ROLE OF CALCIFYING BACTERIA ON DURABILITY PROPERTIES OF CARBONATED CEMENT MORTAR

A

DISSERTATION

Submitted in the partial fulfillment of the requirements for the Award of the Degree of

Masters of Science

(Biotechnology)

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CERTIFICATE

Certified that the thesis "**Role of calcifying bacteria on the durability properties of carbonated cement mortar**" which is submitted by Ms. Gurvinder Kaur, in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology in the Department of Biotechnology, Thapar University, Patiala is record of the candidates's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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I hereby declare that the work presented in this thesis entitled as "**Role of calcifying bacteria on the durability properties of carbonated cement mortar**" submitted by me for the requirement of award of degree in Masters of Science in Biotechnology in the Department of Biotechnology, Thapar University, Patiala is true and original record of my own independent and original research work carried out under the supervision of Dr. M Sudhakara Reddy, Professor Dept of Biotechnology, Thapar University. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

This is an authentic record of my own work during the period of six months from January 2014-July 2014.

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I dedicate this thesis to my parents who have always supported me, no matter what path I chose in my career, and to my maternal grand parents whose support and reassurance throughout this process was a great help. Thanks for all your love and support.

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Abstract

Current concern about degradation of concrete, and ageing infrastructure, impose an unprecedented demand on construction industry, which requires sustainable technologies to meet society's need in a cost effective manner. Microbial Concrete which is perfect blend between biology and engineering presents an highly efficient method to remediate, building and construction materials. Different research groups have proposed the ureolytic microbial calcium carbonate precipitation by different calcifying bacteria. Although urea hydrolysis presents a straight forward model for studying microbial $CaCO_3$ precipitation but it also possess certain limitations. The large amount of ammonia released with large amount of nitric acid production can cause detrimental effect to the environment and produces pungent odours. Therefore, in the present study our aim was to develop an effective carbonation process in presence of microbes, based on Microbially induced calcium carbonate precipitation (MICCP) to enhance the durability properties of building materials and structures. The effect of initial carbonation curing of 7 days was examined on ordinary Portland cement (OPC) mortars. A bacterium *Bacillus megaterium* have been successfully used to optimize different parameters (pH ,Carbonic Anhydrase activity, $CaCO_3$ precipitation) in presence of pure CO_2, in *in-vitro* conditions, and to improve the durability properties of cement mortars *ex-vitro*. In *in-vitro* conditions, a particular conc. of CO_2 i.e. 0.112g/L was optimized to induce maximum carbonate precipitation. For field study, bacterial mortar cubes of dimension 70.6 x 70.6 x 70.6 were subjected to bacterial culture and carbonation curing to see the enhancement in durability properties. The results indicated that microbial process involving ureolytic pathway increased the compressive strength by almost 36%, and Microbial process involving Carbonation curing increased compressive strength by 38% with reference to their respective controls. Bacterial deposition of a layer of calcite on the surface of specimens, and carbonate precipitates formed between cement and sand particles resulted in almost 3 times decrease in water absorption and permeability compared to control cubes without bacteria. It shows that bacterial carbonation can yield significant results in enhancing the durability of construction materials without the use of urea.

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Abbreviations

Abbreviation	Word (s)
MICCP	Microbially induced Calcium carbonate precipitation
UA	Urease
CA	Carbonic anhydrase
CO_2	Carbon dioxide
OPC	Ordinary Portland Cement
DIC	Dissolved inorganic carbon
RCA	Recycled concrete aggregates
NBU	Nutrient Broth Urea media
SEM	Scanning electron micrograph
XRD	X-ray Diffraction
EDX	Electron dispersive X-ray diffraction
ANNOVA	Analysis of Variance
CHS	Calcium silicate hydrate

Chapter 1

Introduction

Concrete, widely used construction material is considered as indestructible because of their longer service life but due to variety of reasons including material limitations, design gaps and construction practices, exposure conditions it can be destroyed (Samudre *et al.,* 2014). Continuous exposure of hard weathering, dissolution of mineral matrix and ingress of aggressive gases leads to an increase of porosity and consequently increase in mechanical damage (Achal *et al.,* 2011). Current concern about the degradation of concrete and the economic impact of its repair and maintenance, have drawn attention to adopt methods to slow down its deterioration or to diminate concrete degradation (Hewlett., 1990).

Undoubtedly, broad range of products are available for the protection of concrete surfaces. Like, impregnation of cracks with epoxy based fillers, latex binding agents such as acrylic, polyvinyl acetate, organic and inorganic coatings etc. But these traditional methods are associated with several problems in vogue like different thermal expansion, weak bonding, degradation with time, need of constant maintenance and environmental pollution. Both water repellants and consolidants have been applied to protect stone but due to incompatibility with the stone both have often been reported to accelerate stone decay (Delgado Rodriguez., 2011). Secondly these organic treatments results in the formation of harmful Biofilms. And due to usage of large quantities of organic solvents while manufacturing, they contribute to pollution.

All above drawbacks and ageing infrastructure impose an unprecedented demand on infrastructure, which requires sustainable technologies to meet society's need in a cost effective and low impact manner (Dejong *et al.,* 2011). MICCP provides an alternative method to potentially, uniformly improve the strength and enhancement in the durability characteristics of construction materials. Microbes as they are eco- friendly, self healing, energy efficient can be used to enhance the durability of concrete and may bring new approaches in the concrete/construction industry. This unique way of concrete design which crossbreds between biology and engineering study is called microbial concrete (Wang *et al.,* 2010). Microbial concrete makes the use of calcifying bacteria isolated from calcareous sites. Many biological reactions can result in the production of carbonate or carbonate species. But because of its simplicity and the lack of an excess proton production, the most commonly studied

system of applied MICCP to date is urea hydrolysis via the enzyme urease, in a calcium- rich environment.

$$CO(NH_2)_2 + 2H_2O \longrightarrow 2NH_4+ + CO_3^{2-}$$

$$Ca^{2+} + CO_3^{2-} \longrightarrow CaCO_3$$

It is true that Microbially induced calcium carbonate precipitation via urea hydrolyses has been most widely used and offers unlimited advantages in field of carbonate precipitation and it gives better results in less time but due to certain factors it can be harmful for the environment. Certain limitations of this process include

- large amount of NH_3 is released in the environment causing pungent odours
- Nitric acid production.
- In pilot applications, highly concentrated NH_4Cl is produced as a byproduct.

An alternative system to produce calcium carbonate precipitates with the aid of bacteria, of replacing urea with pure CO_2 can be thought of. MICCP via urea hydrolysis as mentioned above possess certain limitations. In order to overcome these problems and to utilize living systems in calcium carbonate precipitation and concrete durability enhancement, an alternative called carbonation can be used. Carbonation can be defined as the direct influx of CO_2 into the bacterial medium. Addition of CO_2 in the presence of calcium chloride induces the formation of $CaCO_3$ crystals by the following reaction.

$$CO_2 + CaCl_2 + H_2O \longrightarrow CaCO_3 + HCl$$

The alternate curing method for carbonate production can be carbonation curing which uses high purity carbon dioxide (99.5% of CO_2) for accelerated hydration and durability improvement. In construction industry carbonation of concrete is traditionally defined as the chemical reaction between atmospheric carbon dioxide and products of cement hydration particularly $Ca(OH)_2$, according to this reaction.

$$Ca(OH)_2 + CO_2 \longrightarrow CaCO_3 + H_2O$$

Other hydration products like calcium silicate hydrate present in concrete also react with CO_2 and appears to be converted to $CaCO_3$. Sufficient amount of $Ca(OH)_2$ is present in Ordinary Portland cement (OPC) hydrated matrix to buffer the low pH due to formation of carbonic acid. Therefore carbonation of calcium silicate hydrate (CSH) would have only subtle effects on the hydrated cement matrix (Borges *et al*, 2010). However carbonation curing has never been adopted in large scale production. This was due to the reason that flue gas (14% CO_2) carbonation was not effective in hydration acceleration and pure gas carbonation was expensive. But the later situation may change in near future. Large quantities of purity, low cost

CO_2 could soon be available as, regulations requiring reductions in CO_2 emissions are developed. In this case, pure gas carbonation can simultaneously accelerate strength, stabilize the dimension, and enhance the durability (Hasan *et al.,* 2014). By reducing the hydroxyl ion and precipitating calcium carbonate on the surface layer, carbonation curing could improve the concrete resistance to sulfate attack (Rostami *et al.,* 2011). Since carbonation is CO_2 uptake process (Shao *et al*., 2006), recovered cement kiln carbon dioxide can be recycled into concrete products to make contribution to carbon emission reduction.

CO_2 collected from cement kiln can be beneficially utilized in precast production to reduce the carbon emission, accelerate early strength and improve durability of the products. Over all the carbonation process can decease the porosity of the matrix, thus strengthens the weak surface and lower the water absorption of the porous cement mortar. (Lange *et al.*, 1997)

Currently strategies on CO_2 mitigation are focused on removal, recovery and disposal of CO_2 at the control source. Utilization of CO_2 recovered from stack gases has been explored for urea production and enchanced oil recovery, mineral trapping etc.

Objectives

- Optimization of CO_2 flux for calcium carbonate precipitation
- Evaluation of durability properties on bacterial treated cement mortar
- Influence of bacteria on durability properties of carbonated cement mortar

CHAPTER 2

Review of Literature

2.1 Microbially Induced Calcium carbonate precipitation

Microbially induced calcium carbonate precipitation (MIICP) has experienced an increased level of interest in recent years, for applications such as bioremediation (Ferris 2003; Fugita *et al.,* 2001), waste water treatment (Hammes *et al*., 2003), strengthening of concrete (Ramachandran *et al.,* 2000) and selective plugging for enhanced oil recovery (Ferris & Setehmeir., 1992; Gollapudi *et al*., 1995; Nemati & Voordouw., 2005).

Reseachers have proposed the precipitation of carbonates through biofilms, extracellular polymeric substances, photosynthetis, ammonification, denitrification, sulfate reduction, anaerobic sulfide oxidation and hydrolysis of urea. (Bachmeier *et al.,* 2002; Castanier *et al.,* 2000).Subsurface bacteria can induce calcium carbonate precipitation by several bio-geochemical processes such as urea hydrolysis, nitrate reduction, sulfate reduction. (Castanier *et al*., 1999, 2000; Riding., 2000). Subsurface bacteria can promote calcium carbonate precipitation by increasing the subsurface alkalinity (Kohnhauser 2007). The most commonly used system in MICCP is urea hydrolysis via the enzyme urease in a calcium rich environment. The hydrolysis if urea presents several advantages over the other carbonate generating pathways, as it can be easily controlled and it has the potential to produce high amounts of carbonate with a short period of time. Calcium carbonate precipitation is mainly governed by four key factors (Hammes and Verstraete.,2002).

- The calcium concentration
- Dissolved inorganic carbon (DIC) concentration
- pH
- Nucleation sites on bacteria

2.2 Enzymes Involved in MICCP

2.2.1 UREASE (UA)

Most microorganisms are ureolytic in nature. Urease UA; EC (3.5.15) is a nickel containing metalloenzyme found in a wide range of microorganisms and plants. As a clinical perspective this enzyme can indicate increased virulence properties in pathogenic bacteria (Collins & Orazio., 1993; Provorov & Vorobyov., 2000) and as a general nitrogen volatisation phenomenon

in agricultural soils (Pettit *et al.,* 1976). The role of urease in MICCP is well documented by researchers (Stocks- Fischer *et al*., 1999; Ramakrishnan *et al*., 2007; Paassen *et al*., 2009). Bacterial urease catalyzes the hydrolysis of urea to CO_2 and ammonia resulting in increase of pH and carbonate concentration in the bacterial environment.

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

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3$$

These products equilibrate in water to form bicarbonate, 1 mol of ammonium and hydroxide ions which give rise to pH increase

$$H_2CO_3 \longrightarrow 2H^+ + 2CO_3^{2-}$$

$$NH_3 + H_2O \longrightarrow NH_4^- + OH^-$$

$$Ca^{2+} + CO_3^{2-} \longrightarrow CaCO_3 \ (Ksp = 3.8 \times 10^{-9}$$

Bacteria has been ascribed to their ability to create an alkaline environment through various physiological activities (Figure 2.1).

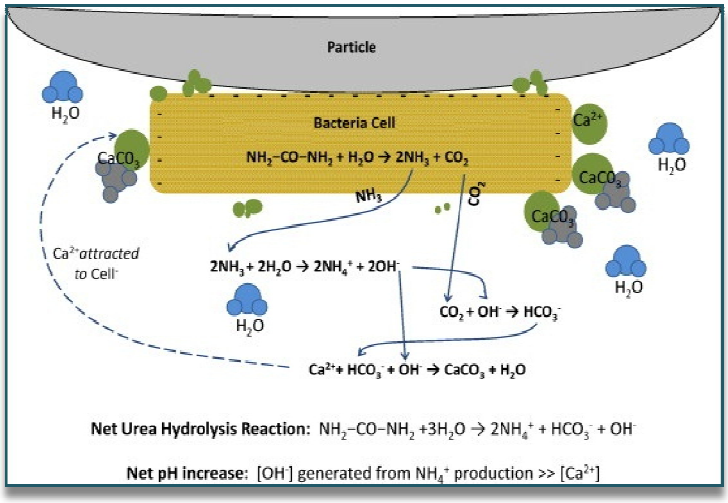


Figure 2.1 Bacteria serving as nucleation site for $CaCO_3$ precipitation in the sand particles (Source: DeJong *et al.,* 2010)

Due to the presence of several negatively charged groups on bacterial surfaces, positively charged metal ions could be bound on them, favoring hetrogenous nucleation (Douglas & Beveridge., 1998; Baurerlein., 2003).

Figure 2.2 shows that commonly, carbonate precipitates develop on the external surface of bacterial cells by successful stratification (Castainier *et al*., 1999) and bacteria can be embedded in the growing crystals. (Rivadeeyrya *et al*., 1998; Castainier *et al*., 1999).

Possible biochemical reactions in urea-$Cacl_2$ medium to precipitate $CaCO_3$ at the cell surface can be summarized as follows:

$$Ca^{2+} + cell \longrightarrow cell\text{-}Ca^{2+}$$

$$Cl^{-} + HCO_3^{-} + NH_3 \longrightarrow NH_4Cl + CO_3^{2}\text{-}$$

$$Cell\text{-}Ca^{2+} + CO_3^{2}\text{-} \longrightarrow Cell\text{-}CaCO_3$$

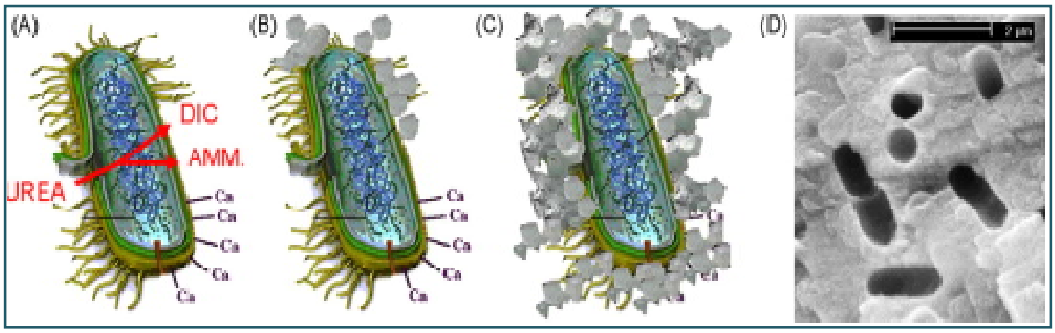


Figure 2.2 Simplified representation of the events occurring during the microbially induced carbonate precipitation. (A). Presence of calcium ions results in local super saturation and heterogeneous precipitation of calcium carbonate on the bacterial cell wall (B). Whole cell becomes encapsulated (C). Limiting nutrient transfer, resulting in cell death. Image (D) shows the imprints of bacterial cells involved in carbonate precipitation. (Source: Hammes & Verstraete., 2002).

2.2.2 CARBONIC ANHYDRASE (CA)

Another enzyme that facilitates the interconversion of CO_2 and HCO_3^{2-} and plays a role in calcium carbonate precipitation is carbonic anhydrase EC (4.2.1.1). Both UA and CA seems to play a synergistic role in enzyme catalysed reactions in calcification (Botre & Botre., 1989) In case of bacterial system, Carbonic anhydrase activity was first reported in *Neisseria sicca* by Veitch and Blankenship (1963). Recently, CA from *Bacillus pumilis*, *Citrobacter Freundii*, *Bacillus mucilaginous, Bacillus megaterium, Aeromonas caviae, Pseudomonas fragii* (Sharma *et al.,* 2009) has also been reported. This enzyme can also induce the process of biomineralization or $CaCO_3$ precipitation. The active site of most Carbonic anhydrase contains a zinc ion, and hence classified as metalloenzymes. This enzyme has been ubiquitously distributed in organisms

(Smith & Ferry., 2000). This enzyme catalyzes the interconversion of CO_2 and H_2O to bicarbonate and protons.

This enzyme employes a 2 step mechanism:

- Firstly there is a nucleophilic attack of a Zn bound hydroxide ion on CO_2
- Secondly regeneration of active site by ionization of Zn bound H_2O molecule and removal of proton.

CA catalyzes the hydration reaction in CO_2 sequestration and therefore has been proved as one of the most potential biological catalyst for this reaction and aides in large amount of CO_2 sequestration.

$$CO_2 + H_2O \longrightarrow H_2CO_3 \qquad HCO_3^- + H^+$$

This prokaryotic enzyme has also been shown to function in cyanate degradation and the survival of intracellular pathogens within their host (Smith & Ferry., 2000).

The biomimetic approach for carbon dioxide capture (figure 2.3) using microorganisms capable of fixing CO_2 through metabolic pathways or via use of an enzyme such as carbonic anhydarse has been widely used these days. CA offers advantages over other methods, due to specificity for CO_2 and its eco- compatibility.

Botre & Botre., (1989) reported that in enzyme catalysed reactions, increase in the rate of removal of CO_2 from solution facilitated by CA increases the rate of production of NH_3 consequent from urea dissociation. Achal & Pan (2011) recently found that along with Urease, CA might also be involved in $CaCO_3$ precipitation. Li *et al.,* (2011) hypothesized following reactions involving UA and CA that might be taking place during $CaCO_3$ precipitation:

$CO_2 (g) \longrightarrow CO_2 (aq)$ Dissolution

$CO_2 (aq) + H_2O \longrightarrow H_2CO_3$ Hydration

$H_2CO_3 \longrightarrow H^+ + HCO_3^-$ Dissociation

$HCO_3^- \longrightarrow H+ + CO_3 2-$ Carbonate formation

$Ca^{2+} + CO_3^{2-} \longrightarrow CaCO_3$ Precipitation

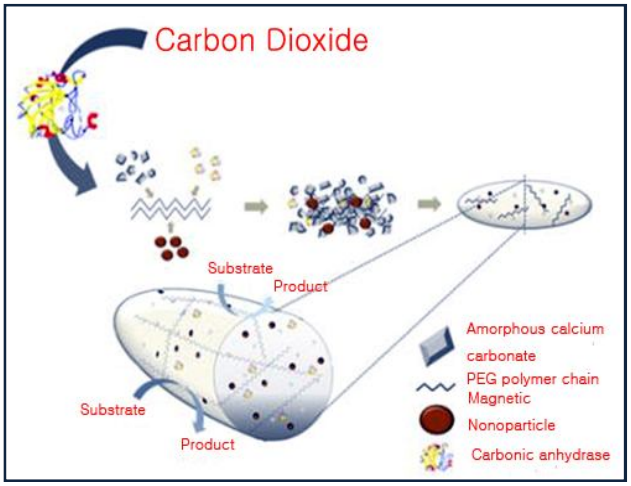


Figure 1.3: Carbon dioxide sequestration by bacterial Carbonic anhydrase (source: Celera carbonate technology)

The sequestration of CO_2 into $CaCO_3$ by bacterial CA has paved way for application of this enzyme in bio-mineralization of bacterial carbonates.

2.3 Microbial carbonates in construction materials

Microbial metabolic activities lead to production of relatively insoluble organic and inorganic compounds intra- and/or extracellularly, which remain in the environment for a long time even after cell death. Some microbes produce variety of polymers and glycocalyx outside the cell wall (Lappin-Scott *et al*., 1988; MacLeod *et al*., 1988), while some accumulate inorganic compounds such as phosphorites, carbonates, silicates, iron, and manganese oxides in cytoplasm (Beveridge *et al*., 1983; Ghiorse, 1984; Knoll, 1985; Ruiz *et al*., 1988; Rivadeneya *et al*., 1991). The potential of MICCP technology in restoration of cement mortar cubes, sand consolidation and limestone monument repair, reduction of water and chloride ion permeability in concrete via urea hydrolysis has been investigated by many researchers (Stocks-Fischer *et al*., 1999; Bang *et al*., 2001; Dick *et al*., 2006; Ramakrishnan 2007; De Muynck *et al*., 2008a,b; Sarda *et al*., 2009; Achal *et al* ., 2009a,b; 2011a,b; Chu *et al*., 2011).

When concrete is mixed with bacteria, the bacteria go into a dormant state. When any cracks or minor damage occurs to concrete, it provides space for water or air entry within concrete and then spores of bacteria initiate calcite precipitation process. Bacterial deposits increase the impermeability of both water and other aggressive substances. Microbial addition will provide an alternative and high quality concrete sealant that is cost effective and environmentally safe (Achal *et al.*, 2010).

2.3.1 Microbial Calcification in soil cement blocks and ground improvement

Formation of coherent calcite by the calcifying bacteria *Bacillus megaterium* (SS3) encouraged its use as microbial calcite for applications in low energy soil cement blocks (Dhami *et al.,* 2013). Mitigation strategies such as various methods of compaction or ground improvement methods such as jet grouting or soil mixing are not suitable or require high amount of energy, high costs and materials with significant impact on the environment (Passen *et al.,* 2010).

MICCP by urea hydrolysis has shown promising role for ground improvement. Recent research initiatives (whiffin *et al.,* 2005; Dejong ., 2006) have shown that calcite crystals form cohesive "bridges" between existing sand grains, increasing strength and stiffness of sand with limited decrease in permeability. Recently the techniques which aim at changing soil properties on demand by stimulating natural (bio-chemical) processes *in-situ* has been found and called as bio-grouting i.e., *in-situ* soil strengthening technique, involving MICCP (Meurs *et al.,* 2006). In this case reagents and catalysts are injected and transported to the location where strenghthening is required. Improvement in strength and sand columns upon bacterial carbonates was also reported. Kantaz *et al.,* (1992) reported that the sand consolidation by *Bacillus pasteurii* reduced porosity by upto 50% and permeability by 90% in the areas where cementation took place. Dhami *et al.,* 2013 investigated the effect of carbonate crystals on the surface of bacterial sand columns. It was observed that there was 23% decrease in waster absorption compared to control sand column.

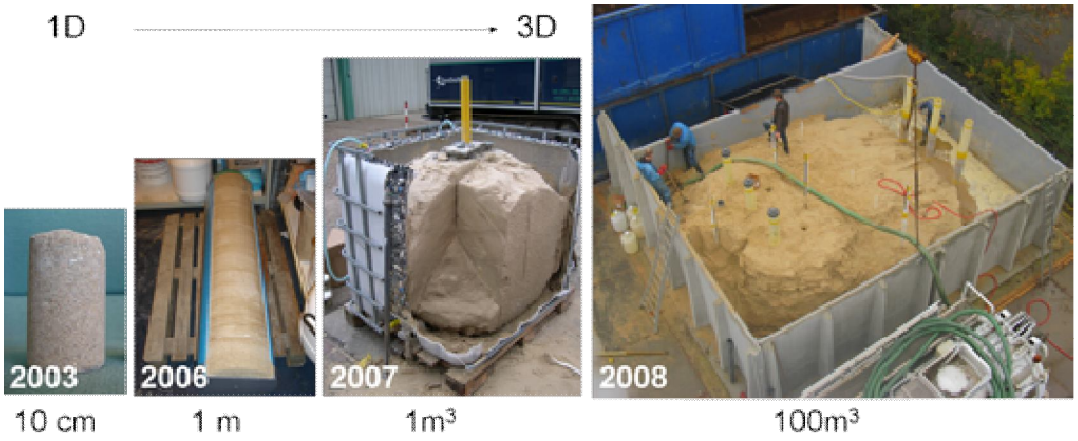


Figure 2.4 Biogrout process subjected to experimental investigation between 2003 and 2008. The volume of sand being cemented was steadily increased during this period. (Source: Deltares)

2.3.2 Microbial calcite in cementious materials

Compressive strength is one of the most important characteristic of concrete durability. It is considered as an index to assess the quality of concrete. Ramachandran *et al.,* (2001) observed the increase in compressive strength of cement mortar at 7 & 28 days using various conc. of *Bacillus pasteurii.* Ghosh *et al.,* (2005) studied the positive potential of Shewanella on compressive strength of mortar specimens. They also concluded that choice of microorganism plays the prime role in improvement of strength characteristics. Achal *et al.,* (2009a) treated mortar cubes with *Sporosarcina pasteurii* and observed 17% improvement in compressive strength. Achal *et al.,* (2011) treated mortar cubes with Bacillus sp. CT-5 and reported nearly six times less absorption of water as compared to untreated specimens. Awvolusi *et al.,* (2013) investigated the effect of *Bacillus subtilis* on the compressive strength of microbial laterized concrete, they observed that the compressive strength of concrete showed a significant increase of 20% and 41% for concrete cured in water and NB respectively at 28 days comparison with control concrete.

Permeability is another important characteristics of concrete that affects its durability. De Muynck *et al.,* (2008b) studied the effect of biodeposition of calcite on permeability characteristics of mortar by *B. sphaericus.* The presence of biomass contributed to a large extent in the overall decrease of the gas permeability. Significant differences in carbonation depth between treated and untreated specimens were noticeable after 2 weeks of accelerated carbonation in treated mortar specimens. Achal *et al.,* (2011a) reported the due decrease in water permeability of bioremediated cement mortar cubes treated by *Sporosarcina pasteurii.* The lower permeability of the bioremediated cubes compared with that of control cubes was probably due to a denser interfacial zone formed because of calcite precipitation between the aggregate and the concrete matrix. Fly ash acts as a partial replacement material for both Portland cement and fine aggregate, it also helps to increase impermeability and durability properties when mixed with bacteria (Achal *et al.,* 2011). Qian *et al.,* (2010a) also reported that compressive strength of treated specimens could be restored to 84%.

2.3.3 Microbial calcite in crack remediation

Biomineralised calcium carbonate has proved its efficacy in the case of sealing the cracks in concrete structures. Microbially enhanced crack remediation has been reported by Bang and Ramakrishnan (2001) where *Bacillus pasteurii* was used to induce calcium carbonate precipitation. Bang *et al.,* 2001 encapsulated ureolytic bacterial cells in polyurethanes (PU) and reported positive potential of microbially enhanced crack remediation by PU immobilized bacterial cells. Recently Achal *et al.,* (2013) reported the positive potential of bacterial calcite in crack remediationh by *Bacillus* sp.CT-5

2.4 MICCP via Carbonation

Carbonation is widely recognized as a significant cause of corrosion of reinforcement in concrete. The basic mechanism whereby atmospheric carbon dioxide reacts with components of the hydrated cement and destroys alkanity is well understood (Illston., 1994). The chemistry of pore solution is not however, the only property of concrete which affects reinforcement corrosion. Various transport properties have been shown to have a significant effect on the permeability of concrete materials as they control the supply of aggressive species, such as chlorides, to the site of deterioration.

Carbonation alone is commonly done in concrete industry to enhance the durability properties, and has both negative and positive aspects. Glass *et al.,* 1991 pointed out that presence of even a small amount of chloride in carbonated concrete enhances the corrosion rate resulted from carbonation of concrete. Carbonation reduces pH value and destroys the passive film around the steel, but it seems to densify the concrete surface and reduce chloride ion permeability, reduce surface porosity and hence sorptivity in concrete.

Many authors described quantitatively the effect of carbonation on the permeability and pore volume of typical concrete mixes. Samples of two concrete mixes with different water to cement ratios were prepared with both wet and dry curing and exposed in a carbonation chamber for upto 140 days. Results show that carbonation reduces the permeability and pore volume of concrete mixes and air cured specimens after extended exposure to carbonation, showed a higher increase in the impermeability index compared to water cured ones made from the same mix.

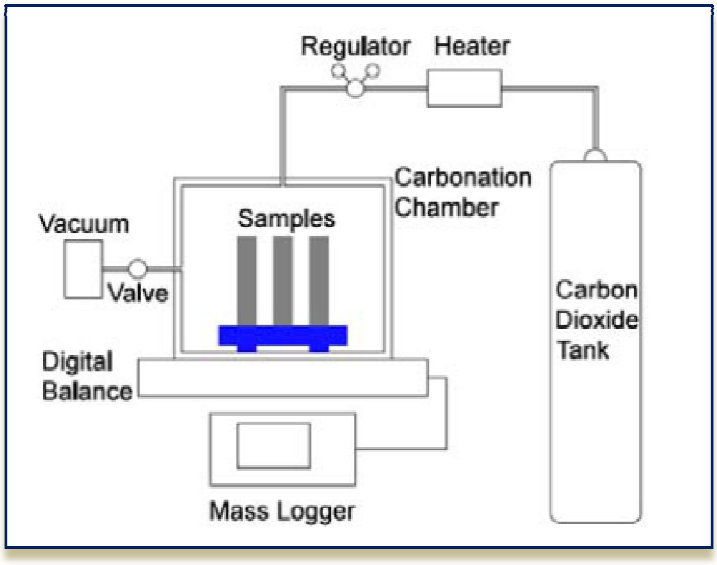


Figure 2.5 concrete carbonation chamber (source: Stienour, 1995)

At full carbonation, theoretical maximum of CO_2 uptake could reach to 50% by the mass of cement (Stienour., 1959). Concrete slab samples after initial curing were placed in a sealed chamber in Fig 2.4 which was then vacuumed to about 0.7 bars and filled with carbon dioxide gas to a pressure of 1 bar. (Shao *et al.,* 2006).

Carbonation leads to a reduction in porosity of the exposed concrete surface as the volume of the reaction product ($CaCO_3$) exceeds that of the original reactants (Parrott., 1987).

Dewaele *et al.,* 1991, examined the effect of carbonation on permeability and porosity of cement grout, but due to problems in the test set up only small carbonation depths were achieved. After 140 days of exposure to CO_2, Carbonation leads to approximately 37% and 4% increase in impermeability index for mix M1 (low strength) and M2 mix (high strength) respectively i.e. in low strength mix, the effect of carbonation was more pronounced. This may be explained by the fact, that the carbonation depths for M1 were more than four times those for M2.

Properties of Recycled concrete aggregates (RCA) can also be enhanced by CO_2 curing as a retreating method for further structural concrete production. (Baojian *et a*l., 2013). This study shows that CO_2 curing process can densify the mortar adhered on the RCA and there was significant reduction in water absorption and porosity of the RCA. Concrete is well known to be reactive with CO2. When this carbonation reaction occurs in freshly cast cement and concrete, this process has been shown to offer mechanical and durability properties (Young *et al*., 1974).

CO_2 curing process can replace steam to reduce embodied energy in concrete products, utilize sufficient amount of carbon dioxide in the vicinity of CO_2 sources. (Shao *et al.,* 2006). When measuring the compressive strength, in comparison to hydration reference of 1.9 MPa, carbonation strength reached 5.6MPa, and steam strength 6.3 MPa.

Carbonation could have both positive and negative effects on concrete durability. It could densify the concrete and reduce chloride ion permeability and on other hand reduces pH and destroys the passive film around steel and accelerating uniform corrosion.

2.5 Polymorphism of carbonate crystals

Biomineralization of calcium carbonate results in the production of different phases of carbonate as anhydrous polymorphs: calcite, aragonite and vaterite or two hydrated-crystalline phases, monohydrocalcite ($CaCO_3.H_2O$) and ikaite ($CaCO_3.6H_2O$) (Rodriguez Navarro *et al*., 2012; Dhami *et al*., 2013b) (Fig. 2.5). Studies suggested that phase, amount, and morphology of calcium carbonate crystals depend on supersaturation, temperature, pH and $(Ca^{2+})/(CO_3^{2-})$ ratio. Calcium carbonate precipitation in microbial systems

typically occurs when the saturation index (with respect to calcite) is above 1 (Arp et al., 2001; Mitchell et al., 2006).

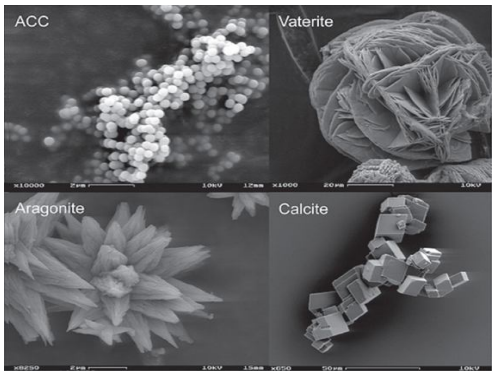


Fig 2.6 Polymorphs of Calcium Carbonate precipitated by MICCP. (Source: Niedermayr & Immenhauser.)

Studies conducted on precipitation and growth morphology of $CaCO_3$ induced by *Myxococcus xanthus* shows that calcite and vaterite formed in a gel medium display a range of morphologies that depend on whether the bacteria are dead or alive. Metabolic activities of bacteria induced i)aggregates of calcified bacteria formed at maximum supersaturation. ii) Vaterite spheres, dipyramide. Dead *M.xanthus* cells induced heterogeneous precipitation of calcite with rhombohedral morphologies at low supersaturtation. Bacterially induced alkanization appears to be a prerequisite for the development of spherimitric and dipyramidor dispenoid like forms in natural biofilms and microbial mats.

Chapter 3

Material and methods

Screening of calcifying bacteria

Sample collection

Different bacterial isolates were collected from alkaline soils and maintained at 4° C for further use.

3.1 Screening of bacteria producing maximium Urease and Carbonic anhydrase activity

All bacterial isolates were grown on Nutrient Agar plates (Casein hydrolysate 15.0g/L, peptone 5.0g/L, NaCl 5.0 g/L and Agar 20.0g/L having pH 9.0). The plates were then incubated at 2 temperatures (28°C and 37°C). The grown bacterial colonies on the plates were subcultured many times on the same medium from where it was picked. Finally, random colonies were transferred on to Urea Agar Base medium (HiMedia, india) to check the production of Urease based on the intensity of pink color. This was followed by quantitative Urease production test.

3.1.1 Preparation of Ammonium chloride and para-nitrophenol standards

Ammonium chloride (50-1000μM) standard was prepared according to the phenol-hypochlorite assay method (Nataranjan., 1995). para-nitrophenol standard (100 μM- 1 mM) was prepared according to spectrophotometric assay of Smith anf Ferry (1999).

3.1.2 Enzyme Assay

For estimation of Urease and Carbonic anhydrase activity in urease, the positive isolates which gave qualitative results on urea agar plates , the bacterial cells (1% OD_{600} = 1.0) of each strain were inoculated into 250 ml flasks containing autoclaved NB media supplemented with 2% urea and 10 μM ZnSO4, and incubated at 37° C upto 5 days.

Urease Assay

1 ml culture broths from each flask were taken at an interval of 24 hrs, centrifuged (8000 rpm for 5mins) and the supernatant was collected. Urease activity was determined for all the bacterial isolates by measuring the amount of Ammonia released from urea according to the phenol-hypochlorite assay method (Natarajan., 1995)

- 1ml of 0.1M Potassium phosphate buffer (pH 8.0) and 2.5 ml of urea (0.1M) were mixed in the cleaned, autoclaved test tubes.
- 250 μl culture supernatant was added to the above mixture.
- Incubated at 37°C for 5 mins.
- 1 ml each of alkaline hypochlorite and phenol nitropruside was added.
- Above assay mixture was incubated at 37°C for 25 mins.
- Optical density was measured at 626 nm.

The Urease activity was calculated with reference to a calibration graph plotted from the results obtained by standards. 1 unit of urease is defined as the amount of enzyme hydrolyzing one μmole urea/min.

Carbonic anhydrase (CA) Assay

Activity of CA was determined according to spectrophotometric assay of (Smith and ferry, 1999)

- 1.8 ml of phosphate buffer (pH 7.0) and 1 ml of 3mM p-nitrophenyl were added in cleaned dry test tubes.
- 200μl of culture filtrate was added to above mixture .
- The increase in A_{348} nm was recorded for 5 mins.
- The A_{348} minute was computed using the maximum linear rate for both the test and blank.
- 1 unit of CA activity is defined as the amount of enzyme required to form 1 μmole of para- nitrophenol/min.

3.1.3 Preparation of Calcium chloride and calcium carbonate standards

Calcium chloride standard (5mM- 25mM) was prepared for soluble calcium estimation in each of the sample.

- 5ml of $CaCl_2$ (different concentration), or 5 ml of culture supernatant in case of test sample was mixed with 4 ml of 5N NaOH in a titration flask.
- Made up the volume upto 50 ml with distilled water
- Hydroxyl napthol blue was used as an indicator.
- It was titrated against 0.05M EDTA and end point of pink to blue was noted.

Amount of EDTA used in ml corresponds to amount of insoluble calcium present in sample.

Calcium carbonate standard was prepared in the similar way, 3N HCl was used to dissolve the crude precipitate which is achieved after centrifugation. 5N NaOH was added to it and volume raised to 50 ml with distilled water, and titrated with 0.05M EDTA using hydroxyl napthol blue.

Amount of $CaCO_3$ = Volume of EDTA used X 0.005 X 1000/gm

Bacillus megaterium was choosen for further studies due to its maximum urease and carbonic anhydrase activity among all the isolates.

3.2 Influx of pure carbon dioxide, an alternative to replace urea in MICCP

In order to avoid urea and replace it due its certain disadvantages, 99.5% pure CO_2 with constant flow rate of approx 20ml/min was flushed into bacterial medium NB for different time periods. Samples were taken out at regular time periods to check for pH reduction, dissolved carbon dioxide in order to make conditions feasible for bacteria to grow and cause calcium carbonate precipitation.

Figure 3.1 Apparatus used to flush carbon dioxide in the flasks.

3.2.1 Optimization of pH via CO_2 influx, required for calcium carbonate precipitation

99.5% pure carbon dioxide cylinder was used for CO_2 bubbling. Constant flow rate was set (20ml/min) using a soap bubble meter. Erlenmeyer flasks containing 100ml of autoclaved bacterial medium (NB pH 8.0) were used for flushing CO_2 for different time periods in duplicates. One in which CO_2 was not flushed was considered as control. 5 ml sample from each of the flask was taken out at 0, 15, 30 and 45 min respectively to note down the change in pH. pH drop recorded in this experiment was too high for bacteria to grow, and such acidic conditions could not prevail Calcium carbonate precipitation. In next set up, pH of Nutrient broth was raised initially to 9 and 10, using 0.1 N NaOH and whole experiment was repeated again as described above, but still the pH recorded was too low to cause calcium carbonate precipitates. Saturation level was seen in pH after a particular time period of CO_2 influx.

Therefore, in the next set up time period of CO_2 bubbling was reduced to 0, 15sec, 30sec, 1 min and 2 min, and pH was recorded.

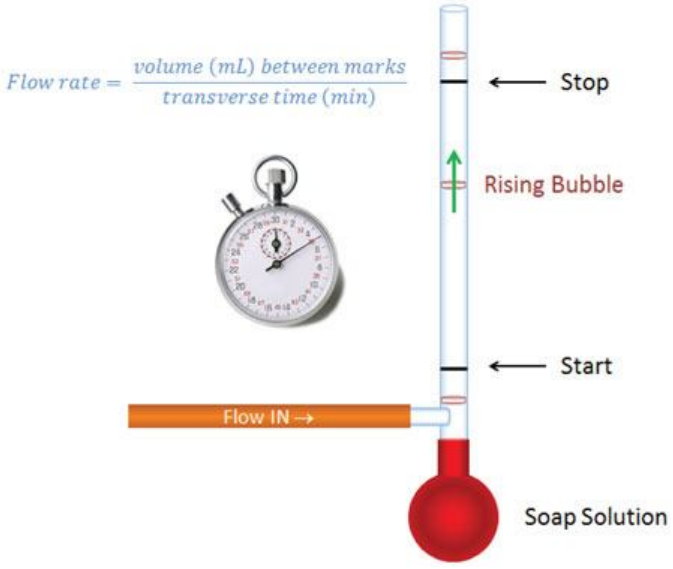


Figure 3.2 Soap bubble flow meter used to set the flow rate of carbon dioxide bubbling

3.2.2 Measurement of CO_2 concentrations in NB at different time periods

The determination of concentration of CO_2 in the bacterial medium was carried out using end point titration method. During flushing 10 ml samples were carefully taken out from flasks at different time intervals of 10 sec, 30sec , 45 sec, 1 min and 2 min. Samples were also taken out from control ones at the same interval. 5 ml of each sample was titrated with the standard sodium hydroxide solution, using phenolphthalein indicator. Free CO_2 in the solution reacts with the sodium hydroxide to form sodium bicarbonate with constant increase in pH. The completion of the reaction is indicated automatically at end point pH of 8.3 and development of pink color. The equivalent concentration of CO_2 in each sample is indicated after the completion of the reaction. All the samples were corrected for amount of CO_2 flushed in the medium by subtracting the values of CO_2 in the control.

Figure 3.3 Development of pink color in the end point titration method of carbon dioxide estimation

Since CO_2 can hydrate and dissociate in H_2O, the reaction scheme may be written as:

$$CO_2 + H_2O \longrightarrow H_2CO_3$$

$$H_2CO_3 \longrightarrow HCO_3^- + H^+ \longrightarrow CO_3^{2-} + 2H^+$$

At pH values less than 8, the concentration of carbonate ions may be neglected and only the following hydration needs to be considered:

$$CO_2 + H_2O \longrightarrow H_2CO_3 \longrightarrow HCO_3^- + H^+$$

When CO_2 reacts to sodium hydroxide,

$$CO_2 + H_2O \longrightarrow H_2CO_3$$

$$H_2CO_3 + NaOH \longrightarrow Na_2CO_3 + H_2O \quad OR$$

$$CO_2 + NaOH \longrightarrow Na_2CO_3$$

3.2.3 Effect of CO_2 on pH, carbonic anydrase activity and calcium carbonate precipitation

Sterile, autoclaved flasks of Nutrient broth of initial pH 10 were prepared and CO_2 was flushed for 10sec, 30 sec, 45 sec and 1 min. one flask without CO_2 was taken as control. Whole study was done taking *Bacillus megaterium* which gave the best urease and CA activity. *B.megaterium* cells with OD_{600} = 1 (1%) were inoculated into flasks . $CaCl_2$ (25 mM) and ZnSO4 (10μM) were added to each of the flask and kept at 37°C for 5 days at shaking conditions. Regularly after 24 hrs 10 ml sample from each flask was withdrawn to see the change in pH, carbonic anhydrase activity, soluble calcium and carbonate content.

10 ml sample was taken out regularly in autoclaved oakridge tubes and checked for pH change immediately. Sample was then centrifuged at 8000 rpm for 5 min at 4°C, to perform CA assay,

insoluble calcium assay and the precipitated carbonates (pallet) were collected and estimated by EDTA titration method as described earlier. **1 ml of EDTA used for titration is equivalent to 5.004 mg of $CaCO_3$ precipitated.**

3.3 Comparison of CO_2 influx with Urea as a source of carbonate precipitation.

Now as CO_2 flushing for 1 min (concentration of CO_2=1056mg/L or 0.105g/L) (99.5% pure, flow rate = 20ml/min) was optimized for good carbonate precipitates, it was compared with addition of 2% urea which is well known to induce calcium carbonate precipitation with microbes. Different sets of CO_2 and urea with their respective controls were used in this study. One bacterial strain *Bacillus megaterium* was used in this study. This experiment was carried out at two different pH (10, 11) i.e. Initial pH was set to 10 and 11 before CO_2 flushing and urea due to its highly basic nature was added before the adjustment of pH.

Different sets made for this comparison

pH 10	pH 11
1. CO_2 1min	1. CO_2 1 min
2. Urea 2%	2. Urea 2%
3. CO_2 (1min) + Urea (2%)	3. CO_2 (1min) + Urea (2%)
4. control	4. control

All the sets were inoculated with 1% culture of *B. megaterium* at OD_{600}=1 and 25 mM Calcium chloride,10 μM ZnSO4, kept at shaking conditions (120rpm), 37° C for 5 days. Sample from each set was taken out regularly after 24 hrs to study all the parameters (pH, CA, insoluble Ca and carbonate precipitation.)

3.4 Investigation of carbonate crystals formed by different sets

Precipitates generated after 5 days of continuous incubation in all the sets were filtered using sterile whattman filter paper of retention size 2.5 μm . The precipitates were collected, dried at 50°C for 1 hr and stored for morphological and minerlogical constituent studies by Scanning electron microscopy, X ray diffraction analysis and energy dispersive X ray analyzer.

3.5 Evaluation of microbes for enhancement of Durability properties of Cement Mortar

3.5.1 Microorganism and cultivation conditions

Bacillus megaterium (isolated from alkaline soil) was used throughout this study. To prepare mortar specimens for compressive strength, water absorption tests, bacterial cells were grown in nutrient broth containing urea (NBU media) and 25 mM $CaCl_2$ as described by Achal et al 2009a. and kept at shaking conditions at 37°C till cell density of 5x 10^7 cells/ml.

3.5.2 Preparation of Cement mortar cubes

Ordinary Portland cement of grade 43 confirming to IS 4031 was used. The standard sand (Enore sand) of grade I, II, III was used as fine aggregates. A cube mould of 70.6 mm x 70.6 mm x 70.6 mm was used per IS 4031-1988. The cement to sand ratio was 1:3 (by weight), and water to cement ratio was kept 0.45. Bacterial culture to cement ratio was also kept 0.45. Sand and cement were thoroughly mixed with water for control cubes, and with bacterial culture OD_{600} =1.5 (5 x10^7 cfu/ml) for test cubes. Cubes were cast and compacted in a vibrator.

Figure 3.4 casting of cement mortar specimens in a mould

3.5.3 Compressive strength test

To study the compressive strength test of cement mortar, the most efficient bacterial isolate *B. megaterium* (based on higher microbial carbonate production), grown in Nutrient broth urea (NBU) medium was used. After de-moulding, all specimens were cured in corresponding medium at room temperature until compression testing at the intervals of 3, 7 and 28 days. Media were replenished at a regular interval of 7 days. Control samples (without bacterial cells) were also prepared in similar manner, and cured in NB without cells. One set with bacterial cells were also prepared which was cured by spraying NBU medium daily till compression testing

intervals. Compression testing was performed using automatic compression testing machine, COMPTEST 3000.

3.5.4 Water Absorption Test

To determine the increase in resistance towards water penetration, a sorptivity test, based on the RILEM 25 PEM (II-6), was carried out on mortars. The mortars were prepared exactly in the same way as for compressive strength tests. The mortar specimens were cured in media for 28 days. After curing the specimens were dried at 45° C in a ventilated oven, establishing a mass equilibrium of less than 0.1 % between two measurements at 24 h intervals. The specimens were then exposed to 10± 1 mm of water (water level just 2mm above the base of specimen). This was done in an atmosphere of 20° and relative humidity of 60%. At regular time (15 min, 30 min, 1 h, 1.5 h , 3h, 5h, 8h, 24h, 72h, 96h, 120hand 144) the specimens were removed from the water and weighed, after drying the surface with the wet towel. Immediately after the measurement, the specimens were submerged again. The sorptivity coefficient, *k* [$cms^{-1/2}$], was obtained by using the following expression:

$Q/A=k\sqrt{t}$

Where Q is th amount of water absorbed [cm^3]; A is the cross section of the specimen that was in contact with water [cm^2]; t is the time[s], Q/A was plotted against the square root of time.

3.6 Effect of Carbonation on durability of cement mortar cubes

Here an attempt was made to avoid urea and utilize CO_2 to cause microbial carbonate precipitation. Carbonation could have both negative and positive effects on concrete durability. Carbonation in most cases seems to densify the concrete surface and reduce the chloride ion permeability.

3.6.1 Preparation of specimens for carbonation

Cubes were prepared in exactly same the way as described in section 3.6.2. The only difference lies in the fact that this time bacterial isolate *Bacillus megaterium* cells used for mixing were grown without urea in the presence of 25mM $CaCl_2$ till OD_{600}= 1.5. Ordinary Portland cement and standard ennore sand was used in the ratio of 1:3 and culture to cement ratio was kept 0.45. Four different sets were made, two of culture mix and two of water mix and one from each was cured separately in CO_2 chamber and water for comparison.

- Bacterial mix in carbon dioxide chamber (BM)
- Water mix in carbon dioxide chamber (WM)
- Bacterial mix in water (BM)
- Water mix in water (WM)

3.6.2 Curing procedure

CO_2 curing was carried out together with conventional hydration (water) curing and bacterial curing as comparison. CO_2 cylinder of 99.5% CO_2 was used for carbonation process. Cement mortar specimens (bacterial mix and water mix) directly after demoulding were placed inside the sealed chamber as shown in figure, which was then vacuumed to about 0.7 bars and filled with carbondioxide gas to a pressure of 1 bar. The cubes were kept inside the chamber for 7 continous days and pressure guage was checked regularly to see the inside pressure. The pressure was almost kept constant and was not allowed to drop below 1 bar. For control, each of these set is cured in water simultaneously.

Figure 3.5 Carbonation curing chamber for curing cement mortar cubes.

The effect of carbonation curing was evaluated on the basis of compressive strength and water permeability tests.

3.7 SEM-EDX and XRD

The morphology and chemical constituents of bacterial carbonate consolidated specimens were analysed with SEM-EDX and XRD. Samples were dried at room temperature, broken pieces of cubes were crushed using pestle mortar. Samples were collected and stored till analyses. For SEM, samples were examined at accelerating voltages ranging from 15 to 35 kV, which was equipped with an energy – dispersive X- ray analyzer for elemental analysis.

Concrete samples were tested in granulated form. XRD- spectra were obtained using an X'Pert PRO diffractometer with a Cu anode (40 KV and 30 ma) and scanning from 5 to 80° 2 θ.

3.8 Statistical analysis

Data were statistically analyzed by an analysis of variance (ANOVA) and when observed differences were significant, the means were compared by Tukey's honestly significant different test. All the experiments in this study were performed in triplicates. Graph pad prism (5.8) software and co-stat (statistical tool) was used for all the analysis.

Chapter 4

Results and Discussions

Alkaline soil represent/provide a prime habitat of alkaliphilic bacteria and are areas of specific interest in case of applied research applications. Alkaliphilic ureolytic bacteria represent the most **favored pathway** for the precipitation of carbonates. (Figure 4.1.1)

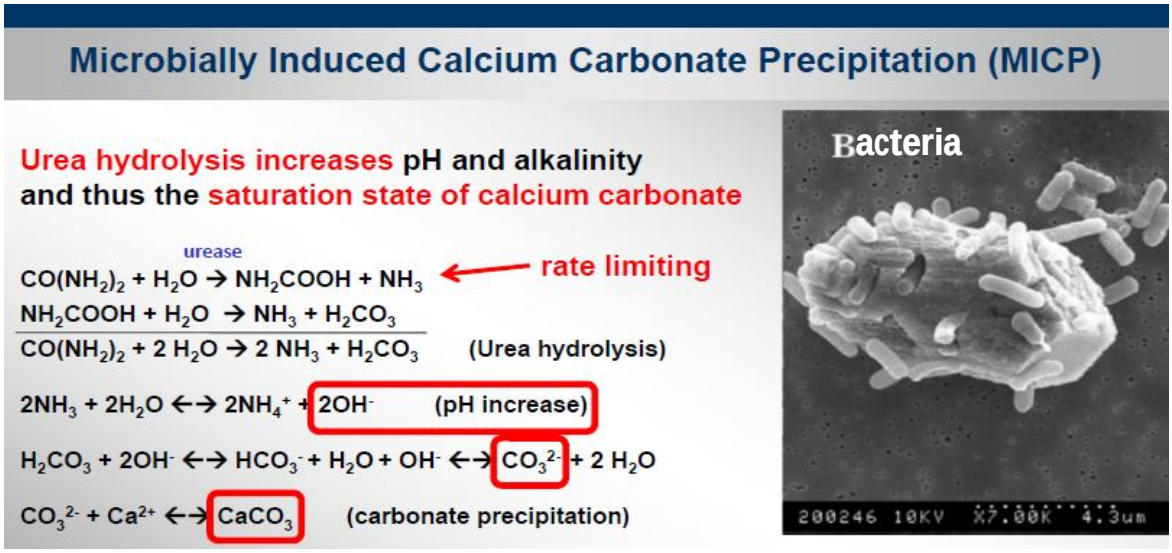


Figure 4.1.1 Microbially induced calcium carbonate precipitation via ureolytic pathway. (Source: Robin Gerlach., MSU(Montana state university., 2012)

Ureolysis is one possible way to manipulate the saturation state of carbonates. (Mitchell & Ferris *et al.,* 2006)

4.1 Screening of calcifying bacteria

All the bacterial isolates were grown on Nutrient Agar plates supplemented with urea for primary screening. After enriching the growth of ureolytic bacteria in the urea enriched nutrient agar, the efficient bacteria were screened for the production of urease in urease selective media i.e. Urea Agar media. Phenol red present in this media is degraded by ureolytic organisms to give pink color in the alkaline environment. (Andrews *et al.,* 1995). Based on this primary screening (Figure 4.1) seven different bacterial isolates were selected for urease and carbonic anhydrase activity.

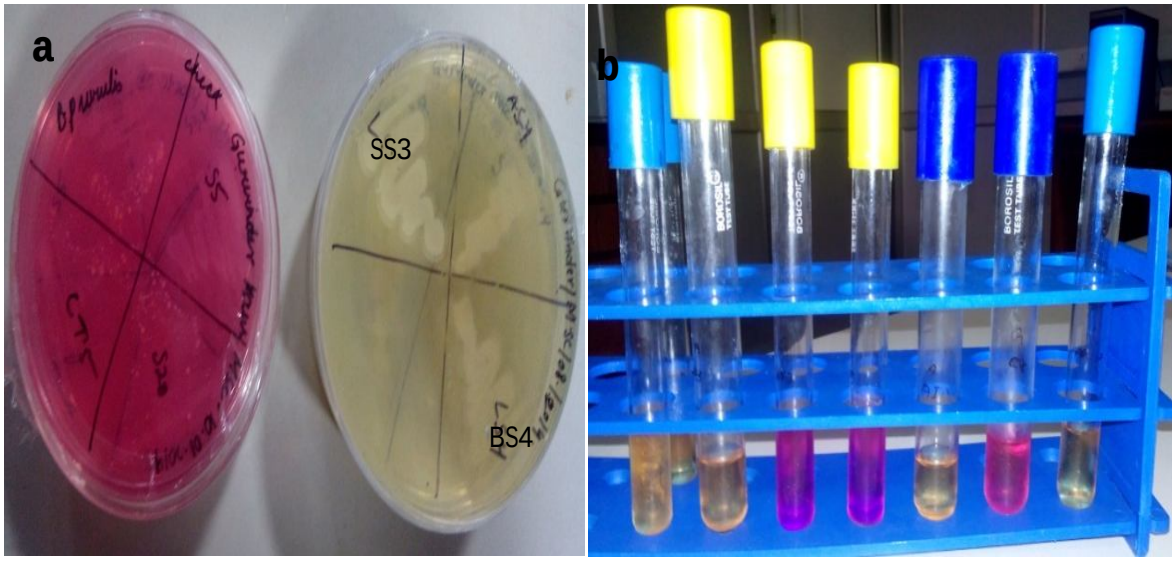


Figure 4.1.2 Primary screening of different isolates on (a) on nutrient agar plates supplemented with urea (b) in urea broth

Of all the bacterial isolates tested, seven of isolates showed positive results after primary screening. They were named as: SS5, BS, BS1, BS2, BS3, BS4(SS3), BS5. The actual names of these species were characterized when identified by 16S rDNA sequence analysis. (Dhami *et al.*, 2012).

BS1- *Bacillus cereus* BS2- *Bacillus thirungiensis* BS4- *Bacillus anthracis* BS5- *Bacillus amyloliquefaciens,* SS3- *Bacillus megaterium*.

These seven bacterial isolates were checked for quantitative production of urease and carbonic anhydrase activity according to procedure described above in section 3.1.2. Urease and carbonic anhydrase activity activity of these seven isolates can be dipicted as:

Table 4.1.1 Comparison of Urease activity (U/ml) and CA (U/ml) by different isolates. Values bearing different letters in the same column are significant at $P < 0.05$. values are mean ± SD (n=3).

Bacterial isolates	Urease production (U/ml)	CA production (U/ml)
SS5	17.095 ± 1.36^{d}	147.6 ± 1.21^{c}
BS4	18.475 ± 0.87^{d}	144.24 ± 6.36^{c}
BS1	31.935 ± 2.51^{c}	188.17 ± 4.44^{ba}
BS2	44.295 ± 4.32^{b}	220.74 ± 12.12^{a}
BS3	35.650 ± 1.57^{bc}	$194.89 \pm 9.89a^{b}$
SS3	74.315 ± 3.61^{a}	207.63 ± 7.26^{ab}
BS5	19.305 ± 2.58^{d}	150.31 ± 1.01^{c}

Bacillus megaterium (SS3) has the highest urease activity of 74.315U/ml followed by BS2.

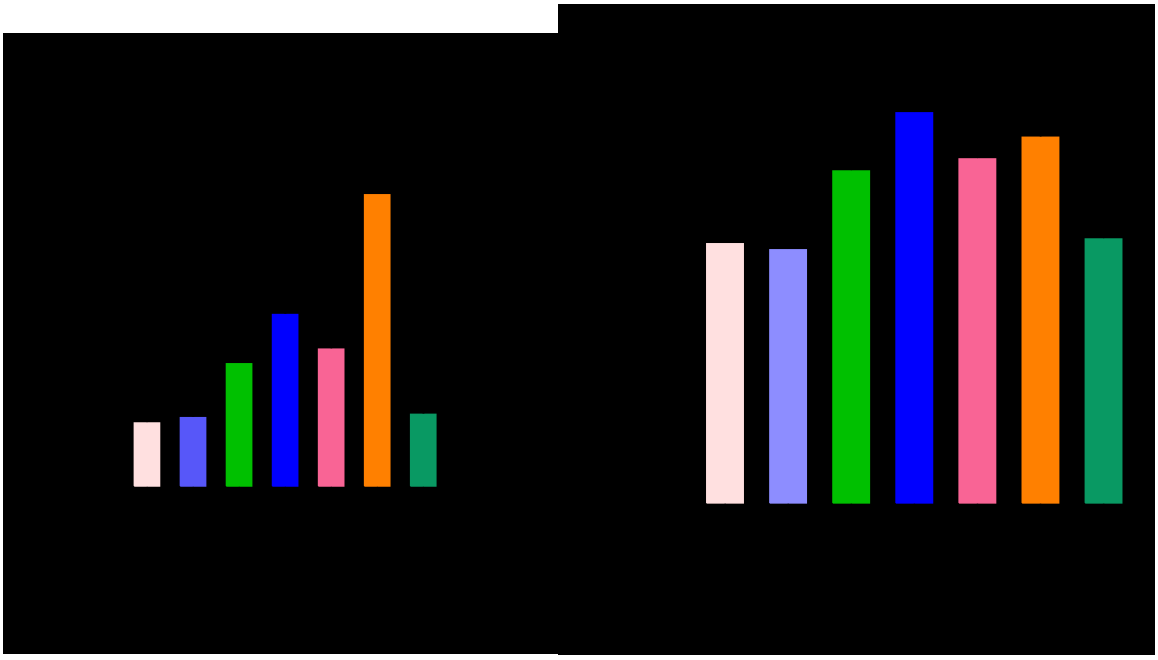


Fig 4.1.2 Urease and Carbonic anhydrase activity (U/ml) of different bacterial isolates. Bars represent mean ± SD (n=3)

Bacillus megaterium (SS3) due to its high ureolytic activity and carbonic anhydrase was taken for further studies.

4.2 Influx of pure CO_2, an alternative to replace urea in MICCP

The ultimate role of bacteria by hydrolysis of urea is to produce carbonate ions for the precipitation of calcium carbonate. Urea hydrolysis results in subsequent rise of pH which is the main factor governing $CaCO_3$ precipitation. If carbon dioxide is directly flushed into the bacterial media and conditions favors for growth of bacteria, bacteria may serve as nucleation sites and we can achieve suitable amount of calcium carbonate precipitation. Here in this study an attempt was made to optimize pH and particular CO_2 concentration which could induce sufficient amount of carbonate precipitates.

4.2.1 Optimization of pH

99.5% pure carbon dioxide with a constant flow rate (adjusted by soap bubble meter) of 20ml/min was flushed into the autoclaved bacterial medium NB (Himedia, India) of pH 8.0 for different time periods. Change in pH at different time periods was recorded.

Table 4.2.1 Change in pH after flushing CO_2 in NB of initial pH 8.0 for different time periods of 0, 15, 30, and 45mins.

Time period(min)	pH (CO_2 +ve)	pH (control)
0	8.03	8.01
15	5.9	7.96
30	5.89	7.82
45	5.62	7.83

In this case, there is a great reduction in pH i.e., saturation is achieved after 15 minutes of CO_2 flushing. There is no further change in pH if CO_2 is flushed for more time. This decrement in pH is of no use, as such an acidic environment cannot prevail calcium carbonate precipitation. In the next set, for better results we initially raised the pH of Nutrient broth to 9 and 10 using 0.1N NaOH and reduced the time of CO_2 bubbling for 10 mins. (All the sets were performed in duplicates). In this case too, the pH drop was too high to induce calcium carbonate precipitation. In that case, in next set up we raised the pH of NB to 10 and reduced the time period of CO_2 bubbling to just seconds, keeping the flow rate constant (20ml/min).

Table 4.2.2 Change in pH after flushing CO_2 in NB of initial pH 10 for the time periods of 1min, 45 sec,30sec and 10sec.

Time period	pH
1 min	8.91
45 sec	8.84
30 sec	9.49
10 sec	9.78

This drop in pH was sensible and could induce calcium carbonate precipitation if enriched with suitable bacteria and calcium source.

4.2.2 Measurement of CO_2 concentration

We cannot measure CO_2 in time periods. For comparison, it is important to know that how much concentration of CO_2 is being dissolved when flushed for different periods in different flasks.

This was done using end point titration method by methodology explained in sec 3.2.2

Calculation of carbondioxide present (mg/ml) =

$$\frac{\text{Normality of NaOH X Equivalent weight of } CO_2 \text{ X 1000 X ml of NaOH used}}{\text{ml of sample taken}}$$

Table 4.2.3 Concentration of CO_2 being flushed at different time periods at constant flow rate

Time period	Carbon dioxide conc.(g/L)
2 min	0.127
1 min 30 sec	0.116
1 min	0.112
45 sec	0.108
30 sec	0.105
10 sec	0.037

4.3 Effect of prudent concentrations of CO_2 on various parameters of $CaCO_3$ precipitation

Before looking for biotechnological applications of microorganisms, it is prerequisite to optimize the various parameters affecting the microbial product. We need to optimize a particular concentration of CO_2 flushing for desired pH and carbonate precipitation. 100 ml bacterial media (NB pH10) was autoclaved in 5 Erlenmeyer flasks . CO_2 was flushed in these flasks for 0, 10sec, 30sec, 45sec and 1 min at constant flow rate. One without CO_2 was considered as control. Bacterial isolate *B. megaterium* of OD_{600}=1 (1%) was inoculated in all the flasks. 25 mM $CaCl_2$ and 10µM $ZnSO_4$ were also added and flasks were kept at 37°C for 5 days under shaking conditions. After every 24 hrs samples were withdrawn to check the pH, CA activity, soluble calcium and calcium carbonate precipitates. Figure 4.3.1 depicts the ph profile for carbonate precipitation at different concentrations of CO2.

The enzyme activity of the extracellular carbonic anhydrase secreted in the supernatant by bacterial metabolism was carried out using p-NPA assay. It was found that CA activity increased with time in all the concentrations of CO_2 till 4rth day, and declined after that(Figure 4.3.2). CA activity in CO_2 conc. with 0.108 g/l declined after 3 day. Maximum linear increase in CA activity was seen with 0.112g/l CO_2 conc. and CA activity was found to be 289.2 U/ml on 4 day.

Soluble calcium estimation can directly tell us that how much of calcium has been converted to calcium carbonate. Insoluble calcium corresponds to carbonate precipitates. The amount of

soluble Ca^{2+} in the supernatant and insoluble Ca^{2+} in the pellet was quantified according to the EDTA titration method (APHA et al 1998), described in sec 3.1.3.

Table 4.3.1 Effect of different CO_2 conc. on pH with increase in time.

	CO_2 Concentrations				
Days	**0.037**	**0.105**	**0.108**	**0.112**	**0.000**
1	9.53 ± 0.042^{aB}	7.60 ± 0.007^{dE}	8.21 ± 0.007^{Fc}	7.94 ± 0.007^{eD}	$10.18\pm.092^{aA}$
2	7.57 ± 0.014^{bD}	7.62 ± 0.007^{dD}	8.26 ± 0.014^{eC}	8.42 ± 0.007^{dB}	10.06 ± 0.057^{abA}
3	7.78 ± 0.000^{eD}	7.84 ± 0.014^{cD}	8.41 ± 0.014^{dC}	8.67 ± 0.028^{cB}	9.92 ± 0.042^{bA}
4	8.05 ± 0.007^{dD}	7.92 ± 0.028^{cE}	8.53 ± 0.014^{cC}	8.70 ± 0.007^{cB}	9.64 ± 0.019^{cA}
5	8.43 ± 0.028^{cC}	8.05 ± 0.007^{bD}	8.92 ± 0.007^{bB}	8.96 ± 0.021^{bB}	9.69 ± 0.007^{cA}
6	9.33 ± 0.014^{bD}	9.125 ± 0.007^{aE}	9.42 ± 0.021^{aC}	9.90 ± 0.007^{aB}	10.00 ± 0.012^{bA}

Values bearing same letters (lowercase) in a column, and same letters (uppercase) in a row are not significant at $P<0.05$, values are mean ± SD (n=3)

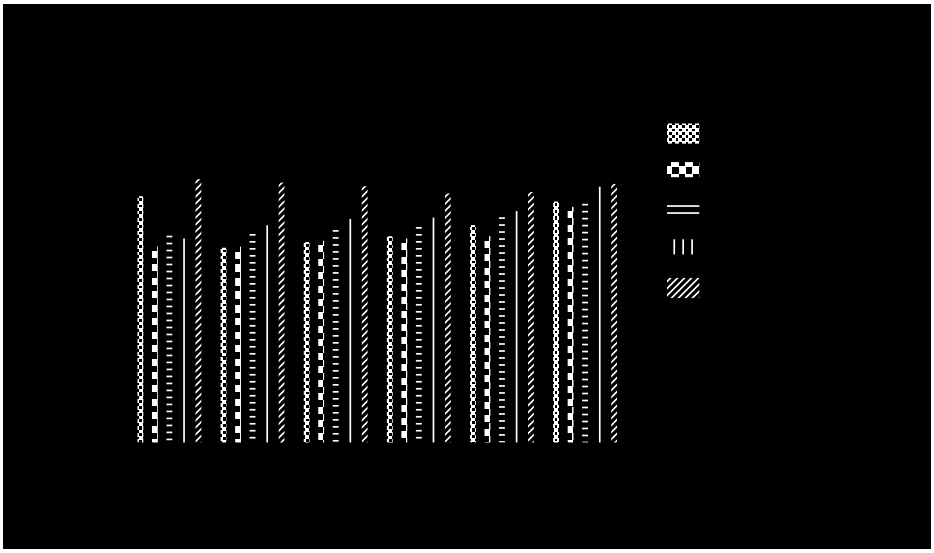


Figure 4.3.1 pH profile for calcium carbonate precipitation at different concentrations of CO_2.

Figure depicts that carbon dioxide caused an influential effect on pH, decreasing the alkalinity of the media, but with time as the bacteria grows and utilize calcium chloride and CO_2 to form carbonates, the pH slightly increases for all the conc. of CO_2, but out of four 1 min CO_2 conc i.e., 0.112 g/L showed the highest increment in pH, making conditions suitable for calcium carbonate precipitation.

Table 4.3.2 Production profile of CA activity by *B. mega* at different concentration of CO_2.

CO_2 Concentrations					
Days	**0.037**	**0.105**	**0.108**	**0.112**	**0.000**
1	206.23 ± 1.40^{dC}	244.81 ± 4.26^{cdB}	254.26 ± 0.08^{cB}	248.59 ± 0.05^{cdB}	320.44 ± 0.10^{bA}
2	220.04 ± 0.33^{cE}	265.37 ± 0.07^{abC}	273.64 ± 0.02^{bB}	251.90 ± 0.05^{cdD}	329.59 ± 0.08^{aA}
3	251.97 ± 0.04^{aD}	272.01 ± 0.69^{aBC}	287.19 ± 0.04^{aB}	258.04 ± 0.54^{bCD}	313.63 ± 6.27^{abA}
4	256.81 ± 5.30^{aB}	277.86 ± 4.32^{aAB}	258.27 ± 0.24^{cB}	289.29 ± 2.72^{aA}	252.18 ± 0.05^{cB}
5	238.49 ± 0.62^{bB}	250.26 ± 1.08^{bcA}	238.24 ± 0.22^{dB}	251.37 ± 0.02^{bCA}	220.65 ± 0.01^{dC}
6	209.11 ± 0.03^{dD}	235.60 ± 0.73^{dAB}	221.63 ± 0.02^{eBC}	240.27 ± 0.41^{dA}	208.66 ± 0.19^{dCD}

Values bearing same letters (lowercase) in a column, and same letters (uppercase) in a row are not significant at $P<0.05$, values are mean ± SD (n=3)

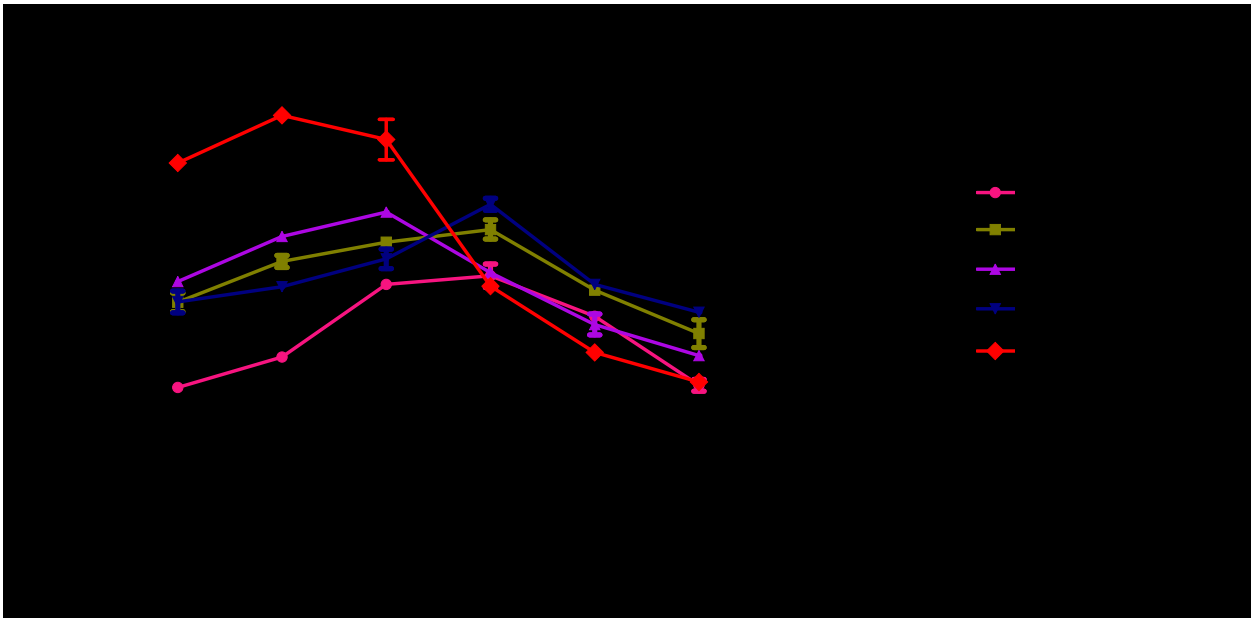


Figure 4.3.2 Carbonic anhydrase activity by *Bacillus megaterium* at different conc. of carbon dioxide.

pH plays a very important role in affecting the activity of an enzyme. As CA is a zinc containing enzyme that dramatically catalyzes the reversible hydration of carbon dioxide, addition of Zinc may have pronounced effect on the production/activity of this enzyme. After CO_2 flux , 10 mM $ZnSO_4$ was added in all the flasks for the reversible hydration of CO_2. Raman *et al.*, (2009) in their study on *citrobacter freundii* SW3 isolated from environmental waste found significant

enhancement in the CA activity upon supplementation of Zinc. In order to investigate the optimum CO_2 conc. to produce maximum CA activity by *B. megaterium*, different CO_2 concentrations were checked for CA profile for the period of 6 days. It was noticed that maximum enzyme activity was produced in culture with CO_2 conc. 0.112g/L. It was also observed that there was a regular increase in CA activity with time at all the CO_2 conc. maximum CA activity was observed on 4^{rth} day (289 U/ml) followed by CO_2 conc. of 0.105 g/L (277 U/ml). CA activity in all the culture increased linearly till 4^{rth} day and declined after that. It can be seen that there is a direct correlation between pH and CA activity caused due to action of bacteria in the presence of CO_2.

Table 4.3.3 Soluble calcium estimation in the supernatant with increase in time.

	CO_2 Concentrations				
Days	**0.037**	**0.105**	**0.108**	**0.112**	**0.000**
1	5.70 ± 0.4^{aAB}	4.45 ± 0.7^{aB}	4.79 ± 0.2^{aB}	4.60 ± 0.0^{aB}	7.73 ± 1.6^{aA}
2	5.17 ± 0.2^{bA}	4.28 ± 0.0^{abB}	3.77 ± 0.4^{bB}	3.74 ± 0.3^{bB}	5.42 ± 0.5^{bA}
3	4.26 ± 0.0^{cA}	4.27 ± 0.2^{abA}	2.64 ± 0.2^{cC}	2.76 ± 0.1^{cC}	3.54 ± 0.5^{cB}
4	2.63 ± 0.2^{dB}	4.15 ± 0.2^{abA}	3.06 ± 0.4^{cB}	1.99 ± 0.1^{dC}	2.76 ± 0.3^{cB}
5	2.00 ± 0.0^{eC}	3.54 ± 0.0^{bA}	2.00 ± 0.0^{dC}	1.24 ± 0.2^{eD}	2.76 ± 0.0^{cB}
6	1.24 ± 0.1^{fC}	2.73 ± 0.0^{cA}	1.99 ± 0.0^{dB}	1.24 ± 0.1^{eC}	2.00 ± 0.1^{cB}

Values bearing same letters (lowercase) in a column, and same letters (uppercase) in a row are not significant at $P<0.05$, values are mean ± SD (n=3)

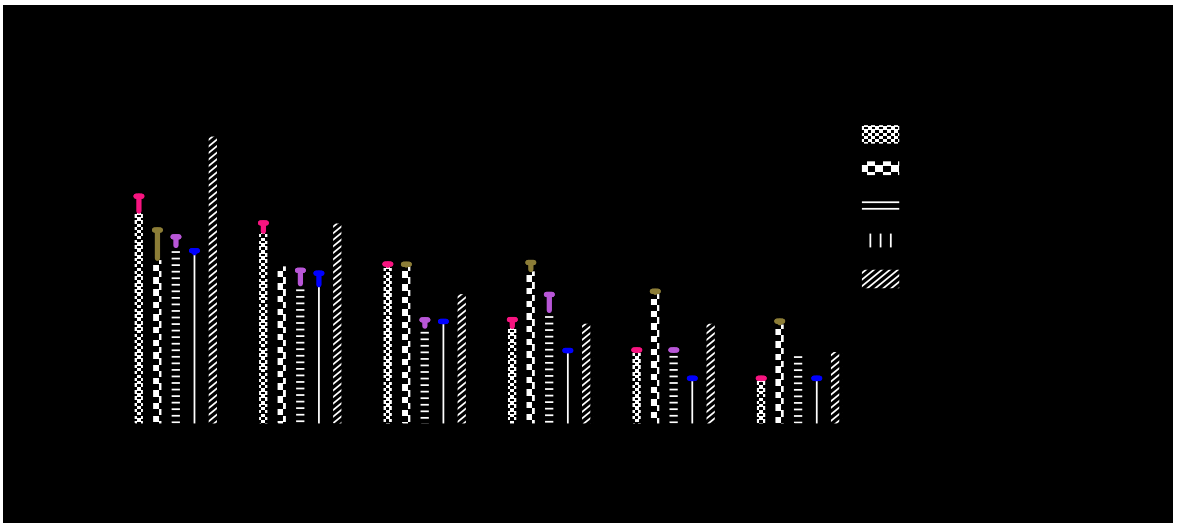


Figure 4.3.3 Effect of *B.megaterium* on the Ca^{2+} concentration (mM) at different concentrations of CO_2 (g/L).

The decreasing trend in the concentration of concentration of Ca^{2+} was observed in all the test sets of experimental systems (Figure 4.3.3). The concentration of Ca^{2+} decreased sharply during the first three days of experiment and then decreased gradually and approached equilibrium. It was observed that there was linear decrease in Ca^{2+} conc. in the set up with initial CO_2 conc. of 0.112g/L. It explains the fact, that calcium source added and the amount of CO_2 flushed initially had significant effect on carbonate precipitation.

CA activity also came out be maximum at this particular concentration, which shows that CA enhances the rate of reaction. After 24 hrs, the Ca^{2+} in the sample reduced to almost 4.5mM and 7.8mM in test and control samples respectively. After 6 days of incubation Ca^{2+} conc. reduced to approx 2.0mM. This shows that maximum, or almost whole of the calcium was utilized for carbonate precipitation. Further in order to confirm the amount of amount of carbonates formed in respective samples, (Figure 4.3.4) precipitates were collected at the end of incubation time and weighed (g/100ml).

4.3.1 Calcium carbonate precipitates

At the end of 6 day, left over culture was filtered using quantitative whatmann filter paper of retention capacity of 2.5μm. Precipitates accumulated over the filter paper were dried in oven for overnight and their weight was noted. Actual weight of precipitates can be estimated by subtracting the weight of filter paper. It was seen that maximum amount of allmost 0.109g/L of Carbonate precipitates were produced in test sample with 0.112g/L of CO_2, with respect to control and different Carbon dioxide concentrations. Significant amount of precipitates were also formed with CO_2 conc of 0.108g/L. i.e. around 0.106 g/100ml.

Table4.3.4 Calcium carbonate precipitates accumulated in different concentration of CO_2 in presence of bacteria *B megaterium.*

Calcium carbonate precipitates at different CO_2 concentrations					
CO_2 concentration (g/l)	0	0.037	0.105	0.108	0.112
Carbonate Precipitates(g/100ml)	0.040 ± 0.01^{b}	0.050 ± 0.01^{b}	0.079 ± 0.03^{ab}	0.106 ± 0.01^{a}	0.109 ± 0.03^{a}

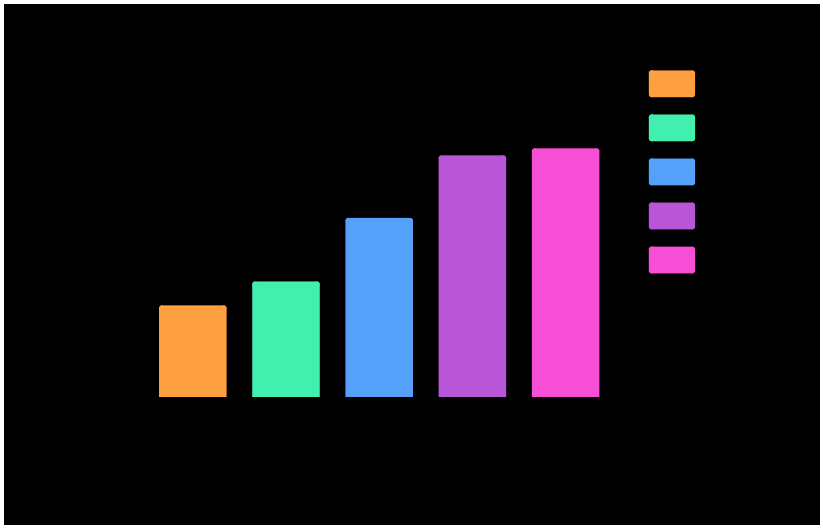


Figure 4.3.4 Effect of different CO_2 concentrations on calcium carbonate precipitates (g/L)

4.4 Comparison of CO_2 flushed with 2% Urea as a source of MICCP

Urea has been used extensively in the process of MICCP driven by its ureolysis via enzyme urease. But due to certain limitations of urea (mentioned above), we made an attempt to replace it with pure CO_2. Various parameters affecting the rate of microbially induced calcium carbonate precipitation were studied at different concentrations of CO_2. Best results pertaining to maximum carbonate precipitation came out with CO_2 flux of 1 min i.e., with CO_2 concentration of 0.112g/L. And now that we have optimized a particular concentration of CO_2 which can drive MICCP in the presence of bacteria, urea can be replaced and its disadvantages can be overcome. To confirm further we compared the different parameters (pH, CA, soluble CA) in both the cases. 4 sets of experiments at two different pH 10 and 11 were made in order to compare different activities.

- CO_2 flushed for 1 min (0.112g/L)
- CO_2 flushed for 1 min with urea added 2%
- Urea 2%
- Control(no urea, no CO_2)

After introducing particular amount of CO_2 and urea in different sets, *Bacillus megaterium* grown overnight (OD_{600}=1) 1% was inoculated in all the flasks. Calcium chloride 25μM and Zinc Sulphate 10μM was also added. Flasks were incubated at 37°C under shaking conditions. Regularly after 24 hrs till 4 days samples were withdrawn and different parameters were checked.

pH change

As for the precipitation of carbonates to occur it is necessary that pH of the medium should increase with time. From the data recorded, it was observed that the presence of urea and CO_2 both in the culture gave almost same increase in pH as given by CO_2 alone (Figure4.4.1)

Table 4.4.1 Effect of CO_2, urea on the pH pertaining to carbonate precipitation.

pH 10				
Days	**CO_2**	**CO_2 +UREA**	**UREA**	**CONTROL**
1	6.94 ± 0.07^{cC}	8.05 ± 0.00^{aA}	8.06 ± 0.00^{cA}	7.63 ± 0.02^{cB}
2	7.66 ± 0.16^{bB}	7.85 ± 0.00^{bB}	8.29 ± 0.04^{bA}	8.16 ± 0.07^{bA}
3	7.86 ± 0.02^{abC}	7.79 ± 0.02^{cC}	8.28 ± 0.07^{bA}	8.07 ± 0.02^{bB}
4	8.09 ± 0.07^{aC}	7.70 ± 0.01^{dD}	8.39 ± 0.08^{aA}	8.28 ± 0.01^{aB}
pH 11				
Days	**CO_2**	**CO_2 +UREA**	**UREA**	**CONTROL**
1	7.15 ± 0.05^{dD}	7.88 ± 0.02^{cC}	9.05 ± 0.06^{cA}	8.72 ± 0.02^{bB}
2	7.65 ± 0.05^{cD}	8.11 ± 0.00^{bC}	9.08 ± 0.05^{cA}	8.90 ± 0.07^{aB}
3	7.97 ± 0.02^{bC}	7.93 ± 0.03^{cC}	9.25 ± 0.02^{bA}	8.53 ± 0.06^{cB}
4	8.10 ± 0.02^{aC}	8.23 ± 0.04^{aC}	9.58 ± 0.04^{aA}	8.47 ± 0.05^{cB}

Values bearing same letters (lowercase) in a column, and same letters (uppercase) in a row are not significant at $P<0.05$, values are mean ± SD (n=3)

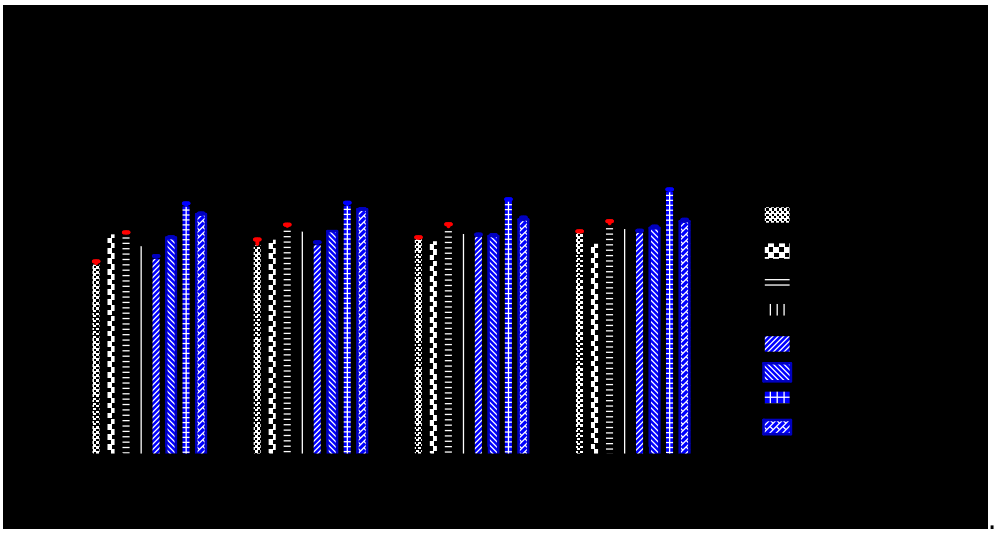


Figure 4.4.1 Effect of different carbonate source (CO_2 and urea) on the pH of two different sets.

The highest pH rise was shown by urea driven samples. It was observed that pH of set 2 (initial pH 11) reached from 9.05 to 9.58 in the presence of urea till the end of 4^{rth} day, and reached from 7.15 to 8.10 in presence of CO_2. It can be seen that influx of CO_2, causes sudden decrease in the pH, and hence alkaline conditions develops with the passage of time. It is clear from the above experiment that if we raise the initial pH of NB to 10 before flushing of CO_2, we can achieve optimum amount of pH rise required for carbonate precipitation.

Carbonic anhydrase activity (U/ml)

Table 4.4.2 Effect on different carbonate sources (CO_2 and urea) on CA activity of bacterial strain *B.megaterium*. Values are mean ± SD (n=3).

		Carbonic anhydrase activity at pH 10		
Days	**CO_2**	**CO_2 + UREA**	**UREA**	**CONTROL**
1	274.60±1.53^{dC}	311.64±0.86^{bA}	294.98±1.15^{cB}	297.45±0.59^{cB}
2	291.24±1.47^{cC}	331.58±0.38^{bA}	324.63±7.07^{bA}	340.45±0.85^{bA}
3	324.64±1.46^{bA}	271.75±9.49^{cC}	298.93±8.07^{cB}	293.34±0.44^{dB}
4	496.10±3.03^{aA}	447.76±5.52^{aB}	421.55±0.53^{aC}	490.50±0.34^{aA}
		Carbonic anhydrase activity at pH 11		
Days	**CO_2**	**CO_2 + UREA**	**UREA**	**CONTROL**
1	303.77±2.67^{cC}	316.07±0.66^{cB}	324.23±0.20^{cA}	298.39±1.92^{cD}
2	353.18±0.18^{bA}	317.65±1.44^{cC}	331.17±1.01^{bB}	316.23±0.89^{bC}
3	350.99±14.3^{cAB}	344.91±1.04^{bA}	305.05±1.10d^{BC}	287.89±1.63^{dC}
4	519.96±4.35^{aA}	491.44±0.28^{Ab}	439.96±1.26^{aD}	469.35±1.26^{cC}

Values bearing same letters (lowercase) in a column, and same letters (uppercase) in a row are not significant at P<0.05, values are mean ± SD (n=3)

In present case maximum CA activity was found to be 446.10U/ml and 519.96U/ml in case of CO_2 induced microbial carbonate precipitation. It was observed that CO_2 can enhance the activity of bacterial carbonic anhyrase and lead to optimum carbonate precipitation. Figure 4.4.2 shows the rise in CA activity till 4 days of incubation. The continuous rise was seen in all the samples, with almost higher activity in CO_2 samples. From the figure it was seen that CA activity was almost same in first three days of incubation with just small rise in activity with increase in time. Sudden increase in CA activity was seen at 4rth day. Maximum activity was seen in control set up with no urea and carbon dioxide, but only bacteria.

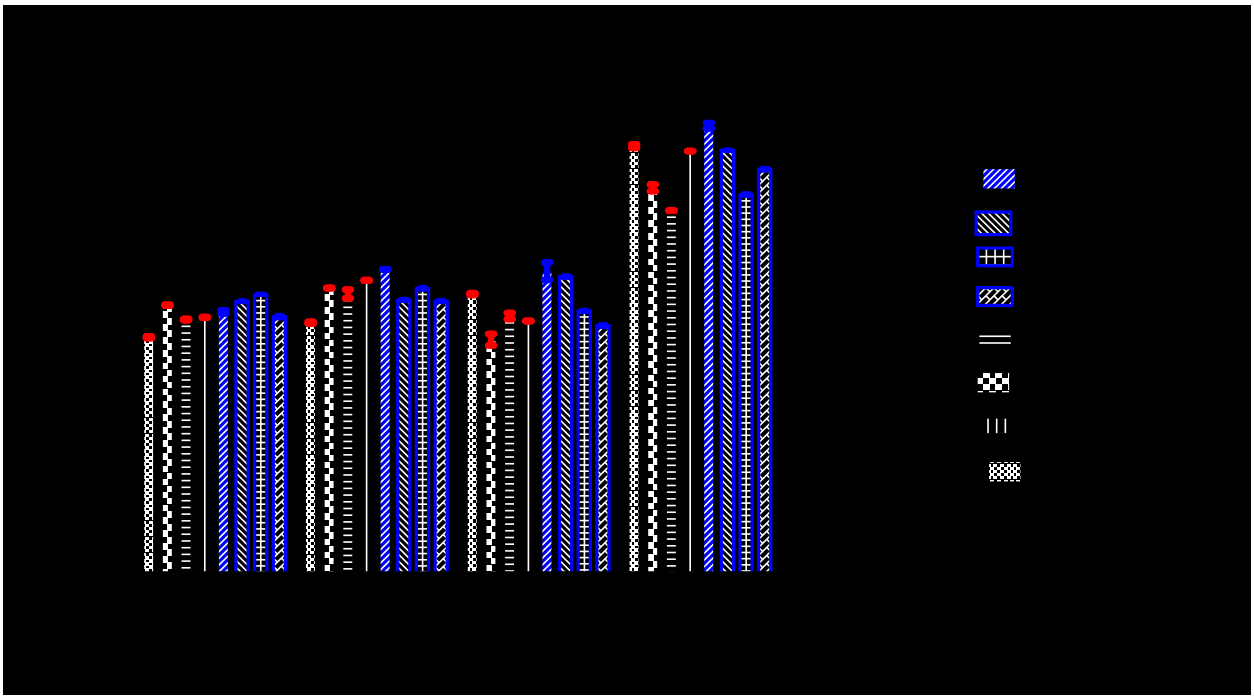


Figure 4.4.2 Effect on different CO_2 and urea on CA activity of bacterial strain *B.megaterium*.

Soluble calcium estimation

As discussed earlier that soluble calcium estimation can directly or indirectly tell the amount of calcium carbonates precipitated in the culture under different conditions. Figure 4.4.3 shows The descending trend in the conc. of Ca^{2+} in all the test sets of experimental systems. It can be seen from the table 4.4.3 that in both the sets (pH 10 & pH 11) the maximum decrement was observed in presence of urea and carbon dioxide both. In 24 hrs, the amount of Ca^{2+} reduced from 25 mM to 6.92 mM and reduced to 2.95mM till the end of 5^{th} day. Similar trend was seen in other test samples too. This clearly indicates that both urea and carbon dioxide when added together can induce the maximum amount of carbonate precipitates. A linear drop in soluble calcium indicates that with increase in time the level of calcium in the form of carbonates increases simultaneously in the culture. Till the end of fifth day the maximum drop was seen in culture with CO_2 and urea both which shows that maximum carbonates might have been formed under this state. Further the mineralogical state of test samples can be confirmed using EDX – ananlysis.

Table 4.4.3 Effect on different carbonate sources (CO_2 and urea) on the rate of soluble calcium kinetics of bacterial strain *B.megaterium*.

Soluble Ca (mM) at pH 10				
Days	**CO_2**	**CO_2 + UREA**	**UREA**	**CONTROL**
1	8.10 ± 0.02^{aB}	6.92 ± 0.04^{aC}	8.83 ± 0.03^{aA}	8.88 ± 0.04^{Aa}
2	7.37 ± 0.04^{bC}	5.82 ± 0.03^{bD}	8.15 ± 0.08^{bB}	8.55 ± 0.04^{abA}
3	7.29 ± 0.05^{bA}	5.82 ± 0.03^{bB}	6.97 ± 0.01^{cA}	7.14 ± 0.27^{bA}
4	6.56 ± 0.0^{cB}	4.96 ± 0.03^{cC}	5.13 ± 0.05^{dC}	7.16 ± 0.24^{bA}
5	4.55 ± 0.04^{dA}	2.95 ± 0.01^{dB}	4.03 ± 0.11^{eA}	4.76 ± 0.71^{cA}
Soluble Ca(mM) at pH 11				
Days	**CO_2**	**CO_2 + UREA**	**UREA**	**CONTROL**
1	7.45 ± 0.18^{aC}	6.56 ± 0.00^{aD}	8.09 ± 0.00^{aB}	8.90 ± 0.07^{aA}
2	7.36 ± 0.05^{aA}	5.15 ± 0.01^{bB}	7.41 ± 0.00^{bA}	7.27 ± 1.01^{aA}
3	6.57 ± 0.01^{bA}	5.13 ± 0.05^{bB}	6.57 ± 0.01^{cA}	5.20 ± 0.06^{bB}
4	5.22 ± 0.08^{cA}	5.04 ± 0.00^{bB}	5.03 ± 0.02^{dB}	5.16 ± 0.00^{bAB}
5	4.10 ± 0.21^{dA}	4.01 ± 0.09^{cA}	4.12 ± 0.18^{eA}	4.31 ± 0.30^{bA}

Values are mean ± SD (n=3). Values bearing same letters (lowercase) in a column, and same letters (uppercase) in a row are not significant at P<0.05, values are mean ± SD (n=3)

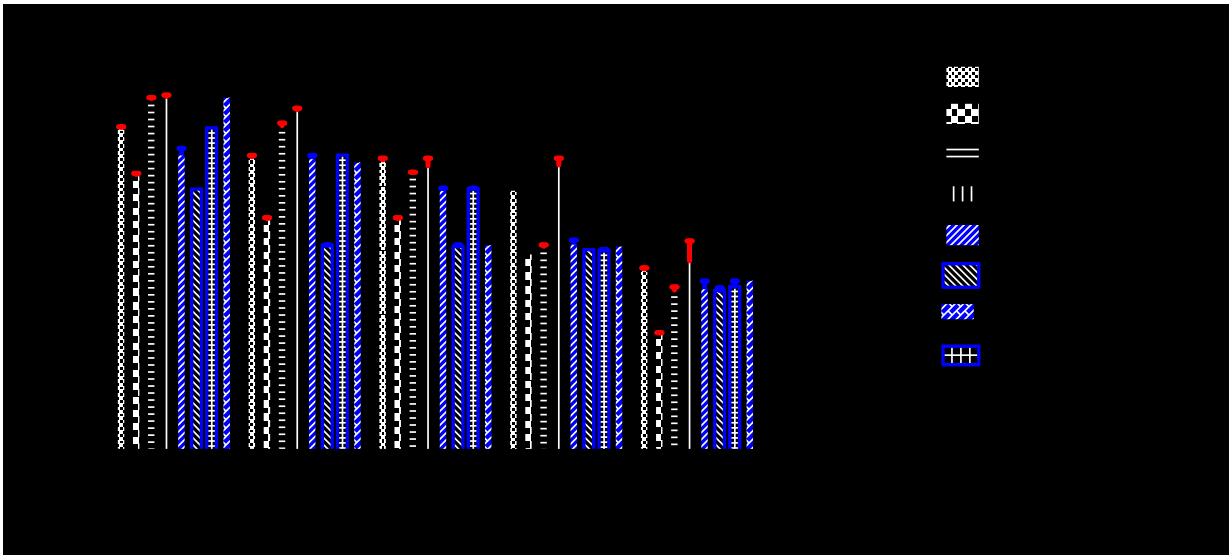


Figure 4.4.3 Effect on different CO_2 and urea on soluble calcium trend with increase in time of bacterial strain *B.megaterium*.

4.4.1 XRD and SEM-EDX analysis

SEM analysis of precipitates formed in presence of bacteria and carbon dioxide in *in-vitro* experiments, shows the presence of different carbonate polymorphs with rod shaped bacteria embedded in between. XRD analysis showed that majority of the carbonate deposits were Calcite, Aragonite and small percentage of Vaterite. Mineral constituents of microbially induced calcium carbonate precipitation were further characterized by Energy dispersive x-ray analyzer. X-ray Diffraction pattern shows that calcium carbonate precipitate is composed of calcite and vaterite crystals.

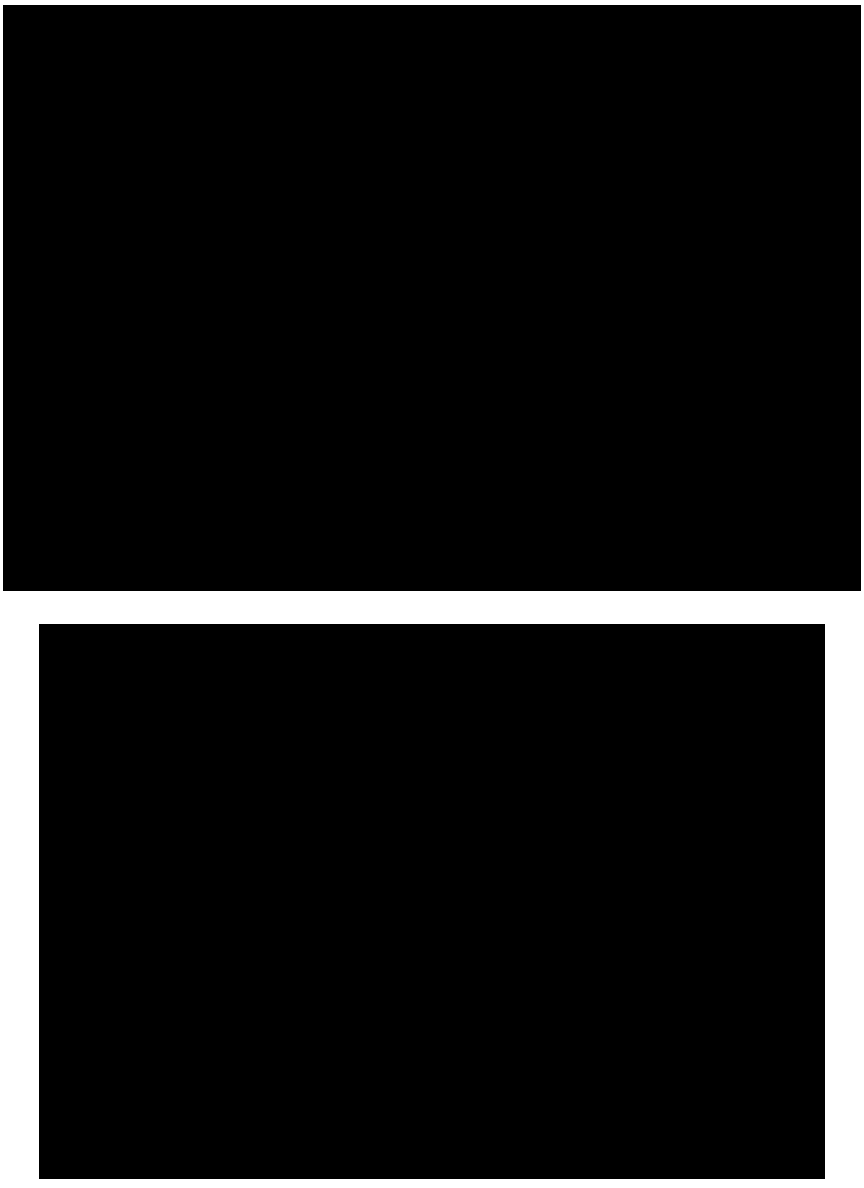


Figure 4.4.4 XRD Pattern of calcium carbonate precipitates at a continuous scanning rate of 2°/min (a) in the presence of 99.5% CO2 B) in the presence of urea and CO_2.

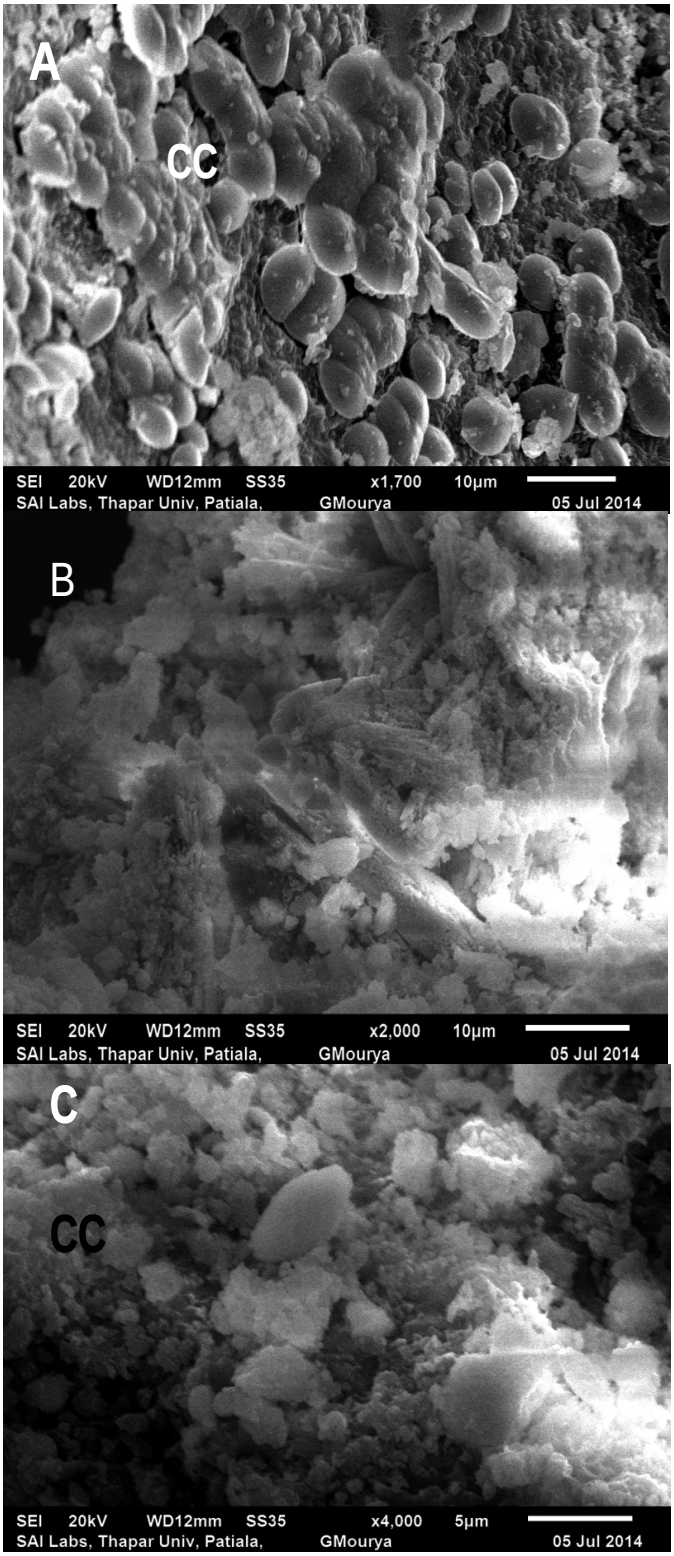


Figure 4.4.5 Scanning electron micrographs of carbonate precipitates formed under laboratory conditions via influx of pure carbon dioxide a) tightly bound spheroidal crystals b) polyhedral and c) aragonite form of crystals.(CC = Calcium carbonate crystals)

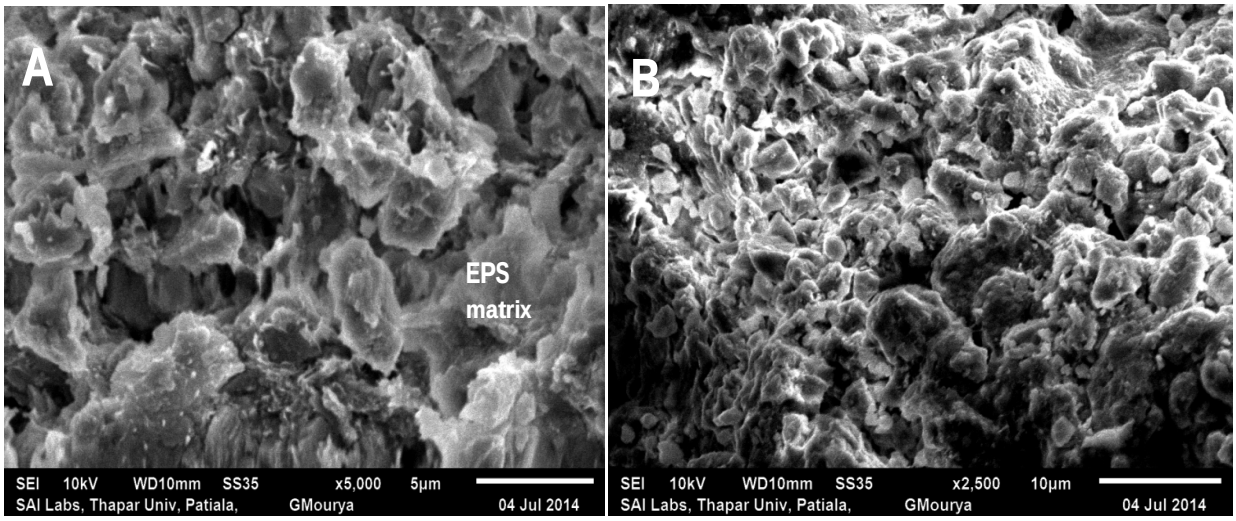


Figure 4.4.6 SEM images of calcium carbonate crystals precipitated by *B.megaterium* in the presence of CO_2 and urea both. A) Fibrous gel like matrix formed by EPS deposition over crystals, B) numerous fluorite type of crystals (cubic) can be seen**.**

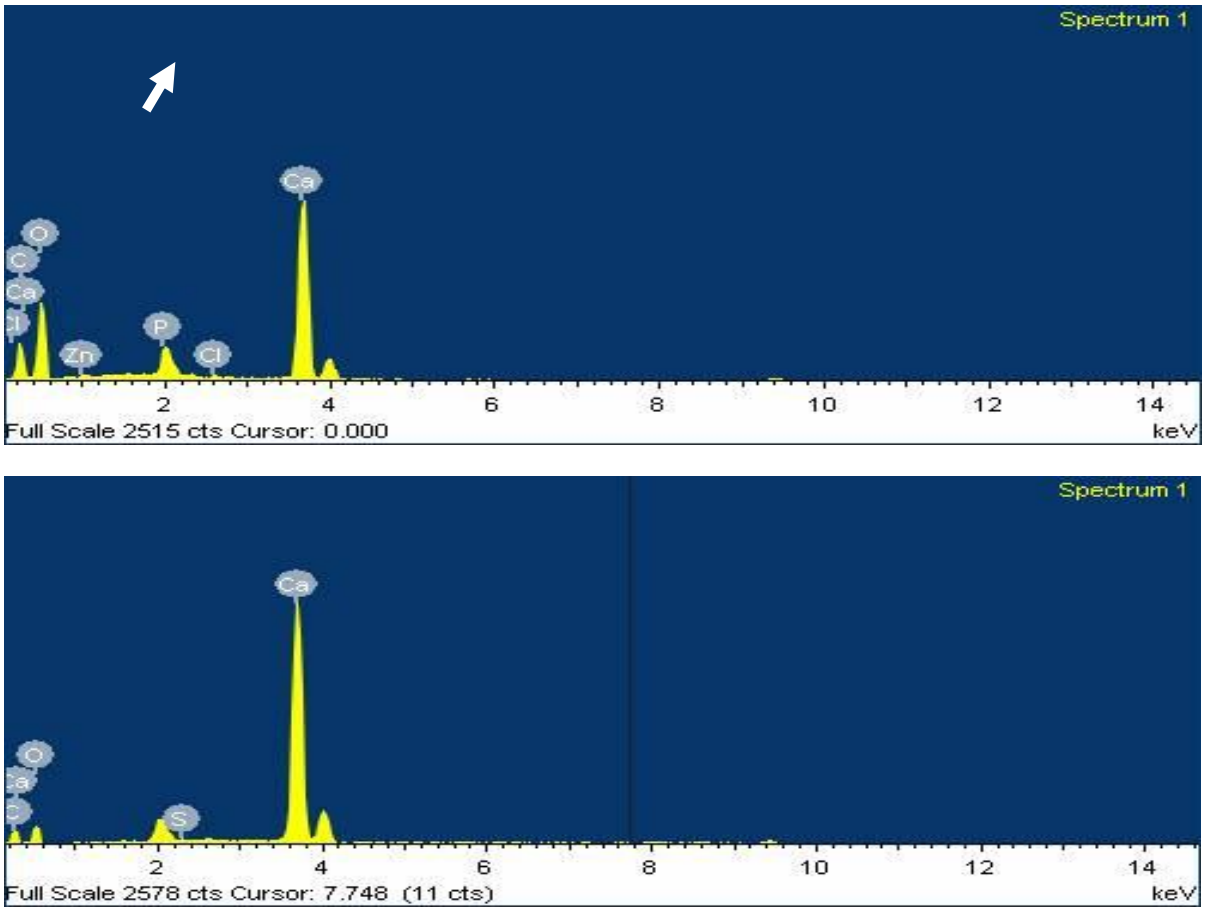


Figure 4.4.7 EDX spectrum of microbial precipitation in *in-vitro* conditions in presence of (a) CO_2, (b) in presence of urea and CO_2 both.

EDX spectrum of the microbial precipitations shows the presence of Ca and precipitation was inferred to as calcite ($CaCO_3$ crystals). In Fig.4.4.4 higher peak of calcium has been obtained which confirms the combined effect of urea and CO_2 than CO_2 alone as in (a)

4.5 Evaluation of microbes for enhancement of durability properties of cement mortar

The limited diversity of the bacterial community in extreme environments like cement or calcareous sludge is not surprising, as these extreme conditions can favour the growth of only few species. The enzyme like urease which these microorganisms produce, precipitate calcium carbonate as one of the main components of concrete, thus reffered to as microbial concrete enzyme. For remediation of building structure, this enzyme needs to be active and stable in alkaline environment (pH 9-11). (Stocks-Fischer *et al*.,1999). Urease in general are not stable under these conditions and therefore emphasis has been given on new sources.

Microbial calcification in cement mortar cubes

Bacterial mediated calcite precipitation is confirmed from the results of present study. 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 surface. Calcite precipitation occurs predominantly in the areas exposed to the air. It is mainly due to the fact that facultatively anaerobic *Bacillus* cells grows at a higher rate in the presence of oxygen and consequently induces the active precipitation of $CaCO_3$ around the surface area (Stocks- Fischer *et al.*,1999). The biodeposition treatment resulted in the formation of a whitish layer on the surface of blocks which was attributed to the formation of carbonate precipitates, while in case of control blocks no deposits were seen.

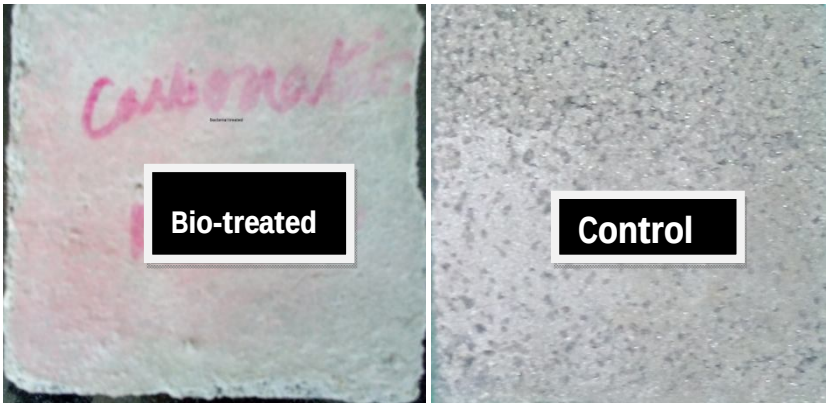


Figure 4.5.1 Calcium carbonate precipitation on bio-treated mortar cube. The biodepostion treatment caused by culture curing resulted in the formation of whitish ($CaCO_3$) layer over the surface of bio-treated specimen (left) with respect to control (right).

4.5.1 Compressive strength based on MICCP

Based upon its higher urease production, *Bacillus* species (*B.megaterium*) was chosen to study the durability parameters. Biocementation was clearly seen on mortars treated with bacterial cells. Table4.5.1 summarizes the 3, 7 and 28 days compressive strength of cement mortar cubes containing bacterial cells and without bacterial cells. Bacterial mixed cubes were made in two sets, one set was cured in bacterial culture, and one was sprayed with nutrient broth daily for 28 days.

Figure 4.5.2 Automatic compression testing machine, COMPTEST 3000.

The highest compressive strength was obtained with mortar cubes that were cured in bacterial culture for 28 days. The control specimens were prepared using water instead of bacterial culture. The maximum compressive strength was obtained in bacterial cubes dipped (cured) in bacterial culture(1%). It showed 36.6% improvement in compressive strength (41.1MPa) with respect to control specimen (30.087MPa) whereas, improvement in bacterial cubes cured by spraying nutrient broth was 32.20% (39.77MPa) compared to control specimen (30.1MPa).

Table 4.5.1 Compressive strength of different sets of mortar cubes at interval of 3, 7 and 28 days.

	Compressive strength (MPa)		
Days	Control(water mix)	Bacteria cured in culture	Bacteria sprayed with NB
3	26.02 ± 3.4^{A}	29.45 ± 3.6^{A}	21.11 ± 2.8^{A}
7	31.40 ± 2.9^{A}	35.65 ± 3.9^{A}	29.16 ± 3.0^{A}
28	30.08 ± 3.7^{B}	41.10 ± 5.2^{A}	39.77 ± 3.7^{AB}

Here values bearing same letter in a row are not significant for P< 0.05 Values are mean ± SD (n=3)

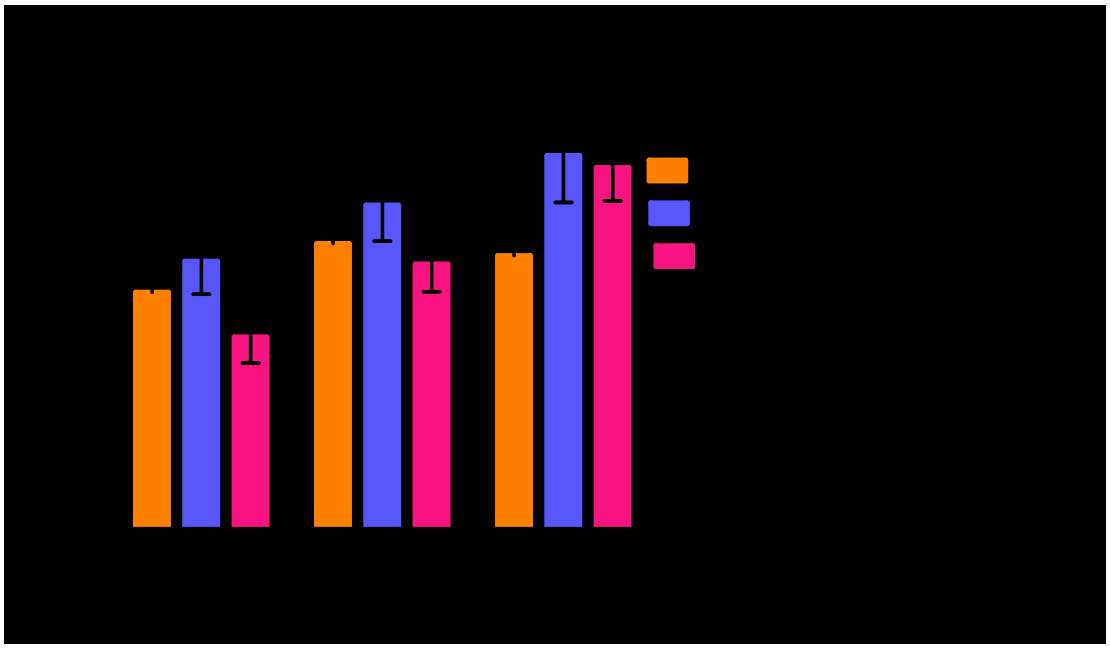


Figure 4.5.3 Effect of *B.megaterium* grown in nutrient media(NB) on the compressive strength of cement mortar cubes cured for 3,7 and 28 days.

It can be concluded that *Bacillus megaterium* significantly improved the strength of mortars. This improvement in compressive strength is probably due to deposition of calcium carbonate on the microbial cell surface and within the pores of cement sand matrix, which plug the pores within the mortar (Ramakrishnan *et al.,* 1998, Ramachandran *et al.,* 2001).

4.5.2 Water absorption

The decrease in permeability of mortar specimen treated with bacteria could be seen from the water absorption test. Figure 4.5.3 shows the influence of the microbially induced calcite precipitation on the water absorption rate in mortar cubes. Over a period of 168 hours, the cubes treated with *Bacillus megaterium* cells, absorbed nearly four times less water than the

control cubes. The presence of bacteria resulted in a significant decrease of the water uptake compared to untreated specimens (control). The deposition of a layer of calcium carbonate crystals on the surface resulted in a decrease of the permeation properties. The lower permeability of the biomineralized cubes were probably due to a denser interfacial zone formed because of calcite precipitation between the sand and the cement matrix compared with that of the control. Decrease in porosity helps to lessen the ingress of pollutants, harmful gases and chlorides. Nemati and Voordouw (2003) noticed a decrease in the permeability of sandstone cores after injecting calcium carbonate forming reactants.

In this study, control and biotreated specimens after 28 days of curing were tested for water absorption test. Two sets were made.

Set 1: First in which we compared the bacterial cubes cured in culture with water cubes cured in NB as control

Set 2: Second, in which we compared bacterial cubes sprayed with NB with water cubes sprayed with NB.

Q/A was calculated for different time periods and graph was plotted between water absorption rate (kg/m^2) and time (hours). Q is the amount of water absorbed (cm^3), and A is the crossectional area (cm^2)

Table 4.5.2 Effect of bacterial isolate *B.megaterium* treatment on the rate of water absorption by cement mortars at different time intervals.

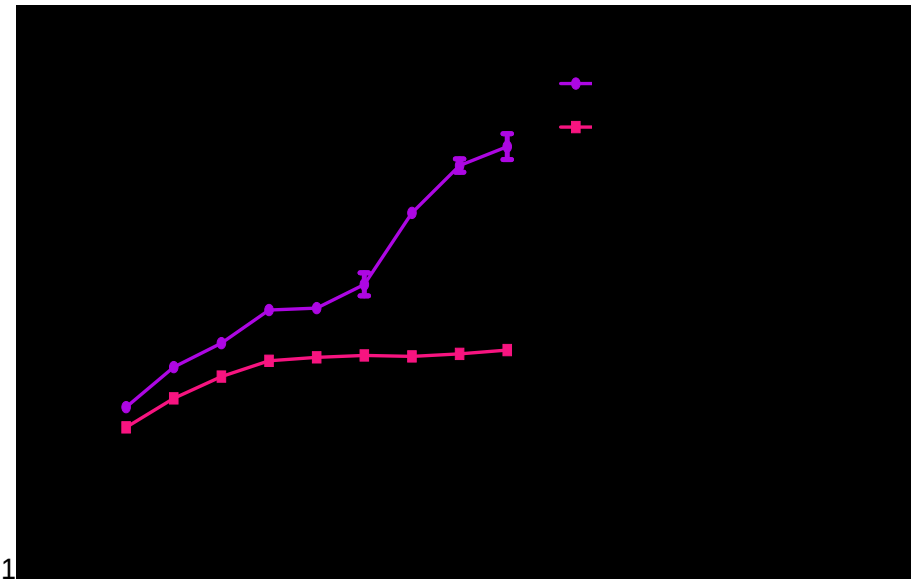
Set 1:

	Time (hrs)								
Treatment	1	4	8	24	36	48	120	144	168
Control	0.47±0.2	1.10±0.5	1.48±0.8	2.0±0.7	2.04±0.3	2.41±0.3	3.54±0.2	4.29±0.1	4.5±0.3
bacterial	0.15±0.7	0.60±0.1	.95±0.1	1.21±0.6	1.26±0.4	1.29±0.3	1.28±0.3	1.32±0.8	1.37±0.7

Set 2:

	Time (hrs)							
Treatment	1	4	8	24	36	48	120	144
Control	0.86±0.5	1.73±1.9	1.90±.14	2.30±.5	2.90±0.14	3.13±.17	3.17±.2	3.22±.4
bacterial	0.39±0.4	0.59±0.6	0.71±0.7	0.76±0.8	0.77±.7	.89±.12	0.93±.13	0.94±.14

Set 1



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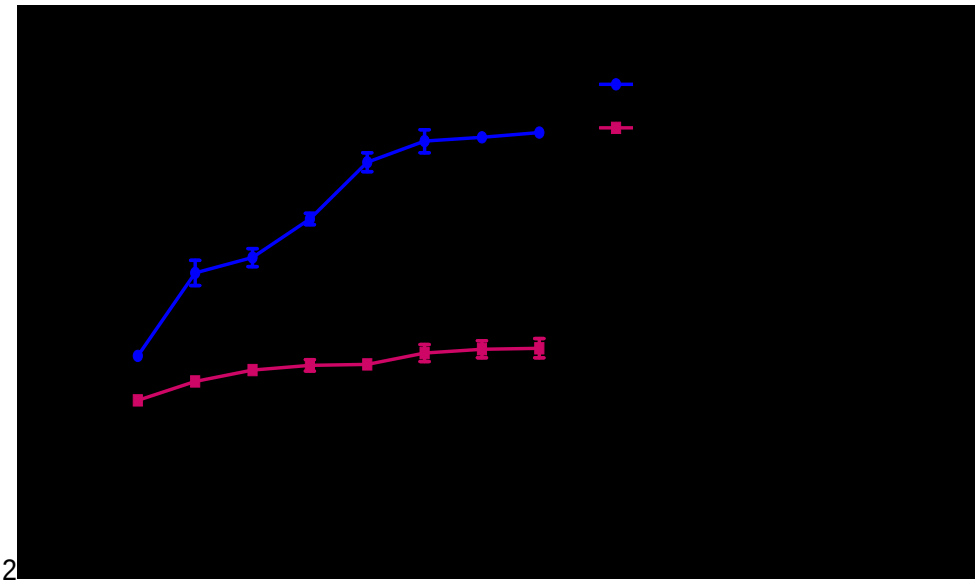

Figure 4.5.4 The influence of MICCP on the rate of water absorption versus time for mortar cubes prepared with *B.megaterium.*

4.5.3 SEM-EDX analysis

Calcite precipitation in cement mortar specimens by B. megaterium was visualized by SEM analysis. A dense growth of calcite crystrals embedded with bacterial cells was observed in the specimens(Fig 4.5.5). On closer observations, rod shaped bacteria associated with calcite crystals were found. This deposition serves as a barrier for the entry of harmful substances, and thus improves its impermeability.

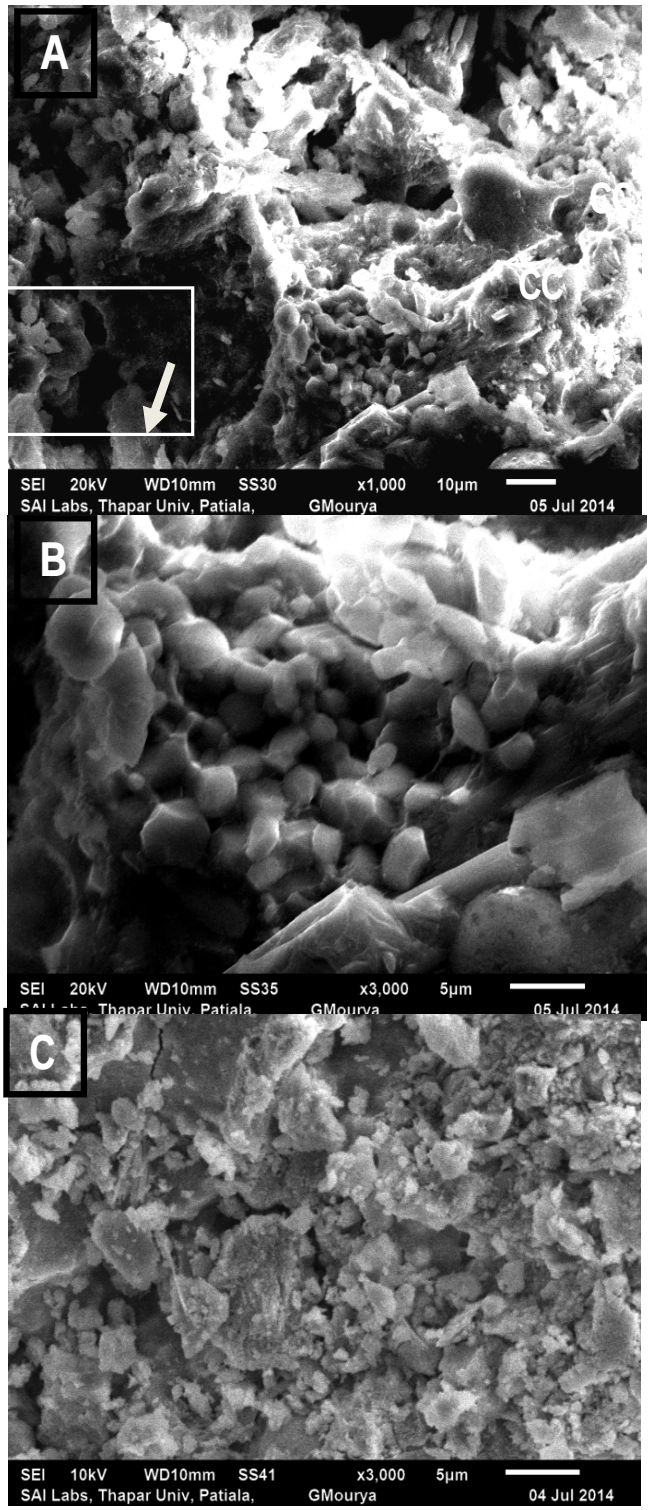

Figure 4.5.5 SEM images of (A) Biotreated specimens, shows the presence of rhombohedra and hexagonal crystals (B) Enlarged section of crystals, which also shows the presence of rod shaped bacterial impressions (BI), (C) Non treated water cured sample of cement mortar shows some imprints of carbonate crystals.

The presence of crystalline calcite associated with bacteria indicates that it served as a nucleation site during the mineralization process (Stocks-Fischer *et al*., Achal *et al.,* 2010b). Based on these results it can be concluded that biocalcification by *Bacillus megaterium* plays an important role in enhancing the durability of any building materials or structures.

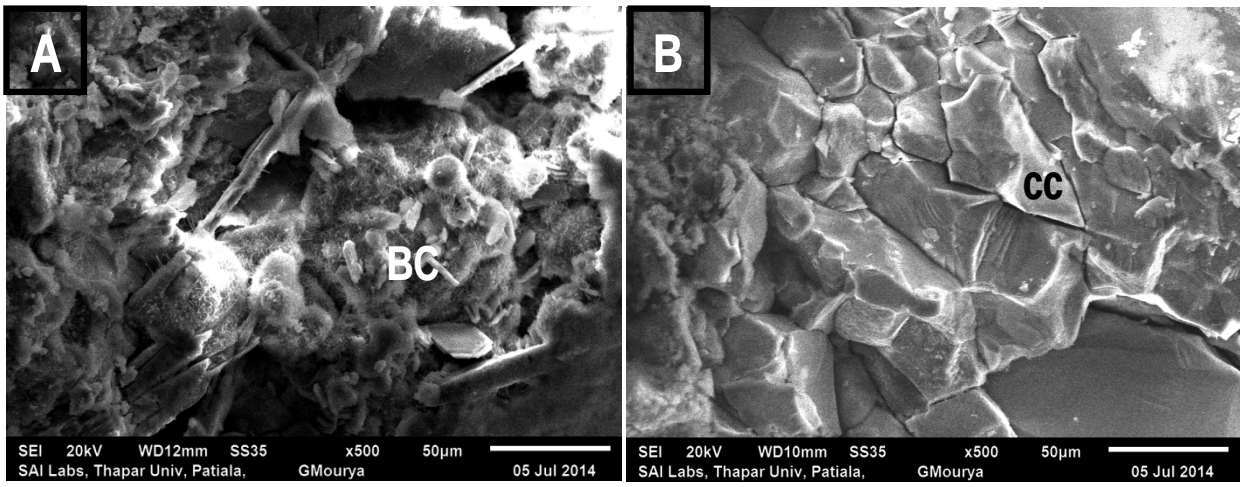

Figure 4.5.6 SEMicrographs of cement mortar (biotreated) cured by spraying Nutrient medium. (A)Shows the presence of fibrous material inside the pores and formation of bacterial calcite (BC), (B) Shows the formation of rhombohedra crystal layer over the surface of cement- sand matrix.

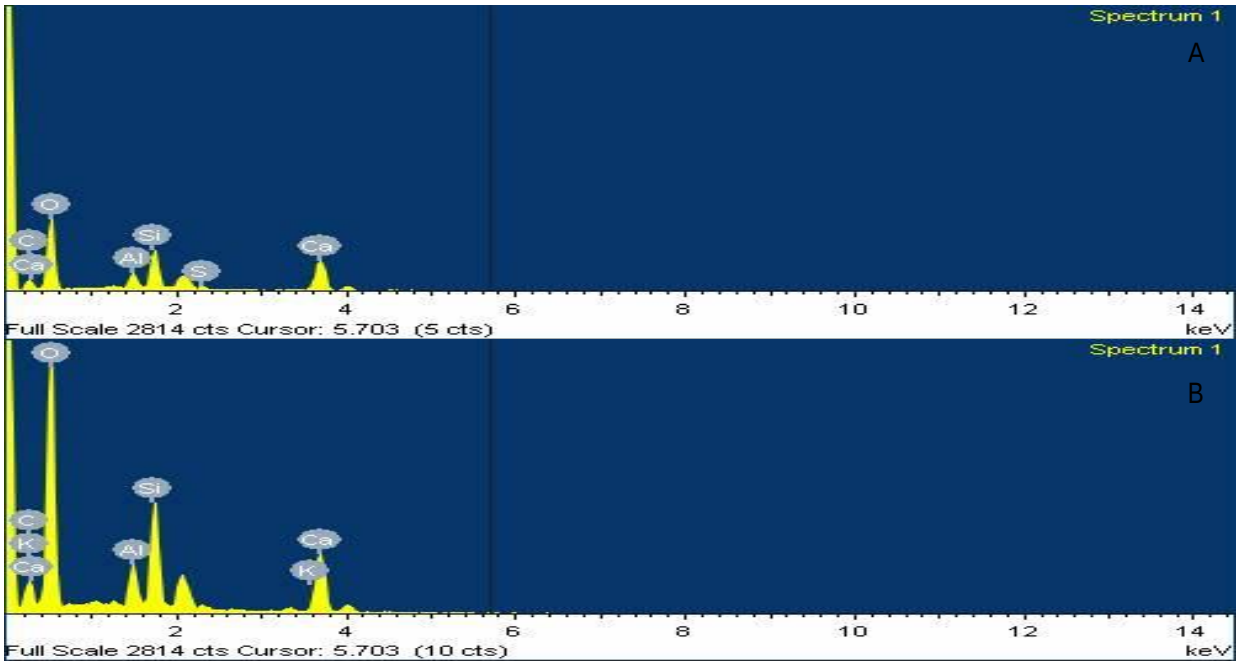

Figure 4.5.7 EDX – spectrum of a) biotreated mortar specimen b) control specimen. Biotreated specimen shows high level of calcium deposition than control sample, which confirms the presence of calcium carbonate deposits.

4.5.4 XRD analysis of cement mortar specimens

The high peaks of the designated elements indicate the presence of calcium silicate hydrates generated in the vicinity of calcium silicates after 24 hours of water curing. Figure 4.5.7 (A) shows the X- ray Diffraction pattern of biologically treated mortar specimens cured in bacterial culture. With reference to control, water mix specimen (B) Biotreated one shows the presence of carbonate precipitation in the form of calcite, while control specimen shows almost 80% of silicates (quartz).

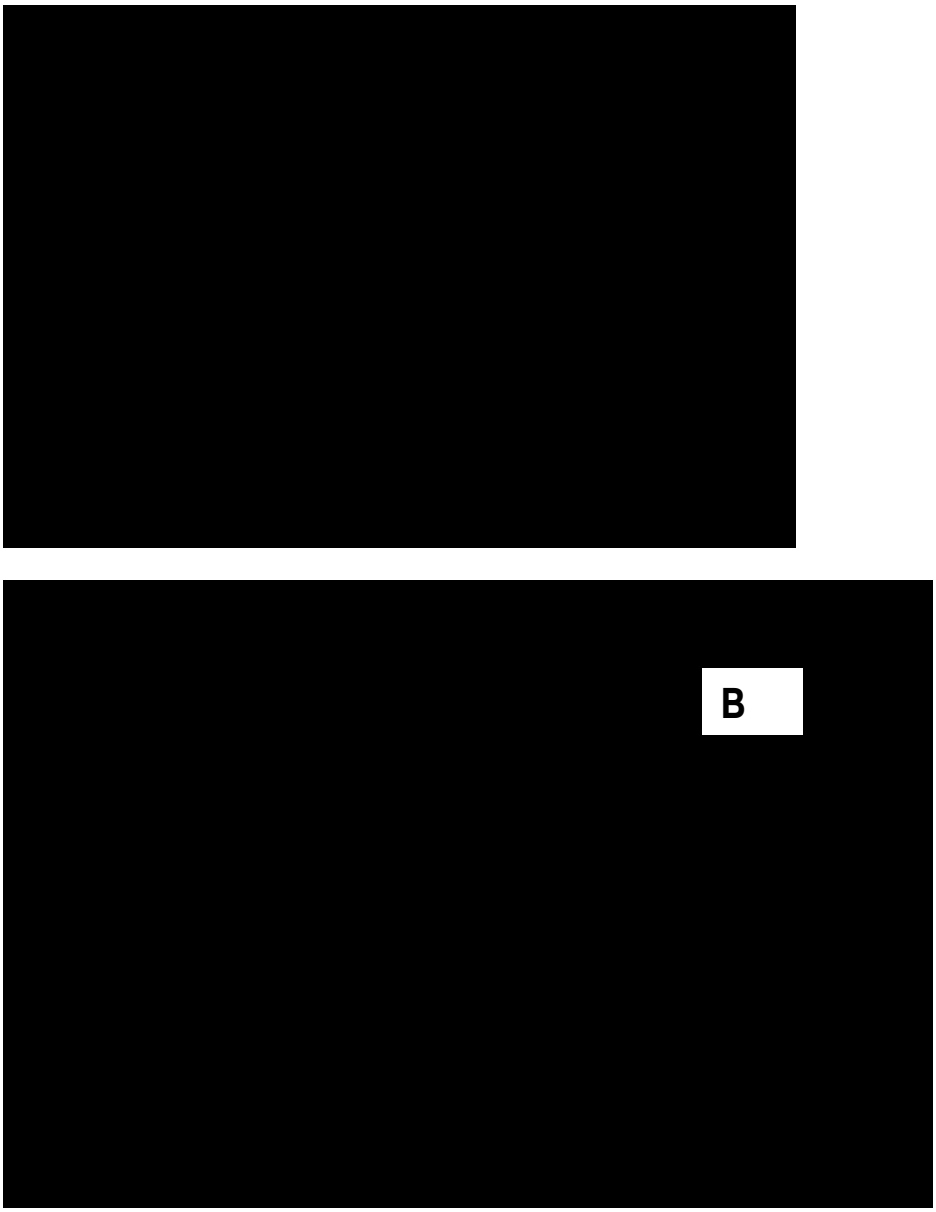

Figure 4.5.8 X-ray diffraction pattern of (A) control specimen (B) biomediated calcium carbonate precipitation in cement mortar.

4.6 Effect of Carbonation on durability of cement mortar

The rate of Carbonation increases with an increase in carbon dioxide concentration, especially for concrete specimens with higher water/binder ratios, the transport of it taking place through the pore system in hardened cement paste.

Carbonation reaction occurs when CO_2 in air dissolves in the pore water of the mortar and reacts with calcium hydroxide. This reaction results in the precipitation of calcium carbonate crystals that build up the microstructure and the pore structure of mortar. This eventually describes the certain properties of cement mortar such as mechanical strength, moisture transport and diffusivity of CO_2. The later stage of carbonation is controlled by the CO_2 diffusion through the sample thickness, because the evaporation of water creates open pore space allowing diffusion paths for carbon dioxide. With increase in CO_2 uptake, calcite precipitation accelerates.

4.6.1 Compressive strength of carbonated cubes

The compressive strength for different mixes (carbonated and noncarbonated) at 7 days of curing are presented in table 4.6.1. It depicts that the compressive strength of carbonated specimens slightly increased in comparison with non-carbonated specimens. In the present study, results showed that bacterial carbonated specimens have slightly less compressive strength than control carbonated (water mix) specimens.

Water mix carbonated cubes have 13% more strength compared to bacterial carbonated specimens. This could be possible due to the reason that, carbonate precipitates formed over the bacterial cells might have not allowed more amount of carbon dioxide to penetrate inside. Bacteria served as nucleation sites for calcium carbonate crystals and might not allow the higher ingress of CO_2. We can also see that bacterial carbonated specimens have 38% (35.61 MPa) more compressive strength than bacterial hydrated specimens and the reason that carbonated specimens have much higher compressive strength than hydrated specimens, may be that calcium carbonate occupies a greater volume than calcium hydroxide, and the surface porosity of carbonated concrete is reduced. Figure 4.6.1 shows the effect of carboantion curing on bacteria mix and water mix cubes with reference to their noncarbonated controls.

Table 4.6.1 Compressive strength of carbonated and hydrated specimens.

Sr.no	Treatment	Compressive strength (MPa)
1	BM + CO_2 curing	35.61 ± 2.8^{ab}
2	BM + hydrated	25.74 ± 2.1^{bc}
3	Water mix+ CO_2 curing	40.90 ± 8.2^{a}
4	Water mix + hydrated	19.86 ± 3.0^{c}

Values bearing different letters in the column are significant at $P < 0.05$ Values are mean ± SD (n=3).

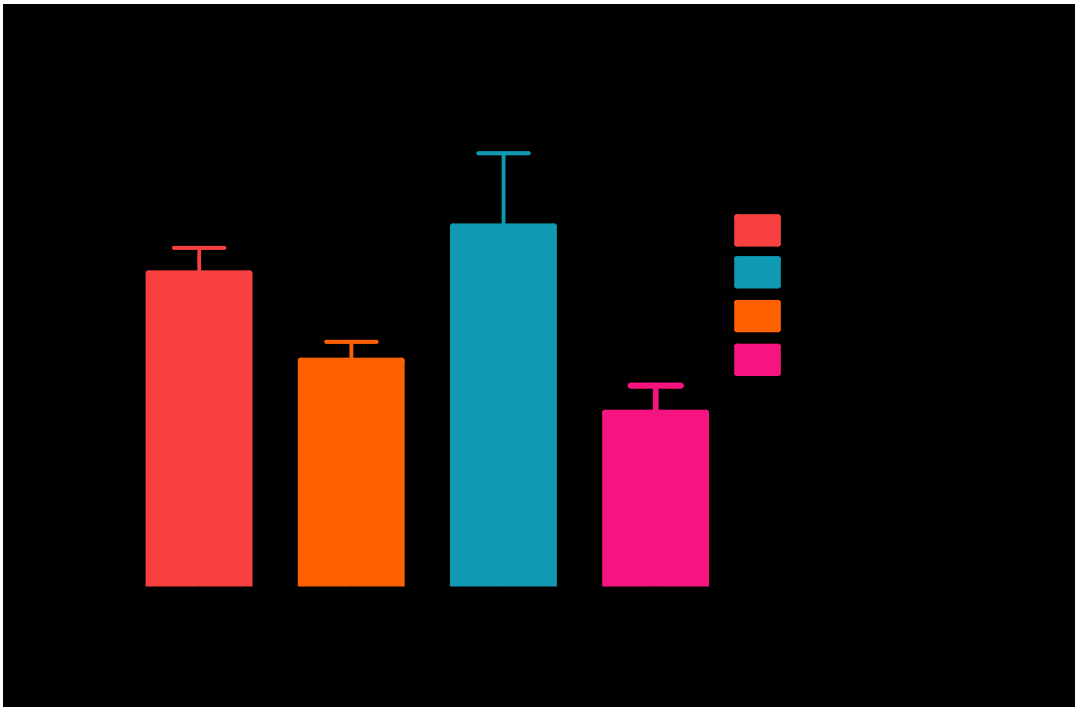

Figure 4.6.1 Effect of Carbonation on the Compressive strength of bacterial and water mix mortar specimens.

4.6.2 Water absorption test

Water absorption test was performed in the same manner as described above in section 3.6.4. Initial carbonation curing is more beneficial than late carbonation after hydration. Concrete carbonated by this manner has more strength and exhibits an enhanced resistance to permeation, sulfate attack and freeze thaw damage. Subsequent hydration after early carbonation attributes significantly to late strength gain and maintains concrete alkalinity above the threshold value. From figure 4.6.2 it can be seen that Bacterial carbonated specimens have

almost 2 times less permeation rate than bacterial hydrated specimens, and water mix carbonated specimens have almost 3 times less permeation rate than hydrated specimens, which clearly explains the fact that carbonation resulted in mineralization, precipitation of carbonates thus reducing permeability.

Table 4.6.2 Effect of Bacterial carbonation on the rate of water absorption of cement mortar specimens at different time intervals

	Time (hrs)							
Treatment	1	4	8	24	36	72	120	144
BM (CO2)	0.41±0.02	0.57±0.07	0.63±0.01	0.76±0.07	0.76±0.04	0.78±0.04	0.78±0.03	0.83±0.07
BM (H2O)	0.29±0.06	0.60±0.11	0.81±0.03	0.98±0.02	1.08±0.02	1.13±0.06	1.20±0.04	1.34±0.02
WM (CO2)	0.25±0.04	0.40±0.02	0.50±0.01	0.58±0.08	0.64±0.05	0.69±0.09	0.74±0.01	0.76±0.01
WM (H2O)	0.11±0.07	0.48±0.09	0.79±0.09	0.84±0.01	1.97±0.03	2.16±0.07	2.63±0.46	3.11±0.14

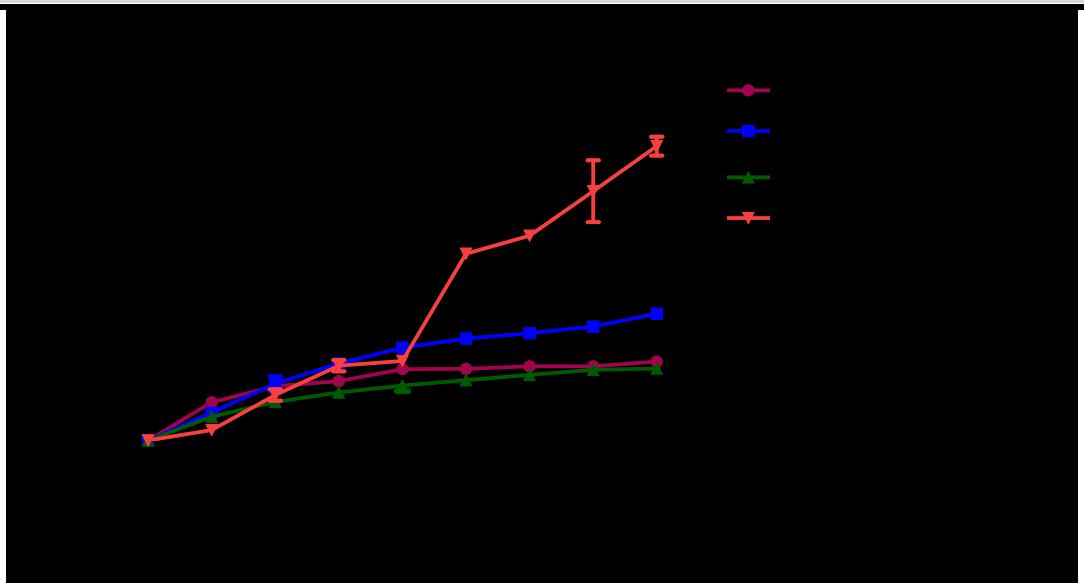

Figure 4.6.2 Water absorption rate of different carbonated and non carbonated specimens.

4.6.3 SEM-EDX and XRD analysis of Carbonated samples

Since carbonated cement mortar had shown an strength gain that was significantly higher than the hydration reference, it is implicated that C-S-H structure formed due to carbonation is stronger owing to the silica dimmers and linear chains integrated with calcium carbonates. SEM analysis shows that, because of carbonation in water mix carbonated specimens, calcium content in C-S-H is reduced, leading to change in silica structure in combination with silica dimers leading to linear parallel chains as shown in figure 4.6.3 with no cross linking, but in

bacterial carbonated specimens, calcium content is relatively higher therefore it shows the formation of carbonate matrix in addition to parallel crystals.

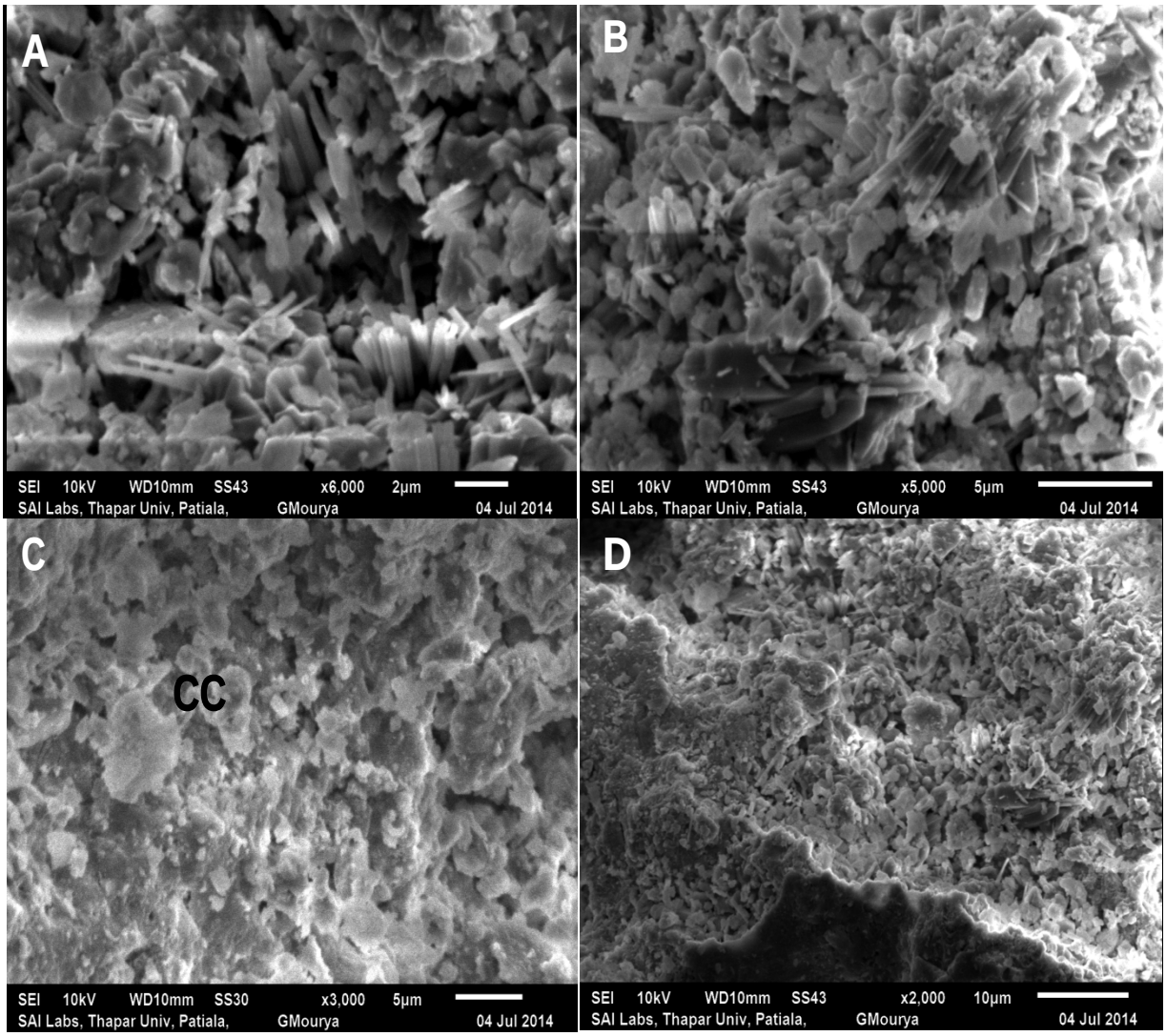

Figure 4.6.3. SEM images of carbonated water mix specimens and carbonated bacterial mix specimens. A) Introduction of CO_2 lead to the formation of linear chains of silica in water mix specimens while B) shows the effect of carbonation on bacterial mix specimens in the absence of urea, parallel crystal morphology can be seen. C,D) carbonate crystals in cement sand matrix.

Quantitative analysis of the chemical composition of samples was also conducted on carbonated specimens using an energy dispersive X- ray analyzer (EDX) at an accelerating voltage of 15 KV. This was done to see the formation of different chemical constituents formed due to influx of CO_2.

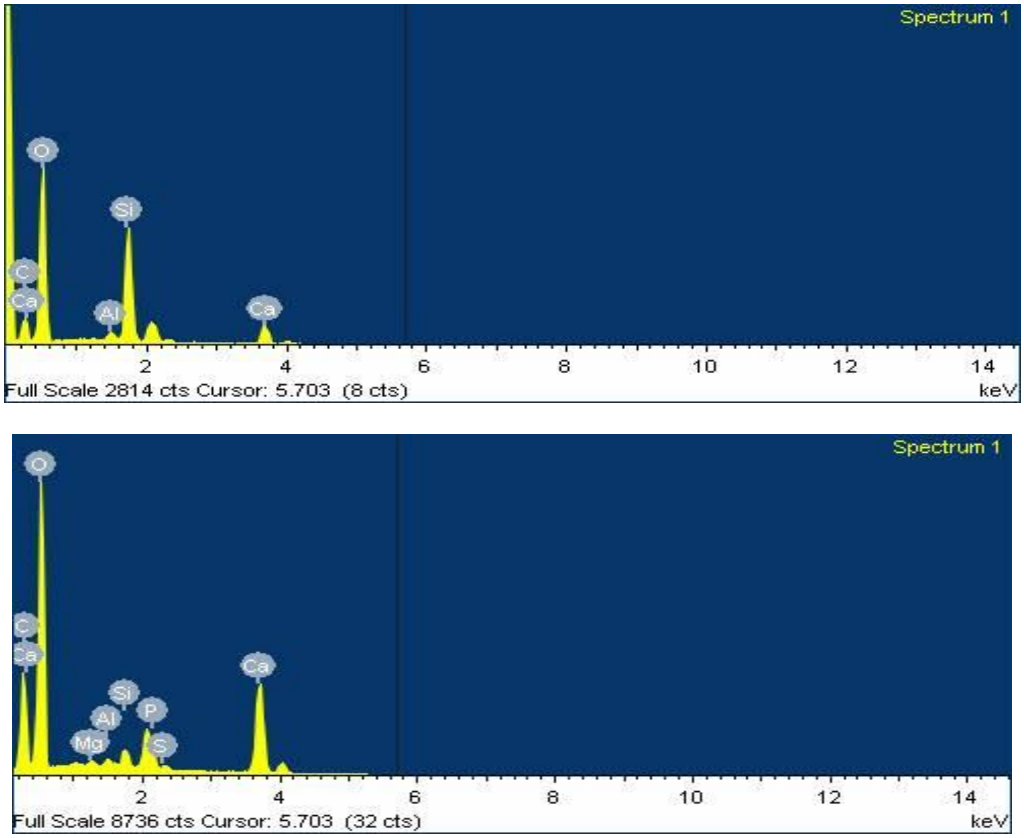

Figure 4.6.4 EDX-spectrum of water mix carbonated specimens and bacterial mix carbonated specimens.

Figure 4.6.5 X-ray diffraction pattern of carbonated specimens. Peaks of Carbon forms indicates the presence of carbon.

4.7 Polymorphism in carbonate crystals

The ultrastructural revelations of the polymorph of crystals suggests that the strain *B.megaterium* used for this study was capable of depositing calcium carbonate, with distinct variations in the quantity and crystal shape of precipitated $CaCO_3$ under different conditions. In *in-vitro* conditions, in lab conditions carbonates precipitated via influx of CO_2, showed different morphologies of carbonate crystals. A huge clustering of spherical crystals, dispersed spherical $CaCO_3$ aggregate particles with rod shaped bacteria embedded in bacteria was seen. Carbonated precipitated via influx of CO_2 and urea both shows the presence of crystals bonded with the extra polymeric substances produced by the bacteria. In *ex-vitro* (field studies) conditions, Biotreated specimens shows numerous morphologies of crystal organization in comparison to untreated control specimens. Biologically treated specimens showed different polymorphs of Calcium carbonate like vaterite (hexagonal), rombohedral (calcite). Biologically treated specimens cured by spraying bacterial media, nutrients shows numerous crystal morphologies and fibrous gel like matrix with bacteria embedded within. Carbonated specimens shows the presence of spiky, sharp needle like crystal morphology. Therefore it can be interpreted that, under different set of optimized conditions and environment, a specific bacteria can produce different types of crystal morphotypes.

Chapter 5

Summary

MICCP via urea hydrolysis is an efficient and straightforward biochemical process used commonly for the precipitation of carbonates. But due to certain disadvantages of urea, there is a need to develop compatible and eco-friendly process which could replace urea in MICCP and produce efficient amount of precipitates. Therefore, our main objective was to establish a carbonation process which in presence of bacteria could enhance the durability properties of cement mortar by accumulation of carbonate precipitates. Furthermore, recommendations to improve the feasibility of MICCP via CO_2 flux in *in-situ* conditions were postulated.

As bacteria acts as catalyst, and biomineralization is based on the ability of bacteria to promote the precipitation of carbonates, *Bacillus* sp. *Bacillus megaterium*, isolated from from alkaline soil, capable of producing high urease and carbonic anhydrase activity was selected for further studies. A suitable mineral precursor compounds which enhance the activity of enzymes and mineral accumulation needs additionally to be incorporated in the matrix to provide highly efficient process. The processs of bacterial mineral formation from CO_2 in presence of calcium source represents an alternative mechanism to the urease- based system investigated in previous studies. In contrast to the latter mechanism, the CO_2 utilization do not result in massive amounts of ammonia which drastically increases the risk of reinforcement corrosion (Neville., 1996) and degradation of the concrete matrix particularly when further oxidized by bacteria to yield nitric acid (Diercks *et al.,* 1991).

In our present study, a novel technique to use pure carbon dioxide to induce calcium carbonate precipitates with the aid of bacteria has been explained. It is clear that flux of CO_2 into the medium reduces the pH state of the system and prevails acidic conditions. And for carbonate precipitation to take place pH of the medium should be 9-11, pertaining to alkaline environment. Therefore in *in-situ* conditions we optimized a particular conc. of CO_2 which could induce optimium amount of calcium carbonate precipitates. To do so, we studied the effect of different concentration of CO_2 on various parameters (pH, CA, Ca^{2+} and carbonate precipitation) of MICCP. We optimized a particular conc. of CO_2 i.e., 0.112g/L which can replace urea and produce optimum results. As a general case we also studied the effect of MICCP via urea hydrolysis on durability properties of cement mortar. A significant improvement in strength was demonstrated after the treatment. Various physico-mechanical properties, compressive strength and water absorption, of the bacteria modified mortar were improved due to the deposition of the calcite material by the bacterial activity. Almost 37% enhancement in

compressive strength was achieved with respect to control specimens. The water absorption rate decresed almost 4 times in bioremediated mortar cubes in comparison to control mix. The decrease in water absorption after a biodeposition treatment is for a large part attributed to the physical obstruction of pores, rather than to stable presence of newly formed calcite layer.

Durability is a major concern for concrete structures for concrete structures exposed to aggressive environments. Many environmental phenomenon are known to significantly influence the durability of concrete structures. Carbonation is one of major factors to cause structure deterioration, mainly in reinforced structure. Carbonation induced corrosion can increase crack development and can decrease concrete durability, but in many cases carbonation can yield significant positive results. It is known that carbonation reduces the pH value and destroys the passive film around the steel, but it also seems to densify concrete surface and reduces chloride ion permeability, reduces surface porosity and hence sorptivity in concrete. In the present Study carbonation of bacterial mix cubes were carrired out in controlled carbon dioxide chamber connected to CO_2 cylinder with constant pressure maintained inside the chamber. The carbonation process proceeded for 7 continuous days and significant results were seen. Carbonation of water mix cubes achieved 13% more increment in compressive strength carbonated bacteria mix cubes on subsequent carbonation. This could be possible due to the reason that carbonate precipitates formed inside or calcite layer formed on the surface of cube might not allow more amount of CO_2 ingress. In water mix, specimens CO_2 curing allowed crosslinking between cement sand particles and hence increase the strength and impermeability of mortars. SEM analysis further shows the formation of parallel silica chains in water mix carbonated specimens which could be a reason of enhancement of strength properties. Results confirmed that CO_2 curing process can cause sufficient carbonate precipitation and densify the sand cement matrix, and cause noticeable enhancement in durability of cement mortar.

Chapter 6

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