Biomineralization using S*ynechococcus pevalleikii* and its applications in building material

A Thesis

Submitted in the partial fulfillment of the requirement

for the award of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

Under the supervision of:

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Candidate's Declaration

I hereby declare that the work which is being presented in this thesis "**Biomineralization using *Synechococcus pevalleikii* and its applications in building material**" submitted by me for the award of the degree of **Master of Technology** 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.S. Reddy, Professor, Department of Biotechnology, Thapar University, Patiala, Punjab, India. 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 in India or Abroad.

Tanul

(Tanul sachdeva)

Place: Patiala

Date: 21, Aug, 2017

CERTIFICATE

Certified that the thesis entitled '**Biomineralization using *Synechococcus pevalleikii* and its application on building material**' submitted by Ms. **Tanul Sachdeva** (601504012) in partial fulfilment of the requirement for the award of the degree of **Master of Technology** in Biotechnology in the Department of Biotechnology, Thapar University, Patiala, Punjab is the record of candidate'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 award of any degree.

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Abstract

Concrete is a broadly utilized building material with a worldwide generation increasing by 2.5% every year and anticipated that would achieve USD 954.7 billion by 2024. However, concrete has a limited lifespan and can be deteriorated easily. Microbially induced carbonate precipitation (MICP) by nitrogen-involved heterotrophic bacteria showed great potential for crack healing and concrete recycling. This study investigated the role of autophototrophs as alternative technology to conquer the pollution created by urea-based MICP. The calcification by *Synechococcus pevalleikii* was studied in concrete solution and cubes, and studied the effect on concrete properties. The pH, calcium concentration and cell viability were examined and scanning electron microscopic studies indicated the morphologies of the minerals produced were majorly cubic and rhombic crystals. Based on laboratory experiments *S. pevalleikii* remained intact in the cement pore solution and a thick layer of calcite and cells were found attached to the concrete cubes. Morphologies and amount of crystals from biotic-formed precipitates differs greatly from abiotic-formed ones. Treatments with UV-killed cells improved the compressive strength and decreased the water absorption. Consequently, the technology of using autophototrophs is promising with great potential for restoring concrete.

Contents

Chapter 1

Introduction

Concrete is the most generally utilized building materials everywhere throughout the world as they are economical, convenient to cast and easily available. Regardless of being the most well known development material, concrete has an inescapable issue of cracking because of physical, chemical and natural weakening. Micro-cracks permit the penetration of water and other impurities such as chloride and sulphate ions in to the concrete matrix leading to premature matrix degradation, corrosion of embedded reinforcement etc. which in turn hinders structural integrity (Basheer et al., 1996; Rivera-Corral et al., 2017; Itagaki et al., 2016). Progressive dissolution of the mineral matrix as a consequence of weathering leads to an increase of the porosity and as a result mechanical properties decreases (Tiano et al., 1999). It is generally accepted that the durability of concrete is related to the characteristics of its pore structure. The downgrading concrete can lead to the ruination of buildings or extensions and the spillage of carbon dioxide in geologic carbon sequestration wells. The concrete must be either repaired or recreated to expand the lifetime of these developments. Despite the fact that a variety of crack filling items are accessible and all have some issues like the requirement for consistent maintenance, ruination after some time and unfriendly consequences for the earth (De Muynck et al., 2008a). The reconstruction brings about the generation of destroyed concrete debris and a higher commitment to carbon dioxide discharge. Therefore technology development to create eco-friendly construction material is necessary to reduce the carbon dioxide production, to increase the endurance of building structures and maintain sustainability. Contribution of microorganism in calcium carbonate precipitation has prompt the advancement of bioprocess innovation and its application in the self healing of concrete and consequently has demonstrated an answer for all the numerous issue confronted by them. Motivated by the use of microbial induced carbonate precipitation (MICP) in soil fortification (Liang et al., 2013) and limestone rebuilding (Castanier et al., 1999). MICP was proposed to tackle the issue of concrete cracking (De Belie et al., 2016) and the concrete aggregates recycling (Qiu et al., 2014). Contrasted with conventional treatments using epoxy, resins, lime mortar and other synthetic mixtures, MICP creates less pollution and forms a rational layer of calcite and minimizes work expenses (De Muynck et al., 2010). Since 1993, utilization of MICP on the walls of Saint-Médard Church i.e under

natural condition has displayed a significant decrease of water absorption (Le Metayer-Levrel et al., 1999). Additionally, enhancement of compressive strength and durability of concrete by MICP were shown on the laboratory scale (Bang et al., 2010; Achal et al., 2011).

Biomineralization is a natural process by which production of minerals by living organisms occurs. Like the creation of limestone, sandstone etc. biomineralization happens at a moderate rate over geological times. Microorganisms have remarkable capacity to precipitate minerals such as calcites, silicates, carbonates, sulphides, phosphates (Barlet-Gouédard et al., 2009). Biomineralization processes, including MICP, have been discovered in a variety of ecological systems (Fujita et al., 2010; Achal et al., 2012). According to the metabolic activities mainly four groups of microbes are recognized to induce MICP: (i) Photosynthetic life forms—for example, algae and cyanobacteria (ii) sulphate diminishing bacteria—responsible for sulphates reduction (iii) microbes utilizing organic acids (iv) heterotrophic microorganisms that are involved in the nitrogen cycle (Stocks-Fischer et al., 1999; Hammes and Verstraete., 2002). Among all, ureolytic microorganisms are the simplest and broadly utilized MICP for concrete technologies (Xu et al., 2014). An alternative MICP technology is required to overcome the drawbacks of ureolytic bacteria. During urea hydrolysis toxic ammonia gas is released as a by-product which may cause environmental and wellbeing dangers. This urea-hydrolysis produces an unpleasing odor. The bacteria form endospores which may lead to the generation of toxic by-products and uncontrolled growth. The urea production creates a lot of carbon dioxide. The bacterial treatment is expensive than the conventional remediation (De Muynck et al., 2013). Use of autophototrophs might overcome these shortcomings and can be more efficient and sustainable. Autophototrophs leads to calcification with an environment-friendly way, they utilizes carbon dioxide and produce oxygen during their growth.

Autophototrophic picocyanobacteria had shown their calcification potential in a scope of natural conditions (Thompson et al., 1997; Dittrich et al., 2004).The precipitation can proceed by either or both of the following reactions:

$$Ca^{2+} + 2HCO_3^- \leftrightarrow CaCO_3 + CO_2 + H_2O \quad (1)$$

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3 \quad (2)$$

The reaction shifts towards right due to oversaturation of $CaCO_3$ during photosynthesis (Jansson and Northen., 2010). Microbes with electronegative cell surface can give nucleation sites for carbonate formation, or creating oversaturation in a thin layer surrounding the bacteria potentially induces carbonate precipitation (Hammes and Verstraete, 2002). Although there are numerous evidences, that the surface of cyanobacteria such as *Synechococcus* strains has exopolymeric substances which serves as the site for mineral nucleation (Obst et al., 2009).

Carbon dioxide sequestration by utilizing carbon dioxide as a carbon source has been tested by cyanobacteria during biomineralization. *Synechococcus* strains were found to sequestrate the largest portion of CO_2 compared to several other cyanobacterial strains (Liang et al., 2013). However, the cyanobacterial cells tried for MICP technology were found to have a great potential to repair or recycle concrete. They were found to survive the tough conditions of cement pore fluid and were able to induce calcium carbonate formation (Zhu et at., 2015).

This study aims to evaluate the calcification capability of cyanobacteria strain *S. pevalleikii BDHKU* in the cement pore solution and on concrete cube surface to create advances in MICP technology. The viability of cells were tested under the harsh conditions of a cement pore solution where pH was as high as 11.7. Growth kinetics of *S. pevalleikii BDHKU* was studied. Experiment on cement mortar cubes was conducted on the laboratory scale to study about the newly formed layers of precipitation on its surface. The performance of cubes was researched which were treated with live cyanobacterial cells under illumination, dark, abiotically, and with UV-killed cells.

Objectives

1. To estimate mineralization efficacy of *S. pevalleikii*
2. Influence of live and UV killed cells to induce calcite formation
3. Effect of cyanobacterial calcification in sand columns
4. Surface treatment of cement mortar cubes with cyanobacterial cells to enhance durability properties.

Chapter 2

Review of Literature

2.1 Concrete history and evolution

Concrete is a strong and relatively cheap construction material and is therefore; presently the most used as construction material worldwide (Emmons and Sordyl., 2006). It is composed of aggregates, binder, water and admixtures in different proportions depending on the required strength and functionality. Concrete was first used by the Egyptians who used mud mixed with straw to bind dried bricks (Mindess et at, 2003; Neville., 1995). Later the Romans made many developments in concrete technology including the use of hydraulic cements made from slaked lime and made famous landmark such as Roman aqueduct and Colosseum that are still standing today. Modern concrete, a $30 billion per year industry in sales alone in the U.S. (National Ready Mixed Concrete Association), is based on hydraulic Portland cement involving sintering of limestone and clay at high temperature (Mindess et al., 2003; Neville., 1996); however, this modern process is still an analogous to that of lime-based mortars that was used by the Romans. The upward trend in global market of concrete is estimated due the growing construction industry across the globe. The upward trends of concrete market from 2014-2020 is shown in fig. 2.1.

Fig 2.1 Expected global market of concrete 2014-2020
(Source: www.transparencymarketresearch.com)

2.2 Damages and repair expenses

There are many construction materials and concrete structures such as limestone, dolostone and marble which lead to deterioration and shorten their service life. There can be several factors which may cause weathering, occurring at any stage of the service life due to volume instabilities with in concrete such as extreme loading, harsh environmental exposures, poor construction procedures or design errors (Saiz-Jimenez et al., 1997; Warscheid and Braams., 2000).

Due to micro- cracks, penetration of water and other impurities such as chloride and sulphate ions in the concrete matrix which leads to premature matrix degradation, corrosion of embedded reinforcement etc. which in turn hampers structural integrity (Basheer et al., 1996). There can be progressive dissolution of the mineral matrix because of weathering which may also leads to an increase of porosity, hence the mechanical features decreases (Tiano et al.,1999). Generally it is acceptable that the durability of concrete is related to the characteristics of its pore structure. Degradation mechanisms of concrete often depend upon the way potentially aggressive substances penetrate into the concrete which may cause damage. The porosity and the connectivity of the pores are affected by permeability of the concrete. Vulnerability of the degradation mechanism of the material depends upon the pore structure of the concrete. The deterioration of concrete structure mainly consist of movement of aggressive gases and or liquids from the surrounding environment into the concrete, followed by physical and or chemical reaction within its internal structure which may leads to irreversible damage (Claisse et al., 1997) For decreasing the susceptibility of deterioration, many conservation treatments had been applied, however these treatments are subject of controversy due to non reversible action and their limited long term performance (De Muynck et al., 2010) For the maintenance and repair of concrete structures huge expenses are incurred.

Koch et al., (2002) and Emmons and Sordyl., (2006) reported that the estimated damage due to corrosion of concrete in the US is $276 billion with annual cost in repairs to be $18 – 21 billion. Therefore, it is imperative that crack propagation in concrete must be minimized to extend the longevity thus reducing maintenance. There is a compelling economic incentive to develop a concrete that can treat and repair the damage all by itself.

2.3 Self-healing concrete

Animals and plants have a natural capability to heal small bodily damages by themselves in a relatively short amount of time without any external influence. Similarly, natural self-healing ability of concrete known as the autogenous healing has been observed for many years (Neville et al., 2002; Li and Yang., 2007). It has been noticed that the micro cracks in old structures were self- healed by the recrystallization of calcite (Edvardsen, 1999). This reveals that under the right environment concrete is able to seal the cracks by itself with the augmentation of some chemical and/or biological additives and with the presence of moisture. In general, under the right environment, carbon dioxide in the air is dissolved in water, and this carbon dioxide reacts with the calcium ions in the concrete to produce calcium carbonate crystals. The calcium carbonate crystallization made in this way is attached and grown on the crack surface. This leads to the reduction in crack width and eventual repair of the whole crack (Ramm and Biscoping., 1998; Edvardsen, 1999). However, depending on this natural process alone, only a limited crack with width up to 100 μm can be repaired (Neville, 2002; Li and Yang, 2007). However, to repair larger cracks with better healing consistence, addition of both chemical and biological amendments might be needed.

Instead of autogenous healing, many researchers had concluded that self-healing behavior can be achieved by the introduction of bacteria into the concrete matrix (Jonkers and Schlangen., (2009); Wang et al., (2012). In brief, it was hypothesized that once moisture enters through freshly formed cracks, dormant but viable bacterial spores immobilized in the concrete matrix becomes metabolically active. Then, these cracks will be healed through microbial calcite precipitation, impeding further ingression of water and other chemicals. Most of the applications of bacterial concrete done so far were for crack remediation treatments, which cannot be considered purely ‘self-healing’ because it was analyzed after artificial crack simulations (De Muynck et al, 2008). In these studies, an efficient plugging of cracks and recovery of mechanical strength was observed which resulted from the presence of adequate amount of organic substances in the matrix due to microbial biomass.

2.4 Biocementation

Biocementation is a process to produce binding material (Biocement) based on microbial induced carbonate precipitation (MICP) mechanism. Biocement is a new ecological material

formed due to the activity of microorganism which results in the deposition of calcium carbonate in the calcium ions rich system. This process can be applied in many fields such as construction, petroleum, erosion control, and environment. In general, mortar refers to ready to use binder material contained a binder, and sand or fine aggregate. Biocementation applied in concrete rift remediation and the production of bacterial concrete has been investigated. Specimen of crack in concrete filled with bio cement shows the significant increment of strength and stiffness value compared with specimen without bio-cement.

De Belie et al., (2016) reported the use of biocement for consolidation, surface protection, external crack repair, and self-healing of cracks and it can remediate cracks in building materials and monumental stones and regain strength. Abo-El-Enein et al., (2012) also found that biocement reduces the permeability and increases the compressive strength of cement mortar and hence enhances the durability. The lower permeability rates resulting from the microbial additive will increase the structures useful life.

2.5 Biominerilization

"Biominerals are everywhere." If we take a look around, we see ourselves surrounded by biominerals whether in the form of beautiful corals, ant hills, caves, shells of mollusks, teeth, bones or rocks (Fig 2.2).

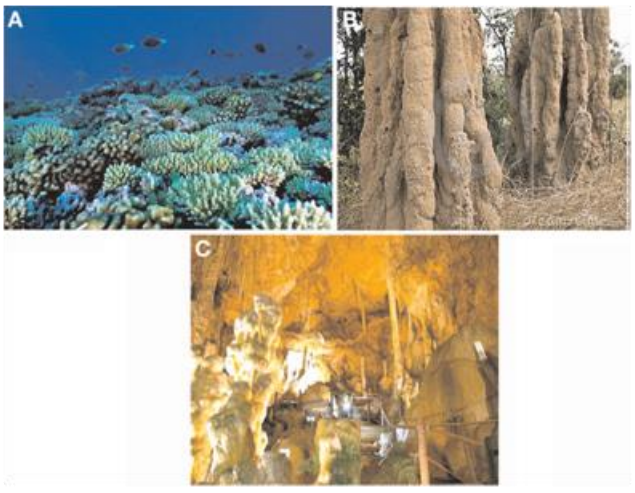

Fig 2.2 Bio-mineralization of calcium carbonates in natural structures (A) Corals (B) Ant hills (C) Limestone caves (Dhami et al., 2013)

Biomineralization is a process by which living organisms produce minerals. These could be silicates in algae and diatoms, carbonates in invertebrates and calcium, phosphates and carbonates in vertebrates. The synthesis of minerals by prokaryotes is broadly classified into

two classes: Biologically controlled mineralization (BCM) and Biologically induced mineralization (BIM) (Lowenstam et al.,1981; Lowenstam and Weiner.,1989). Minerals are directly synthesized at a specific location within or on the cell and only under certain conditions in case of BCM but in case of biologically induced mineralization, the minerals are formed extracellularly as a result of metabolic activity of the organism. The extracellular production of these biominerals invited scientists worldwide for harnessing this capability of microbes for various bioengineering applications. Researchers around the globe are now focusing on harnessing the technical applications of these biominerals in various fields.

Minerals known to be formed via biologically induced mineralization through passive surface-mediated mineralization include Fe, Mn, and other metal oxides, e.g., ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$), hematite (α-Fe_2O_3), and goethite (α-FeOOH); metal sulfates, phosphates, and carbonates; phosphorite; Fe and Fe-Al silicates; and metal sulfides. Among all the minerals that are associated with biomineralization, carbonates are the most widely studied branch of biomineralization which holds promise for variety of fields ranging from biotechnology, geotechnology, paleobiology to civil engineering. It has implications for: (1) atmospheric CO_2 fixation through carbonate sediment formation and lithification (Krumbein et al., 1979; Monger et al., 1991; Chafetz and Buczynski., 1992) and dolomite precipitation (Vasconcelos et al., 1995) (2) solid-phase capture of inorganic contaminants (3) pathological formation of mineral concretions, such as gallstones and kidney stones in humans (Keefe, 1976) (4) the possibility of understanding extraterrestrial biological processes such as Martian carbonate production by bacteria.

2.6 Microbially induced $CaCO_3$ precipitation

Calcium carbonate precipitation is a rather straightforward chemical process governed mainly by four key factors: (1) the calcium concentration, (2) the concentration of dissolved inorganic carbon (DIC), (3) the pH and (4) the availability of nucleation sites (Hammes and Verstraete., 2002). In nature, building and remediation of structures with local materials occurs without any requirement of extreme energy usage. Calcification and polymerization occur at ambient conditions as can be seen from the sustainability of ant hills and coral reefs. This occurs through the application of microorganisms which deposit carbonates (as part of their basic metabolic activities), one of the most well known minerals. These deposits commonly called as calcium carbonate crystals act as binders between loose substrate particles and reduce the pores inside the substrate particles. The use of bacteria for

remediating building materials seems like a new idea, but this conservation method mimics what nature has been doing for years, since many carbonate rocks have been cemented by calcium carbonate precipitation from microbes. The technology of using microbes for calcium carbonate deposition or microbial concrete, called as Microbially induced calcium carbonate precipitation (MICP) can be used for solving various durability issues of construction materials. Microorganisms are abundant in nature, which paves the way for massive production of bacterial calcium carbonate crystals. As the microorganisms can penetrate and reproduce themselves in soil or any such environments, there is no need to disturb the ground or environment unlike that of cement. This technology also offers the benefit of being novel and eco- friendly. Bacterially induced mineralization has recently emerged as a method for protecting and consolidating decayed construction materials.

Biomineralization processes, including MICP, have been observed in a variety of ecological system (Achal et al., 2012; Fujita et al., 2010). Regarding the metabolic activities, three main groups of microbes are capable of inducing MCP: (i) photosynthetic organisms; (ii) sulfate-reducing bacteria; (iii) heterotrophic organisms that are involved in the nitrogen cycle (Castanier et al., 1999; Hammes and Verstraete, 2002). So far, ureolytic bacteria belonging to the third type have mostly been applied for MCP in construction materials (Xu et al., 2014).

High endurance of building material, enhancement of compressive strength and decline in water absorption and permeability were observed by the MICP treatment to a great extent (Ramachandran et al., 2001; De Muynck et al., 2008; De Belie., 2008). Achal et al., (2011) reported increased resistance towards degradation of building material and rate of water absorption declined with the treatment of the MICP. Jonkers and Schlangen., (2009) reported a self replenishing concrete which was developed by *Bacillus* spores and calcium lactate nutrients packed into pellets. During formation of cracks water will come inside and dissolve the pellet causing germination of bacteria and formation of carbonate precipitates.
Gonsalves et al., (2011) showed comparatively positive impact of bioconcrete on environment. Bioconcrete decreased cancer producing compounds by one-thirtieth, reduced ecotoxicity, reduced climate change and fossil fuels.

2.6 Need of alternate MICP

Ureolytic bacteria amongst MICP has been considered as the most easy and energy efficient process (De Muynck et al., 2010). There are uncountable advantages of MICP by urea

hydrolysis but it has drawback too i.e ammonia creation. It have a lot of environmental concerns and also uncertainty of damage to concrete materials (De Muynck et al.,2010). This urea-hydrolysis produces an unpleasing odor. The bacteria form endospores which may lead to the generation of toxic by-products and uncontrolled growth. This has diverted interest in searching of other pathways. Fixing of different carbonate minerals by CO_2 sequestering cells as calcite, magnesite, dolomite have gained interest of many researchers (Dhami et al.,2013). As per various researchers, CO_2 has been stated as a evil molecule which must be reduced. But, due to anthropogenic activities its concentration is increasing day by day. As per the KYOTO protocol, which was signed in 1997 stated that there is an immediate need to reduce level of CO_2 emissions in industrialized nation to 95% (Bolin et al.,1998). Therefore, for the mitigation of CO_2, many researchers have focused on the development of methods. There are many strategies to control CO_2 concentration in the atmosphere. One of them is to reduce its production and release into the atmosphere. The second strategy is to increase the efficiency of the energy use starting from manufacturing to its consumption. But basically, both of these are not practically possible. Hence, researchers came with third option which can capture and dispose the produced CO_2 in a safe manner i.e. sequestration of carbon dioxide (Sharma et al., 2010; Reichle et al., 1999). Although there are number of options of sequestration of carbon dioxide which have been proposed, that includes the infusion of carbon dioxide in deep oceans, in natural formations like deep saline aquifers, oil or gas reservoirs and utilization by advanced chemical and biological and biological processes (Reichle et al.,1999). The alternative for CO_2 disposal in sequestration form paved way for fixing it in the form of carbonate minerals as these are very stable and environmentally benign.

Microbial induced calcium carbonate precipitation (MICP), a local micro-environment is built by microbes allowing chemical precipitation of minerals (Hamilton et al., 2003). A lot of attention is snatched by MICP and use of these precipitated carbonates for structures remediation. Till date majority of work has been done on urease producing heterotropic bacteria (Stocks Fischer et al., 1999; De Muynck et al., 2008; Achal et al., 2009). Not much research has been reported to study the role of autotrophic bacteria like cyanobacteria. Benini et al., (1999) reported the main disadvantage of ureolytic bioconcrete is the production of ammonia and other pathogenic microbes that are in association with ureolytic bacteria. To lower the harsh environmental impact, autophototrophic cyanobacteria were proposed and investigated. They showed a potential to serve as a "green technology" in concrete restoration (Zhu et al., 2015). This was the first study that showed biominerlization by cyanobacteria on the concrete surface, and decline in water absorption.

2.7 Cyanobacteria

Cyanobacteria are Gram-negative, photosynthetic bacteria that carry oxygenic photosynthesis; these are thought as the origin of chloroplasts of plants and eukaryotic algae via endosymbiotic events in the early cambrian or late proterozoic period. Cyanobacteria occupy a wide array of marine, freshwater habitats, terrestrial and it also includes extreme environments like hot springs, bare rocks, permafrost zones and deserts. Some cyanobacteria in their natural environment are frequently exposed to the highest rates of UV irradiance known on the earth. Cyanobacteria also have a large fossil record. Indeed, the oldest known fossils of cyanobacteria are from archaean rocks of western australia, dated 3.5 billion years old. Through the photosynthetic capacity of cyanobacteria, they have been very important in shaping the course of ecological and evolutionary changes throughout the Earth's history, and they also continue to contribute to a large share in harnessing of solar energy and assimilation of CO_2 to organic compounds. For example half of the earth photosynthesis is carried out by phytoplankton, which mostly consists of cyanobacteria. Actually 25% of global photosynthesis can be accounted by the two marine cyanobacterial genera, *Prochlorococcus* and *Synechococcus* in the oxygenic atmosphere. Cyanobacteria generally thrive in high CO_2 levels and are considered as attractive systems for CO_2 capture from flue.

Cyanobacteria are recognized as particularly being responsible for huge carbonate precipitations. It is being estimated that cyanobacteria are the principle contributors in the production of carbonate rocks in almost 70% of Earth history (Altermann et al., 2006). Cyanobacterial stromatolite, a laminated calcareous fossil, was found in various environments, such as, terrestrial areas, marine and fresh water (Krumbein and Giele, 1979; Rodriguez-Martinez et al., 2012). Whiting events shows the high potential of calcification by picocyanobacteria. Whiting events turn the entire water bodies into a milky state (Thompson et al., 1997).

Biomineralization by calcifying cyanobacteria

Calcification is particularly clear in cyanobacterial species. The ecological and geological significance of cyanobacterial calcification is so vast; spectacular examples of cyanobacterial calcification are whiting and stromatolites, very fast and large-scale precipitations of fine-grained $CaCO_3$ together with organic compounds can turn entire water bodies such as the Great Bahama Bank and Lake Michigan into a milky state. Although, understanding of the molecular processes that triggers and controls cyanobacterial calcification is unclear, and

many of the systematic details of proposed models remain contentious, the general process is outlined in fig 2.3.

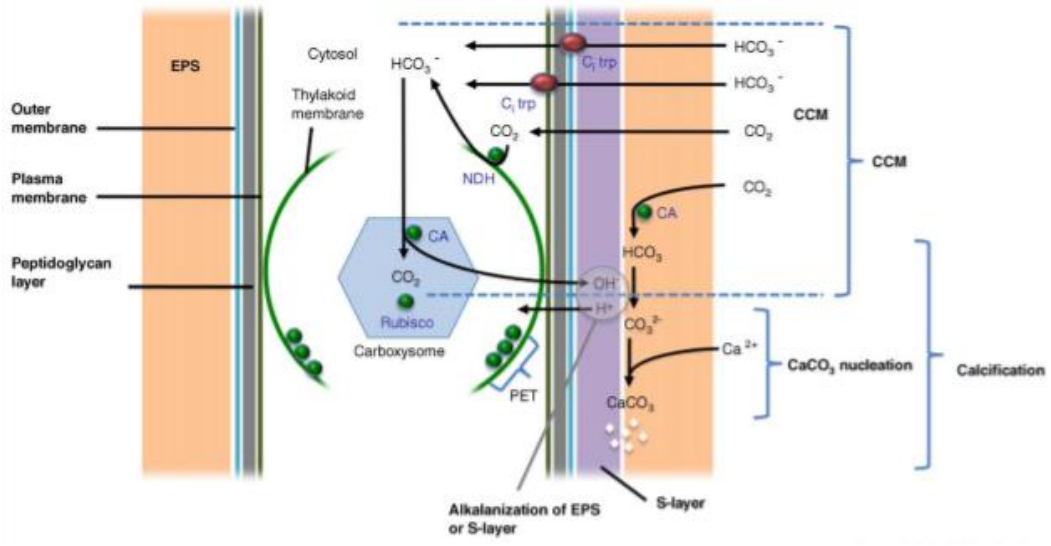

Fig 2.3 Mechanism of calcification in cyanobacteria

Calcification in cyanobacteria is an extracellular process and occurs on in the exopolysaccharide sheath (EPS) or proteinaceous surface layer (S-layer) that surrounds the cells. CO_2 enters the cells mainly via active transport of HCO^{3-} and also through diffusion of CO_2, which is converted to HCO^{3-} during the uptake. Microenvironments of alkaline pH are generated at the EPS or S-layer owing to the CA activity in the carboxysome. The alkaline pH at the EPS or S-layer shifts the equilibria of the bicarbonate buffer system and promotes localized regions of increased CO_3^{2-} concentration at the cell exterior. In addition, both the EPS and S-layer contain Ca_2^{+}– binding domains and serve as nucleation sites for $CaCO_3$ precipitation (Obst et al., 2009; Jansson and Northen., 2010).

The precipitation of calcium carbonates can proceed by either or both of the following reactions:

$$Ca^{2+} + 2HCO_3 \leftrightarrow CaCO_3 + CO_2 + H_2O \quad (1)$$

$$Ca^{2+} + CO_3^2 \leftrightarrow CaCO_3 \quad (2)$$

2.8 Cyanobacteria in concrete restoration

Biominerilization in natural habitat by cyanobacteria was reported by many researchers (Krumbein and Giele., 1979; Wright., 2005; Altermann et al., 2006;) but in field of concrete restoration very few research papers were published. Zhu et al., (2015) reported

cyanobacterial cells were intact in the cement solution, and the biotic-formed precipitates were different in morphology and nearly doubled in amount compared with abiotic-formed ones. The experiments on concrete cubes revealed that a thick calcite-cell aggregate layer adhering to the concrete was formed biotically, while several thin carbonate patches were distributed on the concrete abiotically. This calcite-cell layer decreased the water absorption, and was resistant to sonication.

Chapter 3

Materials and Methods

3.1 Procurement of Cyanobacterial culture

The cyanobacterial strain *Synechococcus pevalleikii BDHKU 21305* was obtained from National marine laboratories, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

3.2. Culture conditions

The cyanobacterial cells were harvested in two different media. Cells were cultured in ASN III and BG 11 media. Batch cultures was maintained in 250 ml of Erlenmeyer flask containing 100 ml of medium, constantly shaken at 110 rpm under the room temperature of 25 ± 2°C with a light intensity of 20 μE m^{-2} $S^{-1}$and a photoperiod of 12:12 h (light:dark). Sub culturing was done after every 20th day.

3.3 Subculturing and Inoculum

Subculturing with 10% inoculum was carried out. Cell division is more active during the mid exponential phase and the cell number is more so culture was taken from this phase. The subculturing was done aseptically in shake flask containing 250 mL of BG11 medium and flasks were placed in temperature controlled orbital shaker at 25 ± 2°C and 110 revolutions per minute (rpm). Culture was grown under illumination (12:12h; light:dark). For maintenance of fresh stock subculturing was done after every 20th day.

3.4 Estimation of dry weight of biomass

To obtain the dry weight of biomass, samples were filtered through Whatman's No. 1 filter paper and dried at 60ºC overnight. The corresponding day optical density of *S. pevalleikii BDHKU 21305* was measured using UV/visible spectrophotometer. For five days dry weight of biomass was noted by this method and after that using a calibration curve OD was converted to dry weight.

3.5 Determination of kinetic parameters

The growth curve was created by plotting the values of biomass against time. Specific growth rate μ (1/d) was calculated according to the Eq (I)

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \qquad (I)$$

here, X_2 and X_1 =are the dry biomass weight (g/L) at time t_2 and t_1 respectively.
Determination of the maximum specific growth rate μ_{max} (1/d) was done from the different values of μ. The doubling time of cell was calculated by Eq (II)

$$t_d(d) = \frac{\ln 2}{\mu_{max}} \qquad (II)$$

X_{max} (g/L) was the maximum biomass obtained. The biomass concentration ΔX (g/L) over cultivation time Δt was calculated as $\Delta X = X_t - X_0$. The overall biomass productivity $P_{overall}$ (g/L/d) was evaluated using Eq (III)

$$P_{overall} = \frac{\Delta X}{\Delta t} \qquad (III)$$

here, X_t = the biomass concentration at time t,

X_o = the initial biomass concentration at inoculation time (t_o).

P_{max} (g/L/d) = maximum productivity.

3.6 Effect of pH

To characterize the survival of cells at high pH, *S. pevalleikii BDHKU 21305* cells were cultivated under different pH regime. 10% of freshly growing culture was inoculated in 200 mL BG11 medium were grown at pH values of 5-12. The culture grown at pH 7 acted as control, all the cultures were grown in triplicates .The cultures were kept under the room temperature of 25 ± 2°C with light for 12:12 h (Light/Dark cycle) for 10 days.

3.7 Microbial carbonate precipitation experiments

To check the microbial carbonate precipitation potential of *S. pevalleikii BDHKU 21305* different experiments were performed at flask level as follows.

Experiment A

Saturated $CaOH_2$ solution was used to simulate the pore water condition of concrete. The saturated $CaOH_2$ solution was prepared by adding reagent grade $CaOH_2$ in distilled water till pH value reaches 11.7. This solution is similar to the fluids present in cracks or in degraded concrete. The mixed solution is denoted as cement pore solution. It was sterilized by filtering the supernatant of the mixture through a sterile 0.2 mm nylon-66 membrane filter just before the precipitation experiments.

The precipitation experiments were performed by injecting 50mM calcium chloride solution through a sterile 0.2 mm nylon-66 filter in 100 mL sterilized cement pore solution in 250 mL flasks (fig 3.2a)

Experiments B

Experiment was run in BG11 medium containing different concentrations of calcium chloride solution and sodium bicarbonate. In order to evaluate the calcification potential of *S. BDHKU 21305,* biotic experiments in the presence of 50 mM $CaCl_2$ were performed in BG-11media (fig 3.2b)

Experiment C

Biotic experiments in cement pore solution were performed without the addition of $CaCl_2$.

Experiment D

Similar experiments in abiotic condition were performed in cement pore solution without the addition of $CaCl_2$.

All trials were conducted in triplicate at 25 ± 1°C with light intensity (20 μE m^{-2} S^{-1}), under constant shaking (110 rpm). Cells initially added to solution were 7.5 x 10^7 cells/ ml and the calcium chloride concentration was 100 mM. The pH was measured after 0, 1, 2, 3, 6, 18 and 24 h. A 5 mL experimental solution was filtered at 0, 6 and 24 hrs, through a 0.45 μm nylon-66 membrane filter to investigate dissolved Ca^{2+} concentration.

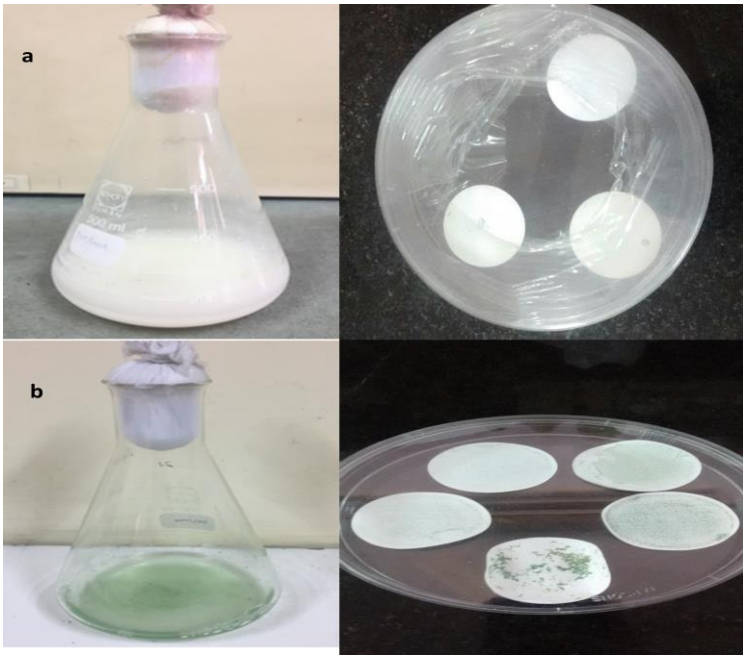

Fig 3.2 Collection of samples from (a) cement pore solution (b) growth medium

3.8 Pilot experiments with concrete cubes

1. Concrete cubes of size 50 x 50 x 50 mm were casted by means of a customized mold. The concrete cubes were demoulded after 24 hours of casting.
2. Cubes were submersed in 50 mM calcium chloride solution along with cells (fig 3.3). The control cubes were submersed in water without cells.
3. The cube specimens were immersed in the beakers in three sets with a light intensity of 20 μE m^{-2} S^{-1} under the temperature of 25± 1°C, and taken out for assessment after 45 days.

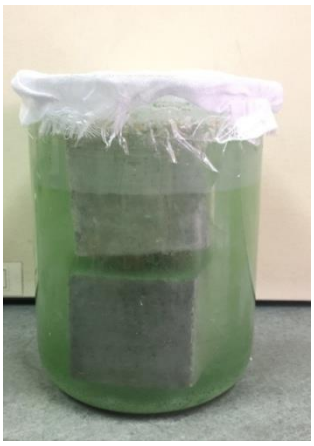

Fig 3.3 Curing of cubes with cyanobacterial cells

Curing set 1 (S1) The solution was kept stationary keeping all other conditions constant. The old solution was discarded and new solution was added after 7 days.

Curing set 2 (S2) The solution was kept constantly shaken with the help of magnetic stirrer. Shaking was performed to ensure the homogenization and aeration of the solution. The solution in the beaker was changed after 7 days.

Curing set 3 (S3) Cubes were kept in the solution with stationary conditions. The addition of fresh solution was done in the preexisting old solution after 7 days.

4. The tests of water absorption, pH changes and compressive strength analysis were recorded on cubes after these treatments.

3.9 Surface treatment by ponding

Surface treatment of mortar cubes with cyanobacterial cells was done by ponding.

1. A small reservoir of dimensions 50 x 50 x 50 mm with the help of acrylic sheet was built to expose the single face of mortar cube as shown in fig. 3.4
2. Cyanobacterial cells were harvested from stationary phase and centrifuged at 8000 rpm for 15 minutes.
3. Cells were washed with 0.1 M $NaNO_3$ solution and supernatant was discarded. Three washes with $NaNO_3$ solution were done. After the last wash, the cells were resuspended in deionized water.
4. The filling of these reservoirs was done with the solution of 10 ml calcium chloride (100 mM) and sodium bicarbonate (50 mM) along with 10 ml of washed live and UV killed cells. The experiments were performed in triplicates.

Fig 3.4 Reservoir made on cube for ponding

5. The solution was renewed after every two days.
6. The curing of cubes was done for 7 days.
7. After curing of cubes, the water absorption test was conducted.
8. The SEM analysis was carried out to identify the crystal structure.

3.10 Sand consolidation

3.10.1 Preparation of sand columns

1. River sand which was clean, dry, well graded, was used in the present study.
2. Sand was sieved from 2 mm size mesh. To remove the bacterial flora the sand was autoclaved thrice after every 24 h.
3. *S. pevalleikii BDHKU 21305*was grown in BG 11 media, batch cultures were being constantly shaken at 110 rpm under the room temperature with a light intensity of 20 µE m^{-2} $S^{-1}$and a photoperiod of 12:12 h (light:dark).
4. The cyanobacterial cells were harvested (7200 rpm for 15 min at 25°C) and washed three times with 0.1 M $NaNO_3$ solution.
5. The cells were suspended into 30 ml sterilized calcification media and mixed as shown in table.3.1
6. Sand columns were prepared by cutting a transparent plastic tube of height 7.8 cm and diameter 3.6 cm. Slurry of sand was filled into the tube. Transparent tube was taken to allow passage for light (fig. 3.5).

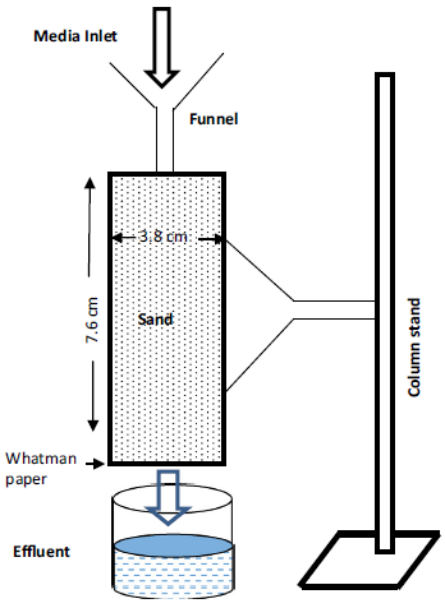

Fig 3.5 Set up for sand consolidation for MICP treatment

7. The bottom surface of every column was blocked by Whatman filter paper.
8. A burette was attached at the top of the column so that media could be dropped at a constant rate through the column.
9. The columns were fed with differently treated cells as shown in table 3.1.
10. The control column contained sterile sand and only calcifying media (without cells).
11. 30 ml calcification media was dropped over columns everyday at room temperature for 14 days.

Table 3.1 Sand consolidation with different calcifying media

Experiments		**Composition of sand coloumn**	**Calcifying media**
Experiment A (Live cells)	I-A set	Cell + $CaCl_2$ (200mM)+ $NaHCO_3$ (50 mM)	$CaCl_2$ (200mM)+ $NaHCO_3$ (50 mM)
	II-A set	Deionised water	Cells + $CaCl_2$ (200mM) + $NaHCO_3$ (50 mM)
	III-A set	Deionised water	Cells + $CaCl_2$ (200mM)
Experiment B (UV killed)	I-B set	Cell + $CaCl_2$ (200mM)+ $NaHCO_3$ (50 mM)	$CaCl_2$ (200mM)+ $NaHCO_3$ (50 mM)
	II-B set	Deionised water	Cells + $CaCl_2$ (200mM) + $NaHCO_3$ (50 mM)
	III- B set	Deionised water	Cells + $CaCl_2$ (200mM)
Control	C set	Deionised water	Deionised water

3.10.2 Examination of the effluent

From the bottom of the sand columns effluent was collected in the beaker at regular intervals of time and divided by the corresponding time period to estimate the flow rate. The effluent was collected at the end of 1, 2, 4, 6, 8 and 10 days and examination of pH and soluble Ca^{2+}

content in order to estimate the activity of cyanobacterial cells within the sand columns. EDTA titration method (Stocks-Fischer et al., 1999) was used to estimate soluble Ca^{2+} content in the effluent.

3.11 Performance tests of the mortar cubes

3.11.1 growth and pretreatment of cells

Cyanobacteria *S. pevalleikii BDHKU 21305*, was grown in BG-11. Batch cultures were being constantly shaken at 110 rpm under the room temperature of 25±2°C with a light intensity of 20 μE m-2 S-1.

Cells were harvested after attaining the logarithm growth stage collected by centrifuging at 8000 rpm for 15 min. Cells were washed by three cycles of centrifugation, disposing the supernatant and resuspension of cells in the sterilized 0.1 M $NaNO_3$ solution. The cells were resuspended in deionized water after the third wash. To kill the cells, one-fourth of cell suspension was exposed to UV light for 3 hours and remaining cell suspension was kept for experiments.

3.11.2 Preparation of the mortar cubes

Mortar cubes of a size of 50 x 50 x50 mm were prepared according to ASTM C109/C109M-13, the international standard test method for compressive strength of hydraulic cement mortars, and were demolded after 24 hours. Cement-sand ratio (1:3) was used in preparing mortar cubes and water to cement ratio was maintained at 0.5. The composition of cement motar per cube: 79 g cement, 239 g sand and 39.8 g water. The mortar cubes were cured for 28 days in water prior to the treatment of bacteria. After curing, the mortar cubes weighted 288.5± 3.7 g.

Fig 3.6 Cement mortar cubes ready for demoulding

3.11.3 Experiments with differently treated *S. pevalleikii BDHKU 21305* on the mortars

1. 5 groups of mortar cubes were prepared with each group containing 6 cubes.
2. The 1st and 2nd groups were subjected to freshly washed cells. The 3rd group was treated with the cells which were UV killed. The 4rth and the 5th groups were not treated with cells.
3. Cell treatment for half an hour was given to cubes by immersing mortar cubes in the cell solution allowing the cells attachment to cubes surfaces.
4. Subsequent to the attachment of cells to the cubes surfaces, the first four groups of cubes were immersed in a 100 ml solution composed of calcium chloride (290 mM) and sodium bicarbonate (200 mM) for 7 days (table 3.2).
5. To stop photosynthetic activities of cells the cubes of 2nd group were covered with a black plastic. The other groups were exposed to light for 12 hours of luminance 20 μE m^{-2} s^{-1}, and 12 h of darkness afterwards.
6. The control i.e 5th group was immersed in deionized water.
7. After treatment of 7 days, the mortar cubes were kept in oven at 72 °C for one day for drying.
8. The general characteristics of mortar cubes were examined, including compressive strength and water absorption tests. Examination was done in triplicates.

Table 3.2 Treatment with different cells and curing solution.

Groups	Cell treatment	Curing solution
1st group	Live cells under illumination	$Cacl_2$ + $NaHCO_3$
2nd group	Live cells under darkness	$Cacl_2$ + $NaHCO_3$
3rd group	Uv killed cells	$Cacl_2$ + $NaHCO_3$
4th group	_	$Cacl_2$
5th group (control)	_	Deionized water

3.11.4 Weight change of the mortar cubes after the experiments

The mortar cubes were weighed before and after the test. The cubes were oven-dried at 78 ºC for one day prior to experimentation and the weight of each cube was taken as '*W*1'. The temperature was maintained at 78 ºC according to EN 1097-6 standard. The mortar cubes were oven-dried again at 78 ºC for one day. The weight of each cube was noted as '*W*2'. Difference in the weight of the treated cubes is calculated as:

W2-W1

Following procedure was followed to measure the weight of the precipitates formed by cells in the bulk solution. 50 ml bulk solution was filtered through 0.45 μm membrane filter. The filter was oven-dried at 78 ºC for one day. Before filtration of bulk solution the weight of the filter was recorded as '*W*3'. The precipitates on the filters were weighed as '*W*4'. The weight of the precipitates in the solution is calculated as:-

(W4-W3) x (V/50)

'V' represents the volume of total bulk solution.

3.12 Compressive strength test

Compression testing was performed using automatic compression testing machine, COMPTEST 3000.To avoid the mistakes in readings because of fluctuation of strength in different directions all the test cubes were put in the same orientation.

3.13 Water absorption test

1. To calculate the change in resistance for water penetration a sorptivity test, which is based on the RILEM 25 PEM (II-6), on mortar cubes was performed (De Muynck *et al*., 2007).
2. After the treatment mortar cubes were oven dried at 72°C for 24 hrs.
3. 5 sides of mortar cubes were coated with epoxy.
4. The exposure of 10±1 mm was given to cement mortar cubes at regular interval of time 15 min, 30 min; 1 h, 1.5 h, 3 h, 5 h, 8 h, 24 h, 72 h, 96 h, 120 h, 144 h and 168 h (fig 3.7).

5. The cubes were taken out from the water and weighed.
6. The surface was dried with a towel immediately after weighing the cubes, the cubes were submerged again.
7. The sorptivity coefficient, k [$cm.s^{-1/2}$], was calculated by the help of following equation

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

Here, Q = amount of water absorbed [cm^3]
A= cross section of the specimen that was in contact with water [cm^2]
t= the time [s]

The graph is plotted between Q/Aand the square root of time from which with the help of slope of the linear relation we can calculate the value of k.

Fig 3.7 Water absorption test conducted on mortar cube specimen

3.14 Estimation of calcium carbonate

Calcium was determined by EDTA titration (fig 3.8). Sodium hydroxide (5N) was added to the solution so that final pH reaches 12–13. A pinch of murexide indicator was added as an indicator and the mixture was finally titrated against 0.05M EDTA. The end point was noted from pink to blue which is easily visualized. 1 ml of EDTA used for titration is equivalent to 5.004 mg of $CaCO_3$ precipitated.

Fig 3.8 Calcium estimation by EDTA titration

3.15 Morphological and chemical characterization of precipitates

The cubes specimen with calcified surface were analysed using Scanning Electron Microscopy (JSM- 6510) equipped with EDX (fig 3.9). Samples were placed on the aluminum holder stub using a double sticky carbon tape. Gold coating of the samples were done. Scanning was done to view precipitates.

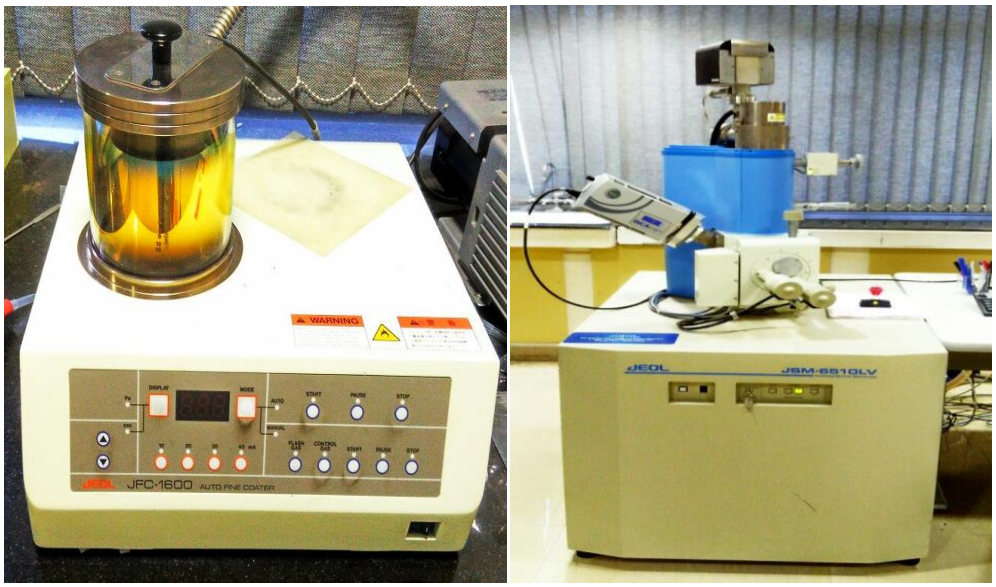

Fig 3.9 Equipments for SEM analysis

Chapter 4

Results and Discussion

4.1 pH optimization

pH of growth medium plays a significant role in the growth of cyanobacteria. The variation in pH affects nutrients uptake, activity of enzyme, and transport of substrates across plasma membrane and electron transport in respiration and photosynthesis. *S. pevalleikii BDHKU 21305* was grown in wide pH range (5-12) to monitor variations in its growth pattern (fig 4.1). Best cell growth was observed at pH 7, where maximum biomass as well as minimum span of lag phase was observed. The cells were found to grow well between pH ranges 7-9. But, when the pH value was lower than 6 a noticeable reduction in growth pattern was observed. Whereas cell growth was cease at pH 5. Reduction in growth was observed from pH 10-11 with a longer lag phase. Negligible growth was found at pH 12. So, for the growth of *S. pevalleikii BDHKU 21305* the best pH range is 7 to 9 and cells showed decent growth till 11 pH.

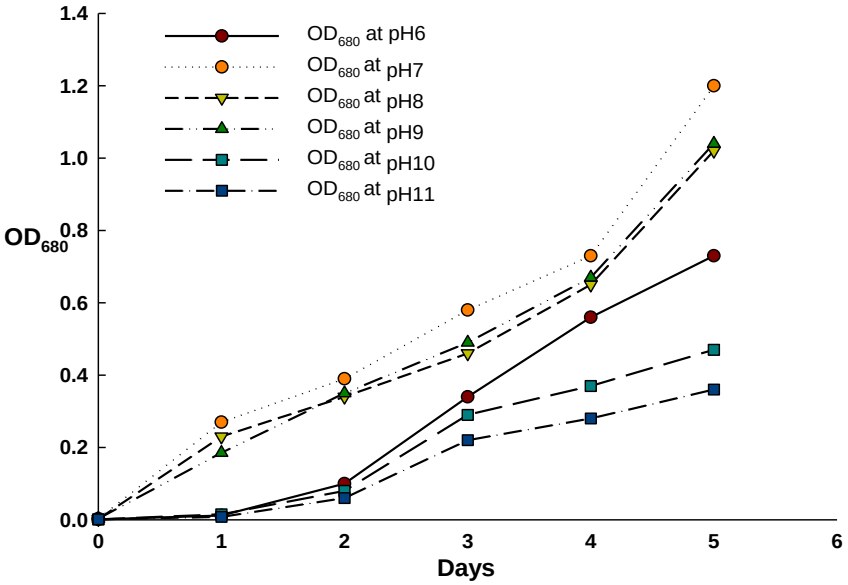

Fig 4.1 Growth patterns of *S. pevalleikii* at different pH

4.2. Dry Weight Calibration Curve of *S. pevalleikii BDHKU 21305*

Fig 4.2 shows the calibration curve of O.D. v/s cyanobacterial biomass concentration. Growth concentration was measured in terms of biomass dry weight (g/L). Biomass can be

more easily measured by using absorbance than by measuring cell dry weight directly. So, the relationship between optical density and cell dry weight for *S. pevalleikii BDHKU 21305* was established by linear regression. It was found that optical density precisely predicted the dry weight (R^2=0.988). Hence, O.D. values were used to calculate the biomass.

$$y = 0.393\ x - 0.01;\ R^2 = 0.998$$

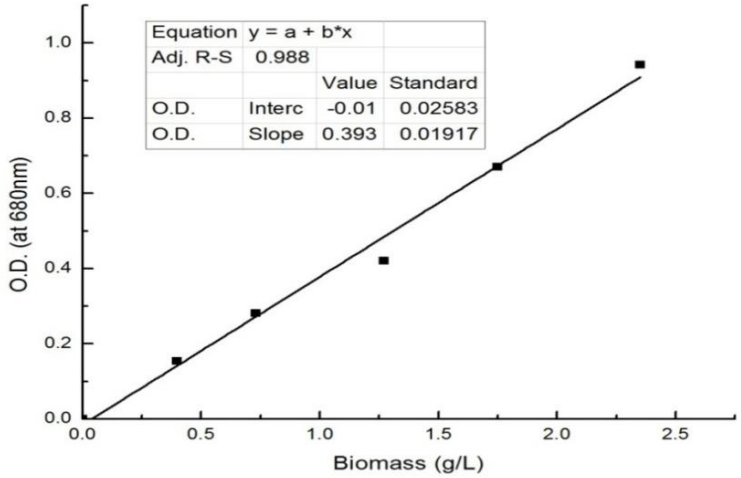

Fig 4.2 Calibration curve of O.D. v/s cyanobacterial biomass concentration

The value x is biomass concentration (g/L). The value of y is optical density of cyanobacterial biomass sample measured by UV-visible spectrophotometer at 680 nm.

4.3. Growth Kinetics of *S. pevalleikii BDHKU 21305*

Fig 4.3 shows the growth pattern of the cyanobacterial cells. Growth was measured in terms of biomass dry weight (g/L). *S. pevalleikii BDHKU 21305* showed the maximum biomass growth.

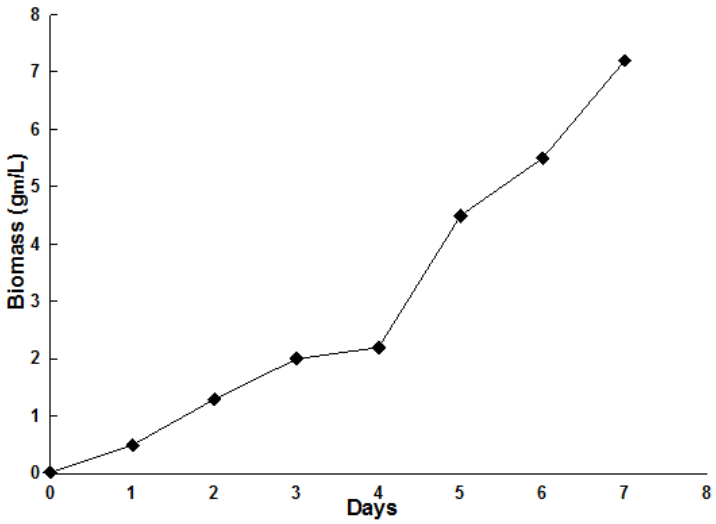

Fig 4.3 Biomass v/s time curve for *S. BDHKU 21305*

The X_{max} value of *S. pevalleikii BDHKU 21305* after 168 hr was found to be 7.86 g/L and the values of μ_{max}, productivity and doubling time (t_d) were calculated as 0.311 l/d, 1.078 g/L/d and 2.22 d respectively**.** It must be understood that it is very unlikely to see stationary phase in autotrophs, however their growth does get affected by depletion of limiting growth factors.

4.4. Dynamics of solution components

The pH of the cement pore solution was as high as 11.7 in the experiments. The pH dynamics showed variations between experiments in media, in cement pore solution and in cement sol (Fig. 4.4A) pH decreased faster in experiments A than in experiments B. Changes in pH and calcium changes in all the experiment are depicted in fig 4.4A and 4.4B respectively.

Experiment A

Due to the addition of $CaCl_2$ solution in cement pore solution the pH dropped from 11.7 to 11.3 initially (Fig.4.4.A). After first 6 h of $CaCl_2$ addition, there was acidification of the solution and pH drop from 11.3 to 10.5. Due to the calcium carbonate formation pH further declined to 7.6 till 12h. The pH value became stable at 7.4 after 18 h. Calcium removed till 24 h was 6mM and 11.5 mM by the end of the experiment. The data supported high calcification potential of *S. pevalleikii BDHKU 21305*at high pH.

Experiment B

The experiment was performed in media only. There was a slight decline of pH in media containing $CaCl_2$. Initially pH of media after addition of $CaCl_2$ was 8.2 which reduced to 7.8 after 6h. pH was decreased to 7.2 and became stable till the end of 24 h. Estimation of the calcification potential of *S. pevalleikii BDHKU 21305* in the cement pore solution was carried out by evaluating the changes in concentration of calcium. The calcium removed was 6 mM at the end of experiment as shown in fig 4.4 B

Experiment C

The experiments performed biotically in cement pore solution without the addition of $CaCl_2$, containing calcium which is solely from the cement solution with the initial concentration of

4.0 mM. The calcium decreased from 4.0 mM to 1.5 mM. This implies *S. pevalleikii BDHKU 21305* has some influence on carbonation of concrete. pH decreased to 8.3 till the end of experiment.

Experiment D

The experiments performed abiotically in cement pore solution without the addition of $CaCl_2$. The calcium decreased from 4.0 mM to 2 mM. There was a slight decrease in pH from 11.7 to 11.5 till 6 h. The value of pH reached 8.5 at 24 h. For this change in pH carbonation of calcium hydroxide is anticipated to be a reason (Papadakis et al., 1991).

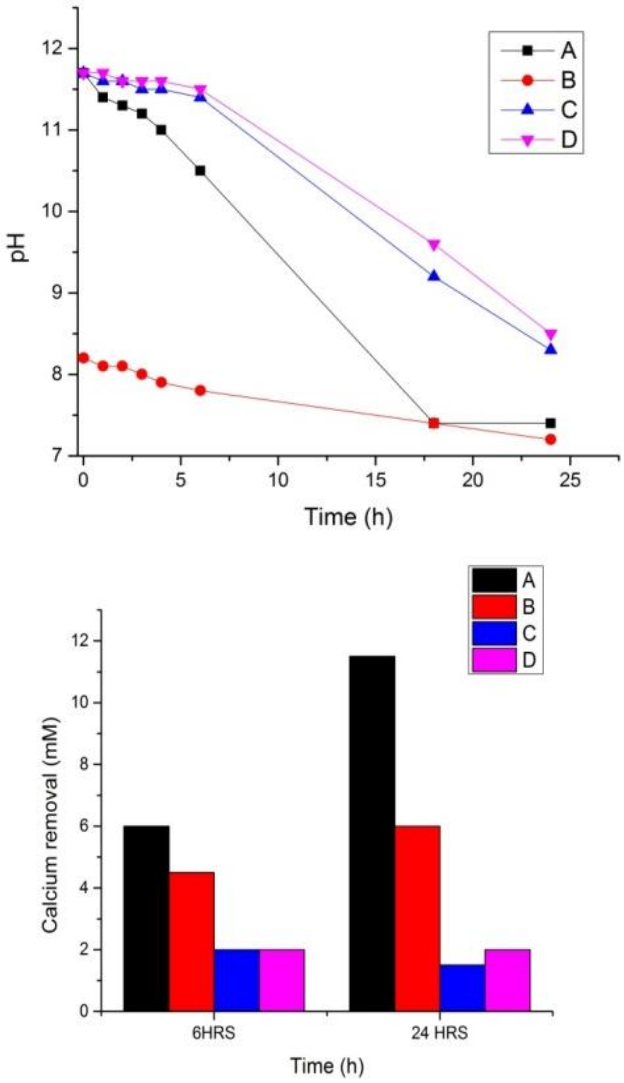

Fig 4.4 (A) Change in pH (B) Calcium removal

It is reported that in 15 days around 47.5 mM of calcium was removed from the bulk solution with an initial calcium concentration of 52.5 mM in M-3 medium by *Myxococcus xanthus*. The removal of calcium resulted in the precipitates formation (Jimenez-Lopez et al., 2008).
In our study calcium is also removed from the solution indicating the formation of precipitates by *S. pevalleikii BDHKU 21305.*

4.5 Sand consolidation

MICP process for soil bioclogging, i.e. to reduce the permeability of soil. Microbially induced carbonate precipitation (MICP) is a common natural process, which is controlled by different mechanisms (Banks et al. 2010; Cacchio et al. 2003; Wright and Oren 2005). One of them is the production of calcite in porous soil by urease-producing bacteria in the presence of urea and calcium ions (Mitchell and Santamarina 2005; Ivanov and Chu 2008).

After the success of laboratory scale experiments *in-situ* soil strengthening at the field (bio-grouting) has been attempted where the biological fluid is injected in the soil. It has been observed that although the technique works well for surface treatments, coarse grained materials and mixed-in-place applications, but in case of fine grained materials the injection well clogs rapidly (Le Metayer et al.,1999). Chu et al., (2012) and Stabnikov et al., (2011) reported considerable reduction in permeability, improved shear strength of soil and reduction of seepage rate due to formation of impermeable microbial carbonate crust. In case of sand plugs, Kantzas et al., (1992) reported that sand consolidation by *B. pasteurii* reduced porosity by up to 50% and permeability by up to 90% in the areas where cementation took place.

In current study cyanobacteria has been used for the sand consolidation. After 14 days the sand column was opened and solid cylindrical consolidated sand was taken out. Fortified sand indicated the bio-calcification by MICP in sand. Experiments I-A and II-A which were live and UV killed cells mixed to sand slurry resulted into complete consolidation of sand in the column (fig 4.5). Sand column treated with UV killed cells was harder than live cells treated column. Partial consolidation appeared in experiment I-B and II-B, 1/2 and 3/4th sand in the column were consolidated respectively. In experiment I-C and II-C there were clumps of sand on the top. The sand came out as it is from the control without any consolidation.

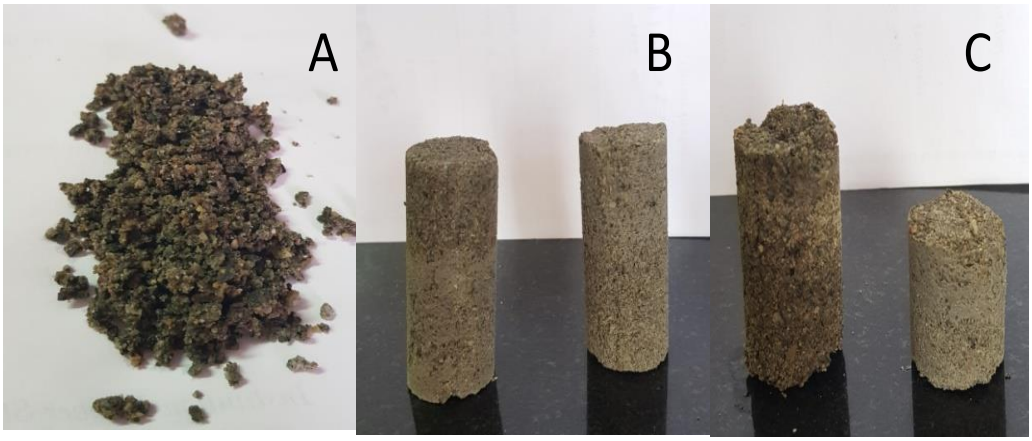

Fig 4.5 Sand consolidation in different calcifying solution (A) control (B) sand consolidated from I-A and II-A (C) sand consolidated from II-A and II-B

4.5.1 Flow rate estimation

Permeation properties of bio-calcified sand coloumns by MICP were investigated (fig 4.6A). Effluent flow rate of calcifying medium was measured regularly interval till 14 days flow rate. The flow rates of cyanobacteria treated columns and the control columns were almost same initially. This shows that at the initial points the pore size of the cells treated columns and control columns were similar. There was a very little reduction in the flow rate of control column i.e 2% in 14 days (fig 4.6B). This shows that there was a marginal change in the structures of pore of the control column due to the flow.

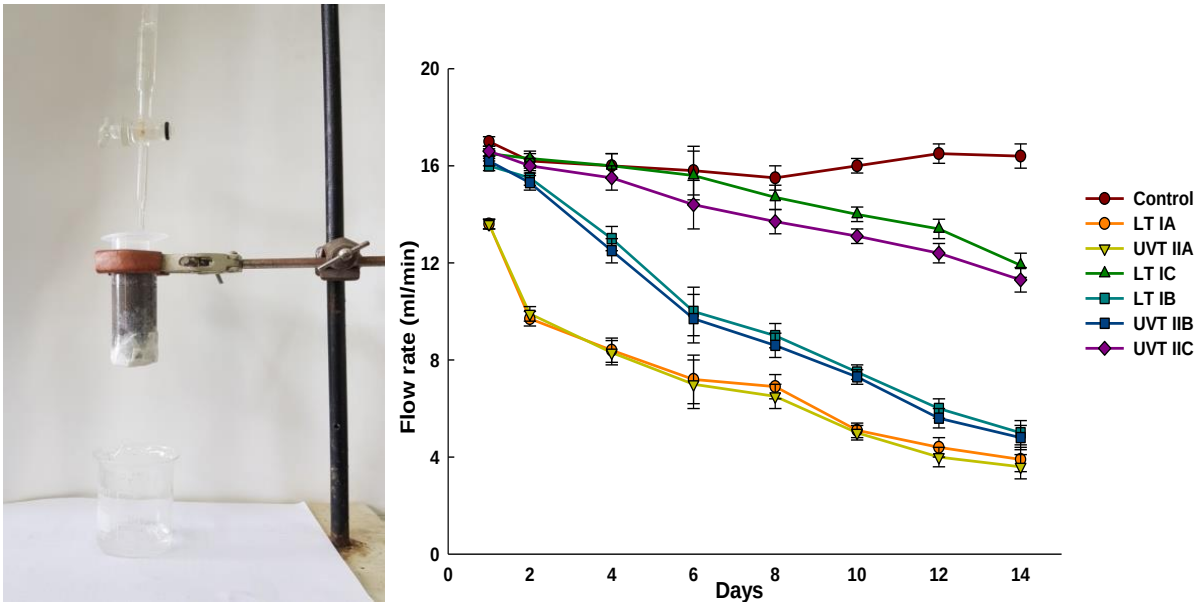

Fig 4.6 (A) Set up for sand consolidation (B) flow rate estimation

In case of the cells treated columns the flow rate decreased very fast which indicates that MICP is causing gradual constraint of the flow channels. The reduction in the flow rate in cells treated columns is primarily because of the carbonates deposition. A little reduction may also happen due entrapped biomass. In 4 days bacterial columns shows the flow rate reduction of 36% while after 14 days it reached to 53%. In the initial stages the flow reduced at a slower rate but in later stages the rate of reduction increased.

4.5.2 pH changes

Change in pH of the effluent was analyzed at 1,2,3,6 and 14 day (fig 4.7). The columns which were treated with live cells were found in the pH range of 7.3 to 7.5. Similarly column treated with UV killed cells were in the pH range of 7.6 to 7.8 at the end of 15th day. The pH of the effluent from control columns, conversely, remained same throughout the duration of the experiment i.e pH 8.1. In every treatment the pH decreased eventually. Initially pH remained similar but afterwards they varied. The pH of the effluent from control and the bacterial treated columns showed great variance, so it can considered as indicator of activity of MICP.

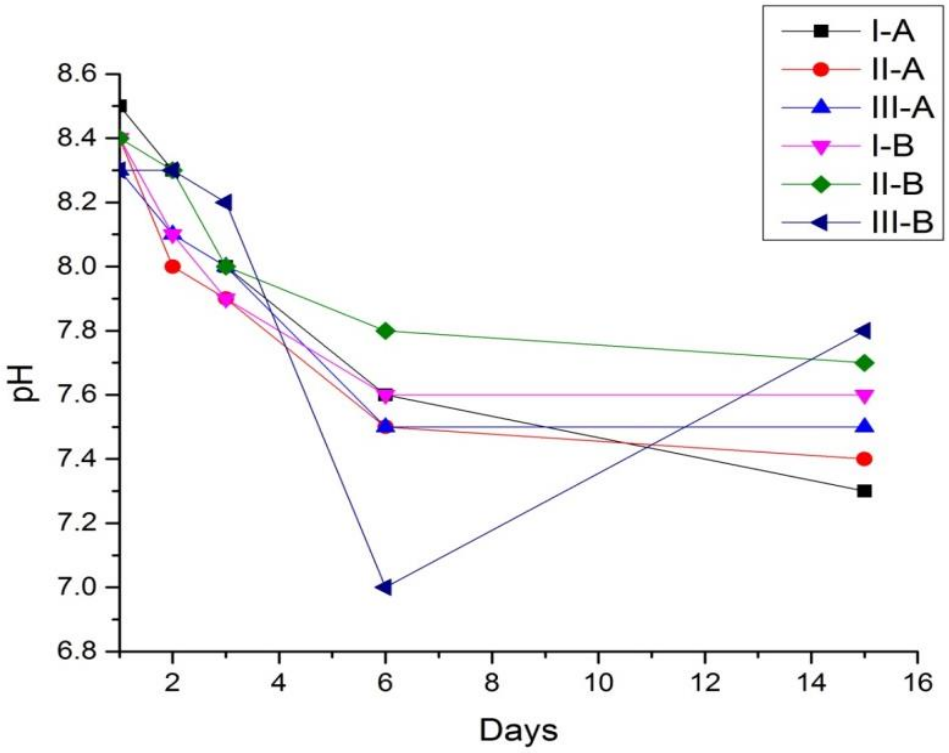

Fig 4.7 pH change of the effluent from sand columns

4.6 Compressive strength and permeability analysis

4.6.1 Compressive strength of cube under different condition for 45 days.

Variation in compressive strength was evaluated for cement mortar cubes under shaking and stationary condition. At the age of 45 days, the compressive strength of control was observed to be 30.9 MPa. Higher compressive strength of 47.96 MPa was observed in mortar cubes treated under shaking condition (fig 4.8). Whereas, compressive strength of 36.3 MPa was observed under stationary condition. Strength of 52.6 MPa was achieved in which media was refilled and was shaken after interval of 7 days, showed strength of 52.6 MPa. The cubes submersed in stationary condition showed minor strength gain.

The enhancement of durability of building materials with the combination of ureolytic bacteria and calcium chloride had been reported by Stocks-Fischer et al., (1999); Bang et al., (2001). The deposition of $CaCO_3$ improved the durability of building materials which were influenced by the ureolytic bacteria and calcium salt had been reported by De Muynck et al., (2008).

In this study, increase in compressive strength is due to the formation of calcium carbonate which is further supported by SEM analysis.

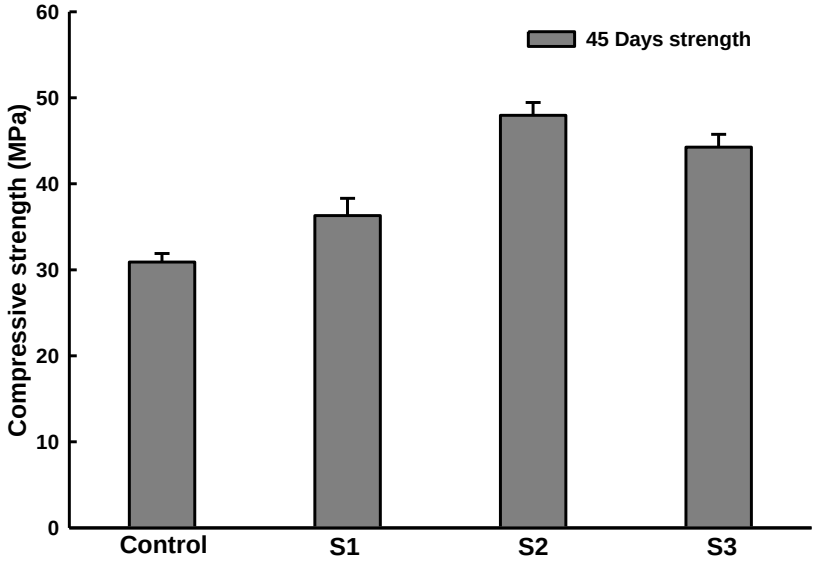

Fig 4.8 Compressive strength **(a)** stationary (S1) **(b)** shaking (S2) **(c)** alternatively shaking (S3) on the cement mortar cubes at the age of 45 days.

4.6.2 Permeability analysis of cubes under different condition for 45 days.

Water resistance was increased under all the different conditions of shaking. Among them, the mortar cubes in shaking condition had the lowest water absorption (fig 4.9). Mortar cubes in shaking condition decreased the water absorption by 4.6 times as that of control. Under stationary and alternatively shaking condition the water absorption of mortar cubes decreased by 3 and 2 times, respectively.

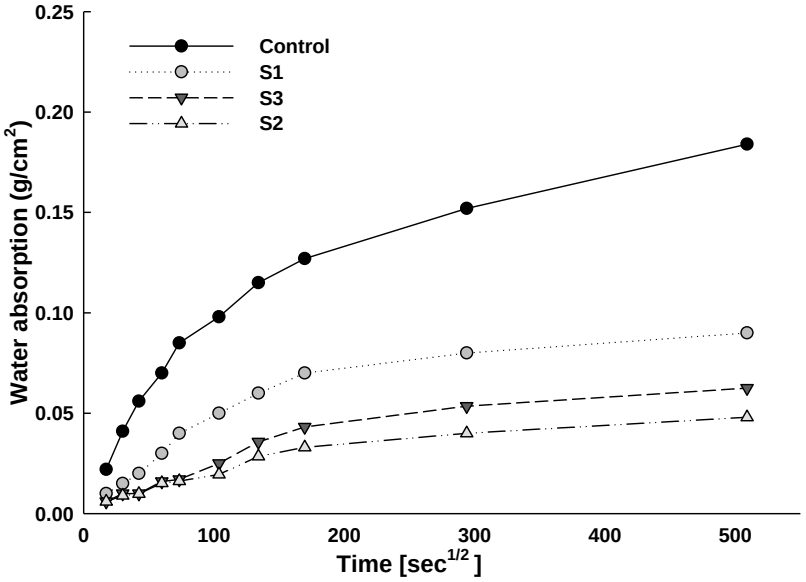

Fig 4.9 Water absorption by cubes under different shaking condition

4.6.3 Compressive strength and permeability analysis of cubes with differently treated cells

The increase in compressive strength of the mortar cubes was found higher when treated with UV killed cells. On the seventh day, the compressive strength of control specimen was 30.2 MPa, and that of mortar cubes treated with UV-killed cells was 36.3 MPa (fig 4.10). On the other hand in abiotically treated specimens, decrease in strength was recorded. The compressive strength of mortar cubes treated abiotically recorded decrease of 4.6% as compare to control specimens. When the mortar cubes were treated with live cells under illumination and in darkness, no major significant difference was observed between them. As compare to control specimens, minimal strength gain was observed in specimens treated under illumination and darkness respectively. further the compressive strength of mortar cubes which were treated abiotically was 28.8 MPa. Within 7 days, the compressive strength of the mortar cubes which were treated with UV-killed rays was increased by nearly 19%.

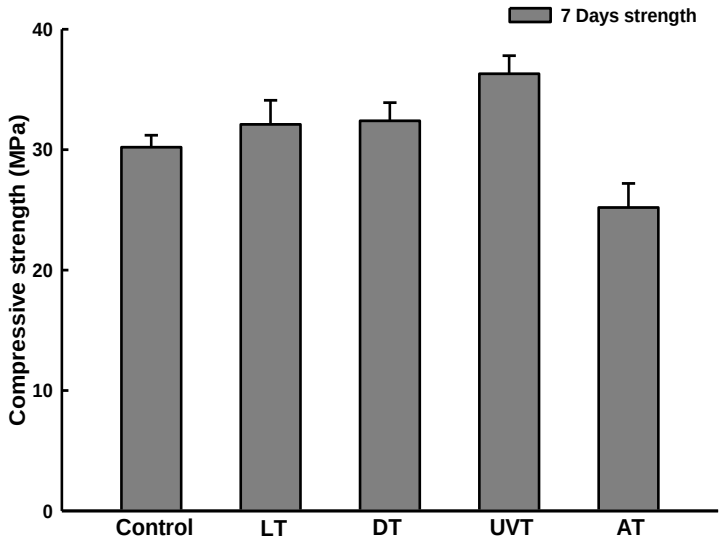

Fig 4.10 Compressive strength of **(a)** live (LT) **(b)** UV killed (UVT) **(c)** under darkness (DT) **(d)** abioticaly (AT) treated cement mortar cubes at the age of 7 days.

A previous study by Zhu et al., (2017) reported specimens treated with live, and UV killed cells showed similar results to this study. The cubes treated with UV killed cells showed significant rise in compressive strength. Drop in strength was observed in case of abiotic treatment. Increment in compressive strength improved by UV-killed cells is comparable to other experiments with live heterotrophic bacteria *S. pasteurii* (Achal et al., 2011); (Achal et al., 2009). 15% increase in compressive strength was observed with *Shewanella* after 7 days (Ghosh et al., 2009). Ramezanianpour et al., (2009) had shown negative impact of calcium chloride on strength of the abiotically treated mortars. This could be a reason for decrement of strength in abiotically treated samples.

4.6.4 Permeability analysis of cubes under with differently treated cells

Compared to the control, all the treatments decreased the water absorption. Among them, the mortar cubes treated with UV-killed cells had the lowest water absorption (fig 4.11). By the end of the water absorption test, UV-killed cells decreased the water absorption by 3.6 times as that of control. Live cells under illumination and darkness decreased the water absorption of mortar cubes by 2.9 and 2 times, respectively.

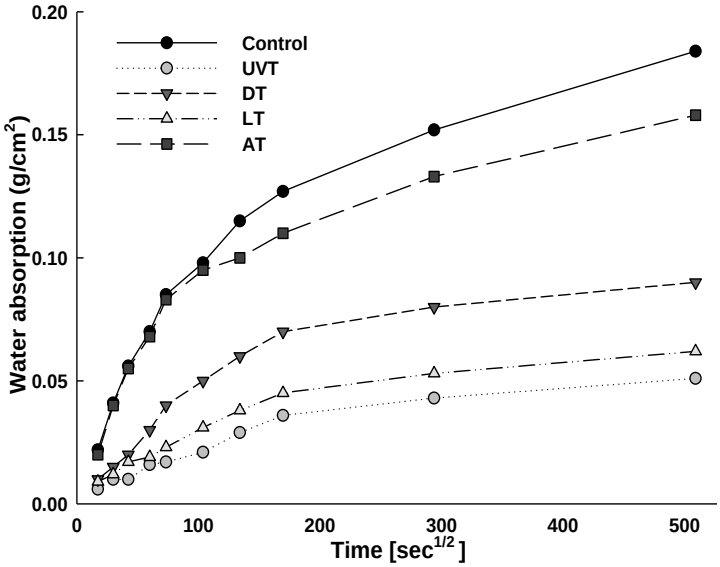

Fig 4.11 Water absorption by cubes of differently treated cells

4.7 Weight change of the mortar cubes

After the 7 days of time period, the control and cubes treated with bacteria has shown no weight gain where as the abiotic cubes had shown the 0.31± 0.03%. So, there was a minor change in weight after the absorption of cells to the mortar cubes. Cubes treated with live cells attained the maximum gain in weight. Weight of all other treated cubes was a little more than the abiotically treated cubes. Weight change after treatment of mortar cubes and precipitates in bulk solution are shown in table

Table 4.1 Weight change of the mortar cubes and the precipitates in the bulk solution.

Weight change	**Control**	**Abiotic**	**Live cells (Illumination)**	**Live cells (Dark)**	**UV-killed cells**
Mortar cubes (%)	0	0.31 ± 0.03	0.38 ± 0.02	0.34 ± 0.02	0.33 ± 0.06
Precipitates (g)	0	0.33 ± 0.01	0.35 ± 0.04	0.35 ± 0.02	0.34 ± 0.02

4.8 Microstructural analysis

4.8.1 Calcium carbonate precipitation on the mortar surface under the shaking condition.

Ability of *S. pevalleikii BDHKU 21305* to precipitate $CaCO_3$ on cement mortar cubes was confirmed in this experiment. Submersion of mortar cubes in the calcifying solution supplemented with cyanobacterial cells for 45 days under shaking condition resulted in formation of layer of bio-calcite on the surface of the cube. Calcified layer was not found on the surface of control cube which was cured in water only. In the biotic experiment, calcite crystals were observed in SEM analysis. Appearance of rhombohedral calcite and spherical shaped vaterite crystals confirmed the presence of calcium carbonate respectively. Presence of calcium carbonate crystals were confirmed by EDX mapping as shown in fig 4.12B. High peaks of principle elements (calcium, carbon and oxygen) indicate the presence of $CaCO_3$. EDX mapping of control sample showed high peaks of calcium and silica along with other elements. This implies no precipitation of calcium carbonate in control samples.

S. pevalleikii can contribute to a better restoration of concrete cracks than in the abiotic condition. Furthermore, the incorporation of microbial organic matter has been shown to enhance the adhesion properties of newly formed calcite-cell aggregates (Hillgärtner et al., 2001; Krumbein, 1979).

This analysis showed that the autophototrophic cells can be a candidate for concrete restoration.

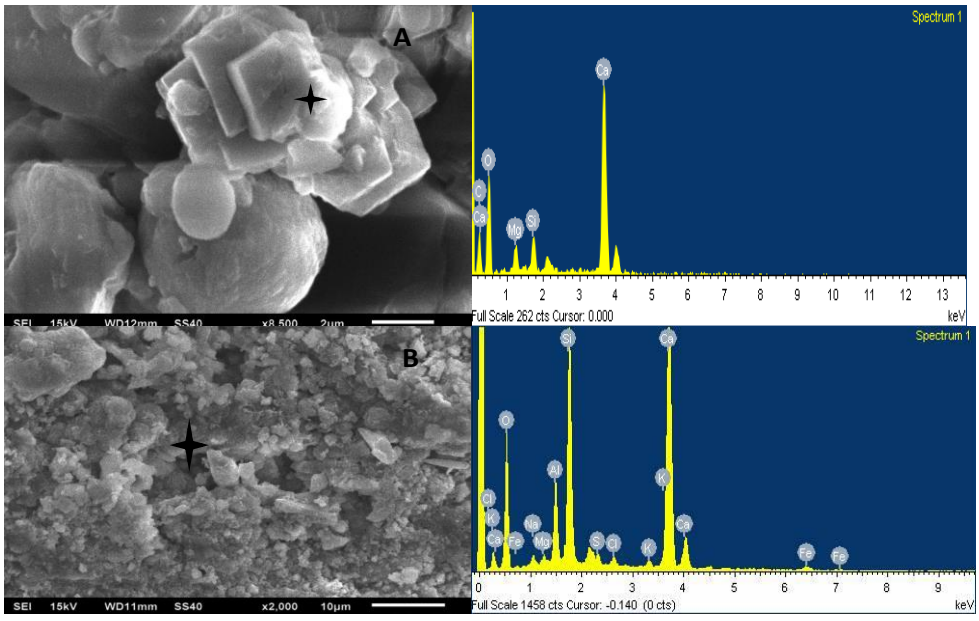

Fig 4.12 SEM images and EDX analysis of the mortar surface

4.8.2 Calcium carbonate precipitation on the mortar surface under the abiotic condition

After being immersed in the $CaCl_2$ solution without addition of cyanobacterial cells for 7 days, the mortar surfaces under the abiotic condition were incompletely covered with calcium carbonates. Unlike microbially formed calcium carbonates, these were loosely attached to the surface in the abiotic condition. Presence of few irregular-shaped $CaCO_3$ crystals was confirmed in EDX analysis as shown in fig 4.13b

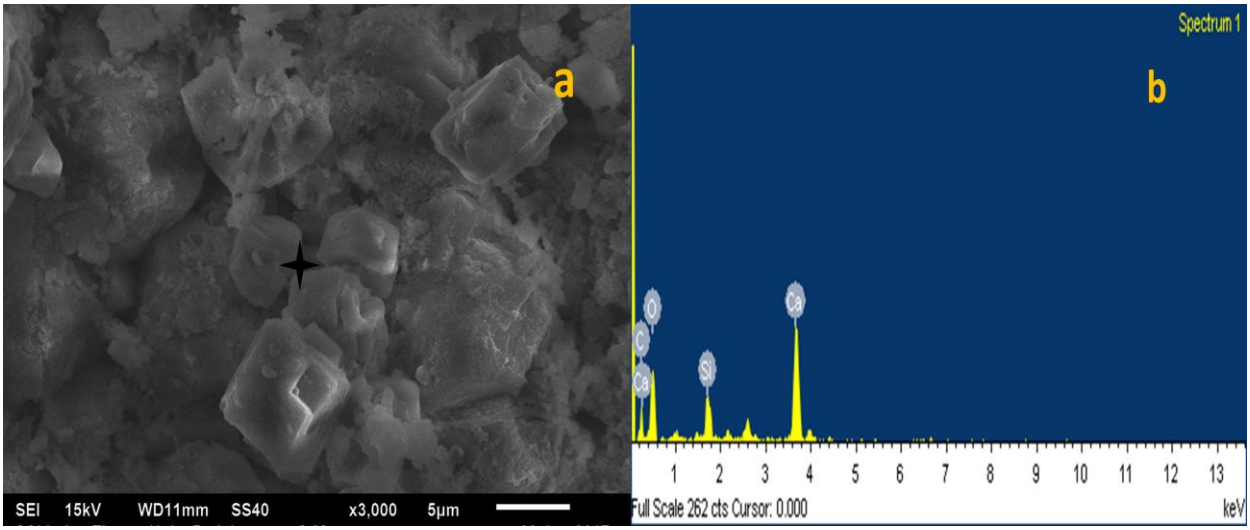

Fig 4.13 SEM images and EDX analysis of the mortar surface treated abiotically.

4.8.3 Carbonate precipitation on the mortar surface treated with live cells

The mortar cubes treated with live cells prior to being exposed to calcifying solution showed a completely different appearance as that of the abiotic condition after the 7-day immersion. The cement mortar cube surface was highly calcified and exhibited rough, fissured and wrinkled features after treatment. SEM images of the mortar specimen treated with live cells showed the presence of stacked rhomohedral calcite (fig-4.14a). EDX analysis further confirmed the presence of $CaCO_3$ crystals with high calcium, carbon and oxygen peaks as shown in fig 4.14b.The irregular shape of crystals is strongly influenced by numerous factors, such as small molecular additives (inorganic ions, small molecular organic species and solvents), soluble and insoluble biomacromolecules, the ratio of the functional groups to the calcium concentration, and temperature (Zhu et al., 2015).

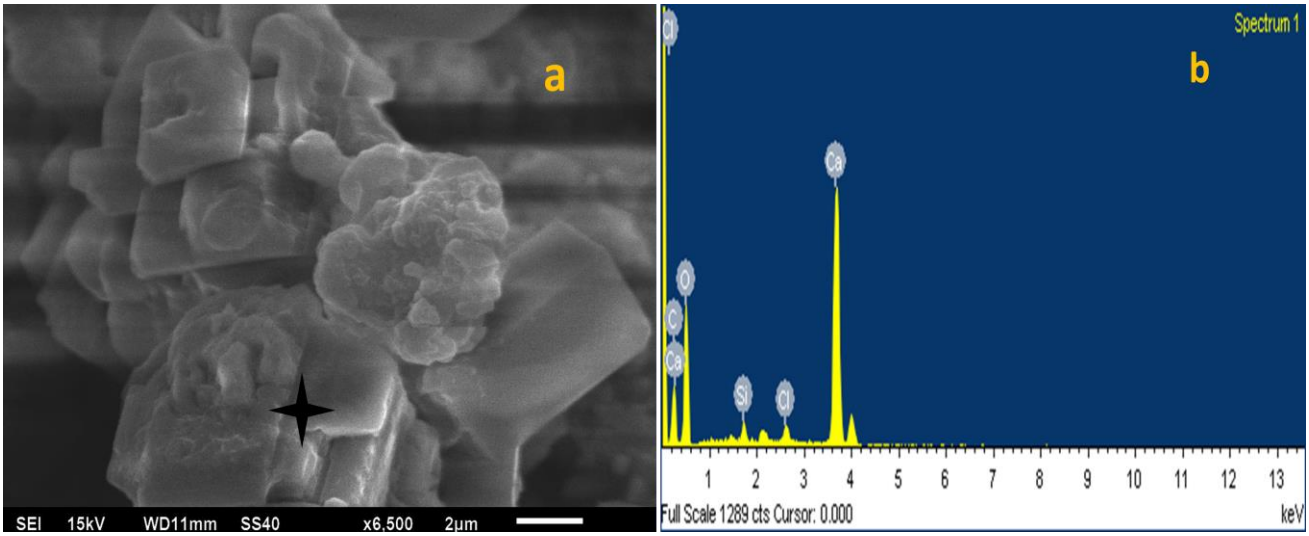

Fig. 4.14 SEM images and EDX analysis of the mortar surface treated with live cells

4.8.4 Carbonate precipitation on the mortar surface treated with UV-killed cells

On comparison UV killed cells also exhibited calcified layer on the surface of cubes. The thin and smooth UV-killed biofilm was covering the surface of the mortar cubes. SEM analysis showed the presence of stacked irregular shaped calcium carbonate crystals having fissures, unlike the perfect rhombohedral calcite crystal formed on mortar specimens treated with live cells.

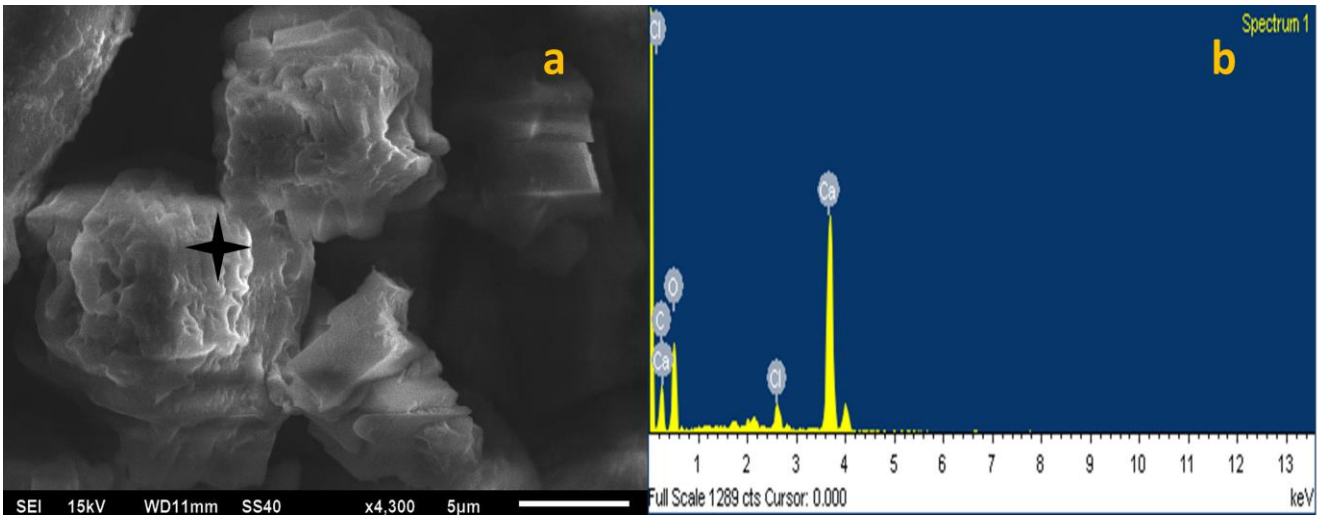

Fig 4.15 SEM images and EDX analysis of the mortar surface treated with UV killed cells

Presence of calcium carbonate crystals due to UV killed cells was also reported recently by Zhu et al., 2017. Cell surface and EPS playing a major role in biomineralization activity by UV killed cells was reported. The EPS promoted cells adhering to the rough mortar surface,

and subsequently formed calcium carbonate above them by attracting the positively charged Ca^{2+} using the negatively charged functional groups, such as carboxylate, amine, phosphory/phosphodiester and hydroxyl, on the cell surface and EPS.

4.8.5 Carbonate precipitation on the mortar surface treated with live cells under darkness

After the treatment with live cells under darkness, mortar cubes exhibited a thin layer of precipitation on its surface. The spherical shaped vaterite crystals appeared in SEM analysis. High peaks of calcium was viewed in EDX mapping, conforming the formation of calcium carbonate.

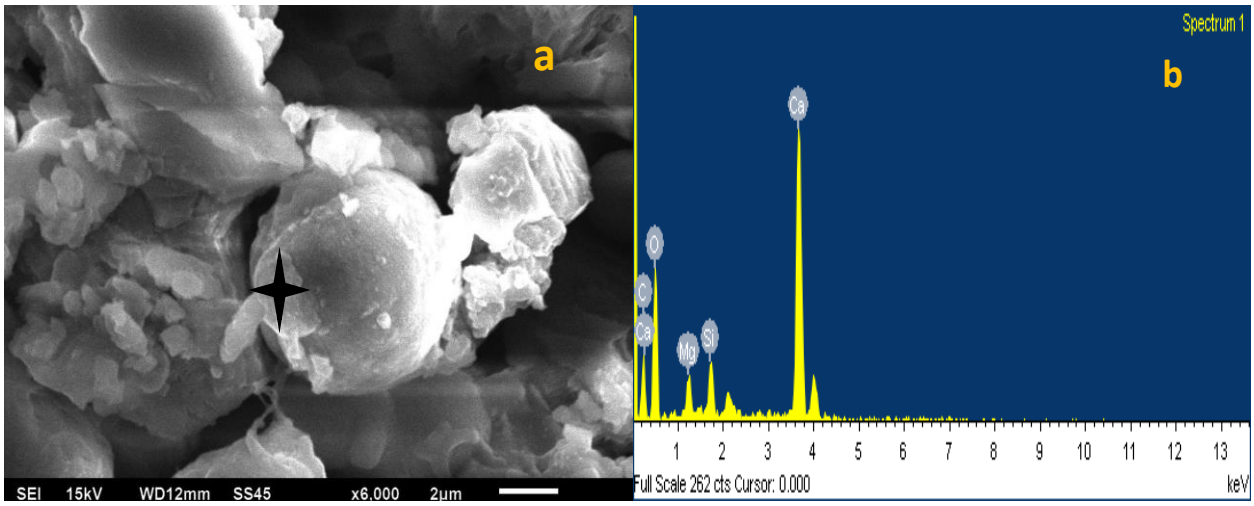

Fig 4.16 SEM images and EDX analysis of the mortar surface treated with live cells under darkness

4.9 Surface treatment

Generally, bio-treatments of concrete structures in lab scale tests are usually conducted by immersing the specimens in bio agents. Instead of immersion some researchers had also proposed spraying method for the surface treatment of on-site concrete samples. Biotreatment of cement mortar sample instead of using immersion or spraying method, ponding method was used in the current study (fig 4.17A). Effectiveness in water resistance of exposed surface was studied after the age of 14 days. Cement mortar cubes treated with UV killed cells showed 5 times higher resistance to water ingress as compared to control specimen as shown in fig 4.17B Water absorption in specimen treated with live cells showed 3 times higher resistance than control specimen.

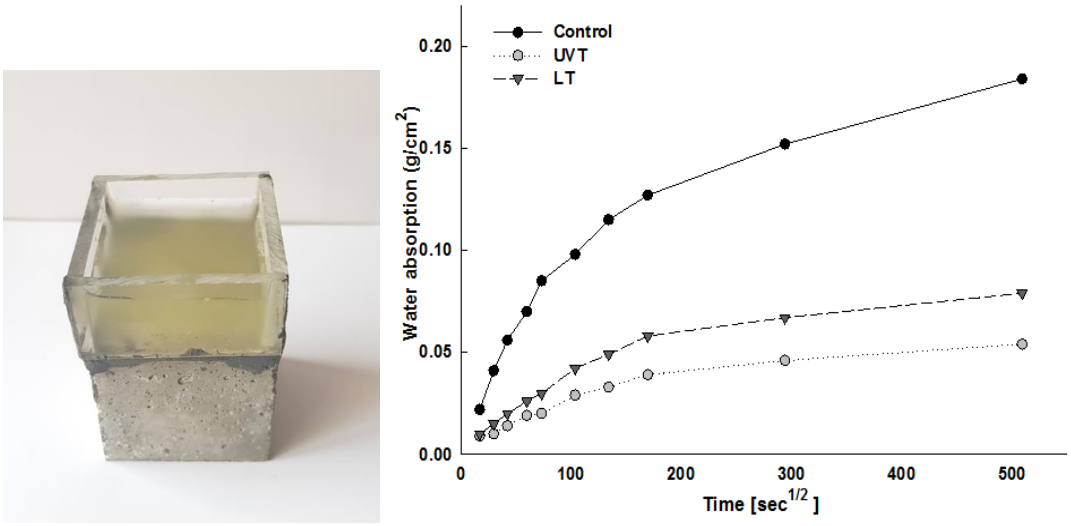

Fig 4.17 (A) Ponding surface treatment (B) water absorption test

A thick layer of precipitates was observed on the surface of mortar cube in both the treatment with UV killed and live cells. The rhombohedral calcite crystals with staircase morphology was observed UV killed cells treated mortar cube. A thick precipitation layer was observed showing more precipitation. Irregular shaped crystals were observed in case of live cells treatment. High peak of Ca, O and C was observed in the EDX mapping of both the samples indicating the presence of calcium carbonate (fig 4.18). EDX mapping revealed the elements Si and Mg, as these elements were present in the cement matrix.

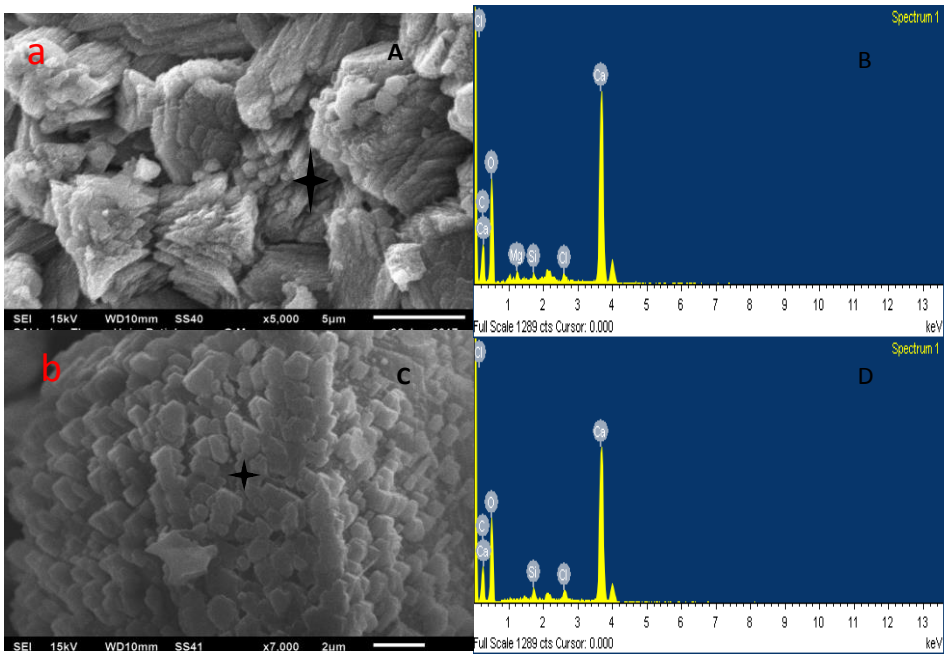

Fig 4.18 SEM and EDX analysis of (a)UV killed cells (b) live cells

At micro scale, positive outcome of low water ingress was observed in ponding surface treatment. Biotreatments on surface of huge concrete structures like slabs and pavements can also be done. This method can also be used at field scale in repairing surface cracks. Various studies has been reported in which ureolytic bacteria was used as pooling solution to heal surface crack at field scale (Richardson et al., 2016). Full scale outdoor application with cyanobacterial surface treatment might be an effective alternate to ureolytic bacteria in outdoor applications.

Conclusion

Employment of cyanobacterial calcium carbonate mineralization offers a novel and self-sustaining strategy in restoration of building material. Inspired from the cyanobacterial recalcitrant calcium carbonate precipitation in geological formations, application of cyanobacterial treatment on building materials was studied. In current study, this property has been exploited for enhancing durability properties of cement mortar cubes. Increase of 10% and 19% of compressive strength was observed in specimen treated with live and UV killed cells respectively. Water resistance increased by 3.6 and 2.6 times in specimen treated with live and UV killed cells respectively. A thick layer of precipitation was observed on the surface of differently treated mortar cube which was further supported by SEM analysis. Stacked rhombohedral calcite polymorphs was observed in specimen treated with UV killed cells. Promising results from this study suggest that microbial treatment with autophototrophic cyanobacteria could provide an alternative eco-friendly way to strengthen the structural properties of concrete.

In conclusion, *Synechococcus pevalleikii BDHKU 21305* has the potential to serve as a green technology in concrete restoration at lab scale. Future studies need to be done in implementing this technology at field scale. It could pave a way to develop a sustainable infrastructure.

References

Abo-El-Enein S. A, Ali A. H, Talkhan F. N, Abdel-Gawwad H. A (2012) Utilization of microbial induced calcite precipitation for sand consolidation and mortar crack remediation. HBRC Journal, 8(3), 185-192.

Achal V, Mukherjee A, Basu P.C, Reddy M. S (2009) Lactose mother liquor as an alternative nutrient source for microbial concrete production by *Sporosarcina pasteurii*. Journal of Industrial Microbiology & Biotechnology, 36(3), 433-438.

Achal V, Mukherjee A, Basu P.C, Reddy M. S (2009) Strain improvement of *Sporosarcina pasteurii* for enhanced urease and calcite production. Journal of Industrial Microbiology & Biotechnology, 38, 1229-1234.

Achal V, Mukherjee A, Reddy M. S (2010) Microbial concrete: way to enhance the durability of building structures. Journal of Materials in Civil Engineering, 23(6), 730-734.

Achal V, Pan X, Fu Q, Zhang D (2012) Biomineralization based remediation of As (III) contaminated soil by *Sporosarcina ginsengisoli*. Journal of Hazardous Materials, 201, 178-184.

Achal, V., Mukherjee, A, Reddy, M. S (2011) Effect of calcifying bacteria on permeation

Altermann W, Kazmierczak J, Oren A, Wright D. T (2006) Cyanobacterial calcification and its rock-building potential during 3.5 billion years of Earth history. Geobiology, 4(3), 147-166.

Bang S, Galinat J. K, Ramakrishnan V (2001) Calcite precipitation induced by polyurethane-immobilized Bacillus pasteurii. Enzyme and Microbial Technology, 28(4), 404-409.

Bang S. S, Lippert J. J, Yerra U, Mulukutla S, Ramakrishnan V (2010) Microbial calcite, a bio-based smart nanomaterial in concrete remediation. International Journal of Smart and Nano Materials, 1(1), 28-39.

Banks E. D, Taylor N. M, Gulley J, Lubbers B. R, Giarrizzo J. G, Bullen H. A Barton H. A (2010) Bacterial calcium carbonate precipitation in cave environments: a function of calcium homeostasis. Geomicrobiology Journal, 27(5), 444-454.

Barlet-Gouédard V, Rimmelé G, Porcherie O, Quisel N, Desroches J (2009) A solution against well cement degradation under CO_2 geological storage environment. International Journal of Greenhouse Gas Control, 3(2), 206-216.

Basheer P. A. M, Chidiact S. E, Long A. E (1996) Predictive models for deterioration of concrete structures. Construction and Building Materials, 10(1), 27-37.

Benini S, Rypniewski W. R, Wilson K. S, Miletti S, Ciurli S, Mangani S. (1999). A new proposal for urease mechanism based on the crystal structures of the native and inhibited enzyme from *Bacillus pasteurii*: why urea hydrolysis costs two nickels. Structure, 7(2), 205-216.

Bolin B (1998) The Kyoto negotiations on climate change: a science perspective.

Cacchio P, Ercole C, Cappuccio G, Lepidi A (2003) Calcium carbonate precipitation by bacterial strains isolated from a limestone cave and from a loamy soil. Geomicrobiology Journal, 20(2), 85-98.

Castanier S, Le Métayer-Levrel G, Perthuisot J. P (1999) Ca-carbonates precipitation and limestone genesis—the microbiogeologist point of view. Sedimentary Geology, 126(1), 9-23.

Chafetz H, Buczynski C (1992) Bacterially induced lithification of microbial mats. Palaios, 277-293.

Cheng L, Cord-Ruwisch R, Shahin M. A (2013) Cementation of sand soil by microbially induced calcite precipitation at various degrees of saturation. Canadian Geotechnical Journal, 50(1), 81-90.

Chu J, Stabnikov V, Ivanov V (2012) Microbially induced calcium carbonate precipitation on surface or in the bulk of soil. Geomicrobiology Journal, 29(6), 544-549.

Cizer Ö, Rodriguez-Navarro C, Ruiz-Agudo E, Elsen, J, Van Gemert D, Van Balen K (2012) Phase and morphology evolution of calcium carbonate precipitated by carbonation of hydrated lime. Journal of Materials Science, 47(16), 6151-6165.

Claisse P. A, Elsayad H. I, Shaaban I. G (1997) Absorption and sorptivity of cover concrete. Journal of Materials in Civil Engineering, 9(3), 105-110.

Dai J. G, Akira Y, Wittmann F. H, Yokota H, Zhang P (2010) Water repellent surface impregnation for extension of service life of reinforced concrete structures in marine environments: the role of cracks. Cement and Concrete Composites, 32(2), 101-109.

De Belie N, De Muynck W (2008) Crack repair in concrete using biodeposition. In Proceedings of the International Conference on Concrete Repair, Rehabilitation and Retrofitting, 291-292.

De Belie N, Wang J (2016) Bacteria-based repair and self-healing of concrete. Journal of Sustainable Cement-Based Materials, 5(2), 35-56.

De Muynck W, Cox K, De Belie N, Verstraete W (2008) Bacterial carbonate precipitation as an alternative surface treatment for concrete. Construction and Building Materials, 22(5), 875-885

De Muynck W, De Belie N Verstraete W (2010) Microbial carbonate precipitation in construction materials: a review. Ecological Engineering, 36(2), 118-136.

De Muynck W, Debrouwer D, De Belie N, Verstraete W (2008) Bacterial carbonate precipitation improves the durability of cementitious materials. Cement and Concrete Research, 38(7), 1005-1014.

Dhami N. K, Reddy M. S, Mukherjee A (2013) Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites. Journal Microbiology Biotechnology, 23(5), 707-714.

Dhami N. K, Reddy M. S, Mukherjee A (2016) Significant indicators for biomineralisation in sand of varying grain sizes. Construction and Building Materials, 104, 198-207.

Dittrich M, Kurz P, Wehrli1 B (2004) The role of autotrophic picocyanobacteria in calcite precipitation in an oligotrophic lake. Geomicrobiology Journal, 21(1), 45-53.

Edvardsen C (1999) Water permeability and autogenous healing of cracks in concrete. Materials Journal, 96(4), 448-454.

Emmons P. H, Sordyl D (2006) The state of the concrete repair industry, and a vision for its future. Concrete Repair Bulletin, 7-14.

Fujita Y, Taylor J.L, Wendt L.M, Reed D.W, Smith R.W (2010) Evaluating the potential of native ureolytic microbes to remediate a 90Sr contaminated environment. Environmental Science & Technology, 44(19), 7652-7658.

Ghosh S, Biswas M, Chattopadhyay B. D, Mandal S (2009) Microbial activity on the microstructure of bacteria modified mortar. Cement and Concrete Composites, 31(2), 93-98.

Gonsalves G. M (2011) Bioconcrete-a sustainable substitute for concrete? (Master's thesis, Universitat Politècnica de Catalunya).

Hamilton W. B (2003) An alternative earth. GSA TODAY, 13(11), 4-12.

Hammes F, Verstraete W (2002) Key roles of pH and calcium metabolism in microbial carbonate precipitation. Reviews in Environmental Science and Biotechnology, 1(1), 3-7.

Hillgärtner H, Dupraz, Hug C.W (2001) Microbially induced cementation of carbonate sands: are micritic meniscus cements good indicators of vadose diagenesis. Sedimentology, 48, 117–131.

Itagaki M, Soukura M, Koike T, Okamoto T, Tokieda H, Hoshi Y, Kato Y (2016) EIS Study on Corrosion Mechanisms of Steel Rebars in Concrete. The Electrochemical Society, 15, 933-933.

Ivanov V, Chu J (2008) Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ. Reviews in Environmental Science and Bio/Technology, 7(2), 139-153.

Jansson C, Northen, T. (2010) Calcifying cyanobacteria—the potential of biomineralization for carbon capture and storage. Current Opinion in Biotechnology, 21(3), 365-371.

Jimenez-Lopez C, Jroundi F, Pascolini C, Rodriguez-Navarro C, Pinar-Larrubia G, Rodriguez-Gallego M, Gonzalez-Munoz M.T (2008) Consolidation of quarry calcarenite by calcium carbonate precipitation induced by bacteria activated among the microbiota inhabiting the stone. International Biodeterioration & Biodegradation, 62, 352– 363.

Jonkers H. M, Schlangen E (2009) A two component bacteria-based self-healing concrete. Concrete Repair, Rehabilitation and Retrofitting II. CRC press, Taylor & Francis Group, Boca Raton, 119-120.

Jonkers H. M, Schlangen E (2009) Towards a sustainable bacterially-mediated self healing concrete. In Proceedings of 2nd International Conference on Self-Healing Materials, Chicago.

Jonkers, H. M, Thijssen A, Muyzer, G, Copuroglu O, Schlangen E (2010) Application of bacteria as self-healing agent for the development of sustainable concrete. Ecological Engineering, 36(2), 230-235.

Keefe W. E (1976) Formation of crystalline deposits by several genera of the family *Enterobacteriaceae.* Infection and Immunity, 14(2), 590-592.

Kirrolia A, Bishnoi N. R, Singh R (2012) Effect of shaking, incubation temperature, salinity and media composition on growth traits of green microalgae *Chlorococcum* sp. Journal of Algal Bio Utililzation, 46-53.

Koch G, Broongers H, Thompson N, Virmani Y (2002) Corrosion Cost and Preventive Strategies in the United States, Federal Highway Administration,773, 115-156.

Krumbei, W. E, Giele C (1979) Calcification in a coccoid cyanobacterium associated with the formation of desert stromatolites. Sedimentology, 26(4), 593-604.

Le Metayer-Levrel G, Castanier S, Orial G, Loubiere J. F, Perthuisot J. P (1999) Applications of bacterial carbonatogenesis to the protection and regeneration of limestones in buildings and historic patrimony. Sedimentary Geology, 126(1), 25-34.

Li V. C, Yang E. H (2007) Self healing in concrete materials. Self Healing Materials, 161-193.

Liang A, Paulo C, Zhu Y, Dittrich M (2013) $CaCO_3$ biomineralization on cyanobacterial surfaces: Insights from experiments with three *Synechococcus* strains. Colloids and Surfaces B: Biointerfaces, 111, 600-608.

Lowenstam H. A (1981) Minerals formed by organisms. Science, 211(4487), 1126-1131.

Lowenstam H. A, Weiner S (1989) Biomineralization. Bulletin of Marine Science, 45(2), 243-252.

Mindess S, Young J. F, Darwin D (2003) Concrete. Prentice Hall.

Mitchell J. K, Santamarina J. C (2005) Biological considerations in geotechnical engineering. Journal of Geotechnical and Geoenvironmental Engineering, 131(10), 1222-1233.

Monger H. C, Daugherty L. A, Lindemann W. C, Liddell C. M (1991) Microbial precipitation of pedogenic calcite. Geology, 19(10), 997-1000.

Neville A (2002) Autogenous healing—A concrete miracle?. Concrete International, 24(11), 76-82.

Neville A. M (1995) Properties of Concrete, London: Longman, 4.

Obst M, Dynes J. J, Lawrence J. R, Swerhone G. D. W, Benzerara K, Karunakaran, Hitchcock A. P. (2009) Precipitation of amorphous $CaCO_3$ (aragonite-like) by cyanobacteria: a STXM study of the influence of EPS on the nucleation process. Geochimica et Cosmochimica Acta, 73(14), 4180-4198.

Papadakis V.G, Vayenas C.G, Fardis M. N (1991) Experimental investigation and mathematical modeling of the concrete carbonation problem. Chemical Engineering Science, 46(5-6), 1333-1338.

Paulo C, Dittrich M (2013) 2D Raman spectroscopy study of dolomite and cyanobacterial extracellular polymeric substances from Khor Al-Adaid sabkha (Qatar). Journal of Raman Spectroscopy, 44(11), 1563-1569.

Qian C, Pan Q, Wang R (2010) Cementation of sand grains based on carbonate precipitation induced by microorganism. Science China Technological Sciences, 53(8), 2198-2206.

Qiu J, Tng D. Q. S, Yang E. H (2014) Surface treatment of recycled concrete aggregates through microbial carbonate precipitation. Construction and Building

Materials, 57, 144-150.

Ramachandran S. K, Ramakrishnan V, Bang S. S (2001) Remediation of concrete using micro-organisms. ACI Materials Journal-American Concrete Institute, 98(1), 3-9.

Ramezanianpour A, Khani M, Ahmadibeni M (2009) Advances in Materials and Corrosion. International Journal of Civil Engineering, (2), 83-91

Ramm W, Biscoping M (1998) Autogenous healing and reinforcement corrosion of water-penetrated separation cracks in reinforced concrete. Nuclear Engineering and Design, 179(2), 191-200.

Reichle D, Houghton J, Kane B, Ekmann J (1999) Carbon sequestration research and development. National Energy Technology Lab, 1.

Richardson A, Coventry K, Pasley J (2016) Bacterial crack sealing and surface finish application to concrete. Sustainable Construction Materials and Technologies, 1716-1724.

Rivera-Corral J. O, Fajardo G, Arliguie G, Orozco-Cruz R, Deby F, Valdez, P (2017) Corrosion behavior of steel reinforcement bars embedded in concrete exposed to chlorides: Effect of surface finish. Construction and Building Materials, 147, 815-826.

Rodríguez-Martínez M, Sánchez F, Walliser E.O, Reitner, J (2012) An upper Turonian fine-grained shallow marine stromatolite bed from the munecas formation, northern Iberian ranges, Spain. Sedimentary Geology, 263, 96-108.

Saiz-Jimenez C (1997) Biodeterioration vs biodegradation: the role of microorganisms in the removal of pollutants deposited on historic buidlings. International Biodeterioration & Biodegradation, 40(2-4), 225-232.

Schultze-Lam S, Beveridge T.J, Des Marais D.J (1997) Whiting events: biogenic origin due to the photosynthetic activity of cyanobacterial picoplankton. Limnology and Oceanography, 42(1), 133-141.

SharmaA, Bhattacharya A (2010) Enhanced biomimetic sequestration of CO_2 into $CaCO_3$ using purified carbonic anhydrase from indigenous bacterial strains. Journal of Molecular catalysis B: Enzymatic, 67(1), 122-128.

Stabnikov V, Naeimi M, Ivanov V, Chu J (2011) Formation of water-impermeable crust on sand surface using biocement. Cement and Concrete Research, 41(11), 1143-1149.

Stocks-Fischer S, Galinat J. K, Bang S. S (1999) Microbiological precipitation of $CaCO_3$. Soil Biology and Biochemistry, 31(11), 1563-1571.

Thompson J , Schultze-Lam S, Beveridge T. J, Des D (1997) Whiting events: biogenic origin due to the photosynthetic activity of cyanobacterial picoplankton, Limnology and Oceanography, 42, 133–141.

Tiano P, Biagiotti L, Mastromei G (1999) Bacterial bio-mediated calcite precipitation for monumental stones conservation: methods of evaluation. Journal of Microbiological Methods, 36(1), 139-145.

Vasconcelos C, McKenzie J. A, Bernasconi S, Grujic D, Tien A. J (1995) Microbial mediation as a possible mechanism for natural dolomite formation at low temperatures. Nature, 377(6546), 220.

Wang J, Van Tittelboom K, De Belie N, Verstraete W (2012) Use of silica gel or polyurethane immobilized bacteria for self-healing concrete. Construction and Building materials, 26(1), 532-540.

Wang J.Y, Van Tittelboom K, De Belie N, Verstraete W (2010, June) Potential of applying bacteria to heal cracks in concrete. Sustainable Construction Materials and Technologies, 1807-1818.

Warscheid T, Braams J (2000) Biodeterioration of stone: a review. International Biodeterioration & Biodegradation, 46(4), 343-368.

Worrell E, Price L, Martin N, Hendriks C, Meida L. O (2001) Carbon dioxide emissions from the global cement industry. Annual Review of Energy and the Environment, 26(1), 303-329.

Wright D. T, Oren A (2005) Nonphotosynthetic bacteria and the formation of carbonates and evaporites through time. Geomicrobiology Journal, 22(1-2), 27-53.

Xu J, Yao W, Jiang Z (2014) Non-ureolytic bacterial carbonate precipitation as a surface treatment strategy on cementitious materials. Journal of Materials in Civil Engineering, 26(5), 983-991.

Zhu T, Paulo C, Merroun M. L, Dittrich M (2015) Potential application of biomineralization by Synechococcus PCC8806 for concrete restoration. Ecological Engineering, 82, 459-468.

Zhu T, Paulo C, Merroun M. L, Dittrich M (2017) Calcification on mortar by live and UV-killed biofilm-forming cyanobacterial *Gloeocapsa PCC731*. Construction and building material, 146, 43-53.