2.6. Effect of pH on bacterial growth

The hydrogen ion concentration of an organism's environment has the maximum influence on bacterial growth. It limits the synthesis of bacterial enzymes responsible for synthesizing the new protoplasm. Each microorganism has its optimum pH. The nutrient broth of different pH ranging from 4 to 12 was prepared in a test tube and sterilized in an autoclave at 121^0C at 103.5k Pascal. One ml of bacterial suspension was inoculated into each tube and incubated at 37^0C in an incubator for 24hrs. The turbidity of each tube was measured at different intervals by using photo colorimeter at 760nm and control tube was used to calibrate the Optical Density (OD) to zero.

2.2.7. Calculation of generation time

The generation time for the different bacterial isolates was calculated by direct method, where the nutrient broth was prepared in a conical flask and sterilized. The different bacterial isolates were inoculated aseptically into different conical flask and un-inoculated broth was kept as control to set the colorimeter to zero. At an interval of every 30mins, OD was taken at 760nm till the OD values doubled. Generation time was calculated by taking the difference of time required for doubling the OD.

2.2.8. Urease assay

The urease activity was determined for all the bacterial isolates in Urease media by measuring the amount of ammonia released from urea according to the phenol-hypochlorite assay method [15]. Ammonium chloride (100µg/ml) was used as the standard. Bacterial isolates were grown in corresponding media and 1% of overnight grown cultures were re-inoculated into urease media and incubated at 37°C. After an interval of 24 hrs, the culture filtrate (250 µl) was added to a mixture containing 1 ml of 0.1 M Potassium Phosphate buffer (pH 8.0) and 2.5 ml of Urea (0.1 M). The mixture was incubated at 37°C for 5 min followed by addition of Phenol Nitroprusside and alkaline hypochlorite, 1 ml each and incubated at 37°C for 25 min. Optical density was measured at 760 nm. One unit of urease is defined as the amount of enzyme hydrolysing one µmole urea per min.

2.2.9. Calcium carbonate estimation

A loop of microbial cultures were inoculated into calcite precipitation media (Urea: 20g/lt. and Calcium chloride: 49g/lt.) in separate conical flasks of 250ml and incubated at 37^0C in an incubator and studied for amount of calcite precipitation at regular intervals. This analysis was done volumetrically by using a characteristic reaction of carbonate compounds, namely their reaction with acids. Calcium carbonate (limestone) is very insoluble in pure water but will readily dissolve in acid according to the reaction 4.

$$2HCl\,(aq.) + CaCO_3(s) \rightarrow Ca^{2+}\,(aq.) + CO_2\,(g.) + H_2O + 2Cl^-\,(aq.) \qquad (4)$$

The above reaction could not be used directly be used titrate the $CaCO_3$ as it is very slow, when the reaction is close to the endpoint. Instead the determination was achieved by adding an excess of acid to dissolve all of the $CaCO_3$ and then titrating the remaining HCl with NaOH solution to determine the amount of acid which has not reacted with the Calcium Carbonate. The difference between amounts of the acid (HCl) initially added and the amount left over after the reaction is equal to the amount used by the $CaCO_3$. The reaction used to determine the leftover acid is represented in Equation 5.

$$HCl\,(aq.) + NaOH\,(aq.) \rightarrow H_2O + Na^+\,(aq.) + Cl^-\,(aq.) \qquad (5)$$

2.2.10. Mass multiplication of the bacterial isolates

About 500ml of nutrient broth was prepared and sterilized aseptically. The pure culture of the different bacterial isolates were inoculated into different conical flasks and incubated at 37^0C for 3-4 days. The cell concentration was measured by direct microscopic method using haemocytometer and further used for application into the concrete specimens.

III. CONCRETE CRACK REMEDIATION

3.1. Preparation of concrete of grade M30 and crack formation

For the current study concrete of grade M30 was chosen. Indian Standards (IS) method of mix proportions was followed for the production of concrete. After the determination of slump, cubes of dimension 100 mm × 100 mm × 100 mm was prepared by casting fresh concrete into cube moulds in three layers. Each layer was compacted using vibrating table. After casting the specimen were de moulded after one day and cured in water. At the time of casting Aluminium foil of thickness 3 mm were used to induce artificial cracks, the foil were inserted into the wet concrete to a depth of 25mm.

3.2. Methods of treatment

3.2.1. Ponding method and injection method

The bacterial cultures which was subjected for mass multiplication in nutrient broth. They were harvested by centrifugation at 5,000 rpm for 15 minutes to obtain a pellet; the pellet was re suspended in saline solution and homogenised. The bacterial culture of $7x10^5$ cells/ml was placed in to the crack by injecting the bacterial injected into the crack which was ponded over the crack. The growth

medium was added at regular intervals without disturbing the already formed calcite layer by slowly injecting using a syringe.

3.2.2. SEM and XRD analysis of microbial remediated crack

The morphology and chemical constituents of bacteria and remediated crack was analysed with SEM and XRD respectively. Calcite layer formed by the bacterial isolates were completely dried at room temperature and then examined by SEM. (Figure. 8a) Samples were gold coated with a sputter coating Emitech K575 prior to examination. XRD-spectra were obtained using an X'Pert PRO diffractometer with a Cu anode (40 kV and 30 mA) and scanning from 3 to 60˚ 2 θ. Calcite layer was crushed and grinded using motor pestle before mounting on to a glass fibre filter using a Tubular Aerosol Suspension Chamber (TASC). The components of the sample were identified by comparing them with standards established by the International Centre for Diffraction data.

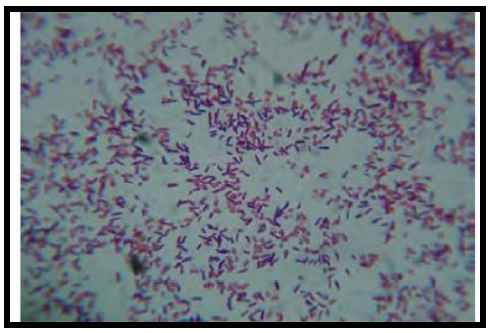
3.2.3. Isolation and identification of calcite precipitating bacteria

The inoculated and incubated plates were observed after 24-48 hours of incubation, colonies appeared on the media [Figure 1a] after 48 hours of incubation, with different colony morphology, all such colonies were named as Isolate-1, Isolate-2, Isolate-3, Isolate-4, Isolate-5 and Isolate-6, and sub cultured on a fresh Calcite precipitation media, and observed for crystal formation around the colony at a regular intervals of 5 days with the help of stereo microscope (Labomed). After around 7 days precipitate formation around were observed.

3.2.4. Gram staining

Gram staining was conducted to determine the Gram reaction and morphology of the isolates. In Gram-positive bacteria primary stain i.e., Crystal violet do not gets decolorized because of the presence of thick peptidoclycan in their cell and do not take up the counter stain safranin hence appear purple. Whereas Gram negative bacteria loses their primary stain on decolonisation with ethyl alcohol because of the presence of thin peptidoclycan and large amount of lipid content in the cell wall and take up counter stain safranin and appear pink. *Isolate-1, Isolate-3, Isolate-5, Isolate-6* were found to be Gram positive *Bacilli*. *Isolate-2, isolate-4* was found to be Gram positive *Cocci*. Photo micrography was also carried out using labomed trinocular microscope CXL-PLUS as shown in the figure 1b. All Gram positive Bacilli were sub cultured and used for further studies.

(a) Growth of bacterial colonies on Calcite precipitation media



(b) Gram positive rods under oil immersion.

Figure 1: Bacterial colonies and gram positive rods

3.2.5. Endospore staining

The *isolates 1,3,5,6* subjected to endospore staining and were visualized under microscope at different magnification for the presence or absence of endospore, position and shape of endospore. It was observed that *isolate-1, 3, 5,* and *6* produced endospores. Photo micrography was also carried out using labomed trinocular microscope CXL-PLUS. Endospore producing isolates were subjected to urease test.

3.3. Biochemical Technique

3.3.1. Urease Test

The *isolate 1*, *Bacillus pasteurii* and *Bacillus sphaericus* were studied for urease activity. The change of the colour of the media from yellow to pink indicates it is urease positive. The isolate-*1, 3, 6* were found to be urease positive and *isolate 5* was urease negative indicating it could not break down urea. All the 3 isolates were found to be Urease positive. [Figure 2]

Figure 2: Urease Test Showing Positive And Negative Reactions.

3.3.2. Molecular Characterisation

ITS sequence of *Isoate-1* was subjected to the BLAST programme to generate the significant alignment and the close matches to the query sequence. ITS sequence isolated from the pure culture of *Isolate-1* showed 99% similarity with *Bacillus flexus*, NCBI accession No.EF157300. The Phylogenetic position confirms that our isolate corresponds to *Bacillus flexus*.

3.3.3 Effect of pH on the growth of bacteria

Growth and survival of microorganisms are greatly influenced by the pH of an environment, the optimum pH required for the growth of all the 3 isolates were determined, *Bacillus flexus* was found to be high pH tolerant were the optimum pH required for its growth was found to be 8, however it even had the ability to grow at pH 11 and 12. Whereas *Bacillus pasteurii* and *Bacillus sphaericus* fails to grow above pH 9. [Figure 3]

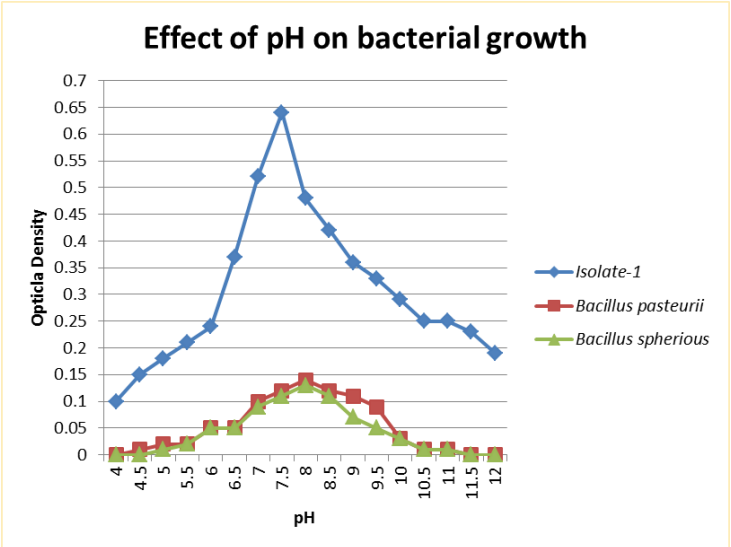


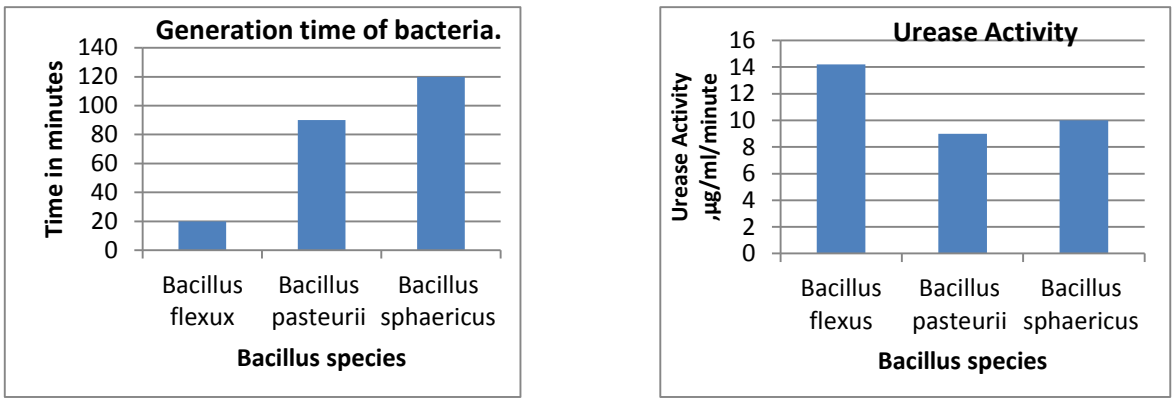
Figure 3: Effect Of pH On Bacterial Growth.

3.3.4 Calculation of generation time

Generation time is the time required for the microbial population to double under standard condition. The generation time of *Bacillus flexus* was found to be 20 minutes, whereas for *Bacillus pasteurii* it was 90 minutes and for *Bacillus sphaericus* it was 120 minutes as represented in graph. [Figure 4a].

3.3.5 Urease assay

The ability to precipitate Calcium carbonate (calcite) is directly related to the amount of urease produced as described earlier in process of calcite formation. So it is regarded as "cementing enzyme". Thus the ability of bacterial isolates to produce urease has been studied. The urease activity of *Bacillus flexus* was found to more when compare to that of *Bacillus sphaericus* and *Bacillus pasteurii* as represented in graph. [Figure 4b].



(a) (b)

Figure 4: Urease activity and generation time

3.3.6 Calcium carbonate estimation

Before microbial application into concrete specimens, the calcite precipitation ability by all bacterial species was studied invitro condition. Calcite production by bacterial isolates in 1000 ml of calcite precipitation media has been shown Table 1; Figure 5, which implies that *Bacillus flexus* has the ability of production of Calcium carbonate in large amount in faster rate when compared to that of other 2 species.

Table 1: Estimation of Calcium Carbonate

Bacteria species	Calcium carbonate gm / lt		
	Day 2	Day 4	Day 6
Bacillus flexus	2.2	5.8	6.6
Bacillus pasteurii	0.68	4.2	5.2
Bacillus sphaericus	0.56	3.9	4.7

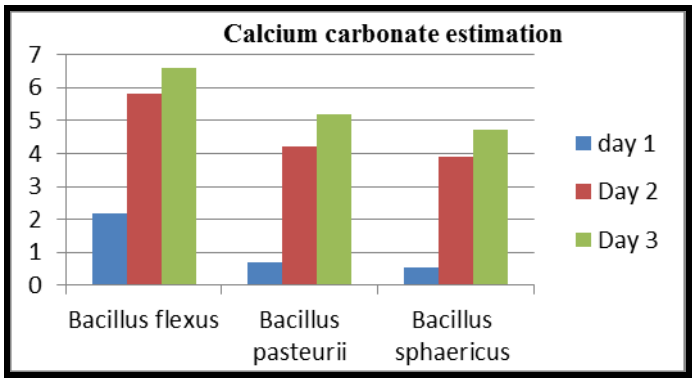
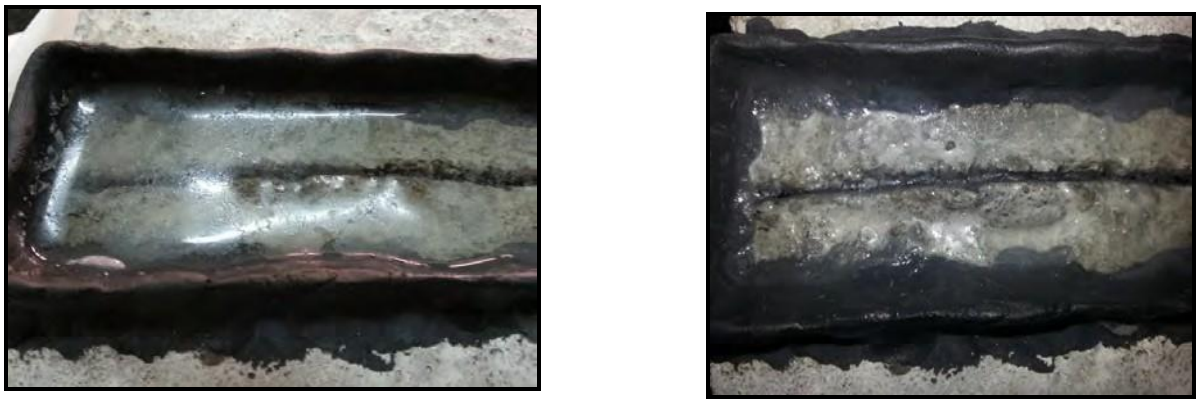


Figure 5: Estimation of Calcium Carbonate

IV. MICROBIALLY INDUCED CRACK REMEDIATION

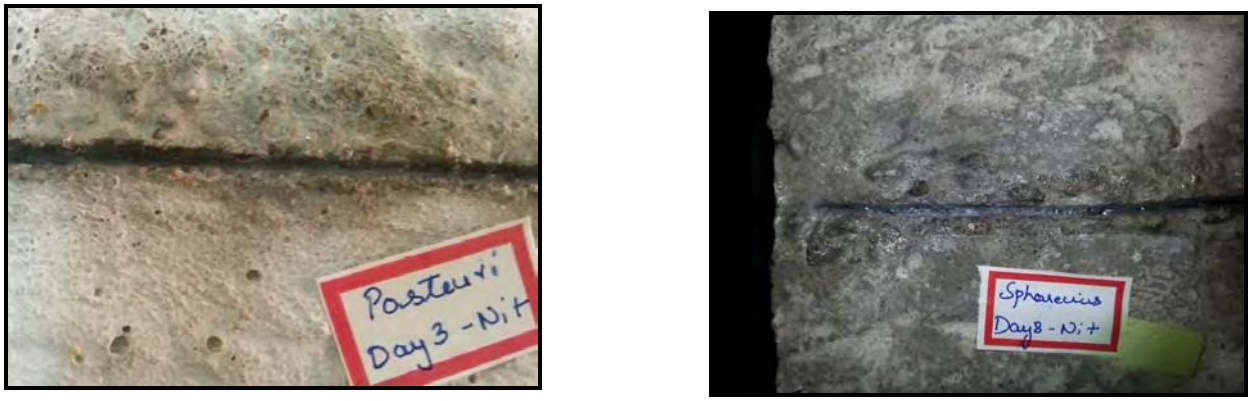
4.1 Ponding method / Injection method

The cured concrete blocks were taken out of curing tank 24hrs priors to treatment. A small raised edges around the crack was created using M-seal [Figure 6a] in order to provide sufficient nutrients for precipitation of calcite. Then after 24hrs the centrifuged bacterial cells of *Bacillus flexus* were injected into the crack, and then calcite precipitation media was flooded over the crack. The precipitation of calcite in visible amount started to appear after 3 days. The precipitate was not confined within the crack but observed all over the edges and surface of ponded area as the bacteria we freely moving in the media. So all the other species we not subjected to ponding method. *Bacillus pasteurii* started to show precipitate after 3 days in small quantities in both calcium chloride and calcium nitrate. *Bacillus flexus* started to show precipitate after 2 days in $CaCl_2$ and Calcium Nitrate. *Bacillus flexus* showed larger quantities of precipitate compared to others but precipitate was maximum in Calcium Chloride source com- pared to Calcium Nitrate and it showed better healing when compared to *Bacillus sphaericus* and *Bacillus pasteurii* [Figure 7] in calcium chloride and calcium nitrate as *Bacillus flexus* was found to be high pH tolerant when compared to that of other two species. However the healing of crack became slow because of the high pH in the concrete block.



(a) Ponding Method (b) Crack Healing By *Bacillus flexus*

Figure 6: Ponding and Crack Healing

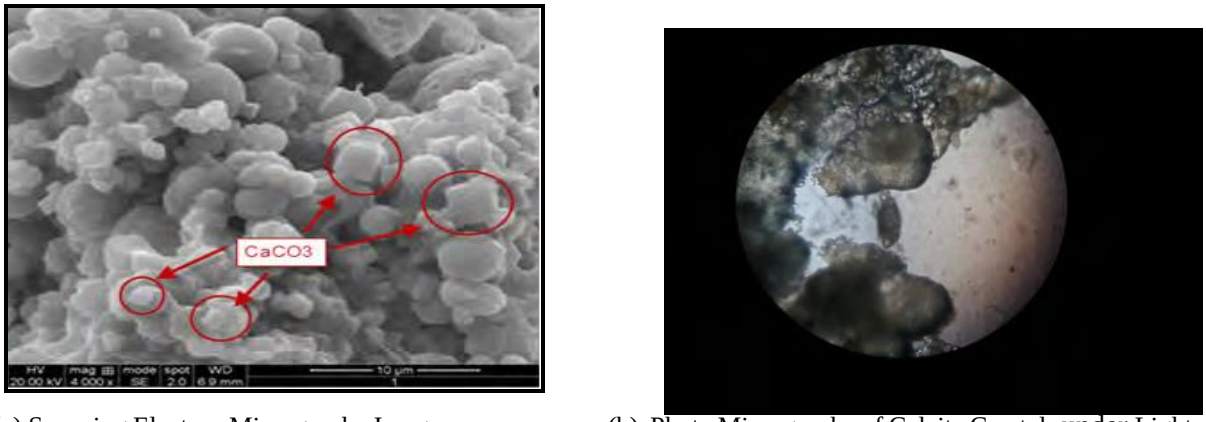


(a) Crack Healing by *Bacillus pasturii* (b) Crack Healing by *Bacillus sphaericus*

Figure 7: Crack Healing of *Bacillus pasturii and Bacillus sphaericus*

4.2 Scanning Electron Micrography (SEM)

SEM analysis on microbial samples is shown in Figure 8a, where distinct rhombohedral shaped (calcite) crystals embedded with round shaped bacterial spores can be found between and on the surface. Mineral constituents of the microbial samples were further characterized by X-Ray diffraction (XRD) analysis.



(a) Scanning Electron Micrography Image (b) Photo Micrography of Calcite Crystals under Light Microscope

Figure 8: SEM and photo micrography

4.3 X-Ray diffraction Analysis (XRD)

Results of XRD confirmed maximum number of calcite peaks. The most abundant mineral present was carbonate deposits were present as calcite crystals as was confirmed by XRD analyses results [Figure 9] were compared with Standard American Mineralogist Database amcsd code 0009873 confirming with SEM Results.

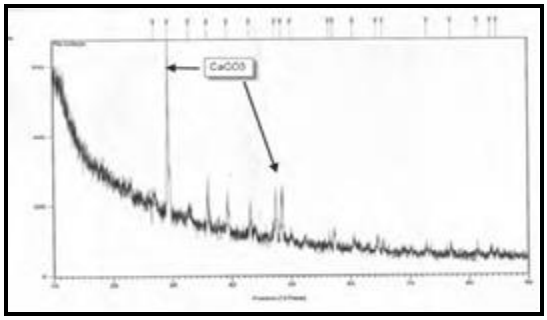


Figure 9: X-Ray diffraction Analysis Data

4.4 Photo micrography

Photo micrography of calcite crystals was also carried out using labomed trinocular microscope CXL-PLUS as shown in the figure 8b. This also confirmed calcite crystal formation due to bacterial activity.

V. DISCUSSION

Urease produced by bacteria is widely known to precipitate calcium carbonate, one of the main components of concrete, thus referred as microbial concrete enzyme. Typically, to remediate building materials urease needs to be active and stable in alkaline environment (pH 9–11) that also include high temperature [1]. Urease in general is not stable under these conditions and therefore, the emphasis has been on newer sources. Keeping these points, urease producing bacteria were isolated from sources such as concrete curing tank and alkaline soils. Isolation and screening of bacteria from these natural environments can be useful for obtaining bacterial strains with the potential of yielding urease enzymes. Also, these areas were selected to isolate indigenous bacteria which can sustain high alkalinity as the aim of the present work was to use these isolates in the remediation of building structures.

The isolated bacteria were screened qualitatively by gram staining, endospore staining and for urease test. All the isolated bacteria of the present study were identified as Bacillus genera and most of the calcifying bacteria belong to the Bacillus genera [16]. The urease media contains phenol red. The

urease producing bacteria utilizes urea present in the media and then degrades phenol red giving pink colour [17]. Based on the intensity of pink colour by naked eyes, thus three efficient urease producers were selected. Salt tolerance, growth temperature range, growth pH range, and extracellular products are important taxonomic criteria which were used to differentiate species in the genus Bacillus [12] The three isolates of the present work also showed the ability to tolerate a wide range of pH and presented ureolytic activity that lead to calcite precipitation, which provides the advantage of uses in various industrial processes. All the three selected isolates were able to grow well in nutrient medium containing urea and $CaCl_2$. *Bacillus flexus*, *Bacillus* sphaericus and *Bacillus pasteurii* was found to be alkaline tolerant, whereas in Bacillus flexus growth was observed above pH 9 up to pH 12.

The aim of this experimental study was to develop an alternate methodology towards the healing of crack for affected structures and in turn increase its lifespan and durability by treating with bacterial cells for their structural rehabilitation. *Bacillus flexus, Bacillus pasteurii* and *Bacillus sphaerius* was selected to study crack remediation. The crack in concrete block loaded with bacteria and calcite precipitation media started healing the cracks from day 3 and continued till 30 days. *Bacillus flexus* showed better healing than compared to *Bacillus sphaericus* and *Bacillus pasteurii* in calcium chloride and calcium nitrate, however there was no precipitate observed in calcium lactate media in any blocks. First, negatively charged functional groups on the bacterial cell walls attract Ca^{2+} to induce a local super saturation so that calcite nucleation takes place on the cell surfaces. The maximum amount of calcite was deposited in the upper layer followed by middle and lower layer. Calcite precipitation occurred predominantly in the areas close to the surface of crack in concrete block. It is mainly due to the fact that facultative anaerobic Bacillus cells grows at a higher rate in the presence of oxygen and consequently induces active precipitation of $CaCO_3$ around the surface area [1] Further precipitation in cracks stopped after 40 days due to very high pH environment deep inside the cracks.

Bacterial mediated calcite precipitation is confirmed from the results of present study. The following model for bacterial mediated calcite bio mineralization can be proposed.

VI. CONCLUSION

The microbial induced calcite precipitation reaction may cause lower amount of capillary pores and clogging of the pores, which reduces chloride ion transport in concrete. The use of bacterial cells has thus become a viable solution not only to some durability problems but also as an environmentally responsible course of action.

Finally, the present study indicates that *Bacillus flexus* can serve as the best option in MICP due to its various special characteristics compared with other species from earlier studies. MICP technique on further optimization in application can be used in remediation of building materials.

VII. FUTURE WORK

1. Investigation of production of bio bricks where instead of burning of moulded bricks to bind the earth, bio cementing is to be tried.
2. Crack filling and its behaviour in other building material like granite, brick and marble.

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REFERENCES

[1]. Stocks Fischer S, Galinat J K, Bang S S, (1999), "Microbiological precipitation of $CaCO_3$", *Soil Biology Biochemistry*, Vol. 31, pp. 1563-1571.
[2]. Meldrum F C (2003), "Calcium carbonate in biomineralisation and biomimetic chemistry", *International Materials Reviews*, Vol. 48, No. 3, pp. 187-224.
[3]. Gollapudi U K, Knutson C L, Bang S S, and Islam M R, (1995), "A new method for controlling leaching through permeable channels", *Chemosphere*, vol. 30, pp. 695-705.
[4]. R V Bang, Sookie S, Galinat Johnna k, Johnna K. Galinat, V Ramakrishnan, (2001), "Calcite

Precipitation Induced By Polyurethane Immobilized *Bacillus Pasteurii*," *Enzyme and Microbial Technology*, vol. 28, pp. 404–409.

[5]. De Muynck W, De Belie N, Verstraete W, (2010), "Microbial carbonate precipitation in construction materials: A review", *Ecol Engg*, Vol. 36, No. 2, pp. 118-136.

[6]. J L Day, A V Ramakrishnan, and S S Bang, (2003), "Microbiologically Induced Sealant For Concrete Crack Remediation," *Quality Assurance*, pp. 1–8.

[7]. Bachmeier K L, Williams A E, Warmington J R, Bang S S, (2002), "Urease activity in microbiologically-induced calcite precipitation." *J. Biotechnology*, Vol. 93, pp. 171-181.

[8]. Muynck De, and De Belie, (2007), " Improvement Of Concrete Durability With Aid Of Bacteria", *Microbial Ecology*, April, pp. 1-11.

[9]. S Al-thawadi and R. Cord-Ruwisch, (2012), "Calcium Carbonate Crystals Formation by Ureolytic Bacteria Isolated from Australian Soil and Sludge," *Journal of Advanced Science and Engineering Research 2*, vol. 2, pp. 12–26.

[10]. K D Arunachalam, K S Sathyanarayanan, B S Darshan, and R B Raja, [10], "Studies on the characterisation of Biosealant properties of Bacillus sphaericus," *International Journal of Engineering Science and Technology*, vol. 2, no. 3, pp. 270–277.

[11]. Varenyam Achal, (2010), "Microbial Remediation of Defects in Building Materials and Structures." Biotechnology Dept., Thapar University, Patiala, Punjab (India).

[12]. Holt, John G, (2001), "Bergey's manual of systematic bacteriology", Second Ed, Springer.

[13]. Ausubel F M, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A and Struhl K, (1994), "Current Protocols in Molecular Biology", John Wiley & Sons Inc.

[14]. Joe Sambrook, David W Russel, (2001), "Molecular Cloning: A Laboratory Manual", Third Edition, Cold Spring Harbour Laboratory Press.

[15]. Natarajan K R, (1995), "Kinetic study of enzyme urease from Dolichos biflorus", *Journal of Chemical Education*. Vol.72, pp. 556-557.

[16]. Ercole C, Cacchio P, Botta A L, Centi V, Lepidi A, (2007), "Bacterially induced mineralization of calcium carbonate: the role of exopolysaccharides and capsular polysaccharides", *Microscopy and Microanalysis*, Vol. 13, pp. 42-50.

[17]. Andrews W H, June G A, Sherrod P S, Hammack T S, Amaguana R M, (1995), "FDA Bacteriological analytical manual", 8th ed. *AOAC International*, Gaithersburg, MD.

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