Microbial remediation of defects in building materials and structures

A Thesis
Submitted in fulfillment of the requirement
for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

By

Varenyam Achal
(Reg No 90600006)

Department of Biotechnology and Environmental Sciences,
Thapar University, Patiala –147004
Punjab (India)

2010

CERTIFICATE

Certified that the thesis **"Microbial remediation of defects in building materials and structures"** which is submitted by Mr. Varenyam Achal, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by him under our supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

(Dr. M. Sudhakara Reddy)
Supervisor & Professor,
DBTES, Thapar University,
Patiala – 147004

(Dr. Prabir C. Basu)
Supervisor & Director-CSED,
Atomic Energy Regulatory Board,
Mumbai – 400094

(Dr. N. Das)
Head,
DBTES, Thapar University,
Patiala – 147004

DECLARATION

I hereby declare that the work which is being presented in this thesis "*Microbial remediation of defects in building materials and structures*" submitted by me for the award of the degree of *Doctor of Philosophy* in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervisions of Prof. M. Sudhakara Reddy, Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India and Dr. Prabir C. Basu, Director-CSED, Atomic Energy Regulatory Board, Mumbai, 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.

(Varenyam Achal)

ACKNOWLEDGEMENT

In pursuit of this academic endeavor, I feel that I have been singularly fortunate because inspiration, guidance, direction, cooperation, love and care - all came in my way in abundance and it seems almost an impossible task for me to acknowledge the same in adequate term.

Yes, I shall be failing in my duty if I do not record my profound sense of indebtedness and heart felt gratitude to my guide, **Prof. M. Sudhakara Reddy**, Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University (TU), Patiala, who guided and inspired me in pursuance of this work. His association with this endeavor of mine will remain a beacon light to me through out my life. Thank you for making me a better person, guiding me and improving silliest and minutest mistakes. His presence was always there whenever I needed whether it was personal or professional life.

I wish to express my deep sense of gratitude with reverence towards my project supervisor, **Prof. Abhijit Mukherjee**, Director, Thapar University (TU), Patiala, for providing inspiration, motivation, encouragement and financial support for the present work. I am highly indebted to him for his support and thought provoking suggestions during many exigent circumstances. His systematic approach and unconditional co-operation made me work hard. Indeed, I shall remain grateful to him forever.

I am also very thankful to my another PhD supervisor **Dr. Prabir C. Basu**, Director-CSED, Atomic Energy Regulatory Board, Mumbai, India, for providing valuable and remarkable support throughout experimental work.

I am very thankful to Dr. N. Das, Associate Professor and Head, DBTES, TU, Patiala for providing suggestions, for thoughts and taking interest in the progress of this work since inception. I express my gratitude to Prof. P. K. Bajpayee, Dean (Research and Sponsored Projects) and Dr. Shusheel Mittal, former Dean (Research and Sponsored Projects), Thapar University, Patiala, for their encouragement and support during my research work in the University.

Constructive suggestions and critical remarks of Dr. Anil Kumar Datta, Assistant Prof.-DBTES, Dr. Shweta Goyal, Assistant Prof., Civil Engineering Dept., TU, Patiala and Dr. Pankaj Krishna, Product Specialist, Ranbaxy, Noida towards amelioration of this work have been of immense help in the progress of this work. I am highly obliged to them for their support in carrying out present research work.

Here the time for the acknowledging friends comes. I was not sure to write this page as I didn't want to end up with a long list of `cliché (which is, anyhow, exactly what I am going to do!). However, I decided to do it because there are plenty of people I want to thank and because I do think that most of the people, like me, enjoy reading their name in the acknowledgements (ha…ha…ha)! The cooperation received through my colleagues namely Dr. Neetu, Diwakar sir**,** Santosh Karn, Navdeep, Santosh Pathak, Giri and Harpreet are thankfully acknowledged. I owe a word of thanks to Mr. Babban (Ram Neval) and Lallan Yadav for their help. Special thanks to friend-cum-brother, Mukesh! I also thank those who could not find a separate name but helped me directly or indirectly.

Above all it's my family members who encouraged me to complete my PhD besides lots of odds and troughs in my life. I owe my personal reckoning of gratitude and benevolence to them for love, care, patience, inspiration, encouragement, motivation and constant driving force which has enabled me to complete my task.

Varenyam Achal

List of Publications

The following publications are the outcome of the present research work:

- **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: 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* 36: 981-988.
- **Achal V**., Mukherjee A., Reddy M.S. (2010). Biocalcification by *Sporosarcina pasteurii* using Corn steep liquor as nutrient source. *Industrial Biotechnology* 6(3): 170-174.
- **Achal V.**, Mukherjee A., Reddy M.S. (2010). Microbial concrete- A way to enhance the durability of building structures. *ASCE-Journal of Materials in Civil Engineering* DOI: http://dx.doi.org/10.1061/(ASCE)MT.1943-5533.0000159
- Mukherjee A., **Achal V**., Reddy M.S. (2010). In search of a sustainable binder in building materials. *Annals Indian National Academy of Engineering Bulletin* 7: 41-51.
- **Achal V**., Mukherjee A., Reddy M.S. (2010). Isolation and characterization of urease producing and calcifying bacteria from cement. *J Microbiology Biotechnology* 20(11): 1571-1576.
- **Achal V.**, Mukherjee A., Reddy M.S. (2010). Effect of calcifying bacteria on permeation properties of concrete structures. *Journal of Industrial Microbiology & Biotechnology* DOI: 10.1007/s10295-010-0901-8.
- **Achal V.**, Mukherjee A., Goyal S., Reddy M.S. (2010). Corrosion prevention of reinforced concrete with microbial calcite precipitation technique. *ACI Materials Journal* (Under revision).

Patent

Mukherjee A., Reddy M.S., **Achal V**. (2008). A novel additive for building materials and methods of preparation & application there of. (Indian Patent No. 2191/DEL/2008)

International conference

- **Achal V.**, Mukherjee A., Reddy M.S. (2010). Microbial concrete- A way to enhance the durability of building structures. *Second International Conference on Sustainable Construction Materials and Technologies, June 28-June 30, 2010,* Università Politecnica delle Marche, Ancona, Italy. (Selected as Outstanding Paper)
- Mukherjee A., **Achal V.**, Reddy M.S. (2010). A sustainable binder for building materials from biological sources. *The 6th International Engineering and Construction Conference (IECC'6), June 28-June 30, 2010*. Housing & Buildings National Research Center (HBRC), Cairo, Egypt.

ABSTRACT

A large number of human activities as well as natural processes, such as weathering, faults, land subsidence, earthquakes, create fractures and fissures in concrete structures that ultimately reduce the service life of the structures. “Microbial Concrete” a novel metabolic byproduct of microbially induced calcite precipitation by way of urease (urea hydrolyzing enzyme) presents a promising novel biotechnology for the enhancement of durability of building materials and structures. The objective of the present research work was to develop an effective, economic and eco-friendly microbial process, based on microbially induced calcite precipitation, to remediate the building materials and structures with self-healing ability and also to enhance their durability. The investigation was carried out on different building materials such as cement mortar, concrete and bricks. The targets were to enhance the compressive strength, reduce the permeability and corrosion protection in case of reinforced concrete.

The ubiquity and importance of microbes in inducing calcite precipitation make “microbial concrete” a most important metabolic product of biomineralization that can remediate and restore such structures. Bacteria were isolated from extreme alkaline environments such as calcareous sludge, cement, alkaline soil and limestone area, so that they can sustain in the alkaline environment of cement. In the present study, a bacterium *Sporosarcina pasteurii* and another bacterial isolated from cement, *Bacillus* sp. CT-5, have been successfully employed to improve the compressive strength of building materials significantly. Microbial process increased the compressive strength by 40%. Further the efficacy of the proposed method in reducing water and chloride ion permeability is established. Initial demonstration of the corrosion protection offered by the microbial concrete is presented. Corrosion rate measurement showed that microbial process lead to around four-fold reduction in the corrosion rate of reinforced concrete specimens. The current work demonstrates that production of “microbial concrete” by urease producing bacteria on constructed facilities could enhance the durability of building materials and at least partially replace the industrial binders and provide a more sustainable alternative.

TABLE OF CONTENTS

List of Figures

List of Tables

Abbreviations

Abbreviation	Word (s)
MICP	Microbiologically Induced Calcite Precipitation
CSL	Corn Steep Liquor
LML	Lactose Mother Liquor
RCPT	Rapid Chloride Penetration Test
SEM	Scanning Electron Micrography
XRD	X-Ray Diffraction
ASTM	American Society for Testing and Materials
IS	Indian Standard (Code)
LPR	Linear Polarization Resistance
RC	Reinforced Concrete
Icorr	Corrosion current
UTM	Universal Testing Machine
ANOVA	Analysis of Variance
EPS	Extracellular Polymeric Substances
MTCC	Microbial Type Cell Culture
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid

Chapter 1

Introduction

1.1 General Introduction

In recent years, it has been pointed out that not only structural safety but also durability is important when designing building or concrete structures. However, in traditional structural design the degradation of structures over long periods of time is not regarded as a serious problem; the destruction of structures has a much greater impact on people or society. Taking these social factors into consideration, it is obvious that we should not consider the problem of durability of building materials as a problem of the past. It is necessary to develop some new eco-friendly self-remediating techniques to support already designed and future constructing buildings to meet various demands to enhance their durability.

Selection of materials and technologies for the building construction should satisfy the felt needs of the user as well as the development needs of the society, without causing any adverse impact on environment. Indian construction industry is one of the largest in terms of employing manpower and volume of materials produced (cement, brick, steel and other materials). Apart from the office, commercial and industrial buildings, $>2 \times 10^6$ residential buildings are built annually in India (Reddy and Jagadish, 2003). Due to high construction costs and the social importance, the durability demands for large building structures are becoming more and more important. Building material is any material which is used for construction purpose. Many naturally occurring substances, such as clay, sand, wood and rocks, even twigs and leaves have been used to construct buildings. Apart from naturally occurring materials, many man-made products are in use, some more and some less synthetic. Concrete is most widely used building material used throughout the world. Current concern about the degradation of concrete and the economic impact of the maintenance and repair of concrete structures, have drawn the attention to processes

of concrete deterioration, and to the methods to slow down or even to eliminate concrete degradation (Hewlett, 1990). Durability of concrete is the ability of a concrete to resist deterioration, particularly deterioration due to weather exposure, chemical exposure or surface abrasion. A durable concrete must maintain its form, quality and serviceability for its prescribed service life.

Natural processes, such as weathering, faults, land subsidence, earthquakes, and human activities create fractures and fissures in concrete structures. These fractures and fissures are detrimental since they can reduce the service life of the structure. Weathering induces an increased porosity, structural weakening of surface layers and unattractive appearance. Synthetic agents such as epoxies and surface treatments with water repellents such as silanes or siloxanes, or with pore blockers are applied for remediation of these structures. However these and other treatments with organic and anorganic products involve some disadvantages, such as different thermal expansion, degradation with age and the need for constant maintenance. Appearance of cracks and fissures is an inevitable phenomenon during the aging process of concrete structures when exposed to weather changes. Such cracking leads to easy passage for aggressive environment to reach the reinforcement and initiate corrosion. Moreover, sometimes repair is carried out in the areas where it is not possible to shut down the plant or it is hazardous for human beings. Hence, in such situations a way should be found out to self healing materials that seal the cracks automatically. Recently, a novel technique has been reported that utilizes microorganisms in remediation of cracks and fissures in natural and man-made structures by precipitation of calcium carbonate.

Calcium carbonate (calcite) is one of the most common and widespread minerals on Earth, constituting 4% by weight of the Earth's crust. It is naturally found in extensive sedimentary rock masses, as limestone, marble and calcareous sandstones in marine, freshwater and terrestrial environments (Klein and Hurlbut, 1999; Hammes and Verstraete, 2002). Bacterial contribution to these extensive formations had been suspected for some time (Drew, 1910) but remained controversial until recent investigations involving the microbial pathways and the required precipitation

conditions, indicated that bacteria have the potential to far exceed the abiotic contribution to calcium carbonate deposition in most environments on Earth (Castanier *et al*., 2000). In addition to this, the huge limestone formations on the sea bottom resulted from great thicknesses of calcareous material from pelargic skeletal organisms (such as coccolithophores and foraminifera) (Klein and Hurlbut, 1999; Morse, 2003). It has been shown that the cellular organelles that are largely responsible for the production of carbonate shells in eukaryotic organisms, are the mitochondria (or chloroplasts). These organelles are widely considered as primitive endosymbiotic bacteria, which further supports the bacterial contribution to carbonate precipitation (Castanier *et al*., 2000). Natural caves, ponds are formed due to erosion and coral reefs, ant hills and dental plaque owing to microbiologically induced precipitation**.** These naturally built ant hills and coral reefs go on increasing in their height and extent. These structures are self supporting and have the property of self healing. Moreover, these formations remain intact even during change of weather. It means they are adapted to the environment and are unaffected by the environmental effects. Even if they are affected up to some extent sometimes healing action starts instantly. The microbial depositions are made so that they maintain their stability against forces induced due to wind, wave action, heat, cold, turbulence and so on. Hence, in nature, structures are built which are capable of growing without any problem. They can acclimatize to environment and have self healing property. Coral reefs and ant hills are very good examples of natural microbial deposits (Fig. 1.1). They also give an idea that strength and durability of any building material can be enhanced by microbial deposits.

Figure 1.1 Coral reefs and ant hills

Concrete is a strong and relatively cheap construction material mostly used worldwide. One drawback, however, is that its massive production exerts negative effects on the environment. The most important aspect of concrete or other building materials is their liability to cracking, a phenomenon that hampers the material's structural integrity and durability. The impact of durability related problems on national economies can be substantial and is reflected by the sums of money spent on maintenance and repair of concrete structures. Although for decades the need to produce more sustainable cement-based products is thus recognized and implemented, the development of sustainable maintenance and repair methods for concrete constructions is still in its infancy. Durability problems such as crack formation are typically tackled by manual inspection and repair, *i.e*., by impregnation of cracks with cement or epoxy-based or other synthetic fillers (Nevile, 1996). However, a promising sustainable repair methodology is currently being investigated and developed in several laboratories, *i.e*., a technique based on the application of mineral producing bacteria. The application of bacteria for bioremediation purposes is becoming increasingly popular as is reflected by recent studies. The applicability of specifically mineral-producing bacteria for sand consolidation and limestone monument repair (Gollapudi *et al*., 1995; Dick *et al*., 2006) and filling of pores and cracks in concrete have been recently investigated (Bang *et al*., 2001; De Muynck *et al*., 2008). In most of these studies ureolytic bacteria of the genus *Bacillus* were used as agent for the biological production of calcium carbonate based minerals. The mechanism of calcium carbonate formation by these bacteria is based on the enzymatic hydrolysis of urea to ammonia and carbon dioxide. Such bacteria added to the cement mixture prior to casting should remain viable for prolonged periods once integrated in the cement matrix and be able to produce copious amounts of minerals needed to plug or seal freshly formed cracks. Integrated bacteria would thus represent an internal self-healing agent which autonomously decreases matrix permeability upon crack formation. An integrated biological healing agent would save manual inspection and repair and moreover increase structure durability.

1.2 Problem and gap in studies

Microbial metabolic activities often contribute to selective cementation by producing relatively insoluble organic and inorganic compounds intra and extracellularly. Microorganisms and microbiologically mediated mineralization processes are active in almost every environment on earth. In a natural setting, precipitation processes continue at slow rate over geological time, plugging cracks in highly permeable rock formation. The bacteria like *Bacillus* species have the ability to induce a mineral plugging in surface fractures and fissures of granite. The bacteria use urea as energy source and produces ammonia which increases pH in the proximal environment, causing Ca^{2+} and CO_3^{2-} to precipitate as $CaCO_3$. The microbial process was found to be most effective in remediating fissures and is a long term remediation tool which is highly feasible for cementation of various structural formations.

Studies related to microbiologically induced calcite precipitation (MICP) and mechanism for the same is not being studied in detail. Researchers have concentrated mainly on *Bacillus* species for urease activity and calcite precipitation. Other species from related or unrelated taxa of *Bacillus* sp. might have the ability for the calcite precipitation in alkaline environment. Alkaline bacteria are diverse in nature, but *Bacillus* sp. has been studied systematically for the exploitation for industrial enzyme and bioremediation. Furthermore, there is need to evaluate the bacterial diversity and the dynamics community structure of microorganisms residing in alkalophilic environment such as cement factory waste, mining areas of limestone and calcite sludge system. Diversity of microorganism in the calcite sludge system will give valuable information about predominant and rare indigenous micro flora inhabiting over there, and will impart valuable information regarding selection of the microbe for the remediation of defects in building materials.

Although work on the use of microbiologically induced calcium carbonate production to remediate fissures and structures were carried out in other parts of the world, only an initial work has been done in India using the same microorganisms procured from other parts (Ghosh *et al*., 2005). A lot of work needs be done before the technology can be implemented in India. The temperatures, humidity, type of

concrete, control of various parameters such as type of mix, concentration of bacteria vary considerably hence a consolidated recommendation can not be arrived. Apart from these, survival of microorganisms isolated from other parts of the world under Indian environmental conditions also needs to be considered. No published work to date has described a sufficient degree towards enhancement in the durability of building structures using microbes by their MICP process. Hence, lot of research is necessary before such technology is ready for field applications. However, the important parameter such as permeation properties was not dealt with. As calcite deposition fills the pores, the extent of permeability reduction must be studied in detail. The carbonation of structures in urban and coastal regions of our country is very high. Carbonated concrete looses protective power of steel. Therefore it accelerates steel corrosion and related problems. Hence, the efficiency of calcite deposition in resisting carbonation and reduction in permeability to improve the corrosion resistance must be studied.

1.3 Aim and Objectives

The aim of the present work was the development of a biological process to enhance the durability of building materials and structures.

To achieve this goal, a better understanding of the bacterial diversity with cementing activity (urease) involved in the remediation process was required. This research contributed with the isolation of these organisms from natural environments (cement, calcareous sludge, limestone areas, alkaline soil), and identification using molecular techniques. Further, all the bacterial isolates were thoroughly characterized based on physiological and biochemical techniques and studied their efficacy to produce urease. Finally the isolates were used to study the quality control of any building material *i.e.* compressive strength, permeability and corrosion test to evaluate bioremediation and durability properties. The objectives of the present investigations are:

- Isolation and identification of efficient bacteria for the production of microbial concrete
- Characterization of microbial concrete producing bacteria based on physiological and molecular characteristics
- Enhancement of cementing activity of these bacteria by strain improvement technique
- Evaluation of microbes for remediation of defects in building materials and structures such as cement mortars, concretes, bricks and reinforced concrete specimens

Chapter 2

Review of Literature

Microbiologically induced calcite precipitation (MICP) is a technique that comes under a broader category of science called biomineralization. It is a process by which living organisms form inorganic solids. MICP is highly desirable because the calcite precipitation induced as a result of microbial activities, is pollution free and natural. The promising results of this process encouraged different research groups from the world to promote MICP. The goal of this review is to provide an in-depth knowledge about the different key factors to understand overall process. In the first part of this chapter, a novel approach of urease based MICP has been discussed. Furthermore, urease producing bacteria and their characterization is discussed. In next part, evaluation of parameters to study durability of building materials and structures is presented. Finally MICP has been explored for the enhancement of durability of building materials and structures.

2.1 Urease and Microbiologically induced calcite precipitation

Microbiologically induced calcite precipitation generally results from a series of complex biochemical reactions involving urease (urea amidohydrolase; EC 3.5.1.5). The commercial demand for urease is not high and currently, urease is only available in industrial quantities from Roche for use in the diagnostic and high technology specialist ceramics fields (Gauckler and Baader, 1999; Roche, 2001). It is thus expensive and is of a higher purity than is required for biocementation. In general, four modes of regulation exist for the synthesis of urease in microbial systems (Mobley and Hausinger, 1989; Mobley *et al*., 1995).

i. Constitutive, where a constant enzyme activity is expressed per cell, independent of external conditions.

ii. Inducible, where a background level of enzyme activity is expressed per cell which can be induced by the presence of an inducer molecule (*e.g.* urea) or other environmental condition.
iii. Repressible, by the presence of ammonia or ammonia precursors including urea. This synthesis is de-repressed (*i.e.* enzyme activity increases) under nitrogen limiting conditions.

iv. Developmental, where an organism in different developmental stages (*e.g.* swarming versus non-swarming) has variable expression of urease (Falkinham III and Hoffman, 1984).

An ideal microbial source of urease for biocementation must be tolerant to high concentrations of urea and calcium. The organism should also have a high level of urease activity that is either constitutively produced (*i.e.* a constant amount of enzyme is expressed per cell) or can be reliably induced.

Urease-producing bacteria can be divided into two distinct groups according to their urease response to ammonium; those whose urease activity is not repressed (*Sporosarcina pasteurii*, *Proteus vulgaris* and *Helicobacter pylori*) and those whose urease activity is repressed (*e.g. Pseudomonas aeruginosa*, *Alcaligenes eutrophus*, *Bacillus megaterium* (Kaltwasser *et al.*, 1972) and *Klebsiella aerogenes* (Friedrich and Magasanik, 1977)). In *K. aerogenes*, the presence of ammonium inside the cell induces the production of glutamine, which prevents further hydrolysis of urea (Mulrooney *et al.*, 2001). Because high concentrations of urea are hydrolysed during biocementation, only those microorganisms whose urease activity is not repressed by ammonium are useful. As well as meeting the needs for biocementation, the organism must also meet the needs for safe environmental application. In order to safely release an organism into the environment, it must be non-pathogenic, non-genetically modified, and not contain any transferable elements that may increase the pathogenicity of environmental strains (*e.g.* antibiotic resistance). Considering both biocementation and environmental constraints, two organisms have potential as sources of urease for biocementation; *Sporosarcina pasteurii* and *Proteus vulgaris*

(Whiffin, 2004). The moderately alkaliphilic organism *S. pasteurii* (formerly known as *Bacillus pasteurii* (Yoon *et al.*, 2001)) is a commonly found in the soil, sewage and urinal incrustations (Sneath, 1986).

Urea is the chief nitrogenous waste produced by vertebrates and is a major nitrogen resource in aquatic and soil ecosystems. In response to the widespread availability of urea in the environment and the universal requirement for nitrogen, a diverse section of the biota has evolved with the ability to hydrolyse urea, through the action of urease. Urease occurs in many bacteria, several species of yeast and a number of higher plants including jack beans (*Canvalia ensiformis*) (Dixon *et al.*, 1980), soybean leaf and seed (*Glycine max*) (Kerr *et al.*, 1983), pigweed (*Chenopodium album*) (El-Shora, 2001) and mulberry leaf (*Morus alba*) (Hirayama *et al.*, 2000). Most organisms with ureolytic ability use urea as a source of nitrogen by actively transporting or passively diffusing urea into the cell cytoplasm, where urease hydrolyses urea releasing two ammonium molecules, which can then be directly assimilated into biomass via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway or by the action of glutamate dehydrogenase (GDH) (Tyler, 1978). To ensure this process is energy efficient, the production of urease in organisms such as *Pseudomonas aeruginosa*, *Alcaligenes eutrophus*, *Bacillus megaterium* (Kaltwasser *et al.*, 1972) and *Klebsiella aerogenes* (Friedrich and Magasanik, 1977), is repressed by the presence of ammonium. There are however some exceptions to this regulation, such as *Proteus vulgaris*, which can produce urease even in the presence of high concentrations of ammonium (Mörsdorf and Kaltwasser, 1989). Some specialist organisms exist that have additional uses for urease, beyond nitrogen assimilation. *Helicobacter pylori,* an inhabitant of the low pH gastric juices in the stomach, not only has intracellular but also extracellular urease that is located on the cell surface. The extracellular urease plays a protective role from the low pH environment of the stomach, by providing a microenvironment of more neutral pH, generated from production of ammonium near the cell surface (Marshall *et al.*, 1990; Dunn and Grütter, 2001; Ha *et al.*, 2001). *Sporosarcina pasteurii* is another specialist organism that has a different use for urease, other than

nitrogen assimilation. *S. pasteurii* is a moderately alkaliphilic organism with a growth optimum at pH 9.25. Alkaliphiles present a special problem for the generation of ATP due to a reversed chemiosmotic proton gradient. In neutrophilic organisms, ATP is produced from the proton motive force that is generated by pumping protons out of the cell from the electron transport chain. This generates a proton concentration gradient (high outside/low inside) and causes protons to be driven back into the cell through the ATP-synthase, resulting in ATP generation (Prescott *et al.*, 1993). When the external environment is highly alkaline, the proton concentration gradient is reversed and the gradient favours protons to be fluxed out of the cell, but not back into it. Alkaliphilic organisms must create a high membrane potential (charge difference across the membrane) by pumping out cations, to drive protons back into the cell against the concentration gradient (Ivey *et al.*, 1998). For *S. pasteurii*, the effluxed cation used to create a high membrane potential to drive ATP synthesis is ammonium, which can be supplied directly as ammonium or indirectly as urea (Jahns, 1996). As the biocementation reaction results in the generation of high concentrations of ammonium, only those bacterial sources where urease is not down regulated by the presence of ammonium are useful. These organisms include *Sporosarcina pasteurii* and *Proteus vulgaris*. For biocementation purposes, an ideal microbial source of urease has the following properties:

i. High urease production capacity
ii. Ability to produce urease in the presence of ammonium
iii. High stability (robust)
iv. Consistent production (reliable)
v. Does not require further down-stream processing prior to use in biocementation

2.1.1 Factors affecting cementation

The precipitation of calcium carbonate in bacterial systems, where nucleation can occur on the bacterial cell surface, is governed by three parameters; (1) the calcium concentration, (2) the carbonate concentration and (3) the pH of the environment (which affects carbonate speciation and calcium carbonate solubility) (Hammes and

Verstraete, 2002).

In order for precipitation to take place, supersaturation of the precipitating species must exist (Randolph and Larson, 1988). Because the solubility product (K_{sp}) of calcite is extremely low (3.3×10^{-9} mol L^{-1} at 25°C (Sawada, 1998)), it is straightforward to achieve the supersaturation. Precipitation can be achieved by simply mixing together moderate concentrations of soluble Ca^{2+} and CO_3^{2-} ions. The crystals that form when the reaction happens rapidly are very small and powder-like with little cementation strength. One of the prime factors controlling the rate of precipitation is the difference between the saturation concentration and the supersaturation concentration (Bodek *et al*., 1988). In general, the higher the supersaturation concentration, the faster the rate and the smaller the crystals that forms, as exemplified by the crystals to form over an extended period with higher cementation strength. The supersaturating product concentration and thus the degree of disequilibrium remains relatively low (compared to the chemical example), which allows larger crystals to form over an extended period with higher cementation strength. The supersaturation level of carbonate ions can be further influenced by controlling the pH. The proportion of total carbonate that exists as CO_3^{2-} in solution at any time is highly dependent on the pH. Below pH 8, the carbonate concentration is very low, which means that if desired, the size of crystals that form can be increased by decreasing the pH or vise-versa, crystal size can be decreased by increasing the pH.

2.1.2 Microbial concrete

Microbial metabolic activities often contribute to selective cementation by producing relatively insoluble organic and inorganic compounds intra- or extracellularly. Use of microorganisms within mortar/concrete leading to the process of biomineralization is now a potential field of research in concrete technology. A novel technique for the remediation of damaged structural formations has been developed by employing a selective microbial plugging process in which metabolic activities promote precipitation of calcium carbonate in the form of calcite. Biomineralization of

calcium carbonate is one of the strategies to remediate cracks in building materials. Biomineralization is defined as a biologically induced precipitation in which an organism creates a local micro-environment, with conditions that allow optimal extracellular chemical precipitation of mineral phases (Hamilton, 2003). Numerous diverse microbial species participate in the precipitation of mineral carbonates in various natural environments, including soils, geological formations, freshwater biofilms, oceans, and saline lakes.

It has been known that calcite (calcium carbonate) has values of technical and industrial applications for the preservation, remediation and restoration of buildings, calcareous stone statuaries and historic monuments. Calcite is needed in high purity and good coherency for better restoration. However, it is a very laborious and expensive process to obtain the highly pure and coherent calcite from natural sources, such as shell crust. Thus, bacterially induced carbonate precipitation has received attention as an environment-friendly method of protecting decayed ornamental carbonate stone and remediation of cracks in building materials, few of such bacteria are *Micrococcus* sp.*, Bacillus subtilis*, *Bacillus pasteurii*, *Deleya halophila*, *Halomonas eurihalina*, and *Myxococcus xanthus* (Rivadeneyra *et al.*, 1991; 1996; 1997; 1998; Tiano *et al.*, 1999; Castanier *et al*., 2000; Cacchio *et al*., 2003; Rodrigues-Navarro *et al.*, 2003).

Use of bacteria in concrete remediation is an unorthodox concept in current concrete research. It is, however, a new approach to an old idea that a microbial mineral deposit constantly occurs in natural environments. Specifically, microbiologically-induced calcite is environmentally innocuous, compared to synthetic polymers currently used for concrete repairs (Ramachandran *et al*., 2001). Like other biomineralization processes, calcium carbonate ($CaCO_3$) precipitation can occur by two different mechanisms: biologically controlled or induced (Lowenstan and Weiner, 1988). In biologically controlled mineralization, the organism controls the process, i.e. nucleation and growth of the mineral particles, to a high degree. The organism synthesizes minerals in a form that is unique to that species, independently of environmental conditions. Examples of controlled mineralization are magnetite

formation in magnetotactic bacteria (Bazylinski *et al*., 2007) and silica deposition in the unicellular algae coccolithophores and diatoms, respectively (Barabesi *et al*., 2007). However, calcium carbonate production by bacteria is generally regarded as "induced", as the type of mineral produced is largely dependent on the environmental conditions (Rivadeneyra *et al*., 1994) and no specialized structures or specific molecular mechanism are thought to be involved (Barabesi *et al*., 2007). Different types of bacteria, as well as abiotic factors (salinity and composition of the medium) seem to contribute in a variety of ways to calcium carbonate precipitation in a wide range of different environments (Knorre and Krumbein, 2000; Rivadeneyra *et al*., 2004).

Calcium carbonate precipitation is a general phenomenon in the bacterial world under appropriate conditions (Boquet *et al*., 1973). Indeed, some bacteria and fungi can induce precipitation of calcium carbonate extracellularly through a number of processes that include photosynthesis, ammonification, denitrification, sulphate reduction and anaerobic sulphide oxidation (Castanier *et al*., 1999, 2000; Riding, 2000). Additionally, the activity of sulphate reducing bacteria has been shown to mediate precipitation of dolomite. The primary role of bacteria in the precipitation process has been ascribed to their ability to create an alkaline environment through various physiological activities (Douglas and Beveridge, 1998).

The evidence of microbial involvement in carbonate precipitation has subsequently led to the exploration of this process in a variety of fields (De Muynck *et al*., 2010). A first series of applications is situated in the field of bioremediation. In addition to conventional bioremediation strategies which rely on the biodegradation of organic pollutants (Simon *et al*., 2004; Chaturvedi *et al*., 2006), the use of MICP has been proposed for the removal of metal ions. Applications include the treatment of groundwater contaminated with heavy metals (Warren *et al*., 2001) and radionucleotides (Fujita *et al*., 2000), the removal of calcium from wastewater (Hammes *et al*., 2003). Another series of applications aims at modifying the properties of soil, i.e. for the enhancement of oil recovery from oil reservoirs (Nemati and Voordouw, 2003; Nemati *et al*., 2005), plugging (Ferris and Stehmeier,

1992) and strengthening of sand columns (De Jong *et al*., 2006; Whiffin *et al*., 2007). Moreover, microbiologically induced precipitation has been investigated for its potential to improve the durability of construction/cementitious materials.

2.2 Bacterial isolation source

‘‘The sure and definite determination (of species of bacteria) requires so much time, so much acumen of eye and judgment, so much of perseverance and patience that there is hardly anything else so difficult.’’

— Otto F. Müller

A variety of microbes inhabit extreme environments. Extreme is a relative term, which is viewed, compared to what is normal for human beings. Extreme environments include high temperature, pH, pressure, salt concentration, and low temperature, pH, nutrient concentration and water availability, and also conditions having high levels of radiation, harmful heavy metals and toxic compounds (organic solvents). Culture-dependent and culture-independent (molecular) methods have been employed for understanding the diversity of microbes in these environments. Extensive global research efforts have revealed the novel diversity of extremophilic microbes. These organisms have evolved several structural and chemical adaptations, which allow them to survive and grow in extreme environments. The enzymes of these microbes, which function in extreme environments (extremozymes), have several biotechnological applications (Satyanarayana *et al*., 2005). Microbial studies pertaining to the artificial alkaline environments such as calcareous sludge, limestone areas or cement are very scarce. Bacterial diversity of such alkaline sites will give valuable information about the predominant microbes, which could possibly be used for microbial remediation in building materials as they might tolerate the extreme pH of cement.

Although microorganisms are perhaps the most diverse (Torsvik *et al*., 2002; Venter *et al*., 2004) and abundant (Whitman *et al*., 1998) type of organism on Earth, the distribution of microbial diversity at continental scales is poorly understood.

Ecologists describing microbial biogeography typically invoke Beijerinck (1913) from a century ago: ‘‘Everything is everywhere, the environment selects.’’ However, few studies have attempted to verify this statement or specify which environmental factors exert the strongest influences on microbial communities in nature (Papke and Ward, 2004).

Cells face many challenges in an alkaline environment. Of greatest significance is the ability to maintain internal pH. If cells are to survive in an alkaline environment, they must make their cytoplasm more acidic to buffer the alkalinity. Moderate environments are important to sustain life. Moderate means environments with pH near neutral, temperature between 20 and 40°C, air pressure 1 atm and adequate levels of available water, nutrients and salts. Many extreme environments, such as acidic or hot springs, saline and/or alkaline lakes, deserts and the ocean beds are also found in nature, which are too harsh for normal life to exist. Any environmental condition that can be perceived as beyond the normal acceptable range is an extreme condition (Satyanarayana *et al*., 2005). A variety of microbes, however, survives and grows in such environments. These organisms, known as extremophiles, not only tolerate specific extreme condition(s), but usually require these for survival and growth. Most extremophiles are found in microbial world. The range of environmental extremes tolerated by microbes is much broader than other life forms. The limits of growth and reproduction of microbes are, –12° to more than +100°C, pH 0 to 13, hydrostatic pressures up to 1400 atm and salt concentrations of saturated brines. Besides natural extreme environments, there are manmade extreme conditions such as cool houses, steam heated buildings and acid mine waters.

In alkaline environments such as soils, increase in pH is due to microbial ammonification and sulphate reduction, and by water derived from leached silicate minerals. The pH of these environments fluctuates due to their limited buffering capacity and therefore, alkalitolerant microbes are more abundant in these habitats than alkaliphiles. Industrial processes including cement manufacture, mining, disposal of blast furnace slag, electroplating, food processing and paper and pulp manufacture produce man-made unstable alkaline environments (Satyanarayana *et*

al., 2005). From neutral soils, alkaliphilic Gram-positive and endospore-forming *Bacillus* spp., and non-sporing species of *Pseudomonas*, *Paracoccus*, *Micrococcus*, *Aeromonas*, *Corynebacterium* and *Actinopolyspora,* Gram-positive endospore-forming *Bacillus* sp. and alkalitolerant fungi have been isolated.

A major impetus that has driven extensive and intensive research efforts on extremophiles during the last decades is the potential biotechnological applications associated with these microbes and their products. The likely potential has been increasing exponentially with the isolation of new microbial strains, the identification of novel compounds and pathways, and the molecular and biochemical characterization of cellular components. Examples of extremozymes that are now commercially used include alkaline proteases in detergents. The extensive and intensive efforts world over in understanding the diversity of extremophilic microbes have indicated that what we know today is just the tip of the iceberg. Many more novel and useful extremophilic microbes are expected to be discovered in the near future. Only sporadic attempts have been made to isolate extremophiles from extreme Indian environments.

2.3 Phenotypic characterization

Reliable identification of bacteria remains an important task in environmental microbiology. Phenotypic approaches using either conventional or commercial systems are still used in the majority of laboratories. The historic way to characterize bacteria is to describe quantitatively as many phenotypic properties as possible, such as morphology, structure, cultivation, nutrition, biochemical metabolism, pathogenecity, antigenic properties and ecology. Phenotypic similarities do not necessarily indicate phylogenetic relationships (relationship based on the ancestry of organisms) (Gillis and De Leg, 1992). In contrast to animals and plants, the morphology of microorganisms is in general too simple to serve as a basis for a sound classification and to allow for reliable identification. Thus, until very recently, microbial identification required the isolation of pure cultures (or defined co-cultures) followed by testing for multiple physiological and biochemical traits

(Amann *et al.,* 1995). Cultural methods will reveal only those physiological and nutritional types compatible with the cultural environment. The potential limitations of this approach are widely acknowledged and accepted (Torsvik *et al*., 1996).

A global picture of microbial diversity has remained elusive, yet it is critical to understanding microbial adaptation to different environments and their function in those environments. Integrating information from environmental surveys, however, has thus far been a formidable obstacle to a global understanding of microbial ecology. Determining physical and chemical factors, such as temperature, pH, or geography, that correlate with differences between diverse microbial communities will reveal how easily microbes tolerate different kinds of environmental change and will increase our understanding of microbial ecology and evolution.

Carbonate precipitation in microbial mats can be seen as a byproduct of microbial metabolism (Des Marais, 1997); it can also function as an energy source through proton production outside the cell membrane (McConnaughey and Whelan, 1997). Additionally, carbonate production may be a result of the production and consumption of extracellular polymeric substances (EPS) and biofilm. Both together can also play a key role in facilitating carbonate precipitation (e.g. Trichet and De´farge, 1995) through multiple mechanisms: (1) by binding bivalent cations, thereby inhibiting carbonate precipitation; (2) by forming heterogeneous microdomains, which support different types of microbial metabolism, thereby facilitating precipitation; and (3) by serving as an energy and carbon source for heterotrophic bacteria, thereby facilitating carbonate precipitation. Hence, EPS and biofilm are attributed with an important role in carbonate precipitation (Reid *et al*., 2003).

2.3.1 EPS (Extracellular polymeric substances) and Biofilm

EPS and biofilm production by bacteria might play a major role towards reducing permeability and reduction in corrosion rate of any reinforcement. When the biofilm produced from *Bacillus subtilis*, was genetically engineered to secrete polyglutamate

or polyaspartate, an additional small increase in corrosion inhibition on aluminium occurred (Ornek *et al*., 2001). Biofilms probably comprise the normal environment for most microbial cells in many natural and artificial habitats such as concrete surfaces, and as such are complex associations of cells, extracellular products and detritus either trapped within the biofilm or released from cells which have lysed as the biofilm ages (Christensen, 1989).

Biofilm formation can occur by at least three mechanisms. One is by the redistribution of attached cells by surface motility. A second mechanism is from the binary division of attached cells (Heydorn *et al*., 2000). As cells divide, daughter cells spread outward and upward from the attachment surface to form cell clusters, in a similar manner to colony formation on agar plates. A third mechanism of aggregation is the recruitment of cells from the bulk fluid to the developing biofilm (Tolker-Neilson *et al*., 2000). The relative contribution of each of these mechanisms will depend on the organisms involved, the nature of the surface being colonized, and the physical and chemical conditions of the environment. In oligotrophic environments mature biofilms may consist of little more than a sparse covering of cells with relatively little structural complexity. Biofilms can take over 10 days to reach structural maturity, based on microscopically measured physical dimensions and visual comparison (Stoodley *et al*., 1999). The biofilm structure appears to be largely determined by the production of slime-like matrix of extracellular polymeric substances (EPS), which provides structural support for the biofilm (Flemming *et al*., 2000). The EPS is composed of polysaccharides, proteins, and nucleic acids.

Microbial biofilms are often sufficiently thick and extensive to be visible to the unaided eye, and they may contain millions of prokaryotic (and sometimes some eukaryotic) cells in arrangements that facilitate the stable juxtaposition of physiologically cooperative organisms. Cells of particular species are found consistently in certain locations, near the colonized surface or at the apices of towers or mushrooms; the sessile cells that comprise single-species biofilms are located within the microcolonies in species-specific distribution patterns. In these single-species biofilms, the preponderance of the sessile cells may be found in the caps of

mushrooms in a highly organized pattern with relatively regular cell-cell spacing, and the stalks of mushrooms formed by some species are virtually devoid of cells.

It is now widely recognized that in natural settings bacterial cells are most often found in close association with surfaces and interfaces, in the form of multicellular aggregates commonly referred to as biofilms. This proclivity towards multicellularity makes bacterial cells similar to many other types of living cells: capable of unicellular existence and yet generally residing within multicellular communities. Biofilms offer their member cells several benefits, of which protection from environmental insults and assaults is foremost (Davey and O'Toole, 2000). Given their ubiquity and importance in the microbial world, it is hardly surprising that biofilms have attracted the attention of the scientific community and might play a major role as a protective agent to combat adverse conditions inside concrete structures.

2.4 Molecular characterization

Traditionally, in the 1980s and before, the diversity of bacteria was assessed by identification with phenotypic tests and numerical taxonomy from collections of isolates obtained from plating on nutrient media and liquid enrichments. However, plate counts of bacteria from natural habitats, such as soil, freshwater and the sea, are much lower than direct total counts and it is accepted that <1 % of these bacteria are culturable. For this reason studying the true diversity of bacteria in nature was impossible (Fry, 2000).

This problem was overcome in 1990 when Stephen Giovannoni and David Ward's research groups first investigated bacterial diversity in the Sargasso Sea and in hot springs using molecular approaches based on sequence analysis of the highly conserved 16S rRNA gene. This was achieved in the Sargasso Sea study by extracting community DNA, PCR amplification of 16S rRNA genes, sequencing and identification of the source of these genes by phylogenetic analysis. Bacterial species can be identified by generating clone libraries of the 16S rRNA followed by sequencing and comparison with databases containing thousands of ribosomal

sequences to allow a phylogenetic affiliation to microorganisms (Muyzer *et al*., 1993). Analysis of 16S rRNA gene is now widely used for analysis of bacterial populations. The macromolecules that is most suitable for this would require the following prerequisites: i) generally present, ii) functionally homologous in all organisms and iii) the sequence in the molecule should equally change as wide the evolutionary distance lower the mutation rate in the sequence. Ribosomal RNA is proposed as one of the best candidate and it has been used by Woese *et al*. (1990) for his studies on bacterial evolution. Major properties of rRNA are: 1) these are old molecules present in the ribosomes, 2) they are functionally constant, 3) have a wide distribution, 4) are well conserved over large phylogenetic distances, 5) they occur in large number in cells (10^4-10^5/cells), 6) in bacteria three types of rRNA molecules are present with different chain length and sedimentation rate(s): 5S rRNA (about 120 nucleotides), 16S rRNA (~1600 nucleotides) and 23S rRNA (~3000 nucleotides). The 5S rRNA molecule is too small and only suitable to distinguish major phylogenetic groups. 23S rRNA is excellent for phylogenetic studies, but so far few studies are available while 16S rRNA has been given most attention.

2.5 Economization for microbial concrete production

The total cost of a biotechnology-based product includes labour, medium, cultivation operating costs, waste treatment and any downstream or upstream processing. In most processes, the medium ingredients are a major cost factor, ranging between 10 to 60% of the total operating costs (Kristiansen, 2001). After the initial savings achieved with economies of scale (buying in bulk quantity), the medium cost is a production cost that increases proportionally with the size of the scale up (Whiffin, 2004). Because of this, it is important to give due consideration to optimization of the medium prior to scale up. Given that the biocementation process does not require ease of removal of medium components or use of a defined medium, we are able to look at a range of more economical components to replace the existing expensive analytical grade chemicals. Several inexpensive alternative protein sources exist which could replace laboratory grade nutrient media or other such media and these are listed in Table 2.1.

Table 2.1 Possible protein sources for large-scale cultivation of microorganisms

Protein Source	Description	Protein content (%)	Cost (per kg/L)
Yeast Extract	Water soluble portion of autolysed yeast	66	$40
Torula Yeast	Autolysed food grade yeast	54	$9
Corn Steep Liquor (CSL)	Concentrated aqueous liquid from steeping corn	20-40	$5-10
Vegemite	Concentrated autolysed yeast paste	40	$4
Brewery Waste Yeast	Yeast from the brewing process	54	$2
Sludge Biomass from WWTP	Concentrated sludge biomass	55	< $1
Lactose Mother Liquor (LML)	Waste collected from dairy products processing	8-15	< $1

Useful metabolites produced from the microbial, plant and animal cells has touched almost the every aspect of requirement in human life and is increasing day by day. In cell growth and metabolite production, the selection of the nutrients and determination of its concentrations in the cultivation media is a very important step. For commercially viable products, quest of cheaper processes are essential. A lot of variations are made in media formulation for the reduction in cost of the media, maximization of the cell growth and/or product formation and easy separation of the products. The costs of the MICP treatments are attributable both to the price of the product and the number of applications required (De Muynck *et al*., 2010). The theoretic price of the product depends on the price of the microorganisms and the price of the nutrients. One kilogram of lyophilized bacteria (10^{11} cfu/gm) costs around $1100 (per kg). As bacteria are applied in a concentration of 2–3 per gm^2 this results in a cost of $2.2–3.3 (per m^2). The costs of the nutrients are estimated to be about $180 (per kg). Depending on the subject to treatment, the dosage for biodeposition varies between 0.04 and 0.08 kg per m^2, bringing the cost of the nutrients to $7–15 (per m^2). This brings the total product cost around $10–17 (per m^2) and the total price of the treatment amounts goes to about $23–28 (per m^2) (De Muynck *et al*., 2010). Thus, finding out of the cheaper sources of raw materials are particularly important for the products which are required in large quantities.

Several studies have showed that these organisms can grow on a wide variety of complex and synthetic media. Urease enzyme produced by different *Bacillus* spp. are produced in large quantities and have many commercial applications in remediation of cracks in building materials as cementing enzyme. To meet the demand, low cost media is required for the production of urease. The contents of the media such as nutrient broth, soluble starch as well as other components are expensive and have to be utilized optimally for the cell growth and enzyme production (Kumar and Jana, 2009). Urease producing bacteria prefer protein containing nutrient source to grow well. The industrial by-products corn steep liquor (CSL) and lactose mother liquor (LML), which are products of corn starch processing and dairy products respectively, are well known protein source and used to grow different bacteria; but their

preference to grow urease producing bacteria is not documented. As these are high protein source which also contain vitamins and amino acids, can be used as alternative nutrient sources.

LML is widely used as cultivation medium to produce lactic acid by different bacteria such as *Lactobacillus salivarius*, *Bacillus megaterium* (Vasala *et al*., 2005). It is a low-cost alternative that can be used as the hydrolyzed lactose source (Wong and Lee, 1998). LML has found manifold uses in microbiology mainly because of its high content in growth substances. CSL has also found considerable use as a source of nutrients for certain molds and bacteria used in industrial fermentations since long time (Wells *et al*., 1939; Moyer *et al*., 1940; Stubbs *et al*., 1940; Moyer and Coghill, 1946). Liggett and Koffler (1948) reported that corn steep liquor alone contains sufficient nutrients for the growth and development of many bacteria, and Winsten and Eigen (1949) and Picken and Bauriedel (1950) found that corn steep contained a growth stimulant for *Lactobacillus leichmannii* strain 313. Kennedy and Speck (1954) reported that corn steep contains a growth stimulant(s) for a number of lactic acid bacteria when grown in either milk or synthetic media that are supposedly complete nutritionally. CSL is a potentially useful substitute corn nitrogen source. Even after an essential pretreatment, this waste, still appears to be economically attractive, and studies have already shown satisfactory results for the product as a bacterial growth medium (Hoch, 1997). The oxidation of ammonia by *Nitrosomonas europaea* is accelerated in mineral liquid media by the addition of small amounts of corn steep liquor (Gundersen, 1958). Besides organic growth substances corn steep liquor contains a variety of inorganic materials. The use of low-cost substrates such as CSL and LML by *Ralstonia eutropha* produced results comparable to those for the conventional sources, allowing a reduction in the processing of polyhydroxyalkanoate production cost (Marangoni *et al*., 2001). Corn steep liquor was substituted for ammonium sulfate, showing good results for specific growth rate. The growth rates and pyrene degradation rates of *Pseudomonas* sp. LP1 and *Pseudomonas aeruginosa* LP5 were increased in CSL (Obayori *et al*., 2010). It can be well said that exploitation of LML and CSL will produce safe, commercially

viable and high value economic products and also it will help in recycling of such by-products to provide a greener environment option.

2.6 Microbiological sand plugging

Sand is the common material used to make most of the building materials and structures. Microbiological process utilizes a biological byproduct, $CaCO_3$, which has shown a wide range of application potential to make sand as a sealant (Bang and Ramakrishnan, 2001). This inorganic sealant not only is environmentally innocuous but also persists in environments for a prolonged period.

Much laboratory work has been directed to the study of microbial selective plugging. Jenneman *et al*. (1984) have shown that nutrients necessary for growth and metabolism can be transported through sandstone cores. Shaw *et al*. (1985) found that bacteria produce an exopolysaccharide film, which contributes to pore plugging by suspending clay particles, cells, and other suspended solids. The decrease in permeability by MICP is believed to be the result of the *in situ* growth and metabolism of microorganisms, rather than abiotic factors (Raiders *et al*., 1989). In natural environments, mineral precipitation processes constantly occur at a slow rate over geologic time, plugging or selectively cementing permeable porous media (Kantzas *et al*., 1992). Microbial metabolic activities often contribute to selective cementation by producing relatively insoluble organic and inorganic compounds intra- and/or extracellularly, which remain in the environment for a long time even after cell death. Some microorganisms produce glycocalyx and a variety of organic polymers outside the cell wall (Lappin-Scott *et al*., 1988; MacLeod *et al*., 1988), while others accumulate inorganic compounds such as phosphorites, carbonates, silicates, iron, and manganese oxides in cytoplasm (Beveridge *et al*., 1983; Ghiorse, 1984; Knoll, 1985; Ruiz *et al*., 1988; Rivadeneya *et al*., 1991). The importance of selective cementation has been widely recognized in Petroleum Engineering, Geological Engineering and Civil Engineering (Updegraff, 1982; Finnerty and Singer, 1983).

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 the cementation took place. Microbial calcite plugging was selective and its efficiency was affected by the porosity of the medium, the number of cells present and the total volume of nutrient added. The sand column loaded with bacteria was so tightly plugged that the column was fractured with a mechanical knife for examining. Microbiological sand plugging based on MICP suggest that it can be successfully employed on building materials to improve its durability.

2.7 Building materials

The most commonly used building materials throughout world are bricks, cement and concrete.

2.7.1 Bricks

The brick, first produced in a sun-dried form at least 6,000 years ago and the forerunner of a wide range of structural clay products used today, is a small building unit in the form of a rectangular block, formed from clay or shale or mixtures and burned (fired) in a kiln, or oven, to produce strength, hardness, and heat resistance (Toy, 1955). The original concept of ancient brick makers was that the unit should not be larger than what one man could easily handle; today, brick size varies from country to country, and every nation's brick making industry produces a range of sizes that may run into the hundreds.

Mud brick, dried in the sun, was one of the first building materials. It is conceivable that on the Nile, Euphrates, or Tigris rivers, following floods, the deposited mud or silt cracked and formed cakes that could be shaped into crude building units to build huts for protection from the weather. In the ancient city of Ur, in Mesopotamia (modern Iraq), the first true arch of sun-baked brick was made about 4000 BC. The arch itself has not survived, but a description of it includes the first known reference to mortars other than mud. As civilization spread eastward and westward from the

Middle East, so did the manufacture and use of brick. The Great Wall of China (210 BC) was built of both burned and sun-dried bricks.

2.7.2 Cement

Cement is a well-known building material and has occupied the most important place in construction industry. A mixture of cement and sand mixed with water to form a paste is known as *cement mortar* whereas the product that is obtained by mixing cement, water, sand and gravel or crushed stone is called *cement concrete* (Gambhir, 2006). The distinguishing property of cement is its ability to harden when water is added to it. Although all materials that go into a concrete mixture are essential, cement is by far the most important constituent because it is usually the delicate link in the chain. The function of cement is firstly, to bind the sand and coarse aggregates together, and secondly, to fill the voids in between sand and coarse aggregate particles to form a compact mass.

Natural cement is obtained by burning and crushing the stones containing clay, carbonate of lime and some amount of carbonate of magnesia. Clay content in such stones is about 20 to 40 per cent. Natural cement is brown in colour and its best variety is known as Roman Cement. It resembles very closely to eminent hydraulic lime. It sets very quickly after addition of water. It is not as strong as artificial cement and hence, it has limited use in practice.

Artificial cement is obtained by burning at a very high temperature a mixture of calcareous and argillaceous materials mixed in definite proportions. Calcined product is known as *clinker*. A small quantity of gypsum is added to clinker and it is then pulverised into very fine powder which is known as *cement*. Common variety of artificial cement is known as normal setting cement. This cement was invented by a mason Joseph Aspdin of Leeds in England in 1824. After setting, this cement closely resembles a variety of sandstone which is found abundantly in Portland in England. It is, therefore, sometimes referred to as Portland cement. The manufacture of Portland cement was started in England in around 1825. America started the same in 1872 and India started the same in 1904. The first cement factory was installed in

Tamil Nadu in 1904 by M/s. South India Industry Limited and then onwards a number of factories manufacturing cement were started. At present there are more than 70 factories producing different types of Portland cement. Portland cements are composed primarily of calcium silicates which constitute about 80% of the mass. The remaining 20%, compromising aluminates, ferrites and other compounds, although contributing little to cementing properties, is essential in enabling cement to be manufactures economically.

2.7.3 Concrete

The word '*concrete*' comes from the Latin '*concretus*' meaning compounded. Concrete as a building material has been around for a very long time. Concrete today is an indispensable part of the fabric of modern society, used for everything from mundane road pavements and high rise building structures to the more exotic such as ferro-cement yacht hulls. Concrete is used more than any other man-made material in the world (Lomborg, 2001). Despite its long history of use, our understanding of the material has only really developed in very recent times, particularly with respect to its durability, and practices which were commonplace only twenty years ago would now be unthinkable. Concrete comprises cement, aggregates (stone and sand of various grades) and water. It typically also contains additives to improve various characteristics such as workability, strength or resistance to chemical attack. It is nearly always reinforced with steel embedded within it, as it has high compressive strength but poor tensile strength.

2.8 Quality control of building materials

The main aim of any specification is to ensure the quality of the final product. Strength and durability are considered to be the requirements for concrete or any other building materials. Nowadays, performance based durability criteria are becoming more prevalent in specifications for building materials. A major goal of any performance based specification is to ensure the durability of building structures. Durability is defined as the ability of the structure to perform satisfactorily with

minimum maintenance over the anticipated service life of the structure. The quality of any building material depends on three major parameters, viz. (i) compressive strength, (ii) permeability and (iii) corrosion. For an efficient microbial concrete it should produce more compressive strength, less permeability and should not affect corrosion of any reinforcement.

2.8.1 Compressive strength

Compressive strength is considered as an index to assess the overall quality of concrete and it is generally assumed that an improvement in the compressive strength results in improvement of all other properties. Hence strength investigations are generally centered on compressive strengths. Concrete mixes are proportioned on the basis of achieving the desired compressive strength at the specified age.

The compressive strength of the concrete is one of the most important technical properties. In most structural application, concrete is employed primarily to resist compressive stresses. In these cases where other stresses (tensile, *etc*) are of primary importance, the compressive strength is still frequently used as a measure of the resistance because this strength is the most convenient to measure. For the same reason, the compressive strength is generally used as a measure of the overall quality of the concrete, even when strength itself may be relatively unimportant. Finally, the concrete making properties of the various ingredients of the mixture are usually measured in terms of compressive strength. The compressive strength of concrete or mortar is usually determined by submitting a specimen of constant cross section to a uniformly distributed increasing axial compression load in a suitable testing machine until failure occurs. The strength is expressed as the ultimate compression load per cross sectional area, usually in psi, Pa or Kg/cm^2.

2.8.2 Permeability

Most deterioration processes have two stages. Initially, aggressive fluids from environment penetrate and are transported through the capillary pore structure of the concrete or other building materials to reaction sites. Hence, porosity of concrete,

through which the ingress of moisture, chlorides and aggressive environment, takes place should be properly controlled. This penetration is followed by the actual chemical or physical deterioration reactions. To assess the ability of the concrete to perform well during the first stage we need tests that measure transport rates, resistance to penetration of aggressive fluids or both. Hence, to improve the durability of concrete structures, we must study the impermeability of water, chlorides for concrete and methods to improve the same particularly in the cover portion. If the atmosphere is conducive to corrosion then the time between the aggressive agents reaching the steel and the onset of corrosion is dependent on the ease with which they can penetrate the concrete cover. Once corrosion has begun, the rate at which it continues and the time taken for visible damage to occur are influenced by the permeability and porosity of the cover concrete.

There are many alternatives available to improve impermeability to enhance the durability of building materials. Addition of mineral admixtures like ground granulated blast furnace slag (GGBS), fly ash and silica fume helps to improve the compactness of concrete leading to improvement in strength parameters. Due to use of cheap waste material the concrete becomes economical and helps for sustainable development. Concrete with mineral admixtures shows improvement in durability properties. Chemical admixtures such as plasticizers, superplasticisers and water reducing agents help to improve the workability by reducing the intergranular friction ultimately affecting the porosity and distribution of pores. Hence the use of mineral and chemical admixtures helps not only to improve the strength but also to improve the durability of concrete.

The permeability of water and pollutants is the major threat to the reinforced concrete sections. Such penetration leads to the ingress of moisture and chlorides which is responsible for early leakages and corrosion of the embedded steel. The corrosion products volume is greater than the uncorroded steel. Hence, after corrosion it exerts pressure on surrounding concrete leading to cracking of the section. If microbial deposition is able to seal the pores in the cover portion of the concrete in top few millimeters, then the life of the reinforced concrete sections will

improve substantially. Most of the distress in concrete occurs because of leakages due to permeability of concrete or corrosion of embedded steel.

Ingress of pollutants from environment particularly chlorides and sulphates depends on permeability of concrete. Deterioration of concrete structures is directly proportional to the ingress of moisture and chlorides. Once the chlorides reach the reinforcement level, it initiates pitting corrosion. Resistance to penetration of chloride ions is one of the most important measures in durability. It is necessary to study how the concrete with microbial deposition reduces the rate of chloride ion penetration by doing tests for the ability of the concrete to resist the chloride ion penetration. There are many promising test methods for concrete testing that have standards and are applicable to performance specifications. These tests are Air void system (ASTM C 457), Sorptivity test (ASTM C 1585), Rapid migration test (AASHTO TP 64), Chloride bulk diffusion (ASTM C 1556). However, rapid chloride penetration (RCP) test (ASTM C 1202) is the most widely used test in the countries where performance specifications are implemented such as Australia, Canada. RCP test has been increasingly adopted for accelerated testing not only for resistance of concrete against chloride penetration but also for reflecting the permeability of very dense cementitious materials.

Electrochemical methods, including measurement of conductivity and its inverse resistivity have been proposed as methods for assessment of transport properties as well as changes in the pore solution and microstructure in cement based materials. Conductivity and resistivity can be measured using a number of techniques but the most widespread method involves placing a sample between two electrodes. This conductivity method is based on materials science and can therefore be used with confidence in durability prediction models.

2.9 Corrosion of steel-reinforced concrete

Corrosion is the destructive attack of a material by reaction with its environment. Corrosion control is achieved by recognizing and understanding corrosion

mechanisms. Following the research of the English scientist Michael Faraday it has been understood that most corrosion is electrochemical in nature. Since corrosion is an electrochemical process, it follows that electrochemical techniques and electrochemical instrumentation can be used to study the corrosion process.

Corrosion requires oxygen and water to take place, so the key to durability in concrete design is to have a mix which ensures that the reinforcement is always well protected from these elements. As long as the steel is cocooned in alkali-rich cement it is not at risk. Control of permeability of the concrete is fundamental to this, which in turn is dependent on the shrinkage which inevitably takes place as concrete dries out and hardens. The volume of cementitious products after they 'set' is less than their original volume when cast. This is compounded by the fact that more water is required to be added to a concrete mix to enable it to be handled in the wet condition than is required by the cement for the chemical process known as hydration. These volume changes result in pores, or worse, cracks, through which water and carbon dioxide can track to reach the steel.

Electrochemical techniques are popular because they are fast, accurate and versatile. Much faster than weight loss coupons, accurate even to the lowest corrosion rates and versatile enough to cope with any test in laboratory or field it is easy to see why electrochemical instruments are the bedrock of corrosion research. A number of electrochemical techniques have been developed specifically for corrosion measurement. Among these techniques are Linear Polarization Resistance, Cyclic Polarization, Potentiostatic testing, Galvanodynamic sweeps, and Critical Pitting Temperature tests. All these tests are very straightforward to employ in a corrosion laboratory or in the field. The ACM Field Machine is one of the best instruments in the world for corrosion testing in the field and has been used in this thesis work.

Concrete provides more than a protective cover to guard against corrosion. It also offers a passivating environment because of the high pH of the pore solution (approximately 12-13). Iron passivity results from the formation of an ultra-thin (< 10 nm), protective oxide or hydroxide film, that slows down the rate of anodic

dissolution to negligible levels (Shreir *et al*., 1994). The formation of a passive film begins with the dissolution of the metal and the corresponding oxygen reduction which uses the electrons generated by the metal dissolution in its reaction, represented by Equations 2.1 and 2.2, respectively.

$$2Fe_{(s)} \longrightarrow 2Fe^{2+}{}_{(aq)} + 4e^{-} \quad (2.1)$$

$$O_{2\,(g)} + 2H_2O_{\,(l)} + 4e^{-} \longrightarrow 4OH^{-}{}_{(aq)} \quad (2.2)$$

The resulting ferrous ions are attracted to the cathodic portions of the steel. The closest cathodic site is where the iron ions combine with hydroxide ions from the cathodic reaction to form the solid product of ferrous hydroxide (Equation 2.3). It is also possible that hydroxide ions may travel to the anodic regions.

$$2Fe^{2+}{}_{(aq)} + 4OH^{-}{}_{(aq)} \longrightarrow 2Fe(OH)_{2(s)} \quad (2.3)$$

Upon exposure of this film to oxygen, other passivating oxides may form (*e.g.*, Fe_3O_4 and Fe_2O_3) on the outer surface of the film. As a result, these passive films consist of layers of iron hydroxides or oxides in different states of oxidation and cause corrosion of reinforcement (Uhlig and Revie, 1985).

2.9.1 Chloride induced corrosion

Chloride ions locally destroy the passive film on steel in concrete. The sources of these ions include: any chlorides originally present within the cement powder, calcium chloride added at the time of mixing as a set-accelerator, contaminated aggregate or mixing water, or those introduced over the service life of the reinforced concrete structure from marine exposure or the use of de-icing salts.

Sufficient chlorides present in the pore solution adjacent to the steel reinforcement can initiate active metal dissolution at local sites, known as pitting corrosion, provided the supply of oxygen and water is also high enough to ensure that the cathodic reactions can take place over the remaining surface of the steel. The presence of millscale typically observed on the surface of reinforcing steel restricts

the attack of the chloride ions to those areas of the steel where the scale is broken. The exact mechanism of chloride-induced pitting in concrete is not known but it is thought that the overall process is schematically illustrated in Figure 2.1. This diagram was developed assuming a neutral aqueous solution but a similar process can be assumed for steel in concrete albeit at a lower rate and without the evolution of hydrogen gas for the conditions typically encountered in concrete.

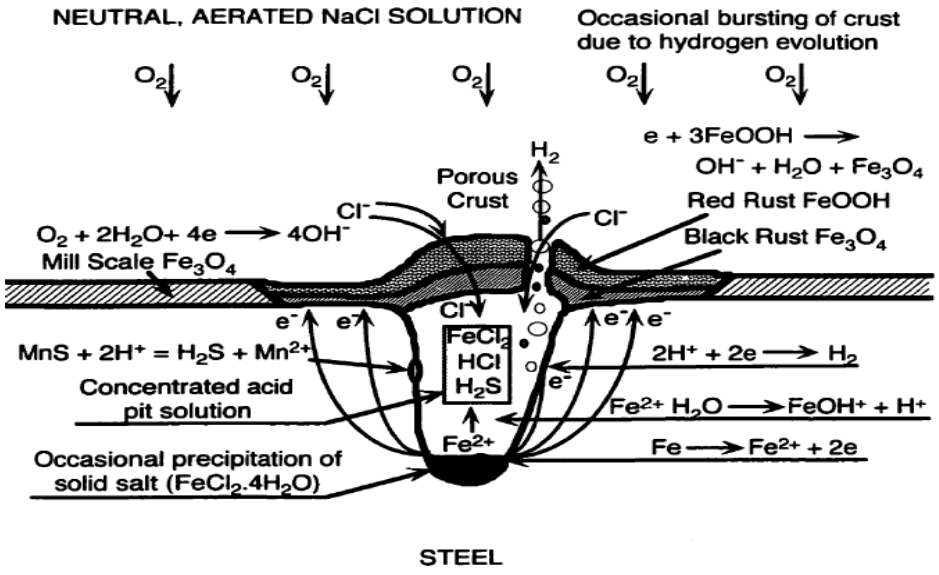

Figure 2.1 Schematic illustration of the chloride-induced corrosion of steel in a neutral, aerated solution (Wranglén, 1985).

Chloride-induced corrosion initiates similarly to the passivation process represented by Equations 2.1 and 2.2. Chloride ions are then attracted to the metal dissolution sites to maintain electro-neutrality and generate soluble iron chlorides. These chloride products can have a range of compositions which are often simplified and reported as ferrous chloride, the thermodynamically favored product, Equation 2.4.

$$2Fe^{2+}_{(aq)} + 4Cl^{-}_{(aq)} \longrightarrow 2FeCl_2.4H_2O_{(s)} \qquad (2.4)$$

It is likely that the presence of a cementitious cover by microbial induced calcite precipitation would affect the initiation of corrosion and the subsequent low corrosion rate but no known research of this type has been published.

2.10 Improvement in the durability of building materials by classical approach

To improve the durability of structures constructed in concrete, engineers started using mineral additives in the form of waste materials from industries such as fly-ash, ground granulated blast furnace slag. Addition of these mineral additives reduced the heat of hydration which helped to do mass concreting jobs. In 1980 chemical admixtures in the form of superplasticisers were developed to improve the workability of concrete and reduce the water cement ratio. Subsequently many chemical additives such as accelerators, retarders and air entraining agents were developed to have some special properties to the concrete required for specific jobs. In 1985, silica fume was introduced so as to fill the micro pores in the cement paste in concrete to give more workability and compactness. Many additives and admixtures were developed for concrete as it is one of the most widely used construction material not only for small elements like concrete sleepers but also in many giant structures which are constructed to satisfy the needs of infrastructure of growing population. In spite of so much development in the ingredients of concrete final product, concrete was not made to react with environment and have the ability of self healing as seen in naturally built structures described above. Most of these additives were passive in nature. More research and development is required to tackle the problems of permeability, ingress of chloride and carbonation to protect the reinforcing material added in concrete from deterioration. There is a need to develop an additive which will react with environment, grow as per needs and will have property of self healing in case of distress.

In existing structures, it is not possible to change the nature of the concrete easily. As deterioration starts to occur, the emotively termed ‘concrete cancer’ steps have to be taken to arrest the decline and repair the damage. Fortunately, in most cases the onset of this deterioration is not necessarily a death warrant for the building, and repairs can be affected. Before this is attempted, it is essential to have a full understanding of the cause and extent of the breakdown. It may already have reached, or be close to the point, where economical repair is not feasible. This decision may be affected by the nature of the building or structure. Economics may hopefully play little part in

determining whether to retain or demolish a national icon. Several tests are available to the engineer for diagnosing the condition of the concrete. The alkalinity of the concrete at the surface and at various depths down to the position of the reinforcement will give an indication of how much and how soon the reinforcement may be at risk. This is known as the depth of the carbonation front. The presence of chlorides can be determined, again at varying depths. The permeability of the concrete will give an indication of its susceptibility to absorption of air and water.

In many cases, the deterioration of the reinforcing steel is a localised problem, caused by an isolated air/water path or where reinforcement or fixings connected to the reinforcement are at or near the surface. In such cases the offending area can be cut out and repaired with a mortar patch. In this regard, significant advances have been made with the design of repair materials in very recent years. The key factor is to ensure that the steel is protected, and the weakest point is the interface between the new patch material and the original concrete. Shrinkage of the patching mortar must be avoided at all costs. Repair mortars now compensate for the natural tendency of the mortar to shrink, also containing latex products which give them some flexibility. A secondary consideration in terms of durability, but understandably often foremost in the mind of the building owner is the appearance of the patch material. Matching the color, texture, sheen and other characteristics of the parent material is often reasonably straightforward, with a bit of trial and error; however there is a tendency for the new and old materials to weather at different rates, so that what looks good today may be a very prominent patch in 5 years' time.

Concrete is the most widely used construction material, with Portland cement as the main ingredient. Portland cement manufacture alone is responsible for about 5% of the global greenhouse gas emissions (Mahasenan *et al*., 2003). Currently, concrete is consumed at the rate of 1 ton per year for every living human being, making it the largest consumed man-made material. There are so many synthetic agents or latex binding agents which are used to avoid any kind of fractures and fissures in the concrete structures or used in repair applications such as the bonding of fresh concrete, sprayed concrete or sand/cement repair mortar to hardened concrete. A

comparative chart of different properties of these latex bonding agents is given in Table 2.2 (Mailvaganam, 1997).

There is reluctance by some repair product manufacturers to adequately address the problem, with a very limited range of appearances available from their materials in different countries. There is a propensity for specifiers to recommend overcoating of the whole building, such as with a high-build acrylic paint in order to achieve a uniform appearance, which is also easily matched in future if further repairs become necessary. This may well be a blessing in disguise for some buildings, but for others the original appearance is their single most important feature, and it should not be discarded lightly. It also needs to be said that a building which is painted may mean a lifetime of higher maintenance costs. It will probably need repainting every ten years, perhaps less, to retain its smartness. Despite the advice to use an appearance-changing coating only as a last resort, these products have their place. Indeed, they may be essential as a means of preventing the absorption of moisture and air, in the latter case being classed as anti-carbonation coatings. In selecting a coating, one should have in mind what is intended to be achieved by that coating, if it is not simply a change of appearance. In particular it must be noted that some are waterproof but permeable to air. These will not necessarily arrest the advancement of the carbonation front. Others are not vapour-permeable, which can lead to blistering of the coating if there is moisture trapped behind it.

The first step in selection of repair techniques and products must always be a thorough investigation to understand the nature of the problem; otherwise a repair exercise may prove to be completely fruitless. We need to look into some other repair techniques to improve the durability of building materials that also should be environment-friendly.

Table 2.2 Latex bonding agents comparative chart (Mailvaganam, 1997)

Property/ test method	Acrylic	Polyvinyl-acetate (non-re-emulsifiable)	Butadiene-styrene	Polyvinylacetate (re-emulsifiable)
Appearance	Milky white	Milky white	Milky white	Milky white or clear
Solids Content	45%	55%	48%	50%
Primary Use	Bonding fresh concrete to old concrete	Bonding fresh concrete to old concrete, thin layer toppings	Bonding fresh concrete to old concrete, concrete admixture, thin layer toppings	Bonding to plaster
Application Methods	Brush, broom, spray, roller as adhesive, trowel as topping	Brush, broom, spray, roller as adhesive, trowel as topping	Brush, spray, roller as adhesive, trowel as topping.	Brush, spray, roller
Applications	<—Underlayments, stucco, grouting-mortar, terrazzo, crack fillers—>			
Cleaning, Surface Preparation	Remove oil, grease; wet surface	Remove oil, grease	Remove oil, grease	
Comp. Strength (MPa) 2 in. (50 mm) cubes ASTM C109	 22.0 28.27	 23.45 24.82	 22.75 27.58	 22.0 (air) 20.68 (wet)
Tensile Strength (MPa) 1 in. (25 mm) thick briquettes ASTM C190	 4.0 4.24	 2.41 3.10	 3.10 4.0	 2.06 (air) 2.83 (wet)
Flexural Strength Bar (MPa) ASTM C-348-61T	 6.55 9.65	 6.90 8.62	 8.62 11.38	 6.38 (air) 5.17 (wet)
Where to Use	Indoor and out-door exposures; on concrete, steel, wood; guniting; thin section topping. May be used as a plaster bond within 45-60 min.	Indoor and out-door exposures; on concrete, steel, wood; guniting; thin section topping. May be used as a plaster bond within 45-60 min.	Indoor and out-door exposures; on concrete, steel, wood; guniting; thin section topping. May be used as a plaster bond within 45-60 min.	Indoor ceilings primarily, limited use as concrete bonding agent
Where Not to Use	Not for extreme chemical exposure, not for conditions of high hydrostatic pressure	Not for extreme chemical exposure, not for conditions of high hydrostatic pressure	Not for extreme accelerators, not for extreme chemical exposure, not for constant water	Do not use as an admixture. Do not use under wet or humid conditions. Do not use at temperatures below 10°C.

2.11 Improvement in the durability of building materials through Microbiologically Induced Calcite Precipitation (MICP)

It's safe to say that without microbes, biotechnology would be an extremely limited science. They not only provide the foundation for much of the basic research involved in biotechnology, they help to create many of the processes which are integral to this science. Ubiquitous in nature, the world of microorganisms fascinates everyone. Man has made many inventions in every century to harvest the potential of these tiny inhabitants of nature to his best advantage. Starting from medical science to agricultural science, man has come a long way in developing protective strategies to enhance the durability of building materials in every way possible. Microbiologically Induced Calcite Precipitation (MICP) has gained interest in the last 20 years, particularly with regard to the potential role marine systems may play as 'carbon sinks' for the increasing global production of CO_2. Three main groups of organisms exist that can induce MICP through their metabolic processes:

1. Photosynthetic organisms such as cyanobacteria and algae that remove CO_2,
2. Sulphate reducing bacteria that are responsible for the dissimilatory reduction sulphate, and
3. Several organisms that are involved in the nitrogen cycle (Castanier *et al*., 1999; Hammes and Verstraete, 2002).

The most common form of MICP in aquatic environments is caused by photosynthetic organisms (McConnaughey and Whelan, 1997). The metabolic processes of algae and cyanobacteria utilize dissolve CO_2 (Eq 2.5), which is in equilibrium with HCO_3^- and CO_3^{2-} (Eq 2.6). The removal of CO_2 induces a shift in this equilibrium, and results in an increase in pH (Eq 2.7) (Ehrlich, 1998). When this reaction occurs in the presence of calcium ions, calcium carbonate is produced (Eq 2.8) (Hammes and Verstraete, 2002).

$$CO_2 + H_2O \longrightarrow (CH_2O) + O_2 \qquad (2.5)$$

$$2\ HCO_3^- \longleftrightarrow CO_2 + CO_3^{2-} + H_2O \qquad (2.6)$$

$$CO_3^{2-} + H_2O \longleftrightarrow HCO_3^- + OH^- \quad (2.7)$$

$$Ca^{2+} + HCO_3^- + OH^- \longrightarrow CaCO_3 + 2H_2O \quad (2.8)$$

Calcite can also be precipitated by heterotrophic organisms, by the production of carbonate or bicarbonate and modification of the environment to favour precipitation (Castanier *et al*., 1999). The abiotic dissolution of gypsum ($CaSO_4.2H_2O$) (Eq 2.9) provides an environment that is rich in both sulphate and calcium ions. In the presence of organic matter and absence of oxygen, sulphate reducing bacteria can reduce sulphate to H_2S and release HCO_3^- (Eq 2.10) (Ehrlich, 1998; Castanier *et al*., 1999; Wright, 1999). If H_2S then degasses from the environment, this results in an increase in pH and favors the precipitation of calcium carbonate (Eq 2.8) (Castanier *et al*., 1999).

$$CaSO_4.2H_2O \longrightarrow Ca^{2+} + SO_4^{2-} + 2H_2O \quad (2.9)$$

$$2(CH_2O) + SO_4^{2-} \longrightarrow HS^- + HCO_3^- + CO_2 + H_2O \quad (2.10)$$

MICP can also be induced by organisms involved in the nitrogen cycle, via ammonification of amino acids, nitrate reduction and the hydrolysis of urea. The simplest of all the mechanism described for MICP is the hydrolysis of urea by the enzyme urease, which results in the production of carbonate ions in the presence of ammonium (Eq 2.11). Calcite is readily precipitated under these conditions, in the presence of calcium.

$$CO(NH_2)_2 + 2H_2O \longrightarrow CO_3^{2-} + 2NH_4^+ \quad (2.11)$$

Urease activity is widespread amongst bacteria and this has been the approach used most often for applied MICP for the production of calcite (Mobley and Hausinger, 1989; Stocks-Fischer *et al*., 1999; Fujita *et al*., 2000).

Besides changes induced in the macro-environment, bacteria have also been reported to influence calcium carbonate precipitation by acting as sites of nucleation or

calcium enrichment (Morita, 1980). Due to the presence of several negatively charged groups on the cell wall, at a neutral pH, positively charged metal ions can be bound on bacterial surfaces (Douglas and Beveridge, 1998; Ehrlich, 1998). Such bound metal ions (e.g. calcium) may subsequently react with anions (e.g. carbonate) to form an insoluble salt (e.g. calcium carbonate) (De Muynck *et al*., 2010). In the case of a sufficient excess of the required cations and anions, the metal salt on the cell surface initiates mineral formation by acting as a nucleation site. The anion (e.g. carbonate) in this reaction may be a product of the bacterial metabolism, or it may have an abiotic origin (Ehrlich, 1998). Furthermore, it has been demonstrated that specific bacterial outer structures (glycocalyx and parietal polymers) consisting of exopolysaccharides and amino acids play an essential role in the morphology and mineralogy of bacterially induced carbonate precipitation (Braissant *et al*., 2003; Ercole *et al*., 2007). The actual role of the bacterial precipitation remains, however, a matter of debate. Some authors believe this precipitation to be an unwanted and accidental by-product of the metabolism (Knorre and Krumbein, 2000) while others think that it is a specific process with ecological benefits for the precipitating organisms (Ehrlich, 1996; McConnaughey and Whelan, 1997).

Concrete in service cracks due to direct stress and sub-stress caused by many kinds of reasons, such as changes of temperature and humidity, inhomogeneous sinking, external loading (dynamic or static loading) (Zhong and Yao, 2008). Cracks not only influence the service durability of concrete structure, but also are harmful for the structure safety. A novel technique for the remediation of damaged structural formations has been developed by employing a selective microbial plugging process, in which metabolic activities promote precipitation of calcium carbonate in the form of calcite. Biomineralization of calcium carbonate is one of the strategies to remediate cracks in building materials. Biomineralization is defined as a biologically induced precipitation in which an organism creates a local micro-environment with conditions that allow optimal extracellular chemical precipitation of mineral phases (Hamilton, 2003). Numerous diverse microbial species participate in the

precipitation of mineral carbonates in various natural environments, including soils, geological formations, freshwater biofilms, oceans, and saline lakes.

It has been known that calcite (calcium carbonate) has values of technical and industrial applications for the preservation, remediation and restoration of buildings, calcareous stone statuaries and historic monuments. Use of bacteria in concrete remediation is an unorthodox concept in current concrete research. It is, however, a new approach to an old idea that a microbial mineral deposit constantly occurs in natural environments. Specifically, microbiologically-induced calcite is environmentally innocuous, compared to synthetic polymers currently used for concrete repairs (Ramachandran *et al*., 2001). Calcium carbonate precipitation is a general phenomenon in the bacterial world under appropriate conditions (Boquet *et al*., 1973). Indeed, some bacteria and fungi can induce precipitation of calcium carbonate extracellularly through a number of processes that include photosynthesis, ammonification, denitrification, sulphate reduction and anaerobic sulphide oxidation (Castanier *et al*., 1999; 2000; Riding, 2000). The primary role of bacteria in the precipitation process has been ascribed to their ability to create an alkaline environment through various physiological activities (Douglas and Beveridge, 1998).

Knorre and Krumbein (2000) concluded that microbiologically induced calcite precipitation (MICP) occurs as a by-product of common microbial metabolic processes, such as photosynthesis, urea hydrolysis, and sulfate reduction. These metabolic processes increase the alkalinity (increase the pH and dissolved inorganic carbon content) of the environment and thereby favor $CaCO_3$ precipitation. Alternatively, it is possible that there are specific attributes of certain bacteria that promote and affect $CaCO_3$ precipitation. The negatively charged nature and specific functional groups of microbial cell walls favor the binding of divalent cations (Ca^{2+}, Mg^{2+}), thereby making microorganisms ideal crystal nucleation sites (Rivadeneyra *et al*., 1998). Microbial extracellular polymeric substances are also an important factor in precipitation, either through trapping and concentration of calcium ions or as a result of specific proteins that influence precipitation. Kawaguchi and Decho (2002)

suggested that specific proteins present in biological extracellular polymeric substances cause the formation of different $CaCO_3$ polymorphs.

Microbiologically induced calcium carbonate precipitation occurs via far more complicated processes than chemically induced precipitation. The bacterial cell surface with a variety of ions can nonspecifically induce mineral deposition by providing a nucleation site. Ca^{2+} is not likely utilized by microbial metabolic processes; rather it accumulates outside the cell. In medium, it is possible that individual microorganisms produce ammonia as a result of enzymatic urea hydrolysis to create an alkaline microenvironment around the cell. The high pH of these localized areas, without an initial increase in pH in the entire medium, commences the growth of $CaCO_3$ crystals around the cell. Possible biochemical reactions in Urea-$CaCl_2$ medium to precipitate $CaCO_3$ at the cell surface can be summarized as follows (Stocks-Fischer *et al*., 1999):

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

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

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

Use of microorganisms also found to be effective in enhancing oil recovery from petroleum reservoirs. Raiders *et al*. (1990) found that *in situ* microbial growth and metabolism increased oil recovery between 10 to 38% of the original oil in place. They studied the ability of indigenous populations of microorganisms in Berea sandstone to improve the volumetric sweep efficiency and increase oil recovery by *in situ* growth and metabolism following the injection of nutrients. They also found a preferential decrease in permeability in the core or slab of sandstones with the higher initial permeability, diverted flow into the lower-permeability core or slab and improved the volumetric sweep efficiency.

2.12 Sand consolidation and MICP

Sand is the common material used to make most of the building materials and structures. As such there is no carbonate mineral found in sand, so it is easy to understand the mechanism of calcite precipitation induced by bacteria in it. There are many reports where various researchers have successfully defined MICP in sand consolidation. The participation of *Bacillus pasteurii* in sand consolidation has been demonstrated by Kantzas *et al*. (1992). Gollapudi *et al*. (1995) further investigated the use of *B. pasteurii* for the plugging of sand columns. Although the bacteria were mixed with the sand slurry, consolidation mainly occurred near the surface. Stocks-Fischer *et al*. (1999) showed that microorganisms directly participated in the calcite precipitation by providing a nucleation site and by creating an alkaline environment which favoured the precipitation of calcite. Zhong and Islam (1995) used the consolidation of sand mixtures for the remediation of cracks in granite. Cracks in granite were packed with a mixture of bacteria, nutrients and a filler material. Among the different materials that were mixed with *B. pasteurii*, the silica fume (10%) and sand (90%) mixture lead to the highest compressive strength and lowest permeability.

2.13 Microbiologically Enhanced Crack Remediation (MECR)

Use of microbial products as a long-term remediation tool has exhibited high potential for crack cementation of various structural formations such as granite and concrete (Gollapudi *et al*., 1995; Stocks-Fischer *et al*., 1999). A novel approach of microbiologically-enhanced crack remediation has been reported (Bang and Ramakrishnan, 2001). They used *Bacillus pasteurii* to induce $CaCO_3$ precipitation. Scanning Electron Micrography (SEM) and X-Ray Diffraction (XRD) analysis has shown the direct involvement of microorganisms in calcium carbonate precipitation. As a further extension to this research, Ramachandran *et al*. (2001) investigated the microbiological remediation of cracks in concrete. The authors proposed MICP as an effective way to seal cracks. The appearance of cracks and fissures is an inevitable phenomenon during the ageing process of concrete structures upon exposure to

weather changes. If left untreated, cracks tend to expand further and eventually lead to costly repair. Specimens with cracks filled with bacteria, nutrients and sand demonstrated a significant increase in compressive strength and stiffness values when compared with those without cells (De Muynck *et al.*, 2010). The presence of calcite was, however, limited to the surface areas of the crack. The authors attributed this to the fact that *B. pasteurii* grows more actively in the presence of oxygen. Still, the highly alkaline pH (12–13) of concrete was a major hindering factor to the growth of the moderate alkaliphile *B. pasteurii*, whose growth optimum is around a pH of 9. To retain high metabolic activities of bacterial cells at such a high pH, immobilization technology (where microbial cells are encapsulated in polymers) can be applied. Polyurethanes have been widely used as a vehicle for immobilization of enzymes and whole cells because of its mechanically strong and biochemically inert characteristics (Klein and Kluge, 1981; Wang and Ruchenstein, 1993). Bang *et al*. (2001) found that physicochemically versatile polyurethane is an effective enhancement tool in microbiologically induced calcite precipitation in concrete cracks. In order to protect the cells from the high pH, Day *et al*. (2003) investigated the effect of different filler materials on the effectiveness of the crack remediation. Beams treated with bacteria and polyurethane showed a higher improvement in stiffness compared to filler materials such as lime, silica, fly ash and sand. According to the authors, the porous nature of the polyurethane minimizes transfer limitations to substrates and supports the growth of bacteria more efficiently than other filling materials, enabling an accumulation of calcite in deeper areas of the crack. No differences could be observed between the overall performances of free or polyurethane immobilized cells in the precipitation of carbonate (Bang *et al*., 2001). In addition to this research, Bachmeier *et al*. (2002) investigated the precipitation of calcium carbonate with the urease enzyme immobilized on polyurethane. As an extension to their research on biodeposition on cementitious materials, De Belie and De Muynck (2008) further investigated the use of microbiologically induced carbonate precipitation for the repair of cracks in concrete by using *B. sphaericus*. Ramachandran *et al*. (2001) studied the effect of microbiological calcite precipitation of cracks of various depths on the compressive strength values in Portland cement

mortar cubes and found an increase of the strength in the presence of *B. pasteurii* in the cubes prepared with the deepest cracks (25.4 mm), whose microbial remediation increased the compressive strength by approximately 61% of that of the control concrete.

2.14 Biological techniques for the cleaning of concrete

Biological techniques are also used for the cleaning of concrete and stone. De Belie *et al.* (2005) treated weathered concrete samples made with Portland cement or with blast-furnace slag cement and fouled by lichens with *Thiobacillus* bacteria and an appropriate nutrient by submersion or sprinkling. They documented the effect of 3 cleaning cycles of 3 days by the use of colorimetry and microscopy and found that application of Thio-S consortium as an efficient cleaner. The precipitation of calcium carbonate is also induced by addition of cyanobacterium cells. Dittrich *et al*. (2003) investigated the role of the solution composition on calcite precipitation induced by cyanobacterium cells *Synechococcus* strain PCC 7942.

2.15 Effect of calcium source on MICP

The influence of microbes on the precipitation and dissolution of minerals has been known for a long time. Active microbiologically mediated mineral precipitation could result in crack-plugging, concomitant decrease in material permeability and increase in compressive strength. As bacteria function as catalyst, a suitable mineral precursor compounds as calcium needs additionally to be incorporated in the material matrix to provide a truly autonomous healing mechanism. However, the maximal allowable amount of mineral precursor compound (using different calcium sources) introduced to the concrete mixture is likely limited as larger quantities may negatively affect other concrete properties such as setting time and final strength. Therefore, the addition of smaller quantities of mineral precursor compounds will probably result in autonomous repair of smaller cracks but not in healing of larger cracks (Jonkers *et al*., 2010). The deposition of calcium carbonate to improve the durability of building materials is influenced by the ureolytic bacteria and calcium

salt (De Muynck *et al*., 2007). Different calcium sources have been used by researchers, throughout the world, to help the effective process of microbial mineral formation.

Cement stone with bacterial spores were able to convert concomitantly incorporated calcium lactate to calcium carbonate based minerals upon activation by crack ingress water (Jonkers *et al*., 2010). The calcium carbonate form due to bacterial metabolic conversion of calcium lactate according to the following reaction:

$$CaC_6H_{10}O_6 + 6O_2 \longrightarrow CaCO_3 + 5CO_2 + 5H_2O$$

The metabolic conversion of calcium lactate does not result in production of massive amounts of ammonia what drastically increases the risk of reinforcement corrosion (Neville, 1996) and degradation of the concrete matrix particularly when further oxidized by bacteria to yield nitric acid (Diercks *et al*., 1991). Boquet *et al*. (1973) showed that most soil bacteria are able to precipitate crystals of calcium carbonate when tested in a medium containing calcium acetate and that calcite production by bacteria is just a function of the media composition. Urzi *et al*. (1999) found a variety of crystal morphologies and big crystals produced by bacteria in medium containing calcium acetate.

A lot of research on biodeposition was conducted with calcium chloride as the calcium source (Adolphe *et al*., 1990; De Muynck *et al*., 1995; Stocks-Fischer *et al*., 1999; Bang *et al*., 2001). Best results were obtained with the combination of ureolytic bacteria and calcium chloride, towards enhancement of durability of building materials (De Muynck *et al*., 2010).

2.16 Compressive strength based on MICP and microbial dose

The compressive strength of cement or concrete is the most common property used by the engineer in designing buildings and other structures. Compressive strength test results are used to determine that the concrete mixture as delivered meets the requirements of the specified strength in the job specification. This result is used for

quality control and acceptance of concrete. By keeping these points in mind, it is very important to study the effect of microbes on the compressive strength.

Besides external application of bacteria in the case of remediation of cracks, microorganisms have also been applied in the concrete mixture. Until now, research has mainly focused on the consequences of this addition on the material properties of concrete, i.e. strength and durability. Ramachandran *et al*. (2001) investigated the use of microbiologically induced mineral precipitation for the improvement of the compressive strength of Portland cement mortar cubes at the age of 7 and 28 days. They found that inclusion of microbial biomass enhanced the compressive strength of cement mortar cubes. They used live and killed cells of different concentrations of *Bacillus pasteurii* and found that the live cells, at lower concentrations, increase the compressive strength of cement mortar with a longer incubation period. The overall increase of strength, therefore, resulted from the presence of an adequate amount of organic substances in the matrix due to the microbial biomass.

To achieve greatest improvement in the compressive strength, cell concentrations/microbial doses needed to optimize. Ghosh *et al*. (2005) demonstrated the positive effect of the addition of *Shewanella* on the compressive strength of mortar specimens. They found that the strength of mortar cubes increased at all levels of anaerobic microbe addition. For these samples, the presence of a fibrous material inside the pores could be noticed. As a result, a modification of the pore size distribution was observed. The positive effect of the addition of *Shewanella* improved with increasing curing times. The greatest improvement in compressive strength occurs at cell concentrations of 10^5 cells/ml for all ages (3, 7, 14 and 28 days). For a concentration of 10^5 cells/ml, an increase of the compressive strength of 17% and 25% was observed after 7 and 28 days, respectively. However, no increase of the compressive strength was observed with additions of *Escherichia coli* (non-urease producing microbe) to the mortar mixture. This led the authors to suggest that the choice of the microorganism plays an important role in the improvement of the compressive strength. Jonkers and Schlangen (2007) investigated that the addition of a high number of bacterial spores ($10^8/cm^3$) by spore forming bacteria (*Bacillus*

pseudofirmus DSM 8715 and *Bacillus cohnii* DSM 6307) resulted in a decrease of strength of less than 10%.

From these findings, it can be concluded that compressive strength of cement mortar increases with an addition of urease producing microbes. This improvement in compressive strength might be due to deposition on the microorganism cell surfaces and within the pores of cement–sand matrix, which plug the pores within the mortar (Ramakrishnan *et al*., 1998).

2.17 Effect of microbes on the permeable properties

Research has indicated that a concrete which is low in permeation properties lasts longer without exhibiting signs of distress and deterioration (Nolan *et al*., 1995). Therefore, the permeation properties have been used principally for the comparison of the effectiveness of different surface treatments enhancing the durability of concrete.

Permeation controls the ingress of moisture, ionic and gaseous species into concrete. As the permeation of concrete decreases, its durability performance, in terms of physicochemical degradation, increases. Carbonation is a chemical reaction between carbon dioxide, in the presence of moisture, and calcium hydroxide present in hydrated concrete to form calcium carbonate. Carbonation occurs at the concrete surface including the surfaces of any cracks throughout the life of the concrete. However, as the depth of the carbonated layer of ordinary Portland cement (OPC) concrete increases, the products of carbonation reduce the rate of further carbonation (Khan and Lynsdale, 2002). The use of blended cements or supplementary cementing materials decreases the permeability, thereby increasing the resistance of concrete to deterioration by aggressive chemicals (Malhotra and Mehta, 1996).

Microbial carbonate precipitation (biodeposition) decreases the permeation properties of mortar and concrete (De Muynck *et al*., 2007). The deposition of a layer of calcium carbonate on the surface of the mortar specimens resulted in a decrease of water absorption and gas permeability (De Muynck *et al*., 2005). The

decrease in permeability of concrete and mortar specimens treated with bacteria can be seen from the water absorption and water vapour diffusion experiments. De Muynck *et al*. (2007) investigated the effects of microbial biomass on the permeability of concrete and mortar. To determine the increase in resistance towards water penetration, they carried out a sorptivity test. They coated the mortar specimens with bacterial biomass (*Bacillus sphaericus*), oven dried then dipped into 10±1 mm of water. They found that the presence of only bacteria resulted in a significant decrease of the water uptake compared to untreated specimens (a reduction of 45%, 43% and 24% with increasing w/c). When a calcium source was added to the medium an additional significant decrease of the water absorption coefficient was noticed. They also studied the durability of the treated surface by measuring the resistance to carbonation and chloride ingress, as chloride ions are one of the corrosion causing agent. They obtained a 19% decrease of the chloride migration coefficient, by the addition of bacterial biomass, compared to untreated cubes. The addition of bacterial biomass resulted in a significant smaller carbonation rate compared to untreated cubes.

Ramakrishnan *et al*. (2001) investigated the effect of this technique on the durability of concrete. The presence of bacteria was observed to increase the resistance of concrete towards alkali, sulfate, freeze thaw attack and drying shrinkage; the effect being more pronounced with increasing concentrations of bacterial cells. The authors attributed this to the presence of a calcite layer on the surface, as confirmed by XRD analysis, lowering the permeability of the specimens.

Many scientists studied the effect of biomineralization in the form of calcium carbonate deposition in sandstone and limestone. Dick *et al*. (2006) studied such deposition on degraded limestone by *Bacillus* species. They concluded that such deposition reduces the water absorption rate of limestone. They studied the capillary water absorption and absorption under vacuum. A decrease in the ability to absorb water will result in a deceleration of the weathering process. Similar observations were made by Tiano *et al*. (1999), who observed a reduction of about 60% from the samples treated with biodeposition. Le Metayer-Levrel *et al*. (1999) studied the

bacterial carbonatogenesis for the protection and regeneration of limestone in buildings, monuments and statues. Their study confirmed the deposition of protective surface due to biocalcification on the stone surface which reduced its permeability for gas without affecting its aesthetic appearance. They further state that biomineralization recreates a material that is remarkably similar to the limestone substrate. It uses natural microbial mediation which follows the same natural process that formed many limestones. Finally, they concluded that biological mortars or cement can be used to affix small pieces broken from statues and to fill small cavities on limestone surfaces.

The transport mechanisms normally associated with reinforced concrete deterioration are: absorption, diffusion and permeation. Any combination of these mechanisms may act to transport aggressive species through the concrete. In experimental methods it is common to limit the flow to one single mechanism, which can be used to derive a transport parameter for the concrete that is being tested. Ingress of chlorides occurs due to the permeation of water through concrete in some form. It was widely recognized that the concrete cover provides protection to the reinforcement against both carbonation and chloride ingress and these are related to different transport characteristics of the concrete. Therefore, in addition to monitoring the advance of the chloride front and the carbonation front, a measure of the transport properties of the concrete cover is important for assessing the durability of reinforced concrete.

Williamson *et al*. (2008) observed the variation in concrete near surface and away from the surface. Near surface concrete is more porous and with coarser pores than the concrete away from the surface. The properties and composition of the concrete that is close to the surface differ from those of the underlying material or heartcrete. It is clear that the quality of concrete in the cover zone is a key factor in reinforced concrete durability. This difference is owing to

a) Air and water move upwards in the fresh concrete creating a variable water/cement ratio and therefore also porosity gradient.

b) The wall effect influences the uniformity of distribution of large particles near to a moulded surface.
c) The effect of different compaction methods and quality.
d) The adequacy of curing – the surface layer of concrete is susceptible to drying effects and hence incomplete hydration.

Their study found aggregate size, bar location, method of compaction and cover thickness influence near surface concrete permeability. If the atmosphere is conducive to corrosion then the time between the aggressive agents reaching the steel and the onset of corrosion is dependent on the ease with which they can penetrate the concrete cover. It was found that, when the specimens were oven dried permeability increased to such a level that the test could not be completed. This was probably due to the formation of micro cracks as a result of rapid drying. Nine days air drying time was chosen for all the samples as by this time, the initial rapid weight loss was complete. Although the moisture content was not zero, it seemed reasonable to assume that the moisture contents and profiles of all specimens tested would nominally be identical. Thus, it can be concluded that bacterial carbonate precipitation helps in improving the resistance of mortar specimens to degradation processes. The deposition of a layer of calcium carbonate crystals on the surface leads to a decrease of the permeation properties. The biodeposition treatment helps in an increased resistance towards carbonation. The presence of the biodeposition treatment also improves the resistance towards chloride penetration.

Hooton *et al*. (2002) suggested that durability should be measured on the concrete system rather than on the individual constituents. Test methods must be developed for determination of the water cementitious material ratio (w/cm) in both fresh and hardened concrete, for concrete penetration resistance (as opposed to permeability) in both laboratory and in place concrete. Test methods should be developed for in place moisture content of concrete. To improve the durable properties of concrete, permeation to different ions, especially chloride ions, must be investigated. Nokken and Hooton (2006) worked towards the permeation of ions to concrete surface. They concluded that the test measures the electrical conduction of all ions not just chloride

ions. For low to moderate water cement ratio the total charge passed correlates well with chloride diffusion (or permeability) coefficient. As water cement ratio increases, heat is generated by ion–ion and ion solid collisions. The resulting increase in temperature increases the conductivity of the pore solution (and therefore the current flow). This may also change the microstructure, producing erroneously high coulomb values. To overcome this current passed through the sample after 1 minute under an applied DC voltage of 60V should be measured and compared. In some cases the material property of conductivity can be related to the results of ASTM C1202. For values less than 3000 coulomb, a linear relationship between initial current and total coulombs has been observed. For greater values heating effects causes significant deviation from the relationship.

Abou-Zeid *et al*. (2003) studied the factors affecting the charge passed through the sample. They concluded that as the water cement ratio increases the charge passed increases indicating the sample to be more permeable. A silica fume content of approximately 7% into concrete can be considered a good initial dosage for achieving a significant reduction in chloride ion penetration. They further found out that the 28 day charge passed test data can be used for some assessment of the 56 day value of the silica fume specimens by assuming the 56 day value is 50 to 60% of the 28 day value.

Zhang *et al*. (2005) studied the potential drop in migration testing. When an external potential was applied to an electrolytic, aqueous solution system, a potential drop will occur in all interfaces between the solids and the solution due to the combined effect of electrical double layer and polarization. This drop was dependent on the physical and chemical properties of the system.

External applied potential = Potential difference across the specimen + total potential drop outside the specimen.

Bickley *et al*. (2006) studied the performance specification for durable concrete from different countries. They gave emphasis on the importance of compressive strength

and permeation properties towards improvement in the durability. They concluded that Australia stands out as a leader in the use of the performance based specifications. Australian code AS 1379 for "Specifications and supply of concrete" has specified two grades of concrete (Normal and special grade). Normal grade concrete was specified primarily by compressive strength, whereas for others limiting values are given for chloride and sulfate contents of the hardened concrete, shrinkage, density and 7 day compressive strength.

With above discussions it can be concluded that chloride ion permeation to the concrete is a major drawback that reduce the service life of a building structure. The rapid chloride permeability test (RCPT) can be performed on samples with diameters ranging from 95 mm to 102 mm. The thickness of the samples should be minimum 50 mm. Vacuum saturation apparatus, vacuum pump and vacuum desiccator with vacuum gauge are required for this test. The reagents required are rapid setting, electrically nonconductive resin coating to coat the circumferential area, sodium chloride solution (3%) and 0.3 N reagent grade sodium hydroxide solution in distilled water. Constant voltage power supply with current measuring device is used. The test measures the charge passed through the specimen expressed in the units of coulombs and this is then related to chloride permeability of concrete. Nevertheless, this test is often used because of its convenience and short-term duration.

2.18 Summary of literature review and future prospective

The promising results on the use of microorganisms for the improvement of the durability of building materials have drawn the attention of research groups all over the world but until now, work on such bioremediation was mainly confined in some countries. A lot of work needs be done before the technology can be implemented. The temperatures, humidity, type of concrete, control of various parameters such as type of mix, concentration of bacteria vary considerably from place to place and country to country, hence a consolidated recommendation of any one's result can not be arrived. Apart from these, survival of microorganisms isolated from other parts of

the world under different environmental conditions also needs to be considered. No published work to date has described a sufficient degree towards enhancement in the durability of building structures using microbes by their MICCP process. Hence, lot of research is necessary before such technology is ready for field applications. Moreover, long term effect of such treatment is not yet reported. Most of the studies based on MICCP were carried out to evaluate compressive strength, water absorption and crack remediation of mortars and concretes. The role of MICCP in reducing the corrosion rate of reinforced concretes has not been studied in detail and needed to look further. Further studies should be carrying out to find some economical nutrient sources from industrial by-products to make overall process green and economic.

Different research groups are working for alternative approaches to obtain a protective layer of calcium carbonate on the surface of building materials. The goal of the present work is to provide an in-depth application of the different methodologies based on literature to fill the gap of current research towards enhancement of the durability of various construction materials.

Chapter 3

Materials and methods

3.1 Isolation and identification of efficient bacteria for the production of microbial concrete

3.1.1 Sample collection

Samples were collected randomly from calcareous sludge, cement, alkaline soils and limestone area. Calcareous sludge was collected from ACC Gaggal Cement Plant, located in Barmana district of Bilaspur (Himachal Pradesh, India). Limestone samples were collected from L&T cement area, Tadipatri (Anantpur, Andhra Pradesh, India). Alkaline soil samples were collected from Thapar University Campus, Patiala (Punjab, India). Cement sample was collected from commercially available bags. All the samples were collected from 0-30 cm of depth by alcohol sterilized implements in sterile containers and kept at 4°C until their use.

3.1.2 Isolation and cultivation of bacterial species

All the samples were first enriched for urease producing bacteria by inoculating one-gram of respective sample in 50 ml of nutrient broth (Casein hydrosylates 15.0 g/L; Peptone 5.0 g/L, NaCl 5.0 g/L, pH 7.5) (HiMedia, India) (pH 8.0) containing 2% urea and incubated at 37°C for 120 hrs under shaking condition (130 rpm). For isolation and enumeration of cultivable bacteria, all the samples were serially diluted in saline (NaCl, w/v 0.85%) and plated on alkaline nutrient agar (Casein hydrosylates 15.0 g/L, Peptone 5.0 g/L, NaCl 5.0 g/L and Agar 20.0 g/L having pH 9), and the plates were incubated at two temperatures (28°C and 37°C). The grown bacterial colonies from the plates were sub cultured several times on the same medium from where it was picked. Finally to select urease producing bacteria, the colonies were randomly selected and transferred onto Urea Agar base medium (Gelatin peptone 1.0 g/L, Dextrose 1.0 g/L, Potassium phosphate 2.0 g/L, NaCl 5.0

g/L, Phenol red 12.0 mg/L, Urea 20.0 g/L and Agar 20.0 g/L having pH 7.5, HiMedia, India) to check the production of urease based on the intensity of pink color.

Two industrial by-products Lactose Mother Liquor (LML) and Corn Steep Liquor (CSL) were collected as economic industrial media to study different parameters. The industrial by-products LML and CSL were analyzed for various important characteristics such as pH (Richards, 1954), solids and metals concentration (APHA, 1992). Nutritional parameters were analyzed by HPLC methods. All the media were supplemented with optimum concentration of urea and calcium chloride (2% urea and 25 mM $CaCl_2.2H_2O$). The pH of the media was adjusted to 6.5 with 1N HCl prior to autoclaving without urea and $CaCl_2$. Filter-sterilized urea and $CaCl_2$ was added later. The final pH of all the media was adjusted to 8.0.

Sporosarcina pasteurii (previously known as *Bacillus pasteurii*) is a well known urease producing and widely used for biocalcification and remediation of cracks in concrete materials (Ramakrishnan *et al*., 1998; Stocks-Fischer *et al*., 1999; Ramachandran *et al*., 2001). This strain (MTCC 1761) was procured from IMTech (Institute of Microbial Technology), Chandigarh, India and was used to optimize various parameters such as temperature, pH, and substrate (such as urea concentration and calcium sources) to measure urease activity. Further, efficiency of this bacterium was also compared with the isolated bacteria of present study in terms of microbial concrete production.

3.1.3 Urease Assay

The urease activity was determined for all the bacterial isolates in all media (nutrient, 10% LML and 1.5% CSL media) by measuring the amount of ammonia released from urea according to the phenol-hypochlorite assay method (Natarajan, 1995) at different time intervals. Ammonium chloride (50-1000 μM) was used as the standard. Bacterial isolates were grown in corresponding media and 1% of overnight grown cultures of OD 0.5 were re-inoculated into corresponding media and

incubated at 37°C under shaking condition (130 rpm). After an interval of 24 hrs, culture was centrifuged at 8000 rpm for 5 minutes. The culture filtrate (250 µl) was added to a mixture containing 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 ml of urea (0.1 M). The mixture was incubated at 37°C for 5 min followed by addition of phenol nitroprusside and alkaline hypochlorite, 1 ml each and incubated at 37°C for 25 min. Optical density was measured at 626 nm. One unit of urease is defined as the amount of enzyme hydrolyzing one µmole urea per min. With the objective of obtaining high yield of urease production, factors influencing the production (such as temperature, time, pH, media and substrate) by the selected strain were also studied.

To optimize the concentration of LML and CSL for optimum urease production by selected bacterial strain, different concentrations of LML (10, 20, 30, 40 and 50%) and CSL (1, 1.5, 2, 3, 4 and 5%) were used. The effect of temperature, pH and substrates on urease production was studied to optimize the specific parameter. To optimize the effect of temperature, the reaction mixture of urease assay (as described above) was incubated at different temperatures (25, 30, 37, 45 and 50°C). Similarly to optimize the pH, potassium phosphate buffer of different pH (6, 7, 8, 9 and 10) was used. The concentration of urea was also optimized for maximal urease production. Different concentrations of urea (1, 2 and 4%) were implemented in the media and urease production by the bacterial strain was monitored. In similar way, different calcium sources were studied to measure urease production. Calcium chloride, calcium nitrate and calcium acetate were implemented in the media to optimize the maximum urease production by bacterial strain.

3.1.4 Microbial sand plugging

To perform microbiological calcite precipitation, all the bacterial isolates were grown at 37ºC under shaking condition (130 rpm) in different media (nutrient, 10% LML and 1.5% CSL media) separately. Absorbance at 600 nm was maintained at 1.0. Microbiological sand plugging was performed to study calcite precipitation. 50 ml of grown culture (OD_{600}1.0) was mixed with 100 g sterilized river sand. Sand

slurry containing bacterial culture was packed into a plastic column (height = 15 cm; diameter = 4 cm) and bottom side of column was blocked by using Whatman filter paper. To ensure a good quality of sand column, plastic pipes were cut into two equal halves and joined by rubber bands, so that it would be remove easily after plugging experiments. A control reaction was packed in column in which sterile sand was mixed with different media only (without cells). All columns were fed continuously with all the three media separately at room temperature to mimic the natural environmental conditions. Flow rate was measured by measuring the volume of media that came out of columns per minute. The experiments in all the sand columns were terminated after ten days. Microbial sand column was divided into three layers (upper, middle and lower layer) and each layer was individually ground and sieved through a 45 μm diameter mesh prior to calcite estimation. Precipitated calcite from each layer was measured by EDTA titration method (AHPA, 1989). One gram of sand sample from each layer of sand column was dissolved with 3 N hydrochloric acid. Four ml of Sodium hydroxide (5 N) was added to the precipitate and final volume was made up to 50 ml using deionized water. Few drops of hydroxyl napthol blue were added as an indicator and the mixture was finally titrated against 0.05 M EDTA. End point was noted from pink to blue which is easily visualized and the amount of $CaCO_3$ formed was calculated by the volume of EDTA used × 0.005004 × 1,000/gm of sample used.

3.1.5 SEM-EDX and XRD analysis of microbial sand plug

The morphology and chemical constituents of bacteria and sand consolidated column was analyzed with SEM-EDX and XRD. Samples were completely dried at room temperature, then examined by SEM (Zeiss EVO50) at accelerating voltages ranging from 15 to 35 kV, which was equipped with an energy-dispersive X-ray analyzer (Bruker-AXS, QuanTax 200) for elemental analysis. Samples were gold coated with a sputter coating Emitech K575 prior to examination.

XRD-spectra were obtained using an X'Pert PRO diffractometer with a Cu anode (40 kV and 30 mA) and scanning from 3 to 60˚ 2 θ. Each sand consolidated sample

was crushed and grinded using motor pestle before mounting on to a glass fiber filter using a tubular aerosol suspension chamber (TASC). The components of the sample were identified by comparing them with standards established by the International Center for Diffraction data.

3.1.6 Extraction of DNA

3.1.6.1 Isolation of genomic DNA

A single colony of bacterial isolate was inoculated into 25 ml of nutrient broth in a 250 ml Erlenmeyer flask and incubated for 14-18 hrs at 37°C under shaking condition (120 rpm). Liquid cultures (2.0 ml) were harvested by centrifugation (Eppendorf microfuge) at 8,000 rpm for 1 min. The cell pellets were resuspended with 800 µl saline-EDTA, and approximately 10 µg crystaline lysozyme were added. During incubation at 37°C for 30 min, the cell suspension was mixed thoroughly by inverting the Eppendorf tube several times. After addition of 200 µl SDS (10%), the cell suspension was incubated again at 65°C for 15 min. The cell suspension was extracted with organic solvents to remove proteins and cell debris: first, with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) solution, and centrifuged 10 min at 12,000 rpm. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). To precipitate extracted nucleic acids, 0.7 volume isopropanol was added to the aqueous phase, followed by 10 min centrifugation at 12,000 rpm. The DNA pellets were washed with 750 µl EtOH (70%) and microfuged another 5 min. Finally, the pellets were resuspended in 40 µl TE buffer/milliQ water and stored at 4°C.

3.1.6.1.1 DNA Purification

The DNA was purified by elution through the Wizard DNA Clean up system (Promega, USA) according to manufacturer's instructions in order to remove contaminants, which can hamper in manipulation of DNA.

3.1.6.1.2 Electrophoresis of DNA on agarose gels

DNA was loaded on agarose gels (0.7% w/v) prepared in 0.5× TBE, pH 8.0 (Appendix I) using a 6× loading dye (Appendix I). Ethidium bromide (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then electrophoresed at 3 volts/cm for 45-60 minutes and visualized on a U.V. transilluminator.

3.1.6.1.3 Spectrophotometric quantification of DNA

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement at A_{260}. For double-stranded DNA suspensions, an OD of 1.0 at a wavelength of 260 nm and using a cuvette with 1 cm light path, is equal to a concentration of 50 µg/ml. The quality of the DNA was evaluated by measurement of the A_{260}/A_{280} and the A_{230}/A_{260} ratios. Ideally, the A_{260}/A_{280} ratio should be 1.8-2.0 while the A_{230}/A_{260} ratio should be 0.3-0.9. Ratios (A_{260}/A_{280}) less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

3.1.6.1.4 Ethidium bromide fluorescent DNA quantification

DNA was migrated electrophoretically in an agarose gel containing ethidium bromide (0.5 µg/ml). The quantity of DNA was visually determined with reference to a known DNA concentration of lambda phage (Fermentas, USA) by comparing the intensity of fluorescence.

3.1.6.2 Amplification of 16S rDNA and Purification of PCR products

The polymerase chain reaction (PCR) provides a rapid and highly sensitive method for the primer-mediated enzymatic amplification of specific target sequences in genomic DNA resulting in the exponential increase of target DNA copies.

For amplification of 16S rRNA gene the following primers were used: Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-

ACGGGCGGTGTGTTC-3' (Weisberg *et al*., 1991). The amplification of 16S rRNA from isolates was carried out with a GenAmp 2700 thermocycler (Applied Biosystem, USA). Reaction mixture for the PCR contained 1X PCR buffer (Fermentas, USA), each dNTPs at a concentration of 200 μM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 μM and 2.5 U of Taq DNA polymerase (Fermentas, USA) in a final volume of 50 μl. PCR conditions were as follows: Preheating at 92°C for 120 s, 35 cycles of 92°C for 60 s, 48°C for 30 s and 72°C for 120 s and final extension 72°C for 360 s. Successful amplifications were confirmed by agarose gel (0.8% w/v) electrophoresis and ethidium bromide staining.

Amplified 16S rRNA was purified using the QIAquick PCR purification kit (Qiagen, USA), following the instructions of the manufacturer. Purified PCR products were eluted from the purification columns by the addition of 50 μl 10 mM Tris buffer (pH 8.0). PCR products, as they resulted from amplification of total DNA from environmental samples, were purified by agarose gel (0.8 %) electrophoresis prior to cloning. After staining with ethidium bromide, a defined band was visualized under UV irradiation and excised. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning. Subsequently, the DNA was extracted from the gel matrix material, using the QIAEX gel extraction kit (Qiagen, USA) as per the manufacturer's instructions. PCR products were eluted with 30 μl TE buffer (pH 8.0) and used for the cloning.

3.1.6.3 Ligation of 16S rRNA in T-vectors

The 16S rRNA PCR products were cloned using the restriction independent InsTA Cloning Kit, following the manufacturer's protocol (Fermentas, USA). The 16S rDNA amplicon was ligated into pTZ57R/T vector. The reaction mixture was prepared as described below and incubated overnight at 4°C.

Plasmid pTZ57R/T (55ng/μl)	3 μl
Insert (75ng/μl)	4 μl

Buffer (5×)	6 µl
T4 Ligase	1 µl
H_2O	16 µl

3.1.6.4 Genetic Transformation using $CaCl_2$ method

A single colony of *E. coli* DH5α from a freshly grown plate was inoculated into 25 ml of LB broth in a 250 ml flask and incubated the culture for 16-20 hrs at 37°C under shaking condition (120 rpm). Aseptically 200 µl of the above-saturated culture was transferred into 25 ml of fresh LB broth in a 250 ml flask. The culture was further incubated with vigorous shaking at 37°C for 2-3 hrs. To monitor the growth of the culture, the OD_{590} was determined at every one-hour (OD_{590} should be ~ 0.5). The above culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0°C by storing the tubes on ice for 10 min. The cells were harvested by centrifugation at 8,000 rpm for 10 minutes at 4°C. The pellet was resuspend in 10 ml of ice-cold 0.1 M $CaCl_2$ and store on ice for 15 min. Further the cells were recovered by centrifugation at 8,000 rpm for 10 min at 4°C. The cell pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$. One hundred micro liter of the suspension of competent cells was transferred to a sterile and prechilled microfuge tube (1.5 ml capacity). The plasmid DNA sample (~100 ng in a volume of 5 µl or less) was added to each tube. The content of the tubes were mixed gently and stored the tubes on ice for 30 min. The tubes were incubated in a circulating water bath that has been preheated to 42°C for exactly 2 min without shaking. The tubes were rapidly transferred to an ice bath and chill the cells for 1-2 min. One ml of LB broth was added to each tube and incubated the cultures for 45-60 min at 37°C to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. One hundred micro liters of transformed cells was spreaded on each 90 mm Luria agar-Ampicillin-X-Gal-IPTG plates and incubated at 37°C. Transformed colonies were observed between 12 and16 hrs.

3.1.6.5 Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5α (Lac Z^-) bacterial host cells were plated on Luria agar medium containing 50 μg/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T vector is located in the multiple cloning site (mcs) of the plasmid's lacZα gene; if no insert is present, functional β-galactosidase is produced, and the transformed bacterial colony is blue. These few blue colonies occur due to the presence of supercoiled vector molecules, which have escaped linearization. However, if the host cell receives a recombinant plasmid containing a 16S rDNA insert in the lacZα gene, the resulting transformant colony is white (Lac Z^-).

3.1.6.6 Isolation and purification of plasmid DNA from recombinant bacteria by alkaline lysis method

The plasmid DNA was isolated based on the alkaline lysis method. A single transformed *E. coli* white colony was transferred into 2 ml of Luria broth medium containing appropriate antibiotic (ampicillin, used in a final concentration of 50 μg/ml) in a loosely capped 15 ml tube and incubated the culture overnight at 37°C with vigorous shaking. 1.5-2.0 ml of the above-saturated culture was transferred into a microfuge tube and cells were harvested by centrifugation at 8,000 rpm for 1 min. The bacterial pellet was resuspended in 200 μl of ice-cold Solution I (Appendix I) by vigorous vortexing to ensure that the bacterial pellet is completely dispersed in this solution. Further 200 μl of freshly prepared Solution II (Appendix I) was added and the contents were mixed by gentle inversion of the tubes, five to ten times. The tubes were stored on ice for 5 min. Finally 300 μl of ice-cold Solution III (Appendix I) was added and mixed by inversion to disperse Solution III (Appendix I) through the viscous bacterial lysate. The tubes were stored on ice for 10 min. The tubes were centrifuged at 12,000 rpm for 10 min in a microfuge. The upper aqueous phase was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). To precipitate extracted plasmid DNA, 0.7 volumes isopropanol were

added to the aqueous phase, followed by 10 min centrifugation at 12,000 rpm. The DNA pellets were washed with 750 µl EtOH (70%) and microfuged another 10 min. Finally, the pellets were resuspended in 40 µl TE buffer/milliQ water and stored at 4°C for further use.

3.1.6.7 Size screening for recombinant plasmids

Clones containing approximately 1.5 Kb 16S rDNA inserts were identified by PCR screening, using the rapid protocol for preparation of template DNA from single bacterial colonies and M13-forward (5'-GTAAAACGACGGCCAGT-3')/M13-reverse (5'-CAGGAAACAGCTATGAC-3') plasmid primers. The amplification products were checked by agarose gel (1.0% w/v) electrophoresis.

3.1.6.8 Sequencing

The 16S rDNA inserts were sequenced for both strands using M13 forward and reverse primers, used for pTZ57R/T vectors. The sequence was generated by chain termination method (Sanger *et al*., 1977) using an Applied Biosystems automatic sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

3.1.6.9 Analysis of sequence data

Sequences were submitted to the CHECK_CHIMERA program of the RDP (Maidak *et al.*, 2001), in order to detect the presence of possible chimeric artefacts generated by PCR. Similarities were calculated for nearly complete 16S rRNA sequences using only unambiguously determined nucleotide positions. The 16S rRNA gene sequences of all the isolates were compared with those available in GenBank/ EMBL databases using BLAST program (Altschul *et al*., 1997) and at RDP-II (Cole *et al*., 2003). The sequences of closely related strains and uncultured bacteria were retrieved from RDP-II and aligned using multiple alignments CLUSTALW program (Thompson *et al*., 1997). The evolutionary distance was calculated by Kimura 2 parameter, phylogenetic dendrogram was constructed by neighbor-joining method

using MEGA software version 4.0 (Tamura *et al*., 2007). For analysis, 1000 bootstrap replicates were performed to assess the statistical support for the tree.

3.1.7 BOX PCR amplification

BOX-PCR (based on primers targeting the highly conserved repetitive DNA sequences of the BOX-A subunit of the BOX element) was conducted to obtain the genomic fingerprinting of the all efficient bacteria.

BOXA1R: 5'-CTACGGCAAGGCGACGCTGACG-3' primer was used to study distinctly different species. The reaction procedure was: an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 90°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 8 min and a final extension at 72°C for 10 min. PCR products were then examined through horizontal electrophoresis in 2% agarose gel containing ethidium bromide at 40 V in 0.5×TBE buffer.

3.1.8 Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to check proteins in the supernatant from all bacterial isolates as described by Laemmli (1970), using a 4.5% (w/v) stacking and a 12% (w/v) separating/resolving gels. The compositions of reagents used in SDS-PAGE are provided in Appendix I. The solution for stacking and separating/resolving gel was prepared as given below.

Reagents	**Resolving gel (12%)**	**Stacking gel (4.5%)**
Distilled water	3.4 ml	6.1 ml
Acrylamide:bisacrylamide solution	4.0 ml	1.3 ml
Gel buffer (pH 8.8)	2.5 ml	2.5 ml
SDS (10%)	0.1 ml	0.1 ml
APS	50 µl	50 µl
TEMED	10 µl	10 µl

The gel plates containing prepared gel were clamped in the electrophoresis tank filled with the reservoir buffer. 50 µl each of samples were mixed with the same amount of sample buffer (Appendix I) and the processed samples were then treated at 90ºC in a boiling water bath for 2-3 min so as to ensure complete dissociation of protein subunits and optimum SDS binding and 30 µl of each prepared samples were loaded in the stacking gel having wells. After loading, the electrophoresis was carried out at constant voltage of 60 V at 4ºC until the dye reached near the bottom of the slab gel. After the electrophoresis was complete, the glass plates were removed from the tank and the gel was taken out from the plates carefully. The gel was stained by keeping in staining solution (Appendix I) while agitating gently on an orbital shaker overnight. After that the staining solution was poured off, the gel was transferred into destaining solution (Appendix I) and was allowed to destain for overnight. The final destaining was done in double-distilled water and gel was photographed.

3.1.9 Zymogram analysis (In gel detection of urease activity)

Electrophoresis was carried out at room temperature under non-denaturing conditions using a Hoeffer (USA) electrophoresis unit. The stacking and separating gels contained 5% and 8% acrylamide solution, respectively. The buffer system of Laemmli (1970) was used. Sodium dodecyl sulphate (SDS) was replaced in each electrophoretic solution by double-distilled water and the urease samples were not heated. Following centrifugation, supernatant obtained from bacterial isolates were mixed with sample buffer and electrophoresis was performed overnight at constant voltage.

At the end of electrophoresis, the gel was washed with double-distilled water for 10 min and then three times with 0.01 and 0.005 M acetate buffer (pH 6.0) for 15 min each with gentle shaking. The washed gel was then immersed in 5 M urea solution and incubated at 37°C for 5 min without shaking. The gel was then transferred to a 1.5% sodium nitroprusside solution and kept for 5 min. A volume (200 µl) of freshly prepared 1 M β-mercaptoethanol was added immediately and mixed to obtain a final concentration of 10 mM β -mercaptoethanol for the staining solution. The gel was

gently shaken and a dark purple red color developed, which became intense within 5 min. Photographs were taken on a BioRad (CA, USA) and were processed with Quantity One software (BioRad, USA).

3.2 Physiological characterization and enhancement of cementing activity of bacteria

3.2.1 Growth kinetics of bacterial isolates

The growth profile of bacterial isolates in all media was tested by taking the absorbance (OD_{600}) at regular time intervals for 30 hrs and corresponding cfu/ml were calculated after incubation at 37ºC.

3.2.2 Morphological and biochemical studies of bacterial isolates

To characterize all the bacterial isolates conventional physiological and biochemical characterization tests were carried out as described in Bergey's Manual of Systematic Bacteriology (Holt *et al*., 2000).

3.2.2.1 Gram staining

Bacterial smear from actively growing cells were spread on a glass slide and heat fixed. Smear was flooded with filtered crystal violet for 10 sec and then washed briefly in water to remove excess crystal violet. Later it was flooded with Gram's iodine for10 sec and washed briefly in water. Smear was decolourised with acetone until the moving dye front has passed the lower edge of the section and washed immediately in tap water. Counterstaining was done with safranin for 15 sec and washed with water to remove the excessive stain. Finally samples were visualized under microscope at different magnification

3.2.2.2 Catalase test

Microorganisms able to live in oxygenated environments produce catalase which neutralizes toxic forms of oxygen. Catalase breaks hydrogen peroxide into water and

molecular oxygen. A small amount of bacterial cells was placed onto a clean microscope slide and few drops of H_2O_2 (3%) were added. A rapid evolution of O_2 as evidenced by bubbling supports positive result. No bubbles or only a few scattered bubbles indicated this test as negative.

3.2.2.3 Oxidase test

One drop of reagent (N,N,N',N'-tetra-methyl-p-phenylenediamine dihydrochloride) was added onto the bacterial culture on an agar plate. Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions were ignored.

3.2.2.4 Nitrate reduction test

Nitrate broth is used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen.

Nitrate broth contained nutrients and potassium nitrate as a source of nitrate. After incubating the nitrate broth, 2-3 drops of sulfanilic acid and α-naphthylamine were added. If the organism has reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. Sulfanilic acid was added; which reacted with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α-naphthylamine to form a red-colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it was considered a positive result for nitrate reduction.

3.2.2.5 Alkalinity, salinity and temperature test

Different sodium chloride concentrations (0, 2, 5, 7, 10, 12 and 15%) were amended in the alkaline nutrient broth (pH 10) to determine the survival of the bacterial isolates in saline conditions. Growth of bacterial isolates was recorded by measuring the absorbance at 600 nm of 24 hrs grown cells. Four temperatures (25, 30, 37 and

45^{o}C) were chosen to test the growth pattern of bacterial isolates. Bacterial isolates were grown in liquid and solid media and incubated at different temperatures as stated above.

3.2.2.6 Fermentation of carbon substrate by bacterial isolates

Total of 35-carbohydrate fermentation tests were performed with isolated bacterial species according to the manufacturer's direction (HiMedia, India). Inoculum was prepared by growing the cells in alkaline nutrient broth (pH 9) at 37°C in shaking condition until the inoculum turbidity was ≥0.5 OD at 600nm. Citrate utilization, lysine, ornithine, TDA, nitrate reduction, acid phosphatase, urease and H_2S production tests were performed with all bacterial isolates by standard methods.

3.2.2.7 Antibiotic profiling of bacterial isolates

Bacterial isolates were grown in nutrient broth until the absorbance reached to 1.0 at 600 nm. The grown bacterial cells were spread on nutrient agar and antibiotic discs were kept on it. These plates were incubated at 37°C and the inhibition zone was noted. Ready precoated twenty antibiotic discs (HiMedia, India) were used to check the sensitivity of the bacterial isolates. These were: Norfloxacin (10 µg), Gentamicin (10 µg), Chloramphenicol (30 µg), Cefuroxime (30 µg), Ciprofloxacin (5 µg), Cefaperazone (75 µg), Ceftazidime (30 µg), Roxithromycin (30 µg), Calaritromycin (15 µg), Co-Trimoxazole (25 µg), Netillin (30 µg), Cefaclor (30 µg), Cephotaxime (30 µg), Cephadroxil (30 µg), Azithromycin (15 µg), Ampicillin/Cloxacillin (10/10 µg), Penicillin (10 units), Amikacin (30 µg), Sparfloxacin (5 µg) and Ampicillin/sublactam (10/10 µg).

3.2.2.8 Urease assay

Urease production by all bacterial isolates in different media was determined as discussed in section 3.1.3.

3.2.2.9 Calcite estimation

Calcite estimation by all bacterial isolates was estimated from different layers of sand column in different media as described in section 3.1.4.

3.2.2.10 Protease assay

Alkaline protease activity was determined according to the method of Fukumoto *et al.* (1971) with little modifications. One ml of the sample was added to 5 ml of 0.6% casein and incubated at 37°C for 10 minutes. The reaction was stopped thereafter by adding 5 ml of a TCA mixture containing 36 ml of 50% (w/v) TCA solution, 220 ml of 1 M sodium acetate solution, 330 ml of 1 M acetic acid solution in a total volume of 1000 ml. The unreacted casein was precipitated, and the resulting precipitate was filtered off using Whatman filter paper No. 1. The optical density of the filtrate was measured at 275 nm against a blank. A standard graph was generated using standard tyrosine solutions of 5–50 µg/ml. One unit of protease activity was defined as the amount of enzyme, which liberated 1 µg tyrosine per min at 37°C.

3.2.2.11 Biofilm production

Biofilm production from all the isolates was established aseptically in nutrient broth containing calcium chloride and urea on glass plates (25×75 mm) using crystal violet (CV) staining method as described by Morikawa *et al*. (2006) with slight modifications. Briefly, an overnight culture of all the bacteria were diluted to an OD_{600} of 0.5 and 1 µl was added to 99 µl nutrient medium onto the glass plate and incubated at 37°C for 24 hrs. Then, media and loosely bound cells were removed from the glass plate by gently rinsing with sterile milliQ water and the remaining cells and matrices were stained with 150 µl of 1% CV solution for 25 min at room temperature. After washing twice with distilled water, the CV attached to the biofilm was solubilized in 150 µl DMSO, quantified by measuring its absorbance at 570 nm and expressed in cfu/mm^2.

3.2.2.12 Extracellular polymeric substances production

Extracellular polymeric substance (EPS) production was tested according to the procedure described by Friedman *et al*. (2001). Briefly, the dye Congo red (CR) was mixed in a bacterial culture (approximately 10^8 cfu/ml) at a concentration of 3.5 mg/l and was incubated for 30 min. The colored culture was centrifuged for 5 min at 8000 rpm to harvest the dyed cells. The cells were washed with 1 ml milliQ water to release adsorbed CR into the water. To remove the cells, the dyed culture was centrifuged for 5 min at 8000 rpm. Optical density was measured at 430 nm. The increase in absorbance is due to the uptake of CR by the EPS producing bacteria. The quantity (nmol) of EPS production was calculated by CR bound/10^8 cfu/ml.

3.2.3 Enhancement of cementing activity of bacteria by UV irradiation

Two bacterial isolates Bp W and CT-5, based on their ability to produce high urease activity, were selected to develop the phenotypic mutants by exposing to UV irradiation. These isolates were grown overnight in nutrient broth containing 2% urea solution at 37ºC under shaking condition. The cells were washed twice with 0.5 M phosphate buffer (pH 8.0) and diluted in same buffer to obtain $\approx 4 \times 10^7$ cfu/ml. Two ml of these suspensions was placed into 60 mm diameter Petri dishes at a distance of 15 cm in dark and irradiated to UV for 0, 5, 10, 15 and 20 min inside the UV chamber using Philips 20 W germicidal lamp. Portions of 0.5 ml of suitable dilutions of bacterial strains were spread on high selection pressure medium and incubated at 37°C overnight and then transferred onto Urea agar base medium (HiMedia, India) to check the production of urease based on the intensity of pink color. The survival percentages were estimated for each treatment. These isolates were cultured at least five times to assure incapability of reverse mutation and qualitative urease production. The isolates were compared with wild type/parental strain for quantitative urease production and calcite precipitation.

3.3 Evaluation of microbes for remediation of defects in building materials and structures

3.3.1 Compressive strength tests of cement mortar cubes

To study the compressive strength test of cement mortar, the most efficient bacterial isolate (CT-5, based on higher microbial concrete production), grown in nutrient medium and CSL medium was used and also compared its efficacy with Bp W (*Sporosarcina pasteurii*). Locally available clean, dry, well graded, natural river sand was used as fine aggregate. Ordinary Portland Cement conforming to IS 8112 (1989) was used. The cement to sand ratio was 1:3 (by weight), and the water to cement ratio was 0.47. A cube mould of 70.6 mm (dimension 70.6 mm × 70.6 mm × 70.6 mm) was used, as per IS 4031 (1988). Sand and cement were thoroughly mixed; adding along with grown culture of bacterial isolates correspondence to OD_{600} 1.0. The water to bacterial culture ratio was also maintained at 0.47. Cubes were cast and compacted in a vibration machine. After de-molding, all specimens were cured in corresponding medium at room temperature until compression testing at the intervals of 3, 7 and 28 days. Media were replenished at a regular interval of 7 days. Control samples (without bacterial cells) were also prepared in similar manner. Compression testing was performed using automatic compression testing machine, COMPTEST 3000.

The effect of different concentrations (5×10^3, 5×10^5, 5×10^7 and 5×10^8 cells per ml) of efficient bacteria was also studied to know the optimum microbial load to get maximum compressive strength as described above.

3.3.2 Compressive strength testing: simulated cracks with variable depths in cement mortar cubes

A cube mould of dimension 70.6 mm × 70.6 mm × 70.6 mm was prepared as described in section 3.1. The cubes were provided with a cut to simulate a crack. The width of the cut was kept at an average of 3 mm (0.118 in.) and the depths were 13.4,

18.8, and 27.2 mm [0.527, 0.74, and 1.07 in.]. The cracks in the cubes were remediated with efficient bacterial cells along with natural sieved sand. Microbial plugging of the concrete cracks was examined with different concentrations of cells (5×10^6, 5×10^7 and 5×10^8 cells per ml) mixed with sand. The cracks in the control specimens were filled with natural sieved sand and water. Both sets of cubes were cured in media containing urea and $CaCl_2$ for 7 and 28 days. The medium was replaced after interval of 7 days. Compression testing was performed using automatic compression testing machine, COMPTEST 3000.

3.3.3 Water Absorption Test

To determine the increase in resistance towards water penetration a sorptivity test, based on the RILEM 25 PEM (II-6), on mortar cubes was carried out (De Muynck *et al*., 2007). The cement mortar specimens were exposed to 10±1 mm of water. At regular time intervals (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), the specimens were removed from the water and weighed, after drying the surface with a wet towel. Immediately after the measurement the test specimens were submerged again. The sorptivity coefficient, k [$cm.s^{-1/2}$], was obtained by using the following expression:

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

where Q is the amount of water absorbed [cm^3]; A is the cross section of the specimen that was in contact with water [cm^2]; t is the time [s], Q/A was plotted against the square root of time, then k was calculated from the slope of the linear relation between the former.

3.3.4 Total Porosity Measurements

The simplest of total porosity measurements is based upon the Archimedes' Principle which states a body wholly or partially immersed in a fluid is buoyed by a force equal to the mass of the fluid displaced. The total porosity (P) of cement-based materials can then be calculated from their water saturated surface dry mass (m_{sat}), their mass suspended in water (m_{water}), and their oven dry mass[2] (m_{dry}).

$$P\ (\%) = (m_{sat} - m_{dry}/m_{sat} - m_{water}) \times 100 \qquad 3.1$$

These moisture states are schematically illustrated in Figure 3.1. In concrete samples the time taken to saturate the samples is typically 24 hrs.

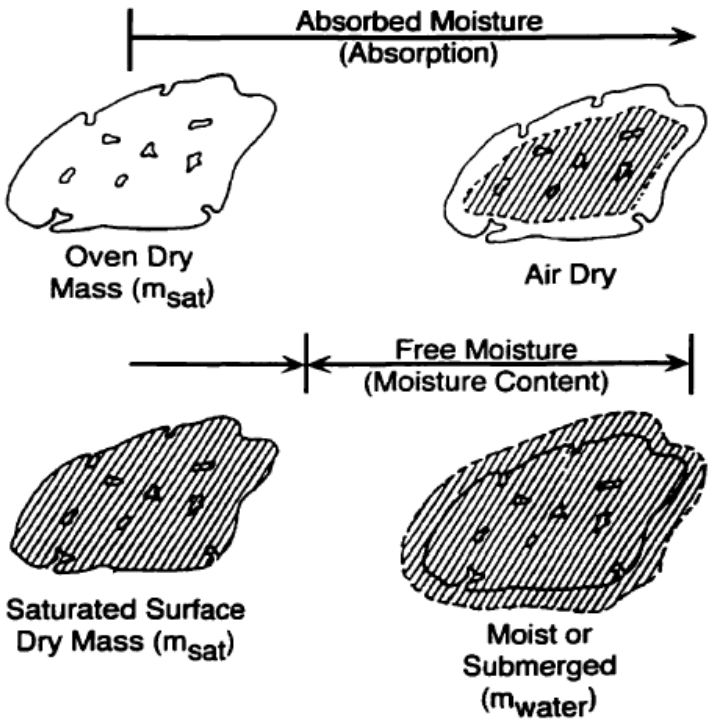

Figure 3.1 Schematic illustration of moisture states of cement-based materials (Neville, 1973)

All mortar cubes (dimension 70.6 mm × 70.6 mm × 70.6 mm) were immersed in tap water for 24 hrs prior to measuring their water-saturated surface-dry mass, and their mass in water. Each sample rested on a mesh stand in approximately 100 ml of water per sample. These conditions ensured that all sides of the sample were exposed to water and that sufficient water was present to enable complete saturation. The water-saturated surface-dry mass was determined by removing each specimen from the water, wiping the excess water off with a damp towel, and immediately weighing the section. Its mass in water was determined by placing the water-saturated surface dry sample in a wire basket which was immersed in water. The mass of the section was calculated by subtracting the mass of the basket in the water alone from the mass of

the basket in the water with the section in it. After the weighing was completed, the samples were dried at 100°C for 24 hrs to remove all of the evaporable water. This time period was determined from monitoring the mass of water lost with time from ten similar samples. Reweighing these dried specimens provided the oven dry mass. From all these measurements, the total porosity was calculated for each specimen using Equation 3.1.

3.3.5 Water Impermeability Test

For conducting the water impermeability test, the concrete cubes of dimension 150 mm × 150 mm × 150 mm (M20 grade) were prepared with grown culture of efficient bacterial isolates correspondence to OD_{600} 1.0 and all specimens were cured in corresponding medium at room temperature until impermeability test. Ordinary Portland cement, fine aggregate (medium-sized natural/river sand) and crushed stone coarse aggregate with maximum size of 20 mm was used in concrete. The ratio of cement: sand: coarse aggregate was 1:1.54:2.86. The water/bacterial culture–cement ratio was maintained at 0.47. Control samples (without bacterial cells) were also prepared in similar manner. After air drying specimens were firmly secured in position in the test apparatus (AIMIL India Ltd, Delhi) and analyzed water impermeability test as per German standard DIN 1048. Water nozzles with adjustable pressure were connected on the surface of the cube. Care was taken so that the gasket was properly fixed between the sample and top, bottom plate, so as to avoid any damage to the sample. The bottom side gasket was properly checked to avoid any leakage of water when the sample was subjected to water under pressure.

Atmospheric pressures of 1, 3 and 7 bar were applied sequentially for 24 hours. When the samples were subjected to 7 kg/cm^2 pressure we have to check if any water leakage occurred or if there was any drop in the pressure, the same was adjusted to 7 kg/cm^2 immediately. Once the pressure of 7 kg/cm^2 was kept for 24 hours the samples were removed and split in to two halves using a wedge and compression testing machine. The penetration depth of water into the concrete was measured and

marked with paint. Resistance to penetration is a measure of impermeability of concrete.

Gravitation aids deposition of calcite on the top surface of the cubes likely to have more deposition while the vertical surfaces receiving significantly less. Visual inspection indicated that the deposition of calcite was significantly less on the vertical sides. Hence, to check its effect or the permeability in the lateral direction, the samples were again mounted on the permeability apparatus so that the vertical side from the split half portion faced the water nozzles. The samples were removed and split to measure the depth of water penetration into the side surfaces of the cubes. This has been termed as side penetration.

3.3.6 Rapid Chloride Permeability Test

3.3.6.1 Preparation of samples

Cylindrical concrete samples (diameter 100 mm, height 50 mm) were prepared with efficient bacterial isolates casted and cured in respective media containing urea-calcium chloride for 28 days by regularly replenishing the media. Ordinary Portland cement, fine aggregate (medium-sized natural/river sand) and crushed stone coarse aggregate with maximum size of 20 mm was used in concrete. The ratio of cement: sand: coarse aggregate was 1:1.32:3.29. The water–cement ratio was 0.47. After curing, the samples were removed from the curing media. The samples were allowed to air dry for 8 days. Control samples (without bacterial cells) were also prepared in similar manner. The typical set up of RCP test is shown in Figure 3.2.

Figure 3.2 Typical set up of RCP test as per ASTM 1202-05 used in this study

3.3.6.2 Conditioning of samples

Water was boiled in a large sealed container and allowed to cool to the room temperature. Rapid setting resin coating material was prepared and applied on the circumferential surface of the specimen with brush. Depressions, if any on the surface, were filled before coating the surface. Additional curing for coating on such spots was done. Sample was kept on a suitable support while coating to ensure complete coating of sides. Care was taken so that the coating does not spill over the cross section which may reduce effective area of chloride penetration. The coating was allowed to cure until it no longer sticks to the hand. Specimens were placed into the desiccator so that both the faces of the specimens were exposed. Desiccator was sealed with cover and the vacuum pump started so that pressure decrease to less than a 1 mm Hg within few minutes. Vacuum was maintained for about 3 hrs. With vacuum pump still running, using stop cock, sufficient cooled boiled water (de-aerated) was drained into the desiccator so as to cover all the samples. Care was taken so that no air goes through this stop cock into the desiccator. Stop cock was closed and vacuum pump was allowed to run for one additional hour. Vacuum line stop cock was closed and the pump turned off. Vacuum line stopcock was turned on to allow air to re-enter the desiccator. Specimens were soaked under water for 18 ± 2 hrs.

3.3.6.3 Procedure for the test

Specimens were removed from water, excess water was blotted off and finally specimens were transferred to a sealed container which maintained the specimen at 95% or higher relative humidity. Specimen was installed into the cell (Fig. 3.3). Suitable sealant along with the rubber gasket was used to make it water tight. Two halves of the test cells were sealed together. One side of the cell containing the top surface of the specimen was filled with 3% NaCl solution, which was connected to negative terminal of the power supply. Other side of the cell was filled with 0.3 N NaOH solution, which was connected to the positive terminal of the power supply. Electrical connections to voltage application device and data reading apparatus were done. Constant voltage of 60 V was applied and the initial current reading after 1 min was recorded. During the test, the air temperature around the specimen was maintained between 20° to 25°C. Current at every 15 mins, was recorded if done manually or recorded automatically by use of data acquisition device. It was ensured that each half of the test cell was filled with the appropriate solution for the entire period of 6 hrs of the test. Test was terminated after 6 hrs and specimen was removed. Cell was rinsed thoroughly with tap water after removing the residual sealant if any. A graph was plotted for current verses time. The area under the curve was measured using trapezoidal rule. The area under the curve gave the charge passed during the test period measuring indirectly the chloride permeability. If the sample used was not of 3.74 in. [95 mm], the charge passed was adjusted by multiplying the value obtained by the ratio of the cross sectional areas of the standard and the actual specimens so as to give the charge passed through the standard sample of 3.74 in. [95 mm]. The charge passed was compared with the standard range given in standard to get the class of chloride ion penetrability.

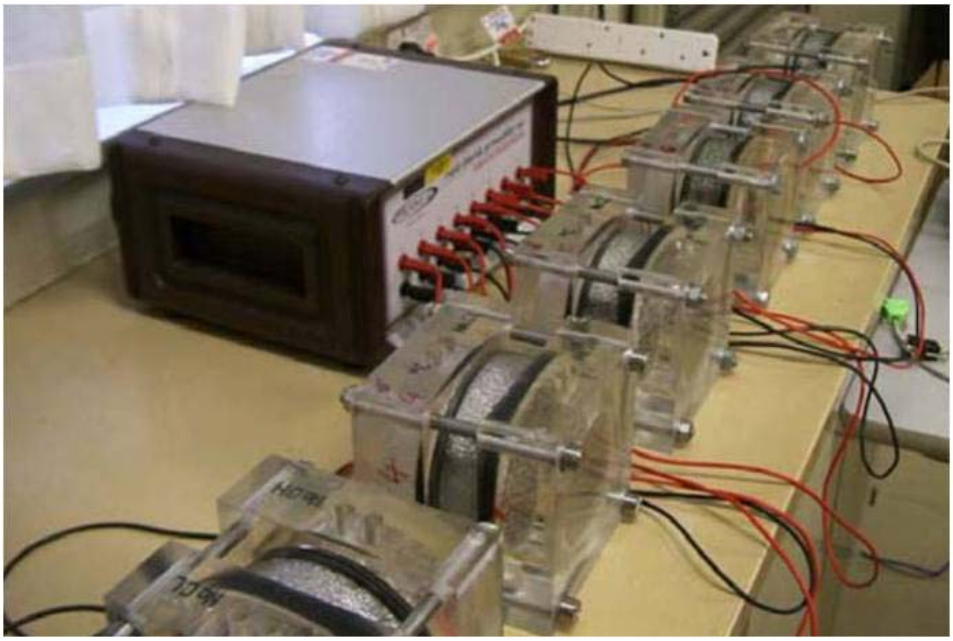

Figure 3.3 Processor and cells used in RCP test

3.3.6.4 Calculation and Interpretation of Results

Current in amperes verses time in seconds was plotted. A smooth curve was drawn through the data and find the area under the curve to obtain ampere-seconds or coulombs of the charge passed during the 6 hrs test period. The total charge passed was a measure of the electrical conductance of the concrete during the period of test. Permeability class will be determined according to Table 3.1.

Table 3.1 Chloride ion penetrability based on charge passed

Charge Passed (Coulombs)	Chloride ion penetrability
> 4000	High
2000 – 4000	Moderate
1000 – 2000	Low
100 – 1000	Very Low
< 100	Negligible

3.3.7 Microbial calcite deposition in bricks

Bricks (of size 228 mm × 108 mm × 62 mm) were procured from a local construction site. Bricks were oven dried at 100°C for overnight, allowed to cool at room temperature and weighed. Microbial calcite deposition on bricks was studied by using CT-5 and Bp W isolates in nutrient and CSL media. Further the dried bricks were immersed into grown bacterial culture (OD 1.0) in these two media for 5 days. After the incubation, bricks were removed, dried at room temperature, weighed and tested for water absorption capacity. For control, bricks were cured in water.

After curing, bricks were saturated overnight in water and weighed. The bricks were then dried in an oven at 100°C for 24 h, cooled and weighed again. Water absorption was calculated by using following formula:

% Water Absorption = $(W_{Saturation} - W_{Oven\text{-}dried})/ W_{Oven\text{-}dried} \times 100$

where $W_{Saturation}$ is the weight of bricks after saturation in water for 24 h, and $W_{Oven\text{-}dried}$ is the weight of bricks after oven drying for 24 h.

After curing, compressive strength tests were also performed using automatic compression testing machine, COMPTEST 3000.

3.3.8 Corrosion analysis

3.3.8.1 Concrete

Concrete mix was prepared using ordinary Portland cement, fine aggregate (medium-sized natural river sand) and crushed stone coarse aggregate with maximum size of 20 mm. The mass ratio of cement: sand: coarse aggregate was 1:1.54:2.86. The bacterial culture-cement/water-cement ratio was maintained at 0.47. The resulting strength of concrete was 20 MPa.

3.3.8.2 Reinforcement

A standard reinforcing tor bar of 300 mm length and 25 mm nominal diameter of Fe 415 grade was used. The bar was shot blasted to Sa 2.5 surface and immediately dipped in oil. The white shining surface was maintained in the laboratory until it was embedded in concrete. A schematic representation of RC (reinforced concrete) specimen is shown in Figure 3.4.

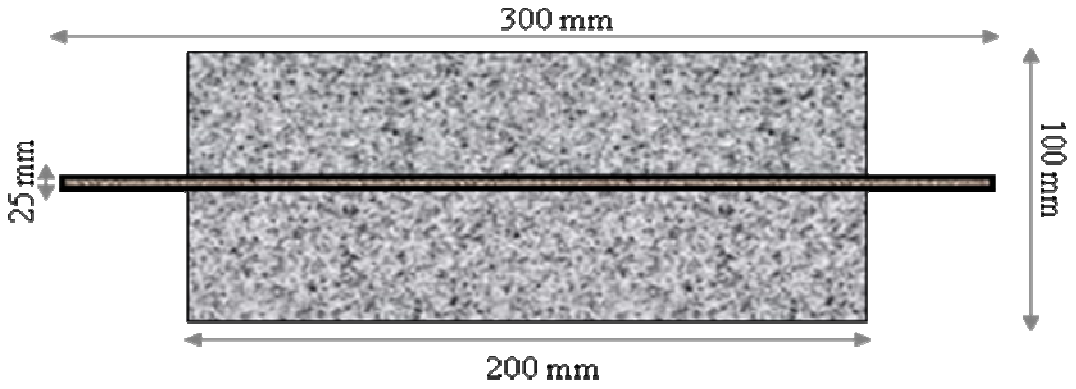

Figure 3.4 A schematic representation of reinforced concrete slab specimen for corrosion analysis test

3.3.8.3 Specimen Preparation

In the present program, concrete slab specimens of dimension 200 mm × 200 mm × 100 mm with an embedded steel bar were used. A special molding system of wooden mold (size 200 mm × 200 mm) was fabricated (Fig. 3.5A) for casting the specimen as shown in Figure 3.5B. Before casting of the test specimens, each reinforcing bar was weighed to 0.1 gm accuracy. All the treatments were performed in triplicates.

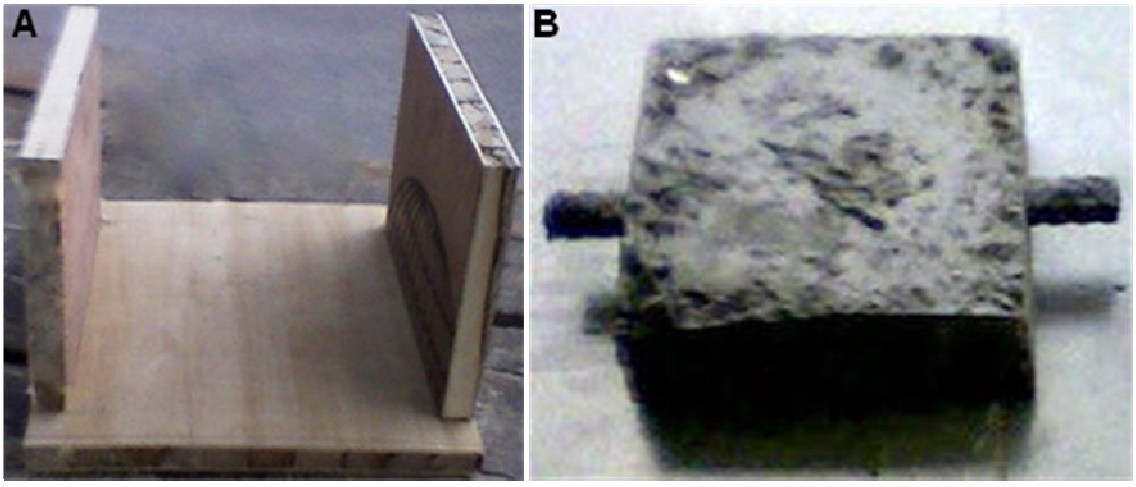

Figure 3.5 Typical size of **(**A) Wooden mold and (B) RC Specimen used in this study to evaluate corrosion experiments

3.3.8.4 Test Procedure

Once the RC specimens were prepared the further test procedure consists of following phases-

- Inducing corrosion in reinforced bar embedded in concrete
- Corrosion monitoring
- Carrying out pullout test on corroded wrapped specimens
- Determining percentage mass loss in reinforcing bar after corrosion
- Tensile strength of bar after corrosion

3.3.8.5 Accelerated reinforced corrosion

The objective of inducing corrosion to the reinforcing bar is to simulate the corrosion damaged concrete. The corrosion process was initiated by applying a constant anodic potential. High voltage (40 V) was used to accelerate the corrosion and shorten the test period. Similar accelerated corrosion test set up was also used by other researchers (Al-Zahani *et al*., 2002; Sujjavanich *et al*., 2005). In this method a constant positive potential was applied to the steel bar embedded in cement and the

current from the reinforcing steel bar to counter electrode is measured periodically. A sharp increase in current indicates the corrosion effect.

The specimens were kept immersed in 3.5% NaCl solution for 24 hrs to ensure full saturation of the test specimen. A stainless steel (SS) mesh rolled around the concrete slab was used as cathode. The exposed reinforced bar was then connected to the positive terminal (making the bar as an anode) of a DC power source while the negative terminal was connected to stainless steel placed near the specimen (Gadve *et al*., 2009). The continuous drip of NaCl solution to the concrete slab was used to induce chloride ion ingress to the specimens. Cotton gauze was rolled around the concrete slab to evenly spread the NaCl solution. The constant potential was impressed for 7 days into all of the specimens. Samples were visually inspected for cracks daily while the current flow was continuously monitored. The crack widths were measured after 2-, 4- and 7-days.

3.3.8.6 Corrosion Monitoring

Several parameters have been monitored during the entire process. Some of these are nondestructive, such as half-cell potential, cell voltages and potentiodynamic scans. These studies were carried out simultaneously with the application of anodic currents. Other parameters were destructive in nature, such as pullout and mass loss tests. These were carried out at the termination of the anodic current exposure.

Corrosion monitoring was done by using ACM Corrosion Analyzer System Field Machine (UK). The Field Machine brings a laboratory out on-site, no sacrifices were made to obtain portability, a Field Machine is very much as capable as it's fixed laboratory counterparts. The field machine enables laboratory precision to be taken outdoors to oilrigs, pipelines, concrete walls and just about anything that needs corrosion monitoring in-situ. The simplest electrochemical test is potential mapping; this gives an idea of corrosion activity but does not give corrosion rate data. More sophisticated tests use the Field Machine to determine corrosion rate from cyclic sweeps and LPR (Linear Polarization Resistance). The rate of corrosion is considered proportional to the corrosion current (Icorr) for the specimen.

3.3.8.7 Pullout Test

After 24 days of exposure to anodic current, pullout tests were carried out on all the specimens. This was done by securing the cylinder in a universal testing machine (UTM) and attaching the grip on to the protruding portion of the reinforcing bar. In case of the control specimen, the bar pulled out of the specimen causing splitting of the cylinder. The pullout force was much larger in the case of bacterial samples. It may be recalled that there was considerable corrosion and consequent loss of metal at the top interface. As a result, the reinforcing bar of all specimens broke off at the interface.

3.3.8.8 Determining percentage mass loss and tensile strength

After completing the pullout test the corroded bars were cleaned off corrosion products as per ASTM G1-90 and weighed to determine mass loss.

After pullout test, tensile strength of the bars was measured in Testing Machine (Hung Ta Instruments, Taiwan). The reinforced bar was cleaned off after completion of pullout test and fit under testing machine between two rings. The strength at which bar broke is measured in this instrument which is expressed in kN.

3.3.9 Statistical analysis

All the experiments were performed in triplicate. The data were analyzed by Analysis of Variance (ANOVA) and the means were compared using Tukey's honestly significant difference test. All the analyses were performed using GraphPad Prism (5.03) software.

Chapter 4

Isolation and characterization of microbial concrete producing bacteria

4.1 Isolation of microbial concrete producing bacteria

Microbial urease hydrolyzes urea to ammonia and carbon dioxide, the ammonia increases the pH of the surrounding environment, which in turn induces calcium carbonate (calcite) precipitation. Due to this calcite precipitation ability such bacteria which produce urease may be regarded as calcite producers. Microbial calcite producing bacteria were isolated from various samples collected from calcareous sludge, cement, limestone area and alkaline soils using nutrient media followed by urea enriched Urea agar media. The results are given in Table 4.1.

The populations of bacteria differ in various alkaline sources. Bacterial population was highest in alkaline soil followed by limestone samples and calcareous sludge. Cement samples recorded very low population of calcite producing bacteria. The isolated bacteria were subjected to primary screening for the selection of efficient strains.

Table 4.1 Population of bacteria in different sources per gram of matter

Sources	Bacteria ($\times 10^3$)
Alkaline soil	4.1
Limestone area	2.4
Calcareous sludge	0.04
Cement	0.005

The primary screening of the microbial concrete producing bacteria included a qualitative test of detecting the ability to utilize urea as an energy source. Results of a first screening demonstrated the presence of an active population of facultative anaerobic, salt tolerant and alkaliphilic bacteria in different samples. The most abundant populations were isolated from the alkaline soil samples. Smaller populations were detected in the samples from the cement. All the bacteria were screened for urease production in urease selective media, Urea agar media and based on pink color intensity (urease producing microorganisms give pink color), out of seventy bacterial colonies, forty different colonies were selected for further studies. *Sporosarcina pasteurii* (previously known as *Bacillus pasteurii*) is a well known urease producing bacterium and widely used by researchers to remediate cracks in concrete materials (Ramakrishnan *et al*., 1998; Stocks-Fischer *et al*., 1999; Ramachandran *et al*., 2001). This strain (*S. pasteurii* MTCC 1761) was procured from IMTech (Institute of Microbial Technology), Chandigarh, India, designated as Bp W.

4.2 Molecular characterization of bacteria

After screening, forty of the isolates were examined by BOX-PCR analysis and further differentiated into separate clusters based on their isolation sources on the basis of DNA banding patterns using BOXA1R primer (Fig. 4.1). Amplification of the regions between highly conserved repetitive DNA sequences of the BOXA subunit of the BOX element of isolate-specific DNA fingerprints was generated. BOX-PCR for all isolates yielded a complex genomic fingerprint consisting of 200 bp to >1kb amplicons of varying intensity. Visual observation of the DNA fingerprints clearly differentiated similarity and dissimilarity among bacterial strains. These results showed complex banding patterns, which reflected a high degree of inter or intra specific genetic unrelatedness among isolates. In case of bacterial strains which were isolated from the limestone and alkaline soil samples, profile showed similarity >80%, so these strains must be identical; although there were some differences in banding patterns among calcareous sludge and cement isolates.

Based on BOX-PCR fingerprints out of forty isolates, six different strains were distinctly differentiated. These isolates were designated as CT-2 and CT-5 (both isolated from cement samples), LS-2 and LS-4 (isolated from limestone), SN-3 (isolated from calcareous sludge) and AS-4 (isolated from alkaline soil).

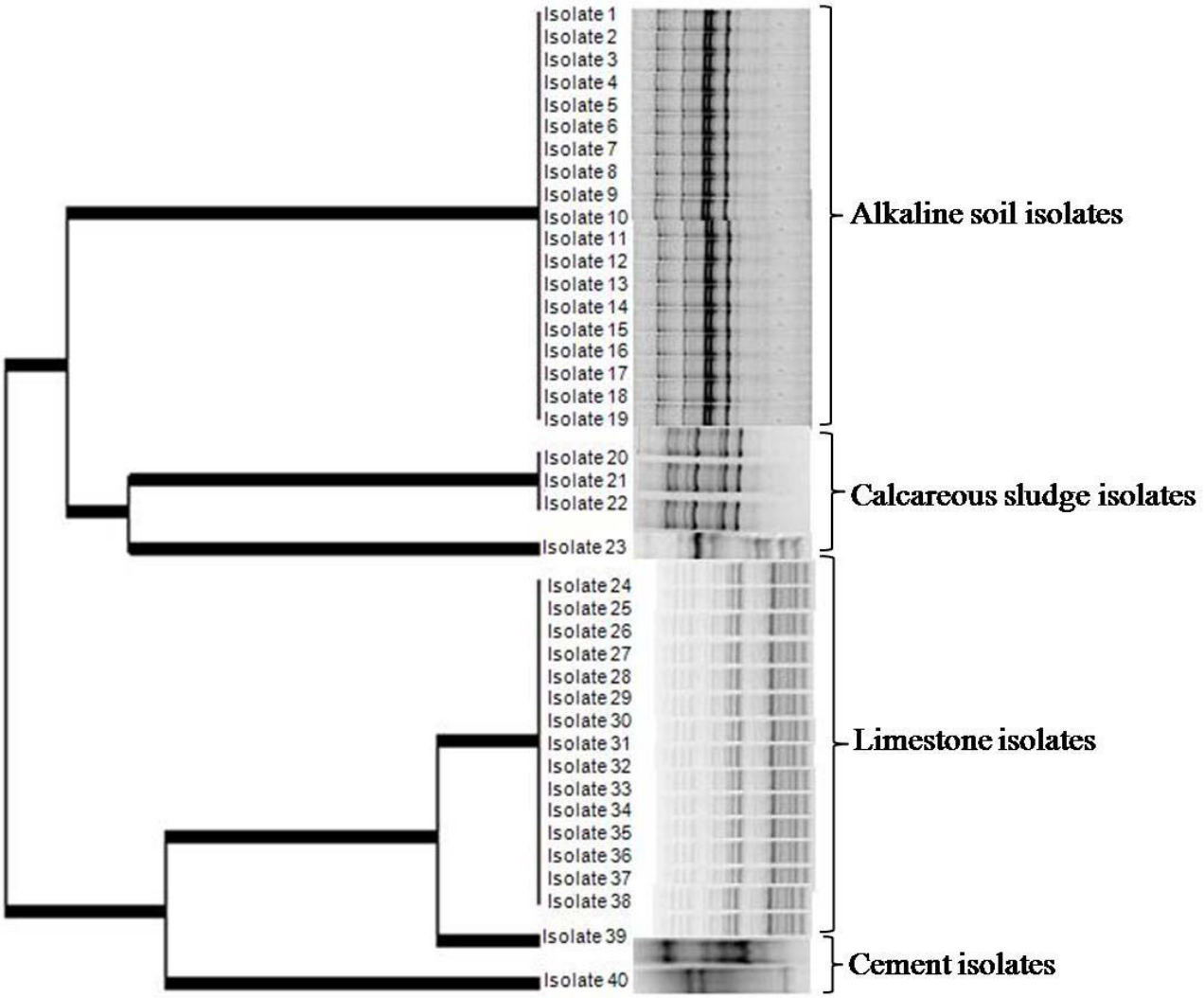

Figure 4.1 Dendrogram generated from BOX-PCR fingerprints of bacteria isolated from different sources

To identify these six bacterial isolates, all were subjected to 16S rRNA amplification using universal primers, and about 1.5 Kb amplicon was observed in all isolates (Fig. 4.2). 16S rRNA PCR products were cloned into pTZ57R/T vector (Fermentas, USA). The plasmid DNA was extracted from different clones and amplified with M13-F and M13-R primers. The 16S rRNA products from selected clones were then sequenced using Applied Biosystems automatic sequencer. Sequencing reactions were performed with the primers M13-F and M13-R.

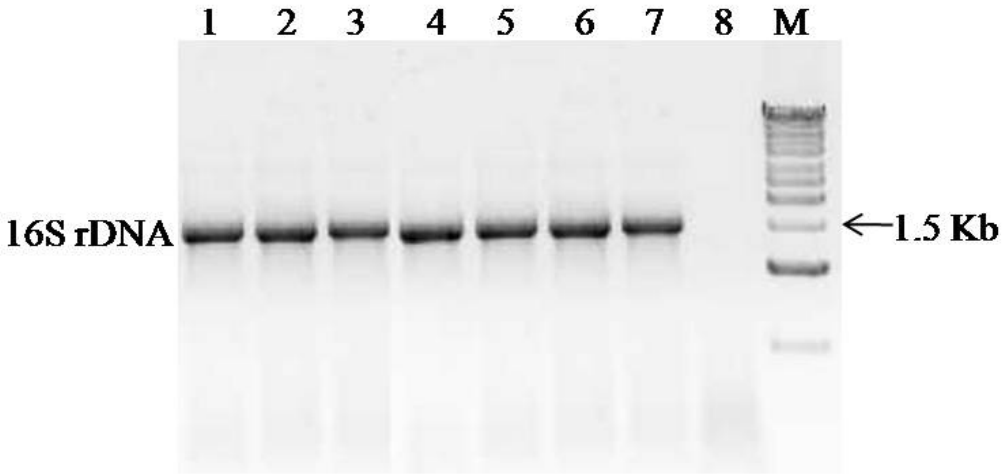

Figure 4.2 16S rDNA amplification of bacterial isolates. Lane 1-7: Bp W, CT-2, CT-5, SN-3, LS-2, LS-4 and AS-4; Lane 8: Control and Lane M: 1 Kb marker (Fermentas)

The sequences were analyzed by multiple sequence alignment to check the similarities among the isolates. The homologies among the sequences varied from 91 to 98% between isolates. Minimum of 91% similarity was found in CT-2 with LS-4 and maximum 98% similarity was found between AS-4 and LS-4 (Table 4.2). None of these isolates had shown 99% or more similarity among themselves. Cement isolates CT-2 and CT-5 had shown 95% similarities.

Table 4.2 Percentage similarity of 16S rRNA sequences of bacterial isolates using multiple sequence alignment (ClustalW)

SeqA	Name	Len (nt)	SeqB	Name	Len (nt)	Score
1	CT-5	1508	2	CT-2	1507	95
1	CT-5	1508	3	AS-4	1503	93
1	CT-5	1508	4	SN-3	1509	93
1	CT-5	1508	5	LS-2	1515	92
1	CT-5	1508	6	LS-4	1512	93
2	CT-2	1507	3	AS-4	1503	92
2	CT-2	1507	4	SN-3	1509	92
2	CT-2	1507	5	LS-2	1515	92
2	CT-2	1507	6	LS-4	1512	91
3	AS-4	1503	4	SN-3	1509	92
3	AS-4	1503	5	LS-2	1515	93
3	AS-4	1503	6	LS-4	1512	98
4	SN-3	1509	5	LS-2	1515	93
4	SN-3	1509	6	LS-4	1512	92
5	LS-2	1515	6	LS-4	1512	94

The phylogenetic tree (Fig. 4.3) constructed with sequences of representative bacteria revealed that all the six isolates clustered with other members of the phylum firmicutes and were associated with *Bacillus* group. The related sequences showing similarity in BLAST were retrieved from GenBank and RDPII and aligned using the program CLUSTALW (Thompson *et al*., 1994). The resulting multiple alignments were optimized visually and the evolutionary distance were calculated by Kimura 2 parameter. Phylogenetic dendrogram were constructed by neighbor-joining method using MEGA 4 package (Tamura *et al.,* 2007). Gaps were treated as missing data. Only unambiguous alignments were used in phylogenetic analyses. The complete 16S rRNA sequence analysis revealed that bacterial isolates had 77% to 100%

similarity with the sequences of NCBI database. The isolate SN-3 was identified as *Bacillus megaterium* as it showed 100% resemblance with it. On the basis of phylogenetic data, isolate LS-2 had close resemblance with members of the genus *Bacillus*. The percentage similarities were calculated between LS-2 and *Bacillus foraminis*, *B. boroniphilus* and *B. niacini* (these species of NCBI database were isolated from marine sediments, Gulf of Mannar) and found to be less than 86%. The isolate AS-4 had shown maximum similarity with *Bacillus subtilis* which is known to urease producing bacterium. The isolate CT-2 showed 97% similarity with two different *Bacillus* species, *Lysinibacillus sphaericus* and *B. fusiformis* (these two species of NCBI database were isolated from alkaline environment (Lonar Lake) and marine sediments area). The percentage differences calculated between isolate LS-4 and three different species of *Bacillus*, *viz*., *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis* were much, so this isolate may represent a new species of genus *Bacillus*. The isolate CT-5 showed maximum homology with two different *Bacillus* species, *B. cereus* and *B. mycoides*. The phylogenetic tree identified this isolate as *Bacillus* sp. It was interesting to find that the sources of all the isolates of NCBI database which were used to make phylogenetic tree were alkaline environment. It matches well with the fact that isolates of present study were also isolated from alkaline environment. It is noteworthy that isolates LS-2, CT-2, CT-5 and LS-4 16S rRNA did not show high identity with sequences in the database. The clones that had sequence identities of over 98% to a known organism may represent the same species. The sequences that share an identity below 98% are usually considered to be part of the same genus (Sadowsky *et al*., 1996). On this basis, these isolates described here probably represent a new member of a known genus (*Bacillus*), which are capable of calcite precipitation.

The 16S rRNA gene sequences of all six bacterial isolates determined in this study were deposited in the GenBank of NCBI data library under accession numbers FJ973470, FJ973471, GU301921, GU301922, GU301923 and GU301924 for CT5, CT2, AS-4, SN-3, LS-2 and LS-4 respectively (Appendix II).

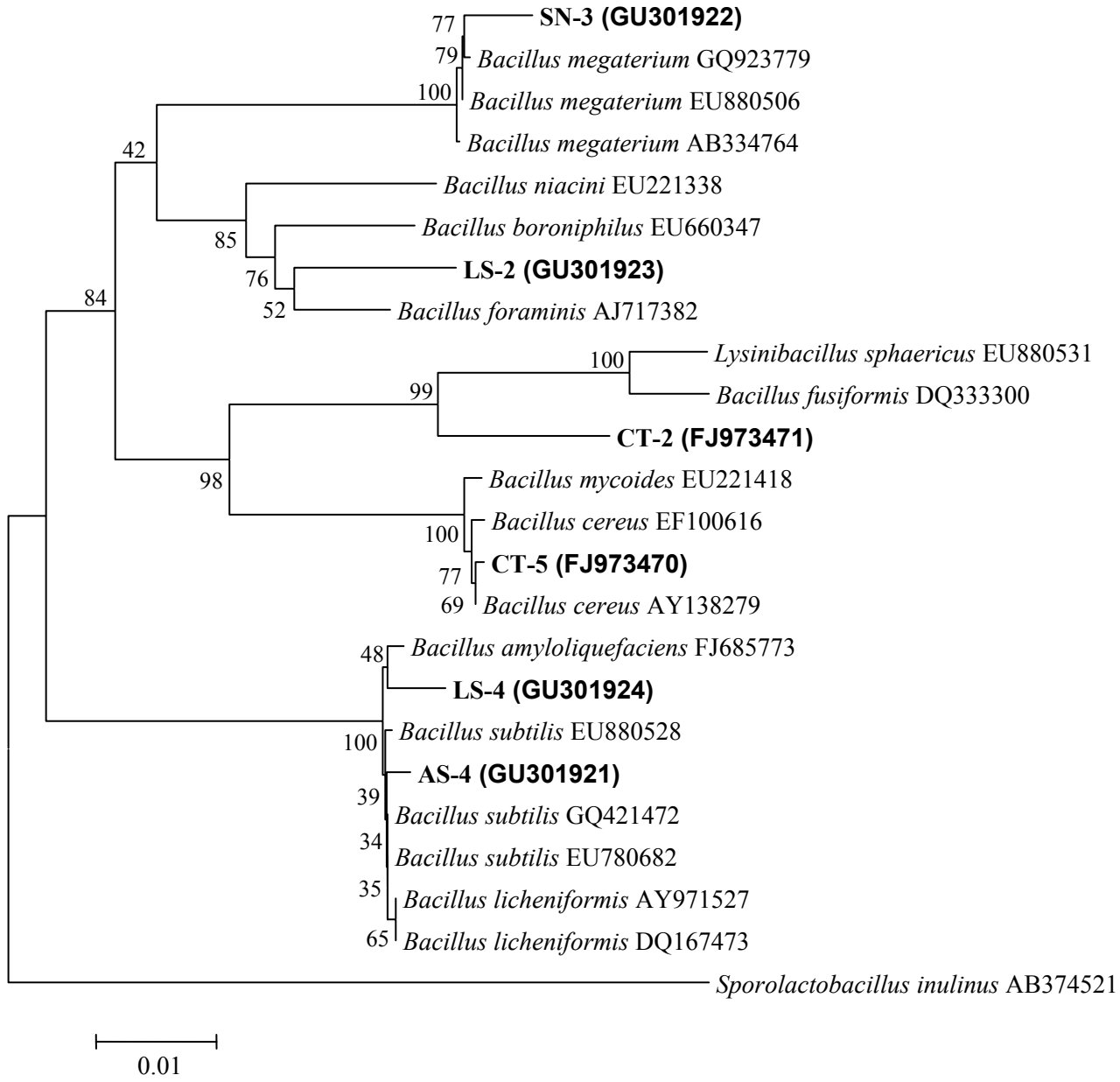

Figure 4.3 Neighbor-joining tree based on bacterial 16S rRNA sequence data from different isolates of current study along with sequences available in GenBank database. Numerical values indicate bootstrap percentile from 1000 replicates. *Sporolactobacillus inulinus* was used as outgroup taxa.

4.3 Physiological characterization of bacteria

The bacterial isolates were subjected to morphological, physiological and biochemical characterization including EPS and biofilm productions ability. All the bacterial isolated (Bp W, CT-2, CT-5, LS-2, LS-4, SN-3 and AS-4) found to be rod shaped, Gram positive and formed opaque creamy colony without any pigmentation on nutrient agar media. All isolates were catalase and oxidase positive and also were able to hydrolyze starch (Table 4.3). None of the isolates were able to degrade tyrosine. Only one isolate *Bacillus* sp. (CT-5) was found to be positive for hydrolyzing esculin. Two isolates *Bacillus* sp. (CT-5) and *Bacillus* sp. (LS-4) utilized citrate and malonate whereas *S. pasteurii* (Bp W), *Bacillus* sp. (CT-5) and *B. subtilis* (AS-4) were able to reduce nitrate and deaminate phenylalanine. *S. pasteurii* (Bp W), *Bacillus* sp. (CT-5), *Bacillus* sp. (LS-2) and *B. subtilis* (AS-4) were found to hydrolyze casein whereas only *S. pasteurii* (Bp W), *Bacillus* sp. (CT-5) and *Bacillus* sp. (LS-2) hydrolyzed gelatin. *Bacillus* sp. (CT-2) and *B. megaterium* (SN-3) hydrolyzed starch only.

When subjected to salinity and temperature tests, *Bacillus* sp. (CT-5) was able to grow well at 10% of NaCl amended media, while other isolates were grown either at 7% or 8% of NaCl amended media (Table 4.4). Eight temperatures (25, 28, 30, 37, 45, 50, 55 and 60°C) were chosen to test the growth pattern of bacterial isolates. All bacterial isolates were able to grow at 30 to 45^{o}C and three bacterial isolates [*Bacillus* sp. (CT-2), *Bacillus* sp. (LS-4) and *Bacillus megaterium* (SN-3)] had shown the growth up to 50^{o}C, whereas *Bacillus* sp. (CT-5) had a wide growth temperature range (25-55°C) (Table 4.4). All bacterial isolates were grown in nutrient media with different pH in buffered media. The growth of isolates was determined in liquid and solid media amended with different buffers. All the isolates were able to grow between pH 6 and 10 and two isolates (*Bacillus* sp. LS-4 and *Bacillus megaterium* SN-3) showed growth between pH 6 and 10.5 (Table 4.4). *Bacillus* sp. (CT-5) had a relatively wide pH tolerance range (6-11).

Table 4.3 Biochemical characterization of the bacterial isolates of present study

Bacterial Isolates	Gram Staining	Catalase	Oxidase	Nitrate Reduction	Citrate Utilization	Malonate Utilization	Tyrosine degradation	Phenylalanine deamination	Hydrolysis of			
									Starch	Casein	Gelatin	Esculin
Bp W	+	+	+	+	-	-	-	+	+	+	+	-
CT-2	+	+	+	-	-	-	-	-	+	-	-	-
CT-5	+	+	+	+	+	+	-	+	+	+	+	+
LS-2	+	+	+	-	-	-	-	-	+	+	+	-
LS-4	+	+	+	-	+	+	-	-	+	-	-	-
SN-3	+	+	+	-	-	-	-	-	+	-	-	-
AS-4	+	+	+	+	-	-	-	+	+	+	-	-

+: positive reaction; -: negative reaction

The ability to ferment various carbohydrates by these bacteria was determined. Majority of the isolates were able to ferment different carbon substrates (Table 4.5). *Bacillus* sp. (LS-2) was able to ferment two carbon sources, *B. subtilis* (AS-4) fermented three carbon sources, *Bacillus* sp. (CT-2) fermented four carbon sources while *B. megaterium* (SN-3), *Bacillus* sp. (LS-4) and *Bacillus* sp. (CT-5) were able to ferment five, eleven and fifteen carbon sources respectively (Table 4.5). *S. pasteurii* (Bp W) was able to ferment sucrose only.

Bacterial isolates were checked for their sensitivity to different antibiotics. Three isolates [*Bacillus* sp. (CT-5), *Bacillus* sp. (LS-4) and *B. megaterium* (SN-3)] were found resistant to some antibiotics, but rest all other isolates were sensitive. *S. pasteurii* (Bp W) and *B. subtilis* (AS-4) were totally sensitive to all the antibiotics used in the present study. *Bacillus* sp. (LS-4) was resistant to 5 antibiotics, *Bacillus* sp. (CT-5) and *B. megaterium* (SN-3) were resistant to 3 antibiotics and *Bacillus* sp. (CT-2) and *Bacillus* sp. (LS-2) were resistant to 1 antibiotic (Table 4.5). Interestingly, the sensitivity patterns to antibiotics among few bacterial isolates [*S. pasteurii* (Bp W), *Bacillus* sp. (CT-2), *Bacillus* sp. (LS-2) and *B. subtilis* (AS-4)] were very similar.

Finally the growth profile of all the six isolates along with *S. pasteurii* (Bp W) was studied up to 30 hrs in nutrient broth amended with 2% urea and 25 mM calcium chloride. Similar kind of growth pattern was observed with all the isolates but *Bacillus* sp. (CT-5) showed relatively rapid growth. The cell growth continued to increase up to 30 hours. The measurement of the optical density allowed the determination of cell concentration in the media. The growth curve showed that cells started to grow after around three hours of inoculation and exponential phase continued up to 30 hours (Fig. 4.4). Growth profiles of isolates of present study matched well with most *Bacillus* sp., which shows longer generation times and significantly shorter lag times (De Siano *et al*., 2006).

Table 4.4 Effect of sodium chloride concentration, temperature and pH on the growth of bacterial isolates

Bacterial isolate	NaCl (%)	Temperature (^{o}C)	Growth in buffered alkaline condition (pH)						
			5	6	9	10	10.5	11	12
Bp W	0 – 7	25 – 45	-	++	+++	++	-	-	-
CT-2	0 – 8	25 – 50	-	+++	+++	+++	-	-	-
CT-5	0 – 10	25 – 55	-	+++	+++	+++	+++	++	-
LS-2	0 – 7	30 – 45	-	+++	+++	++	-	-	-
LS-4	0 – 8	25 – 50	++	+++	+++	++	++	-	-
SN-3	0 – 8	30 – 50	-	+++	++	++	++	-	-
AS-4	0 – 8	28 – 45	-	+++	++	++	-	-	-

-: no growth; +: poor growth; ++: good growth; +++: luxurious growth

Table 4.5 Fermentation of carbon substrates and antibiotic profiling of bacterial isolates of present study

Bacterial isolate	**Carbon substrate fermentation**[#]	**Antibiotic Resistance***
Bp W	Sucrose	-
CT-2	Lactose, Sucrose, Ribose, Citrate	Cx
CT-5	Lactose, Xylose, Fructose, Dextrose, Raffinose, Trehalose, Sucrose, Glycerol, Sorbitol, Mannitol, Ribose, ONPG, Citrate, Malonate, D-Arabinose	Cf, Ca, V
LS-2	Trehalose, Sucrose	V
LS-4	Maltose, Fructose, Dextrose, Sucrose, Inulin, Glycerol, Mannitol, Ribose, Cellobiose, Citrate, Malonate	Cf, On, E, V, Cj
SN-3	Lactose, Fructose, Trehalose, Sucrose, Arabinose,	Cf, Ca, V
AS-4	Lactose, Dextrose, Sucrose	-

[#] Total of 35-carbohydrate fermentation tests was performed with isolated bacterial species using HiCarbo Kit (Himedia Lab., Bombay, India).

* Cx: Cloxacillin (10 μg), Cf: Ciprofloxacin (5 μg), Cj: cefaclor (30 μg), On: Ofloxacin (10 μg), Ca: Ceftazidime (30 μg), E: Erythromycin (25 μg), V: Vancomycin (10 μg); -: no antibiotic resistance observed, out of twenty antibiotics of the current study

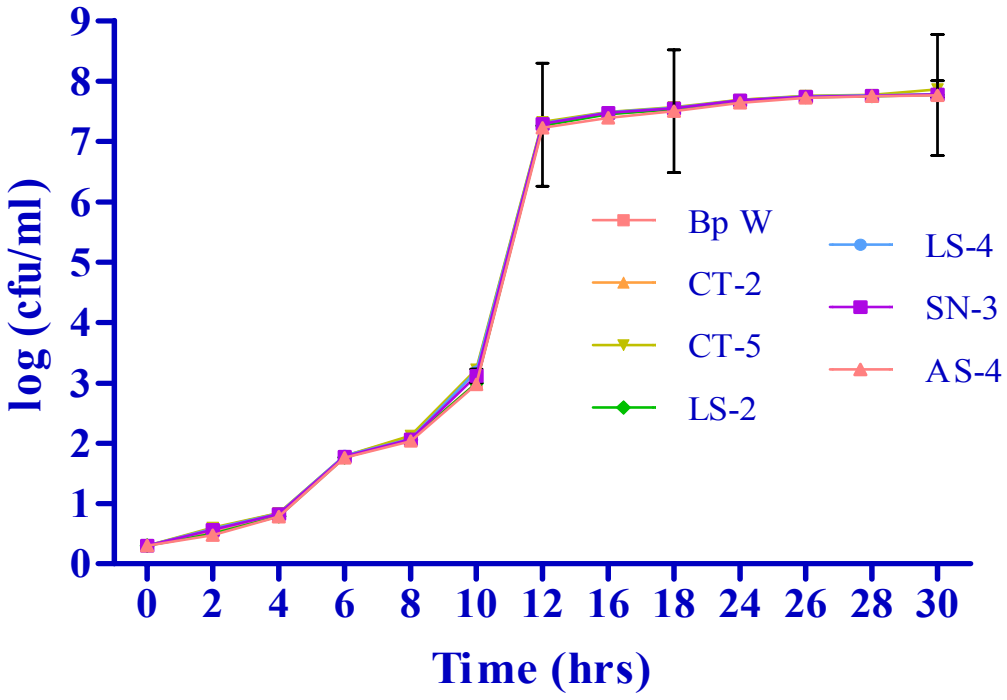

Figure 4.4 Growth behavior of all the bacterial isolates of present study in nutrient broth amended with urea and calcium chloride. Log cfu was calculated by taking absorbance at 600 nm. Values are mean ± SD (n =3)

4.3.1 EPS and Biofilm Production

A significant difference among bacterial isolates was found in terms of extracellular polymeric substances (EPS) and biofilm productions. *Bacillus* sp. (CT-2), *Bacillus* sp. (CT-5), *Bacillus* sp. (LS-2), *Bacillus* sp. (LS-4), *B. megaterium* (SN-3), *B. subtilis* (AS-4) and *S. pasteurii* (Bp W) were able to produce 31.67, 45.13, 27.47, 18.7, 26.67, 20.7 and 18.23 nmol/ml of EPS respectively (Fig. 4.5). A mature sessile bacterial community was observed in case of few isolates due to biofilm production. *Bacillus* sp. (CT-2) and *Bacillus* sp. (CT-5) were highly effective in covering the surface as compare to other isolates and results were highly significant. Monoxenic biofilms of *Bacillus* sp. (CT-2), *Bacillus* sp. (CT-5), *Bacillus* sp. (LS-2), *Bacillus* sp. (LS-4), *B. megaterium* (SN-3), *B. subtilis* (AS-4) and *S. pasteurii* (Bp W) contained 320, 352, 304, 283, 293, 283 and 283 cfu/mm^2 respectively, in nutrient media (Fig. 4.5). EPS and biofilm productions were significantly higher by *Bacillus* sp. (CT-5) followed by *Bacillus* sp. (CT-2) (Table 4.6).

Table 4.6 Comparison of EPS and Biofilm productions by the bacterial isolates

Isolates	EPS Production (nmol/ml)	Biofilm Production (cfu/mm^2)
Bp W	18.23±1.58e	283.33±4.16e
CT-2	33.00±2.09b	320.00±10.0b
CT-5	45.13±1.03a	352.67±15.5a
LS-2	27.47±0.50c	304.00±4.00c
LS-4	18.67±0.29e	283.33±4.16e
SN-3	26.67±0.29c	293.33±5.77d
AS-4	20.67±0.58d	282.67±4.62e

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

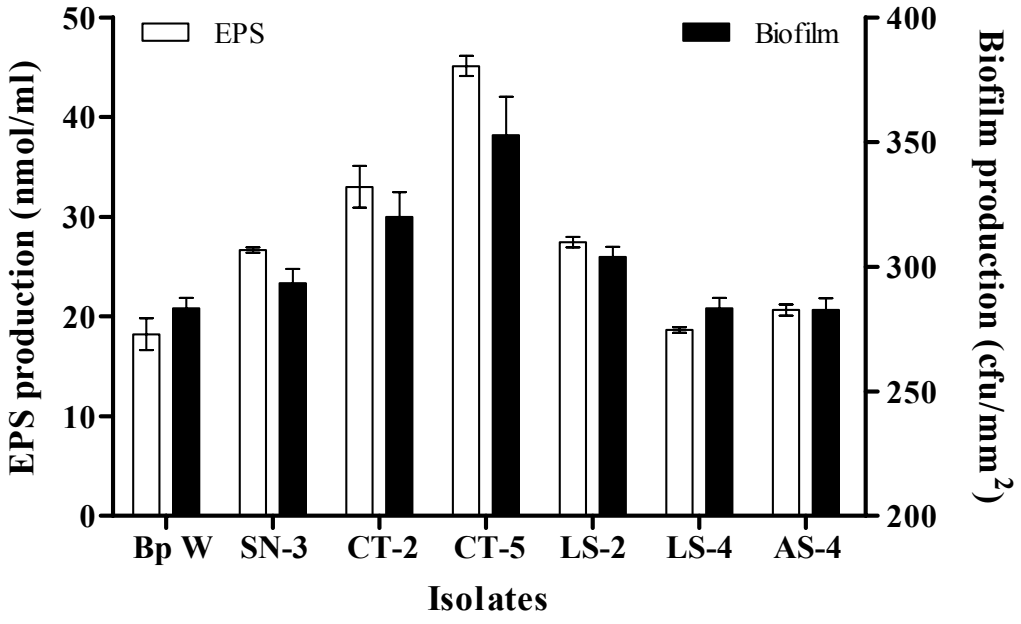

Figure 4.5 EPS and Biofilm productions by different bacterial isolates in nutrient media. Values are mean ± SD (n =3)

4.4 Optimization of parameters for maximal urease production

All the bacterial isolated (Bp W, CT-2, CT-5, LS-2, LS-4, SN-3 and AS-4) were selected to optimize various parameters such as temperature, pH, and substrate to measure urease activity. It was hypothesized that the presence of the enzyme substrate is necessary to maintain a high level of urease activity. To determine if this were the case, *S. pasteurii* was grown with different concentration of energy sources *i.e.*, urea. To determine the effect of high urea concentration on urease activity; 0, 1, 2 and 4% of urea were added in the nutrient media.

Very low amount of urease productions was observed with all the bacterial isolates in the control culture, which did not contain urea, indicating that urea as a substrate is necessary for the urease production. There was significant improvement in the urease production when medium was supplemented with 2% urea (Table 4.7). The urease activity increased more than two fold in the presence of 2% urea, relative to the lowest concentration of urea used (Fig. 4.6).

The efficacy of different calcium sources such as calcium chloride, calcium nitrate and calcium acetate was studied on production of urease by all the bacterial isolates. Calcium sources are required for biocementation reactions. In this experiment, calcium was provided in the biocementation mix as calcium chloride, calcium nitrate and calcium acetate, which ionizes in the water to produce one mole of Ca^{2+} and two moles of Cl^{2-}, NO_3^{2-} and CH_3COO^{2-} respectively. Due to the decomposition of calcium complex after autoclave, calcium sources were tested after filter-sterilization. The maximum urease production by all the isolates was observed using calcium chloride compared to other calcium sources (Table 4.8). Calcium chloride was the preferred calcium source followed by calcium acetate (Fig. 4.7). The differences in urease activity while using different calcium sources might be due to complexity of calcium compound, so that on the availability of free calcium ions and also on the preference of calcium sources by bacteria.

Table 4.7 Optimization of urea concentration for maximum urease (U/ml) production by different bacterial isolates.

Urea conc. (%)	Bp W	CT-2	CT-5	LS-2	LS-4	SN-3	AS-4
0	26.0±4.5d	40.5±4.9d	45.0±1.1d	37.5±6.3d	33.0±1.2d	28.0±5.1d	35.0±6.8d
1	111.5±0.9c	135.5±8.5c	146.5±9.2c	130.5±1.5c	123.0±1.2c	118.0±5.0c	125.5±8.5c
2	237.5±7.1a	258.0±25a	263.0±18a	254.5±20a	247.5±25a	242.5±24a	251.5±25a
4	195.1±6.2b	212.6±19b	218.1±27b	207.6±12b	203.1±10b	200.1±14b	204.6±12b

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

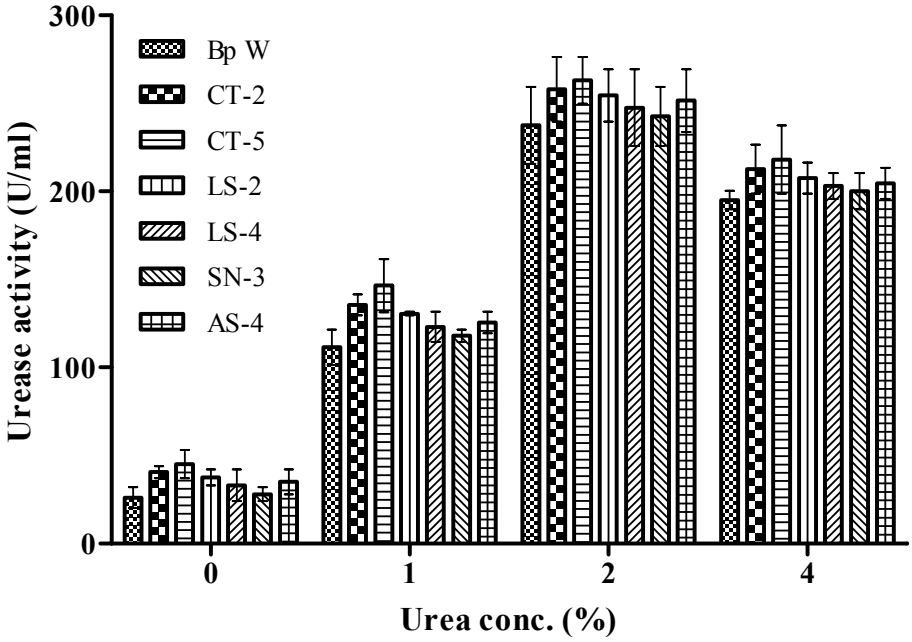

Figure 4.6 Effect of different concentrations of urea on urease activity (U/ml) by bacterial isolates. Values are mean ± SD (n =3)

Table 4.8 Optimization of calcium source for maximum urease (U/ml) production by different bacterial isolates

Calcium sources	Bp W	CT-2	CT-5	LS-2	LS-4	SN-3	AS-4
Ca chloride	97.1±1.5a	101.7±3.5a	103.7±3.4a	100.8±2.3a	98.7±2.0a	97.6±2.2a	99.8±3.7a
Ca nitrate	78.2±2.5c	83.8±4.7c	85.8±7.5c	83.2±3.8c	81.7±5.9c	80.2±5.3c	82.0±5.5c
Ca acetate	87.1±1.3b	91.2±2.1b	93.2±4.9b	90.4±4.6b	88.6±3.5b	87.6±2.1b	89.6±3.5b

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

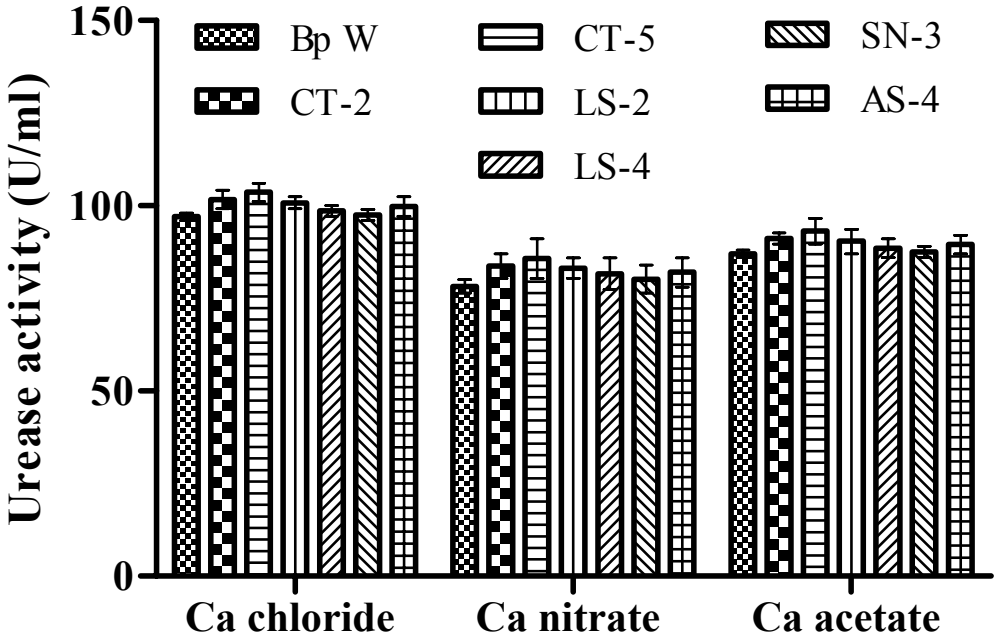

Figure 4.7 Effect of different calcium sources on urease activity (U/ml) by bacterial isolates. Values are mean ± SD (n =3)

The effect of temperature on urease activity produced by bacterial isolates is presented in Table 4.9. The maximum urease production was observed at 37°C by all isolates (Fig. 4.8). Generally above 40-50°C the enzyme activity was decreased because denaturation of proteins occurs at these temperatures. Urease production by all the isolates was lower at 50°C.

The effect of pH on urease activity was shown in Fig. 4.9. Different pH of potassium phosphate buffer was used to measure urease activity. The optimum pH for the maximum urease production was shown at pH 9.0 by all isolates (Table 4.10). For most enzymes the effective pH range was 4-9, beyond these limits, denaturation of enzymes took place. pH is an indication of the amount of hydrogen ions in a solution. pH can directly alter enzyme activity because it may change the shape of the enzyme. This is because there is a change in the chemical make up of the enzyme as bonds are made or broken. When the shape of the enzyme changes, it is no longer specific for the kind of substrate it works on. The substrate molecule would not be able to identify it and thus would not attach to it. Therefore, there would be no reaction. Sometimes the pH may also change the structure of the substrate so that the enzyme does not recognize it (no binding and thus, no reaction). Every enzyme operates best at different pHs. A deviation from these conditions would lead to inefficient enzyme function because of the change of shape of the enzyme. A significant improvement in urease activity was found when incubated at pH 9.0 and after that pH reduced drastically (Table 4.10).

Table 4.9 Optimization of temperature for maximum urease activity (U/ml) by different bacterial isolates

Temp. (°C)	Bp W	CT-2	CT-5	LS-2	LS-4	SN-3	AS-4
25	226.0±6.7c	232.3±7.9c	234.8±11c	230.6±10c	228.0±6.7c	227.0±5.3d	230.0±9.5c
30	237.1±6.9bc	245.9±10bc	248.1±11bc	243.9±7.4bc	240.3±8.0bc	238.1±8.4c	242.9±5.9bc
37	276.7±42a	288.2±30a	289.7±31a	285.4±30a	283.2±30a	281.7±35a	284.7±29a
45	245.4±3.8b	254.1±7.3b	255.7±8.1b	252.0±7.5b	248.4±8.0b	246.4±5.3b	250.0±7.5b
50	215.6±3.7d	224.3±9.7d	228.3±8.3d	221.7±9.3d	218.6±7.9d	217.6±6.5e	220.2±8.6d

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

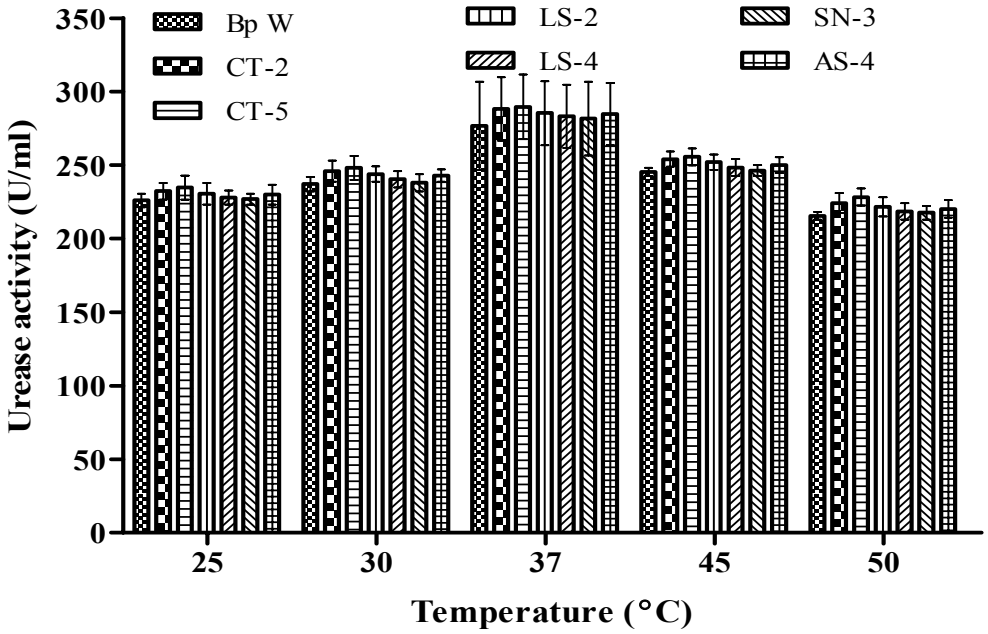

Figure 4.8 Effect of temperature on the urease activity (U/ml) by different bacterial isolates. Values are mean ± SD (n =3)

Table 4.10 Optimization of pH for maximum urease activity (U/ml) by different bacterial isolates

pH	Bp W	CT-2	CT-5	LS-2	LS-4	SN-3	AS-4
6	224.5±4.5d	228.3±1.6e	228.6±1.9d	227.7±0.6e	226.6±2.1d	225.5±3.1e	227.4±1.1e
7	236.2±6.4c	241.1±4.3c	242.0±5.6c	240.4±5.2c	238.7±2.8c	237.6±4.4c	239.4±3.8c
8	262.1±2.9b	266.8±1.2b	267.5±2.1b	265.1±1.3b	263.7±2.4b	263.1±1.5b	265.0±1.5b
9	279.3±3.1a	283.1±2.3a	284.0±2.8a	282.0±2.7a	280.9±2.8a	280.2±1.8a	281.5±3.4a
10	230.3±2.3c	235.3±2.6d	237.0±1.8cd	234.7±2.8d	232.4±4.6cd	231.4±3.8d	233.9±3.9d

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

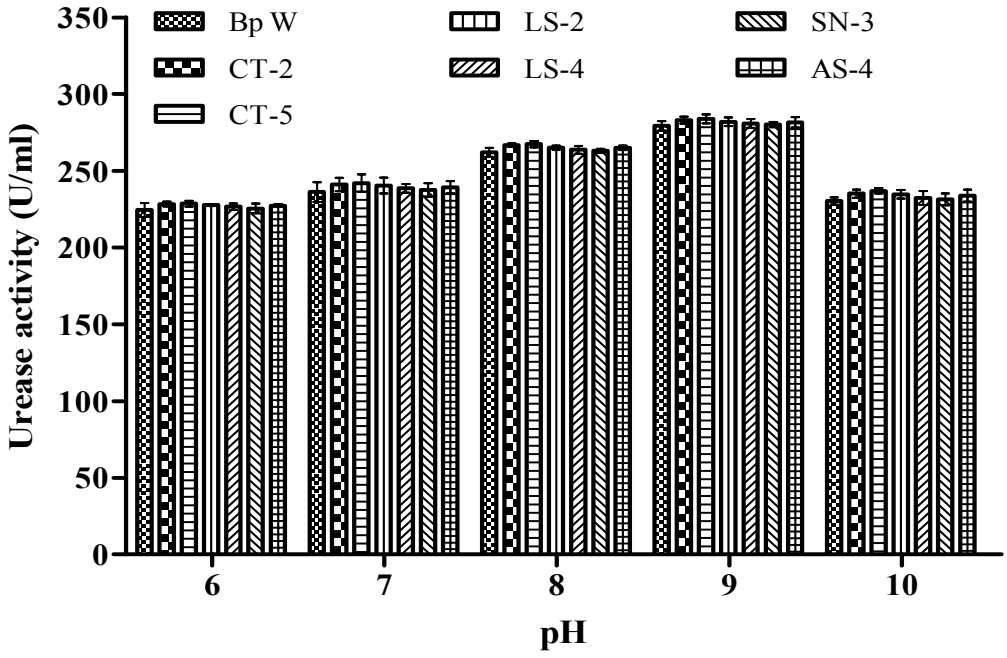

Figure 4.9 Effect of pH on the urease activity (U/ml) by different bacterial isolates. Values are mean ± SD (n =3)

4.5 Microbiological urease production

Calcium carbonate is a major constituent of concrete which makes any building materials or structure stronger. Due to the ability to precipitate calcite, urease is regarded as "cementing enzyme". Thus the ability of bacterial isolates to produce urease has been studied. The urease production by all the six isolates along with *S. pasteurii* (Bp W) was measured in nutrient media. The highest productivities by all isolates were obtained at 120 hrs. The urease productions by *S. pasteurii* (Bp W), *Bacillus* sp. (CT-2), *Bacillus* sp. (CT-5), *Bacillus* sp. (LS-2), *Bacillus* sp. (LS-4), *Bacillus megaterium* (SN-3) and *Bacillus subtilis* (AS-4) were found to be 413, 479, 610, 432, 418, 417 and 423 U/ml respectively (Table 4.11). Among all the isolates *Bacillus* sp. (CT-5) produced significantly higher urease production compared to other isolates. After 120 hrs, urease production was decreased in all cases (Fig. 4.10).

4.6 Protease production

Protease activity produced by bacterial isolates was detected in the culture supernatant from 24 hrs to 168 hrs (7 days) and an increase in its activity was found after 120 hrs, also the highest level was found after 168 hrs. *Bacillus* sp. (CT-5) produced significantly higher protease compared to other isolates (Table 4.12). Protease production by *S. pasteurii* (Bp W), *Bacillus* sp. (CT-2), *Bacillus* sp. (CT-5), *Bacillus* sp. (LS-2), *Bacillus* sp. (LS-4), *Bacillus megaterium* (SN-3) and *Bacillus subtilis* (AS-4) was 38.3, 54.4, 63.7, 45.4, 38.4, 39 and 38 U/ml at the end of 168 hours (Fig. 4.11).

Table 4.11 Comparison of urease activity (U/ml) produced by the bacterial isolates at different time intervals

Isolates	Urease Activity (U/ml)						
	24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**
Bp W	276.7±30f	377.3±7e	400.7±0.7d	412.7±10ef	427.3±0.7e	371.3±0.7f	334.7±1f
CT-2	314.4±2.1d	396.1±6c	410.9±1.5b	479.5±4.8b	575.9±2.9b	541.0±1.0b	467.7±7b
CT-5	344.0±1.7c	431.5±5a	537.3±2.7a	609.8±1.1a	665.2±2.9a	613.8±1.5a	567.7±3a
LS-2	379.9±7.1a	403.7±4b	404.3±6.6c	431.8±4.8c	458.7±1.8c	418.5±5.4c	410.3±6c
LS-4	355.0±2.5b	374.0±5ef	403.6±3.3c	417.8±1.1e	434.1±5.3d	389.9±2.2e	386.9±1e
SN-3	353.5±10b	381.7±4d	385.3±1.3e	417.2±5.3e	457.1±7.3c	408.0±4.8d	399.7±3d
AS-4	286.7±30e	344.7±1f	381.3±0.7def	422.7±10d	437.3±0.7d	407.3±5.2d	387.3±7e

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

Table 4.12 Comparison of protease activity (U/ml) produced by the bacterial isolates at different time intervals

Isolates	Protease Activity (U/ml)						
	24 h	**48 h**	**72 h**	**96 h**	**120 h**	**144 h**	**168 h**
Bp W	27.6±0.3d	28.5±0.2e	29.8±0.3ef	31.5±0.2de	32.9±0.1e	34.4±0.3e	38.3±0.5e
CT-2	31.5±0.5b	34.3±0.4b	38.7±0.3b	40.9±0.4b	43.3±1.0b	47.5±1.3b	54.4±1.6b
CT-5	32.3±0.4a	36.7±0.3a	39.4±0.4a	41.8±0.2a	44.3±0.4a	50.9±0.4a	63.7±0.7a
LS-2	29.8±0.4c	32.6±0.5c	35.5±0.9c	37.7±0.2c	39.5±0.5c	42.0±0.4c	45.4±1.3c
LS-4	27.4±0.6d	28.7±0.2e	30.0±0.3e	31.6±0.2d	32.9±0.1e	34.5±0.2e	38.4±0.3e
SN-3	29.5±0.4c	31.8±0.2d	34.3±0.2d	37.3±0.6c	38.6±0.6d	40.9±0.9d	43.4±0.8d
AS-4	27.7±0.1d	28.6±0.2e	30.1±0.4e	31.7±0.2d	33.0±0.2e	34.6±0.1e	38.4±0.4e

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

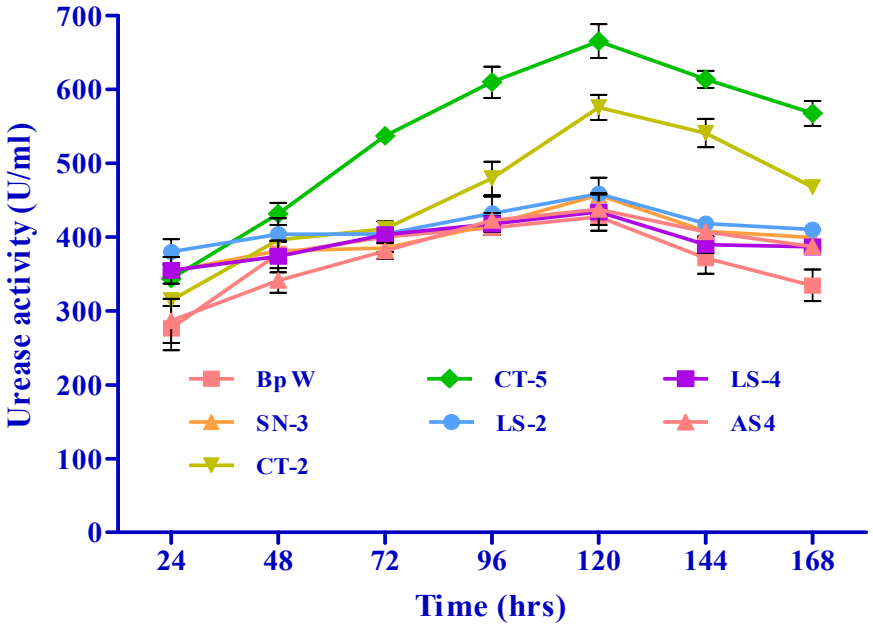

Figure 4.10 Urease productions by all the bacterial isolates in nutrient media at different time intervals. Values are mean ± SD (n =3)

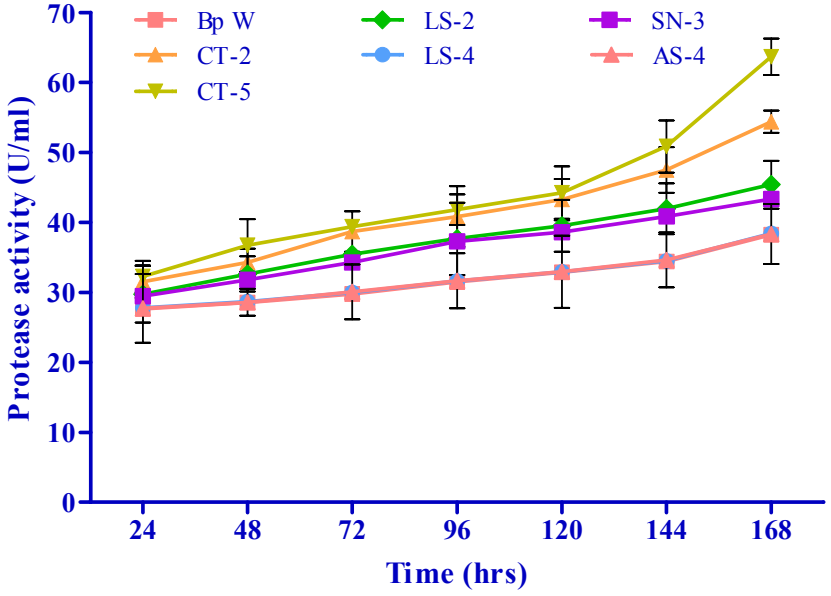

Figure 4.11 Protease activity by all the bacterial isolates in nutrient media at different time intervals. Values are mean ± SD (n =3)

4.7 Microbiological calcite precipitation

Before microbial sand plugging, the calcite precipitation ability by all bacterial isolates were studied at flask level. Calcite production by bacterial isolates in 100 ml nutrient media was expressed as calcite dry weight (mg)/cell dry mass (mg). Separation of the bacterial cell and calcite in the culture broth was made by filtering through a 0.45 μm membrane pore; the calcite was retained on the membrane and the bacterial cell in the filtrate. As shown in Fig. 4.12, *Bacillus* sp. (CT-5) gave the highest productivity of calcite followed by *Bacillus* sp. (CT-2). *Bacillus* sp. (CT-5) produced 1.59 mg calcite/cell dry mass (mg) while *Bacillus* sp. (CT-2) produced 1.3 mg calcite. *S. pasteurii* (Bp W), *Bacillus* sp. (LS-2), *Bacillus* sp. (LS-4), *Bacillus megaterium* (SN-3) and *Bacillus subtilis* (AS-4) produced 0.54, 1.1, 0.56, 0.92 and 0.61 mg calcite/cell dry mass (mg) respectively at the end of 120 h.

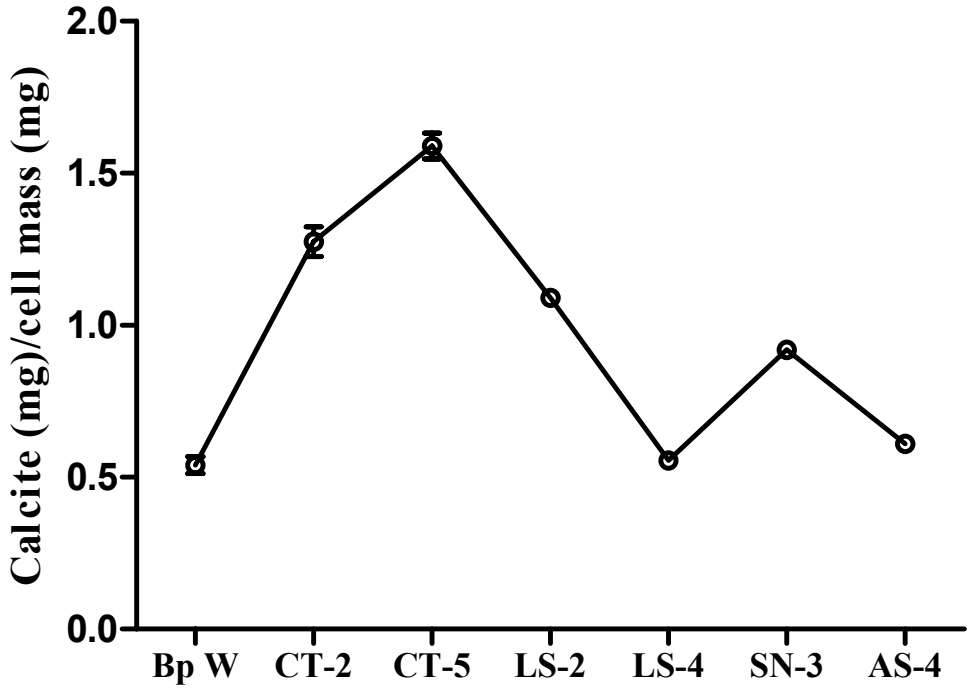

Figure 4.12 Calcite productions by all the bacterial isolates in nutrient media at different time intervals. Values are mean ± SD (n =3)

Calcite precipitation by all the bacterial isolates was evaluated by microbial sand plugging. All sand columns prepared with bacterial isolates were found to be tightly packed except control (without cells) sand column (after removing the plastic, it lost its form and collapsed). Before termination of microbial sand columns, all were fed with nutrient media. The sand columns were assembled as shown in Fig. 4.13. Microbial sand columns (Fig. 4.14) were divided into three layers, namely, upper, middle and lower layer. Calcite content was found to be maximum in case of upper layer of microbial sand column, compared to other two layers (middle and lower) irrespective of bacterial strains used. The maximum amount of calcite was precipitated by *Bacillus* sp. (CT-5). Calcite constituted 30.9 %, 19.59 % and 8.9 % in the upper, middle and lower layer respectively, of the total weight of the sand column plugged by *Bacillus* sp. (CT-5) (Fig. 4.15).

When sand columns were plugged with *S. pasteurii* (Bp W), *Bacillus* sp. (CT-2), *Bacillus* sp. (LS-2), *Bacillus* sp. (LS-4), *Bacillus megaterium* (SN-3) and *Bacillus subtilis* (AS-4) grown in nutrient media, calcite content in the upper layer was found to be 27.64 %, 28.89 %, 28.78 %, 28.42 %, 28.49 % and 28.42 % respectively (Fig. 4.15). There was a significant difference in calcite precipitation in upper and middle layers by different isolates (Table 4.13). *Bacillus* sp. (CT-5) precipitated significantly higher calcite compared to other isolates followed by *Bacillus* sp. (CT-2). Also among all the isolates, sand column consolidated with *Bacillus* sp. (CT-5) isolate was found to be most compact and the tightest one, as it required more physical pressure to break.

To determine the presence of microbial calcite precipitation, the sand consolidated samples were examined under SEM. Based on the maximum calcite production ability; three efficient strains [*S. pasteurii* (Bp W), *Bacillus* sp. (CT-2) and *Bacillus* sp. (CT-5)] were chosen for further analysis by SEM-EDX and XRD.

Figure 4.13 Assembly of microbial sand plugging column

Figure 4.14 Microbial sand plug formed by the bacteria

Table 4.13 Calcite precipitation in different layers of microbial sand column by different bacterial isolates

Isolates	Calcite content (%) in different layers		
	Upper	Middle	Lower
Bp W	27.6 ± 1.1d	17.7 ± 1.6f	8.1 ± 0.3d
CT-2	28.9 ± 2.2b	19.3 ± 1.1b	8.9 ± 0.1a
CT-5	30.9 ± 1.8a	19.5 ± 0.8a	8.9 ± 0.3a
LS-2	28.8 ± 0.2bc	19.1 ± 0.2c	8.9 ± 0.1ab
LS-4	28.4 ± 0.4cd	18.7 ± 0.7e	8.2 ± 0.2c
SN-3	28.5 ± 0.3c	19.0 ± 0.4d	8.4 ± 0.3b
AS-4	28.4 ± 0.4c	18.9 ± 0.7d	8.3 ± 0.3c

Values followed by same letter within the column are not significantly different at $P<0.05$. All values are Mean ± SD (n = 3).

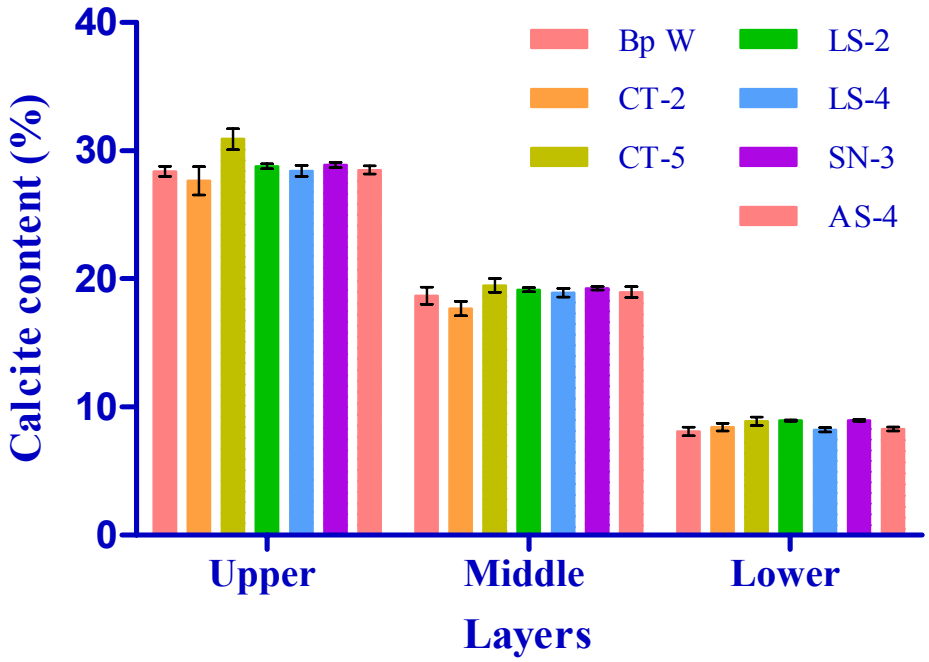

Figure 4.15 Calcite content in the different layers of sand columns consolidated with different bacterial isolates. Values are mean ± SD (n =3)

SEM analysis on microbial involvement in sand consolidation is depicted in Fig. 4.16, where distinct calcite crystals embedded with rod-shaped bacteria can be found between and on the surface of sand grains. Mineral constituents of the microbial sand columns were further characterized by EDX analyses. Presence of high amount of calcium in all the bacterial treated sand columns confirmed that calcium carbonate was present in the form of calcite. Maximum amount of calcium was found to be in sand column consolidated with *Bacillus* sp. (CT-5), which was clearly shown by EDX spectra; further supports that *Bacillus* sp. (CT-5) produced maximum amount of calcite (Fig. 4.17). The most abundant mineral present was silicon which is constituent of quartz, the main component of sand. The majority of the carbonate deposits were present as calcite crystals as was confirmed by XRD analyses although major component was found to be quartz (Fig. 4.18). Results of EDX and XRD confirmed maximum number of calcite peaks and maximum amount of calcium were found in case of *Bacillus* sp. (CT-5) compared to other isolates, this suggests that EDX and XRD analysis results were confirming each other.

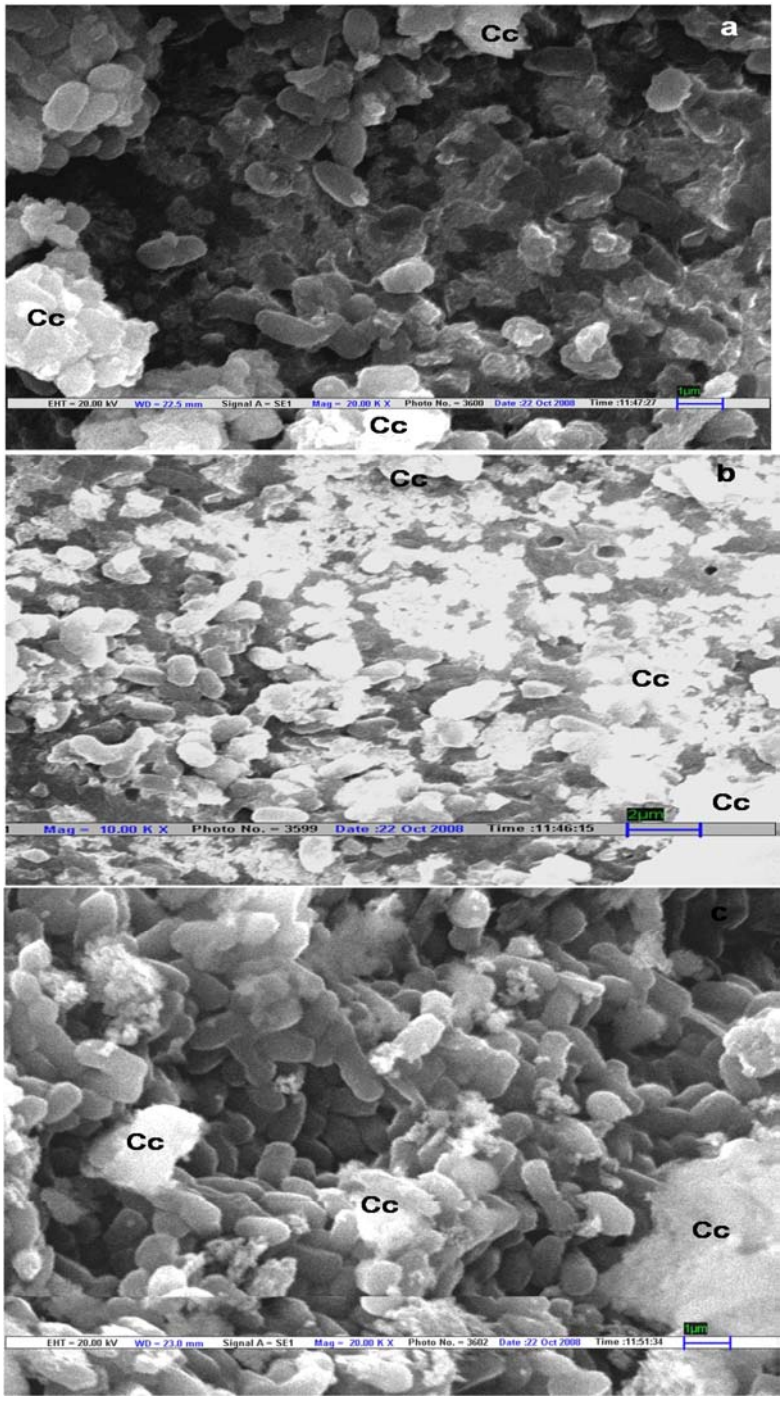

Figure 4.16 Scanning electron micrograph of microbiologically-induced calcite precipitation in sand consolidation by (a) *S. pasteurii* (Bp W), (b) *Bacillus* sp. (CT-2) and (c) *Bacillus* sp. (CT-5) (Cc – calcite crystals)

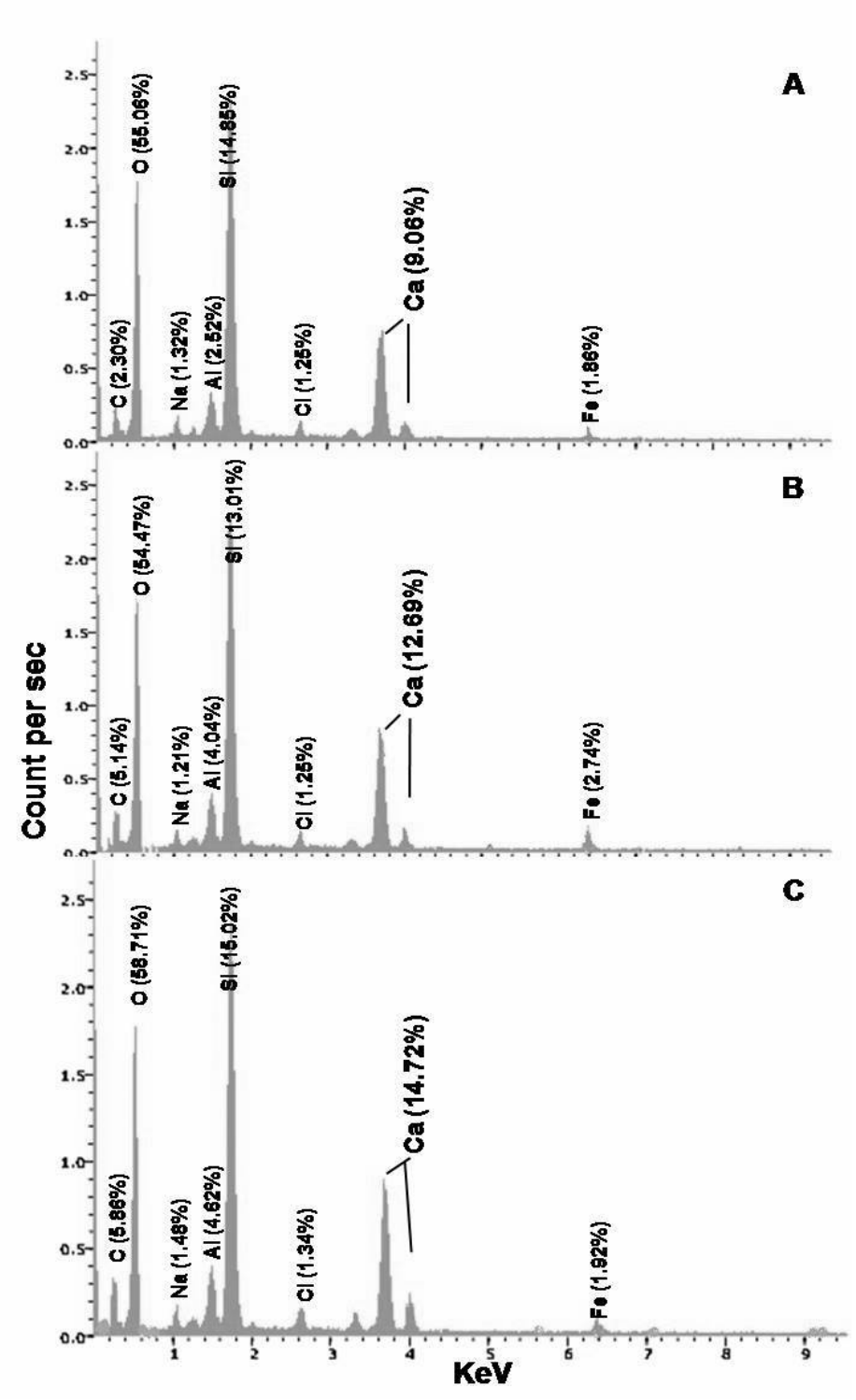

Figure 4.17 EDX Spectra of microbial sand columns prepared with (A) *S. pasteurii* (Bp W), (B) *Bacillus* sp. (CT-2) and (C) *Bacillus* sp. (CT-5). The abundant presence of Ca was evident and the precipitation was inferred to as calcite ($CaCO_3$) crystal.

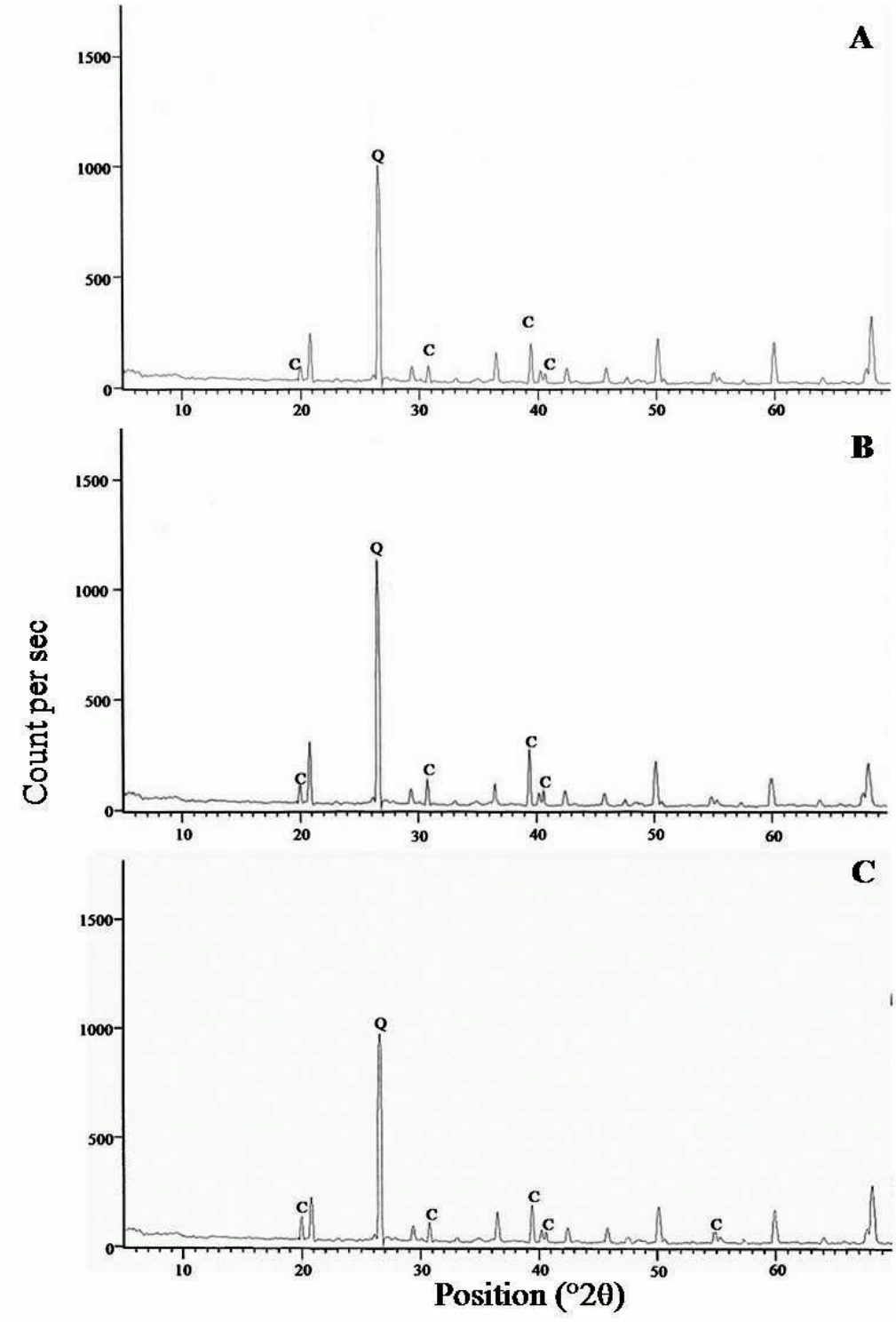

Figure 4.18 XRD spectra of microbial sand columns prepared with (A) *S. pasteurii* (Bp W), (B) *Bacillus* sp. (CT-2) and (C) *Bacillus* sp. (CT-5) (Q – quartz, C – calcite). More numbers of calcite peak in case of *Bacillus* sp. (CT-5) inferred better calcite precipitation as compared to *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-2)

4.8 Polyacrylamide gel electrophoresis and detection of urease by zymography

Towards characterization of all the bacterial isolates, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to check protein profile. Supernatant from all the bacterial isolates showed multiple banding patterns after staining with Coomassie Brilliant Blue R-250. Protein banding pattern was almost similar in all the samples although there was more and highly intense band found in case of *Bacillus* sp. (CT-2) and *Bacillus* sp. (CT-5). The size of proteins band varied between 29 and 205 kD, although majority of bands were between 43 and 97.4 kD (Fig. 4.19).

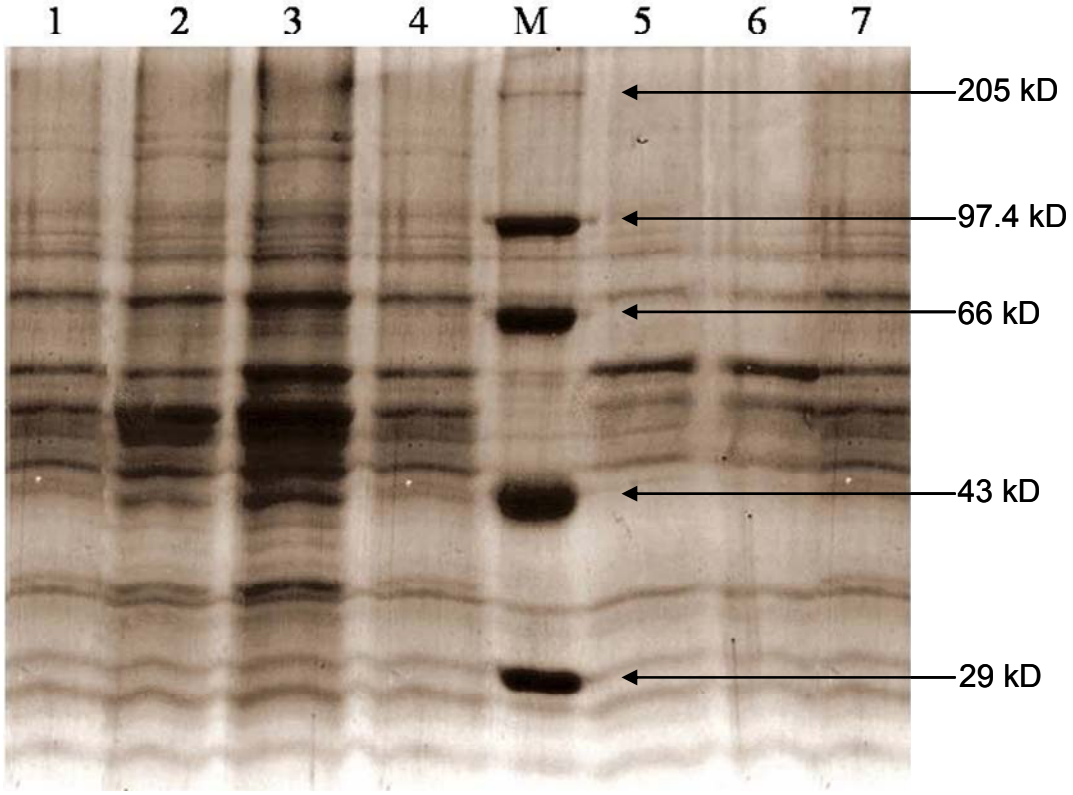

Figure 4.19 Protein bands obtained from supernatant of different isolates using SDS-PAGE. Lane 1- *Bacillus* sp. (LS-2); Lane 2- *Bacillus* sp. (CT-2); Lane 3- *Bacillus* sp. (CT-5), Lane 4- *B. megaterium* (SN-3); Lane 5- *S. pasteurii* (Bp W); Lane 6- *Bacillus* sp. (LS-4); Lane 7- *B. subtilis* (AS-4); Lane M-Molecular mass marker.

Further, zymogram analysis was performed to detect urease in the supernatant of all the bacterial isolates. In the present method, the polyacrylamide gel was incubated with a solution of urea, and this was followed by incubation with β-mercaptoethanol–nitroprusside solution. After an incubation of 5 min purple colored bands developed corresponding to the site of urease activity. The bands that developed due to urease activity were found to be at the same site with all samples. Urease preparations from all the bacterial isolates demonstrated identical electrophoretic mobilities on a non-denaturing activity gel (Fig. 4.20). No color change was observed in the rest of the gel, which leads to the clear visualization of urease activity bands. The color formed after urease action was found to be stable for at least one hour. These results confirmed presence of urease among all the bacterial isolates of present study.

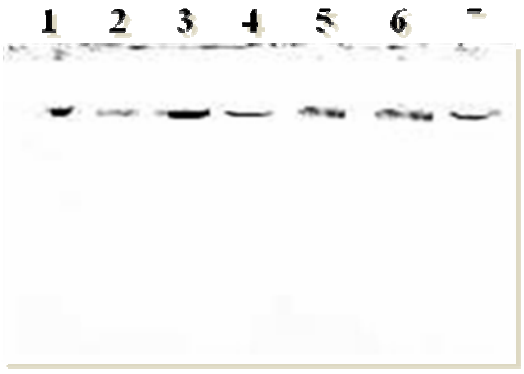

Figure 4.20 Native PAGE of urease developed with different in-gel detection methods for same time length. Lane 1- *Bacillus* sp. (CT-2); Lane 2- *S. pasteurii* (Bp W); Lane 3- *Bacillus* sp. (CT-5), Lane 4- *B. megaterium* (SN-3); Lane 5- *Bacillus* sp. (LS-2); Lane 6- *Bacillus* sp. (LS-4) and Lane 7- *B. subtilis* (AS-4).

Discussion

4.9 Isolation of microbial concrete producing bacteria

Bacteria inhabit an extraordinary array of habitats, from those that offer ideal conditions for most living creatures to those too extreme to support most life forms. They inhabit the relatively benign and nutrient-rich environments of soils, lakes, oceans and other organisms, but they are also found in extreme environments. Complexity in environmental conditions can vary at multiple spatial scales and could potentially influence bacterial diversity (Horner-Devine *et al*., 2004). The limited diversity of the bacterial community in extreme environments like cement or calcareous sludge, alkaline soil or limestone areas is not surprising; because of its extreme alkaline condition and organisms capable of growing in these conditions can only survive at this environment. Urease produced by bacteria is widely known to precipitate calcium carbonate, one of the main components of concrete, thus referred as microbial concrete enzyme. Typically, to remediate building materials urease needs to be active and stable in alkaline environment (pH 9–11) that also include high temperature (Stocks-Fischer *et al*., 1999). Ureases in general are not stable under these conditions and therefore, the emphasis has been on newer sources. Keeping these points, urease producing bacteria were isolated from sources such as calcareous sludge, limestone area, cement and alkaline soils. Isolation and screening of bacteria from these natural environments can be useful for obtaining bacterial strains with the potential of yielding urease enzymes. Also, these areas were selected to isolate indigenous bacteria which can sustain high alkalinity as the aim of the present work was to use these isolates in the remediation of building structures. The population of bacteria differed in these sources. The population of bacteria was more in alkaline soils than other sources. This might be due to the presence of extra minerals as nutrients available in soil other than high pH. Lowest number of bacteria was found in cement and this might be due to very high pH (≈12) and high temperature. Reportedly, certain structural components of the cell wall of some alkalophiles, such as teichuronopeptide, may contribute to pH homeostasis at

alkaline pH and aid bacteria to survive in alkaline environments (Aono *et al*. 1999). The isolated bacteria were screened qualitatively on urea containing Urea agar media. This media contains phenol red. The urease producing bacteria utilizes urea present in the media and then degrades phenol red giving pink color (Christensen, 1946; Andrews *et al*., 1995). Based on the intensity of pink color by naked eyes, total of 40 efficient urease producers were selected.

4.10 Molecular characterization of bacteria

The development and increased availability of techniques in molecular biology have made it possible to obtain information regarding the diversity of bacterial cultures isolated from different habitats (Amarger *et al*., 1994). One such technique, a polymerase chain reaction (PCR)-based assay to fingerprint genomes using BOX is useful for differentiating between bacterial isolates (Welsh and McClelland, 1990). The use of repetitive DNA sequences such as BOX, for bacterial classification is becoming frequent, and has allowed comparisons of possible genetic similarities among different bacterial genomes (Versalovic *et al*. 1991; Louws *et al*. 1994; Selenska-Pobell *et al*. 1995). BOX-PCR was used successfully to generate DNA fingerprints to distinguish between genetically unrelated and closely related bacterial strains. PCR using the primers BOXA1R elicited differentiation at species, subspecies and strains level; hence BOX-PCR was performed to differentiate distinctly different species. It involves the use of primers based on the short repetitive element derived from highly conserved BOXA subunit dispersed throughout the prokaryote kingdom (Laguerre *et al.,* 1996). In the present study, six representative bacterial isolates were found to be genetically dissimilar based on BOX-PCR. The term “genomovar” has recently been introduced to denote phenotypically similar but genotypically distinct groups of bacterial isolates within a species. Polymerase chain reaction (PCR) can produce products to the more highly conserved 5S, 16S, and 23S ribosomal subunits which can potentially differentiate species and also show intraspecific differences (Wakabayashi *et al*., 1999). So to further confirm the genetic variability in the representative isolates, 16S rRNA was

amplified and sequenced. Phylogenetic analysis revealed all the six isolates belonging to genera *Bacillus*. The predominance of this genus led us to hypothesize, according to Rivadeneyra *et al*. (1993), that *Bacillus* spp. plays a major role in the carbonate deposition in natural habitats.

4.11 Physiological characterization of bacteria

The six isolates of the present work also showed the ability to tolerate a wide range of salinity, pH, and temperatures, and presented ureolytic activity that lead to calcite precipitation, which provides the advantage of uses in various industrial processes. Apart from 16S rRNA gene analysis, phenotypic and physiological properties of these isolates also resemble with *Bacillus* species reported previously (Smith *et al*., 1952; Gordon *et al*., 1973). Salt tolerance, growth temperature range, growth pH range, and extracellular products are important taxonomic criteria which were used to differentiate species in the genus *Bacillus* (Claus and Berkeley, 1986). The diverse group of all the six bacterial isolates of present study that thrive well in alkaline environments can be categorized into two broad groups: alkalotolerants and alkalophiles. Alkalotolerants show optimal growth between pH 7.0 and 9.0, but can not grow above pH 9.5. The alkalophiles can be further divided into two groups: facultative alkalophiles and obligate alkalophiles. Bacteria growing in alkaline environments can be divided into two general classes: those with optimum growth ranges above pH 10, termed alkalophiles, and those with optimum growth ranges around neutral pH, but still capable of growth up to pH 9.5, termed alkaline tolerant (Sharp and Munster, 1986; Krulwich and Guffanti, 1989). The antibiotics profile of bacterial isolates was also well matched with *Bacillus* species. There are few studies of the sensitivity of *Bacillus* species to antibiotics even though they relate to the genus taxonomy. Few *Bacillus* sp. has been reported resistance to the antibiotics penicillin G whereas, few also reported to show sensitivity to cephalosporin C, ampicillin, polymyxin B, bacitracin, kanamycin, rifampicin, tetracycline, streptomycin, chloramphenicol and erythromycin (Claus and Berkeley, 1986). All the isolates were able to grow well in nutrient medium containing urea and $CaCl_2$.

This media supported microbial growth to all the isolates. NH_4^+ and Cl^- react with OH^- and H^+ and urea serves as energy source and facilitates growth of bacteria (Stocks-Fischer *et al*., 1999).

All the isolated bacteria were able to produce good amount of EPS and biofilm, although *Bacillus* sp. (CT-5) showed the maximum productions. The matrix of extracellular polymeric substances (EPS) secretions has been described to influence the calcium carbonate precipitation in a positive way (Kawaguchi and Decho, 2002). EPS appears to play a role in the coverage of the surface by biofilms, cell adhesion (Tsuneda *et al*., 2003) and possibly the capturing of the produced calcium carbonate, which might result in a homogeneous layer of calcium carbonate. The importance of a biofilm is to colonize the surface of the stones and react as nucleation site for extracellular calcium carbonate precipitation (Merz-Preiss and Riding, 1999). EPS production and biofilm formation are somewhere correlated. Especially large quantities of EPS are deposited when micro-organisms form a biofilm. They serve as "glue" between the cells. The structure of biofilms is clearly influenced by a number of biological factors such as twitching motility, growth rate, cell signaling, and EPS production (Stoodley *et al*., 2002). The biofilm structure appears to be largely determined by the production of slime-like matrix of EPS, which provides the structural support for the biofilm.

4.12 Optimization of parameters for maximal urease production

The parameters affecting the activity of urease are categorized into two, viz., chemical (amount of substrate and calcium source) and physical (pH of reaction mixture and temperature of incubation). There are certain parameters such as temperature, pH, and substrate on which production of any enzyme depends and that's why it is needed to optimized (Haltrich *et al*., 1993). Temperature can affect a lot of different factors hence its effect on enzyme activity is very complex. It affects the speed of molecules, the activation energy of the catalytic reaction and the thermal stability of the enzyme and substrate. At low temperatures, the rate of enzyme

reaction is very slow. The molecules have low kinetic energy and collisions between them are less frequent and even if they do collide the molecules do not posses the minimum activation energy required for the reaction to occur. It can be said that the enzymes are deactivated at low temperatures. An increase in temperature increases the enzyme activity since the molecules possess greater kinetic energy. The rate of enzyme activity is highest between 0-40°C and this increase is almost linear. After 40°C the rate of reaction starts to decrease. This is because the increase in temperature after 40°C does not increase the kinetic energy of the enzyme but instead disrupts the forces maintaining the shape of the molecule. The enzyme molecules are gradually denatured causing the shape of the active site to change. Temperatures above 65°C completely denature the enzymes (Robinson *et al*., 2005). All the isolates of present study showed similar pattern of growth profile. The necessity for placing cultures into balanced growth prior to collecting data has been stated by Neidhardt *et al*. (1990): "Unless growth is monitored throughout a physiological experiment, the results may not be reproducible....Infact, a physiological experiment done with a poorly characterized culture is *all but useless*." No luxurious growth could be achieved in the absence of urea, which conforms with the coupling of urea hydrolysis and ATP generation in isolates (Jahns, 1996).

Bacillus cells use urea as an energy source, resulting in the production of ammonia and carbonate (Mobley and Hausinger, 1989). This increases the pH and the carbonate concentration near the cells, triggering Ca^{2+} and CO_3^{2-} to precipitate as $CaCO_3$ (Mobley and Hausinger, 1989; Castanier *et al*., 1999; Stocks-Fischer *et al*., 1999; Hammes *et al*., 2003; Udert *et al*., 2003). Considering the effects of substrate (urea or calcium source) concentrations on urease activity, the actual enzyme activity in the precipitating biocementation mixture must change substantially over the course of cementation, as the concentrations of the ions involved change. This means that the rate and hence the size and shape of calcite crystals that form, changes over the course of cementation and either leads to desirable strength or non-desirable strength properties. The length of time that the enzyme works at the optimum rate for strength, will determine the overall strength properties of the cemented material.

Results from these study indicated that varying urea concentration and calcium source produced a great control over urease activity. The urease production profile of the strain as a function of growth was examined in a complex nutrient medium. The production could be seen from the early exponential phase onwards. It was very low during the early stages of exponential phases. The isolates showed a steady increase in urease production with the progression of growth from early exponential to early stationary phase. Different *Bacillus* species have been reported to be producing the maximum enzyme during the late exponential (Atalo and Gashe, 1993), post exponential (Ward, 1983; Manachini *et al*., 1988) and the stationary (Durham, 1987; Purva *et al*., 1998) phases of growth.

The observations made with respect to optimization of growth conditions and process parameters that govern maximal production of urease by different bacterial isolates strengthen the potential of the organism for industrial use or commercialization can be considered in future.

4.13 Microbial urease and protease productions

All the isolated bacteria of the present study were identified as *Bacillus* genera and *Bacillus* species are already known to produce a large amount of urease in soil environments (Mobley *et al*., 1995; Ciurli *et al*., 1996; Benini *et al*., 1999; Bachmeier *et al*., 2002). There was significant difference in urease production among all the isolates when grown in nutrient media containing urea and calcium chloride for different time interval. Bacteria are known to hydrolyze urea by urease for the purposes of: (1) increasing the ambient pH (Burne and Marquis, 2000), (2) utilizing it as a nitrogen source (Burne and Chen, 2001), and (3) using it as a source of energy (Mobley and Hausinger, 1989). The action of urease has two basic effects for bacterial cell (i) it hydrolyses urea to generate high concentrations of ammonium which are required for growth and energy generation, and (ii) it increases the pH of growth environment to alkaline (9-10) which is the optimum growth pH for concrete producer bacteria. In biological systems, many calcareous organisms couple calcification to their metabolic assimilation processes to scavenge protons

(McConnaughey and Whelan, 1997). The subsequent increase of pH in surrounding medium due to the presence of ammonia ions and the additional release of CO_2 from the enzymatic urea hydrolysis further accelerate the rate of the urease induced calcite precipitation. Thus, an active participation of urease is of essence in biochemical calcite precipitation.

Protease production increased significantly with increase in time in all isolates, but after certain time interval urease activity declined. Mehrotra *et al.* (1998) also observed maximum production of protease during the late exponential phase of growth of *Bacillus* sp. The decline in urease production after its optimal level might reflect depletion of available nutrients and also due to high pH. Protease might have adverse effect on urease activity as its accumulation in the culture media may cause breakdown of urease and ultimately lowered urease production. Urease proteins are constantly degraded after 120 hrs and to facilitate this recycling, a complex system for the controlled turnover of proteins might evolved. Damaged proteins like ureases, after certain time interval, are marked for destruction by the covalent attachment of chains of a protein or by selective hydrolysis (Berg *et al*., 2002).

4.14 Microbiological calcite precipitation

The maximum amount of calcite was deposited in the upper layer followed by middle and lower layer irrespective of cells used to prepare the column. Calcite precipitation occurred predominantly in the areas close to the surface of the sand column. It is mainly due to the fact that facultatively anaerobic *Bacillus* cells grows at a higher rate in the presence of oxygen and consequently induces active precipitation of $CaCO_3$ around the surface area (Stocks-Fischer *et al*., 1999).

Bacterial mediated calcite precipitation is confirmed from the results of present study. The following model for bacterial mediated calcite biomineralization can be proposed. First, negatively charged functional groups on the bacterial cell walls attract Ca^{2+} to induce a local supersaturation so that calcite nucleation takes place on the cell surfaces. This is possible because, albeit different in the source of charge

occurrence among different strains, bacterial cell walls are usually electronegative (Schultze-Lam *et al*., 1996). For Gram-positive bacteria, the electronegativity is derived primarily from the secondary polymers teichoic acids that contribute extra negative charges from carboxyls and phosphates (Hogg, 2005) to the existing peptidoglycan framework, and both groups bind strongly with Ca^{2+}. Although we did not rinse the cells prior to the crystallization experiments, Ca^{2+} adsorption on the cell surfaces may still be possible because any pre-sorbed ions (presumably from the culture media) should have been washed off once the cells were immersed into the experimental solutions. This stage may be best described as epicellular growth (Mann, 1988) in which the crystallographic controls dominate and the calcite crystals maintain the rhombohedral morphology. If the concentration of bacteria is high to result in a bacterial scaffold that bears a large area of cell walls, epicellular and intercellular growth originated from the colony will outpace the homo-epitaxial growth in the surrounding area. This happens because the local environment within the colony may have a higher saturation state than the bulk solutions. It was suggested that the aqueous microenvironments associated with microbes are different from those around larger organisms, and one of the differences is that ionic solutes tend to concentrate in the hydrodynamic boundary layers surrounding the bacterial cells due to bacterial sequestration (Thompson *et al*., 1990; Schultze-Lam *et al*., 1992; 1996).

The sand column loaded with bacteria was so tightly plugged that the column was fractured with a mechanical knife for examining. Biologically produced by ureolytic activity, the calcite layer will clog the pores of sand to decrease the water absorption rate and ultimately leading to solid sand column. The growth related microbiological urea degradation is the main source of inorganic carbon dioxide in calcium and carbonate oversaturated medium and the cause of calcium carbonate precipitation (Merz-Preiss and Riding, 1999; Yates and Robbins, 1999, Warren *et al*., 2001). The isolated bacteria of present study were belonging to genera *Bacillus* and most of the calcifying bacteria belong to the *Bacillus* genera (Ercole *et al*., 2001).

4.15 *In-gel* detection of urease activity

Zymogram analysis was done to detect urease activity. Zymogram reveled presence of urease in the supernatant of all the bacterial cultures. Zymogram, or activity staining in non-denaturing polyacrylamide gel, is an established method to demonstrate the presence of different catalytically active molecular species of urease (De Martin *et al.*, 1989). Screening of urease activity by means of a zymogram can also be used to monitor the relative expression of this enzyme in different live system (Sharma *et al*., 2008). Sodium nitroprusside reacts with thiols and forms a purple colour complex in alkaline medium (Szacilowski *et al.*, 2002). Transformation of the yellow colour of nitroprusside-thiol solution essentially requires alkaline pH to develop into the purple colour complex. The local alkalinity produced by ureolytic activity can theoretically be used to develop purple colour due to nitroprusside-thiol reaction. While developing the zymogram, β-mercaptoethanol was used as a thiol compound for colour development. Zymogram reveled presence of urease in the supernatant of bacterial cultures. Urease activity staining in native PAGE can be used to understand the polymeric status of this enzyme under different ionic strengths, pH and storage conditions. The method also reports a new, sensitive and inexpensive method to assay urease in polyacrylamide gels after separation on PAGE under native and non-denaturing conditions.

4.16 Conclusion

In this study, the bacterial groups associated with microbial calcite formation were assessed from different environments (alkaline soil, limestone area, calcareous sludge and cement), by producing urease using culture-dependent approach after enrichment. The bacterial typing by BOX-PCR and the identification by sequencing of the 16S rRNA identified the main culturable bacterial groups found in these environments. The main groups found were *Bacillaceae* which associate with calcite precipitation. One of the cement isolates *Bacillus* sp. (CT-5) was able to produce maximum amount of urease and calcite. Summarizing, new data about bacterial

groups which inhabit the extreme environments were presented. Enzymes from alkalophiles are expected to show optimal activities in extreme conditions; thus, the possibility to have a wide variety of alkalophiles producing concrete enzymes *i.e*., urease will be of valuable importance for biotechnological applications. A rapid, simple and economical zymogram method to detect urease activity in bacteria was established. This method may be used to demonstrate charge/size isoforms of urease, which will help in the better understanding of the distribution of urease in the microbial system. All the urease producing isolates were able to produce EPS and biofilm too, which enhanced their property towards calcite precipitation. Further, the results from this study suggest that bacteria are enhancing calcium carbonate precipitation by providing a well-matched surface to seed calcium carbonate precipitation as well as promoting supersaturated conditions. This work presents a new application for microbial carbonate precipitation as a cementing technique.

Chapter 5

Exploitation of industrial by-products as alternative nutritive sources

5.1 Industrial by-products

Corn steep liquor (CSL), an industrial by-product of corn starch processing industry and lactose mother liquor (LML), an industrial by-product of dairy industry collected from Bharat Starch Industries Ltd., Yamunanagar, Haryana, India and Cepham Milk Specialties Pvt. Ltd., Derrabassi, Punjab, India respectively were used as nutrient sources to grow efficient microbial concrete producing bacteria. Nutrient profiles of these by-products based on their physico-chemical properties are presented in Tables 5.1 and 5.2. The concentrations of LML and CSL are 10% and 1.5% respectively in this study.

The objective of this work is to develop an economical, industrial media that is suitable for large-scale production of microbial concrete. In the present study, LML and CSL have been used to replace the standard medium such as nutrient broth to make the process economic. *Bacillus* sp. (CT-5) isolated from cement was characterized by a higher urease production, higher calcite precipitating ability and higher EPS and biofilm productions, compared to other isolates of present study. Based upon these parameters, this isolate was further characterized to test efficacy to utilize industrial by-products as nutrient source to produce concrete. Microbial concrete producing ability of this strain was also compared with *S. pasteurii* (Bp W).

Table 5.1 Physico-chemical characteristics of the 10% lactose mother liquor (LML)

Component	Quantity	Component	Quantity
pH	6.20	Phosphorus (mg/l)	35
Solid (%)	5.50	Potassium (mg/l)	186
Lactose (%)	15.40	Sodium (mg/l)	44
Proteins (%)	8.00	Calcium (mg/l)	353
Fats (%)	2.00	Sulphur (mg/l)	15
Ash (%)	0.53	Chloride (mg/l)	90

Table 5.2 Physico-chemical characteristics of the 1.5% corn steep liquor (CSL)

Component	Quantity	Component	Quantity	Component	Quantity
pH	3.86	Arginine (%)	0.4	Phenylalanine (%)	0.3
Solid (%)	46-50	Cystine (%)	0.5	Valine (%)	0.5
Carbohydrate (%)	5.80	Glycine (%)	1.1	Inositol (mg/100g)	602
Proteins (%)	24	Histidine (%)	0.3	Choline (mg/100g)	351
Fats (%)	1.00	Isoleucine (%)	0.9	Niacin (mg/100g)	8.4
Minerals (%)	8.80	Methionine (%)	0.5	Pyridoxine (mg/100g)	1.1

5.2 Optimization of LML for urease production

Lactose mother liquor an industrial by-product of dairy industry was used as nutrient source to grow bacteria and optimized for maximum urease production. *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) were grown in LML (of different concentrations) media containing 2% urea and 25 mM $CaCl_2$ to measure urease production. The effect of using LML at different concentrations (10-50%) on the urease production is shown in Fig. 5.1. The urease activity decreased as the concentration of LML increased. The maximum urease production by both isolates was observed at 10% with a C:N ratio equivalent to 8:1. With further increase in the concentration, a slight decline in the urease production was observed. At LML concentrations of below 10%, the urease production were neglisable, that's why data have not been shown.

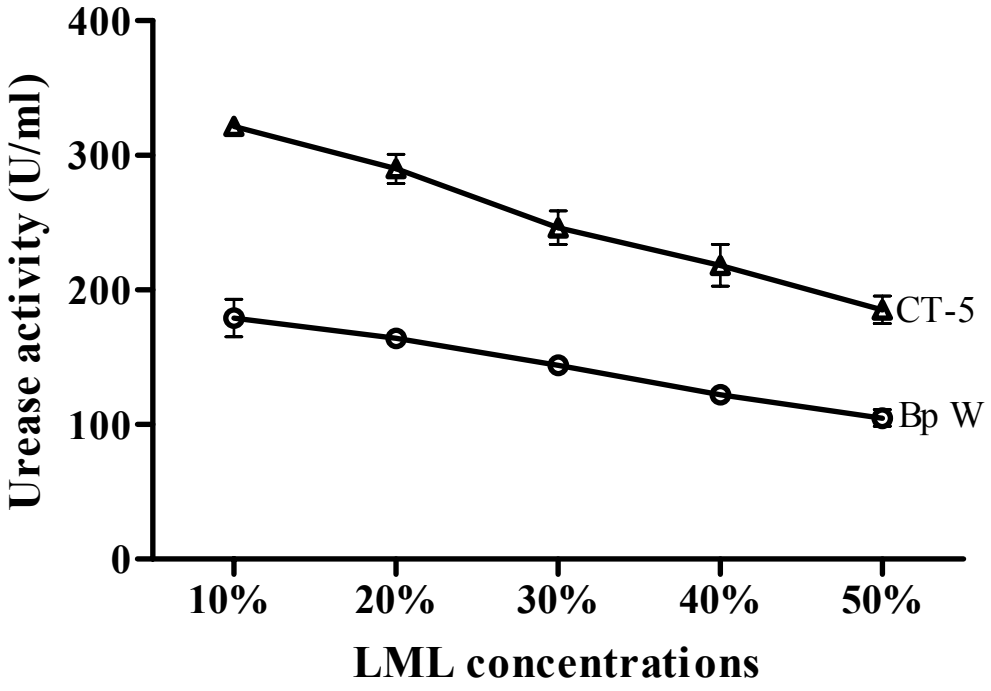

Figure 5.1 Effect of different concentrations of LML on urease production by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5). Values are mean ± SD (n =3)

5.3 Optimization of CSL for urease production

In order to optimize urease production in CSL medium, *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) was grown with various concentrations of CSL. Cultivations included 2% urea and 25 mM $CaCl_2$ to measure urease production. The effect of using CSL at different concentrations (1-5%) on the urease production is shown in Fig. 5.2. The optimum level of urease activity by both isolates was recorded in the culture containing 1.5% CSL. The 1.5% CSL used from concentrated stock contained C:N mass ratio equivalent to 6:1. The higher concentrations of CSL produced significantly less urease activity.

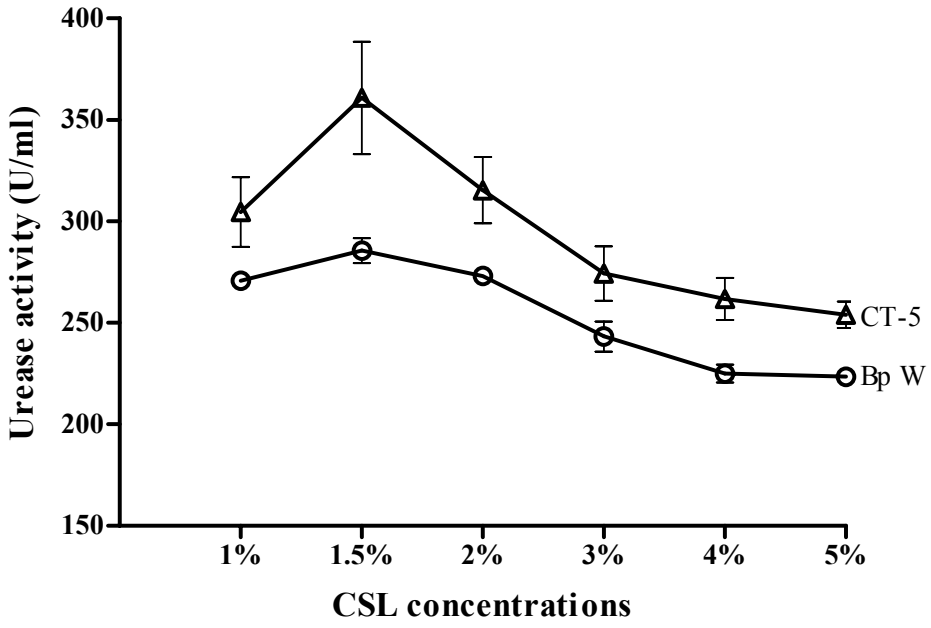

Figure 5.2 Effect of different concentrations of CSL on urease production by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5). Values are mean ± SD (n =3)

5.4 Growth Profile

The ability of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) to use alternative nutrient sources was tested by cultivation in CSL and LML media and compared growth rate with standard nutrient media (NB). The growth profiles of these isolates were studied up to 30 hours in both media. Final pH of media was adjusted to 8.0 prior to supplementation with 2% urea and 25 mM $CaCl_2$. The growth rate of both isolates in CSL medium was comparable with nutrient broth (NB) but was significantly higher than LML media in relation to their cfu/ml (Fig. 5.3). The log cfu/ml was marginally higher in CSL compared to the nutrient media (NB).

Similar kind of growth pattern was observed with both the isolates. *Bacillus* sp. (CT-5) started growing faster in all media compared with *S. pasteurii* (Bp W). It can be, however, speculated that during the initial period of growth, available nutrients in the media favored the growth of bacterial isolates. The visual inspections of growth gave the impression that growth was more rapid in the CSL media which permitted the denser growths.

5.5 Evaluation of industrial by-products for microbial urease production

For large scale production of urease, it is necessary to find an inexpensive substrate for the bacteria to grow on, that still produced a good level of urease activity.

Nutrient media (NB) supported urease production by both isolates very well but no significant difference was observed when nutrient medium was replaced with CSL medium (Table 5.3). The maximum urease production was observed when CSL medium was used to grow the bacterial isolates compared to other media. The highest productivities were obtained at 120 hrs. After 120 hrs, urease production was decreased in all cases. *Bacillus* sp. (CT-5) produced 676 U/ml of urease in CSL media at 120 hrs which was slightly higher than using nutrient media (665 U/ml). It produced 428 U/ml of urease in LML media (Fig. 5.4). The urease production by *S. pasteurii* (Bp W) was found to be 441.5 U/ml, 427.3 U/ml and 354.3 U/ml of urease in CSL, nutrient and LML media respectively.

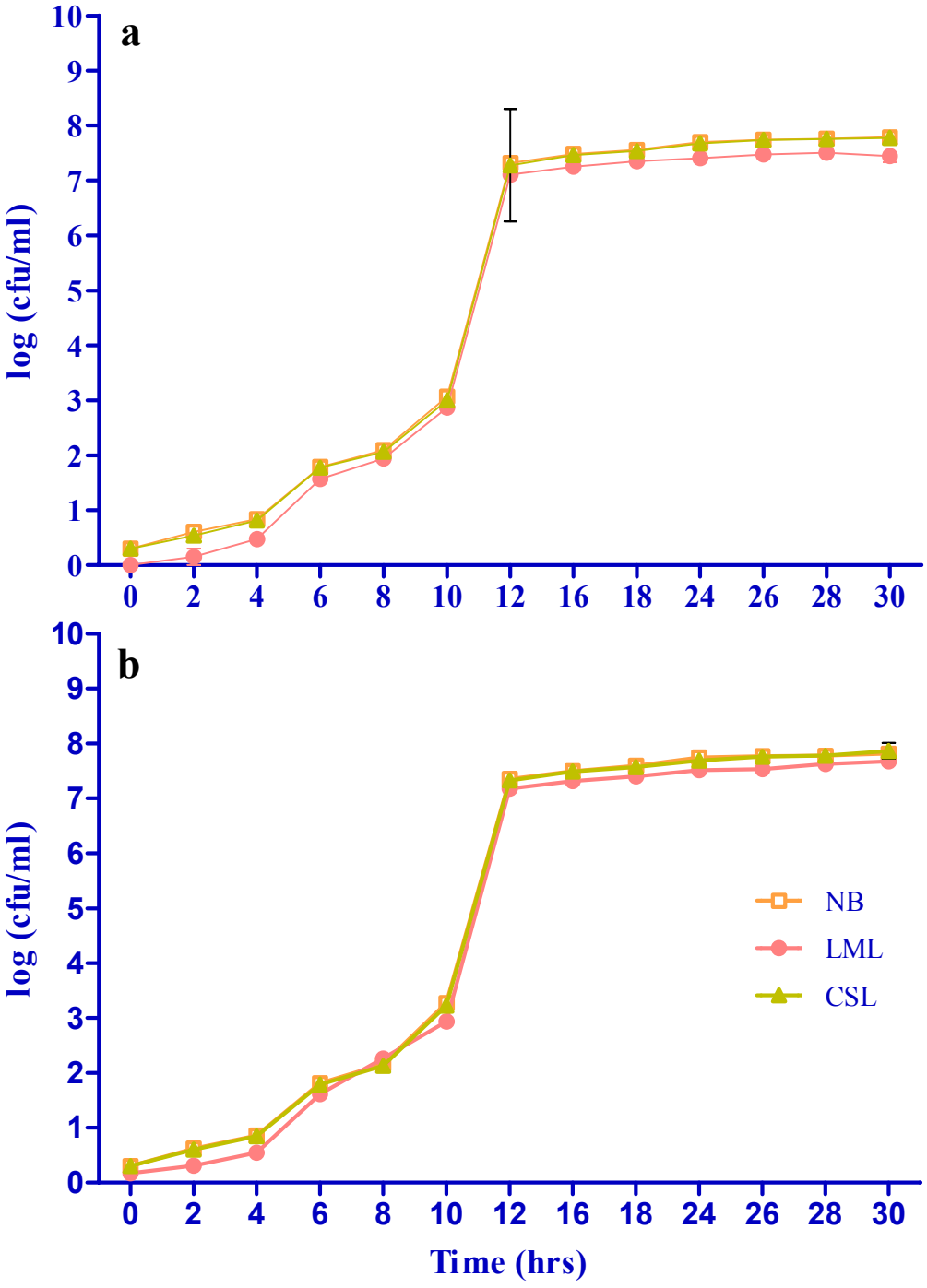

Figure 5.3 Growth rate profiles of (a) *S. pasteurii* (Bp W) and (b) *Bacillus* sp. (CT-5) in CSL, LML and nutrient (NB) media. Values are mean ± SD (n =3)

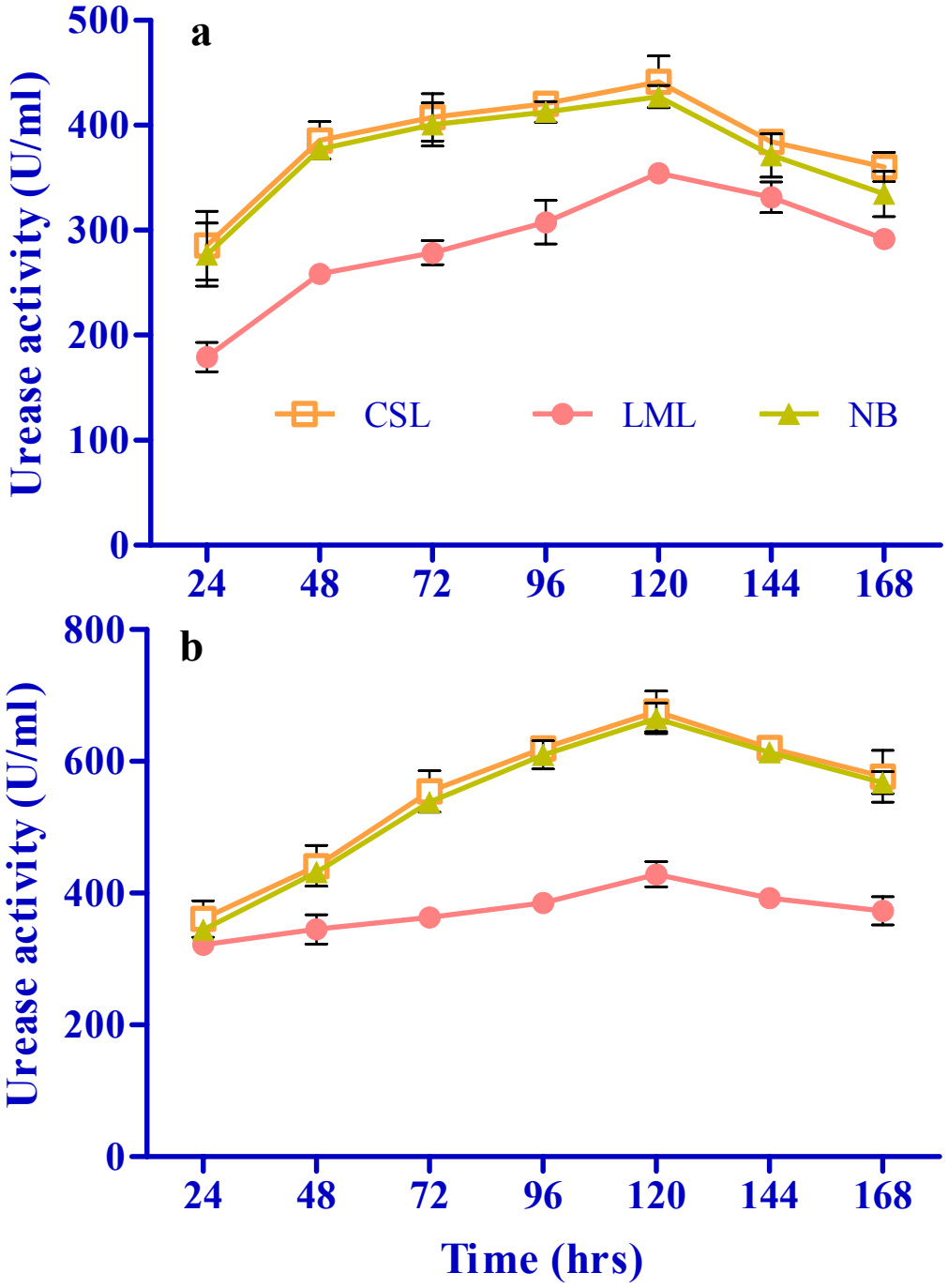

Figure 5.4 Urease productions by (a) *S. pasteurii* (Bp W) and (b) *Bacillus* sp. (CT-5) in CSL, LML and nutrient (NB) media. Values are mean ± SD (n =3)

5.6 Protease production by bacterial isolates

Protease activity produced by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) grown in CSL, LML and nutrient media was detected in the culture supernatant from 24 hrs to 168 hrs and a sudden increase in its activity was found after 120 hrs, also the highest level was found after 168 hrs as observed when nutrient media were used to grow the cells. Protease productions by *S. pasteurii* (Bp W) grown in CSL, LML and nutrient media were 44.2, 35.6 and 41.7 U/ml while *Bacillus* sp. (CT-5) grown in CSL, LML and nutrient media produced 64.7, 38.3 and 63.7 U/ml of protease respectively, at the end of 168 hrs (Fig. 5.5). Production was significantly higher when bacterial cells were grown in CSL media compared to other media (Table 5.3).

5.7 EPS and Biofilm Production

A better understanding of bacterial adhesion process is needed for the production of urease for biocementation process. The ability of bacteria to adhere on surfaces is associated with the extensive production of extracellular polymeric substances (EPS). EPS is responsible for both adhesion and cohesion interactions and play a crucial role in maintaining structural integrity of bacterial biofilms. EPS matrix may enable the bacteria to capture nutrients present in industrial by-products. A significant difference among *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5), grown in CSL and LML media was found in terms of extracellular polymeric substances (EPS) and biofilm productions. *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) (both grown in CSL media) were able to produce 20.9 and 55.3 nmol/ml of EPS respectively (Fig. 5.6). When LML medium was used to grow *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5), they produced 16.5 and 25.7 nmol/ml of EPS respectively while in case of nutrient media; the production was 18.2 and 45.1 nmol/ml respectively. A mature sessile bacterial community was formed by *Bacillus* sp. (CT-5) due to biofilm production but these cells were highly effective in covering the surface when grown in CSL medium followed by nutrient medium. Monoxenic biofilms of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) contained 294 and 366 cfu/mm^2 respectively in CSL media, while in nutrient media it was 283 and 352 cfu/mm^2 respectively (Fig. 5.6). EPS and biofilm productions were significantly higher when CSL was used as nutrient source compared to other media (Table 5.3).

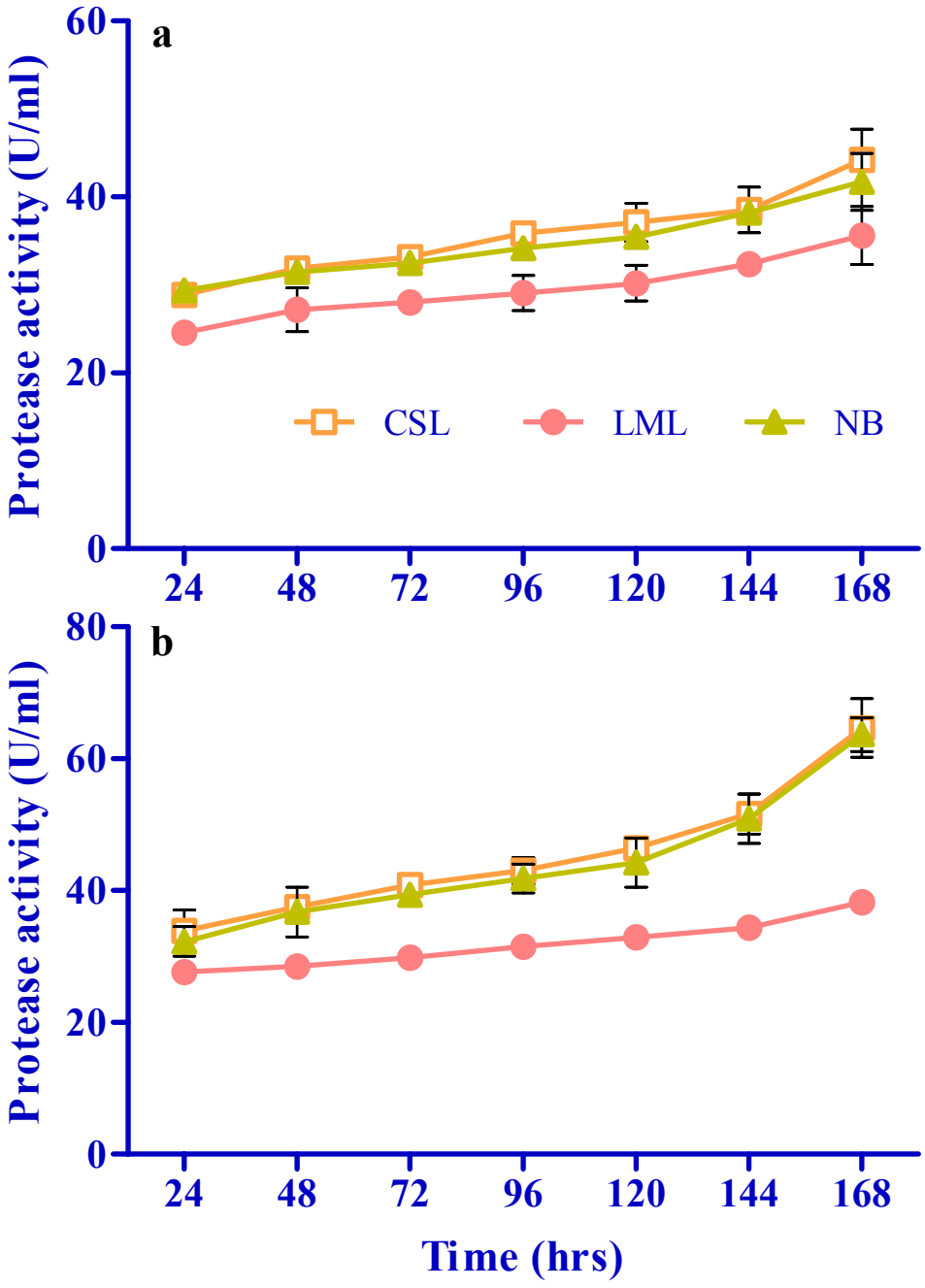

Figure 5.5 Protease productions by (a) *S. pasteurii* (Bp W) and (b) *Bacillus* sp. (CT-5) in CSL, LML and nutrient (NB) media. Values are mean ± SD (n =3)

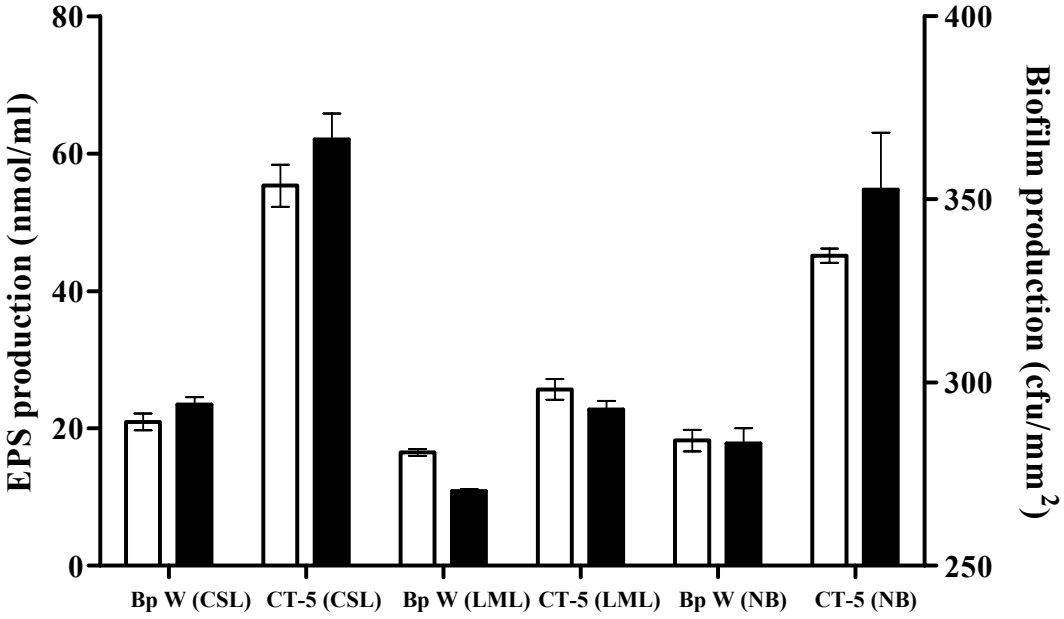

Figure 5.6 EPS (open bar) and Biofilm (filled bar) productions by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) in CSL, LML and nutrient (NB) media. Values are mean ± SD (n =3)

5.8 Microbiological calcite precipitation using industrial by-products

Microbial sand plugging by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) using CSL and LML media were tightly packed. Microbial sand columns were divided into three layers, namely, upper, middle and lower layer. The maximum amount of calcite was deposited in the upper layer followed by middle and lower layer in both cases. Calcite content was found to be maximum in case of upper layer of microbial sand column, compared to other two layers (middle and lower) irrespective of bacterial strains used. Similar kind of result was observed when nutrient medium was used to grow the bacterial isolates. When both isolates were grown in CSL medium, it was more effective in precipitating calcite in sand column. The maximum amount of

calcite was precipitated by *Bacillus* sp. (CT-5). Calcite constituted 32.16%, 27.11% and 30.9% of the total weight of the sand column plugged by *Bacillus* sp. (CT-5) using CSL, LML and nutrient media respectively (Fig. 5.7).

When sand columns were plugged with *S. pasteurii* (Bp W) grown in CSL media, calcite content in the upper layer was found to be 29.75%; while Bp W grown in LML and nutrient media constituted 26% and 27.6% calcite respectively in the upper layer of sand column (Fig. 5.7). The calcite precipitation in the microbial sand columns was significantly higher when both isolates were grown in CSL media compared to other media (Table 5.3).

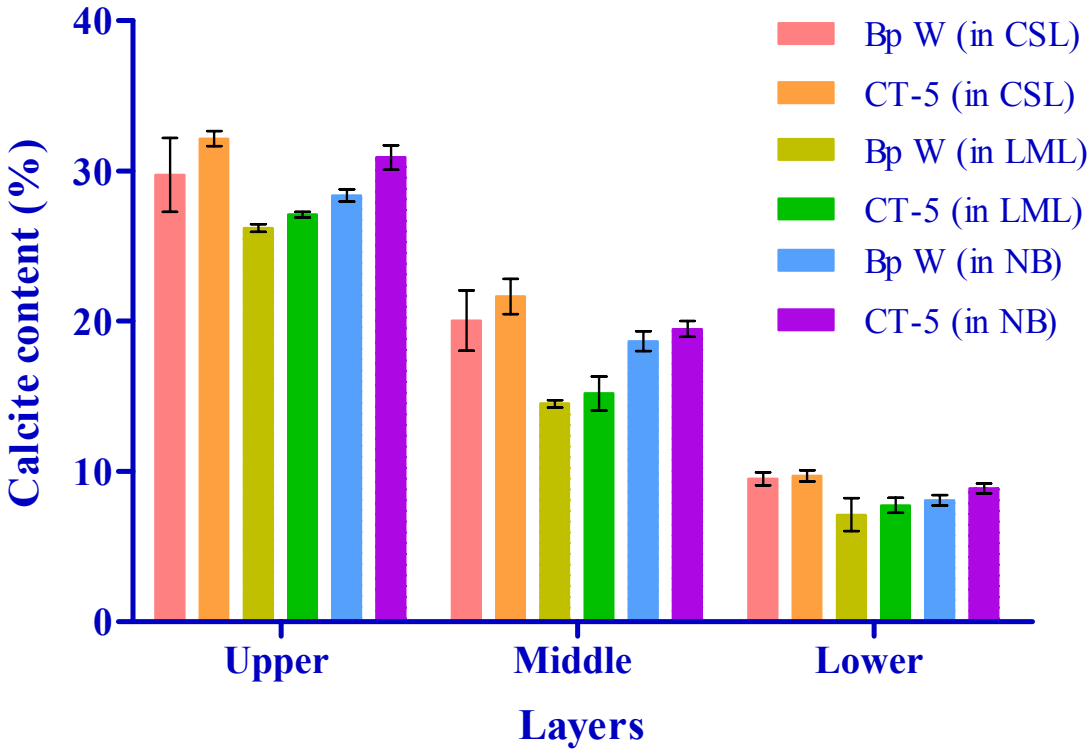

Figure 5.7 Calcite content in the different layers of sand columns consolidated with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) in CSL, LML and nutrient (NB) media. Values are mean ± SD (n =3)

Table 5.3 Comparison of different media for the productions of different parameters by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5)

Parameters	***S. pasteurii* (Bp W)**			***Bacillus* sp. (CT-5)**		
	Nutrient Media	CSL Media	LML Media	Nutrient Media	CSL Media	LML Media
Urease (U/ml)	427.3±0.7b	441.5±7.3a	354.3±7.6c	665.2±2.9ab	675.5±0.8a	428.6±1.2b
Calcite (% upper layer)	27.6±1.1b	29.7±1.5a	26.20±0.3c	30.90±0.8b	32.16±0.5a	27.11±0.2c
Protease (U/ml)	41.7 ± 3.3b	44.2 ± 3.5a	35.6 ± 3.5c	63.7 ± 2.6a	64.7± 4.5a	38.3 ± 0.5b
EPS (nmol/ml)	18.2 ± 1.6b	20.9 ± 1.2a	16.5 ± 0.5c	45.1 ± 1.0b	55.3 ± 3.0a	25.7 ± 1.5c
Biofilm (cfu/mm^2)	283.3±4.1b	294.0±2.0a	270.3±0.6c	352.7±15b	366.3±7.1a	292.7±2.3c

Values bearing different letters in the same row (in different isolates and media) are significant at $P<0.05$. Values are Mean ± SD (n = 3).

5.9 Discussion

In most processes, the medium ingredients are a major cost factor, ranging between 10-60% of the total operating costs (Kristiansen, 2001). Researchers have hitherto used standard nutrient media like nutrient broth to grow the bacteria. The reported nutritional profile for *Bacillus* sp. indicated a high preference for protein-based media (Morsdorf and Kaltwasser, 1989). Process economics reveal that the use of inexpensive with high protein containing industrial by-products such as corn steep liquor (CSL) or lactose mother liquor (LML) can contribute to reduction in the overall production cost. The nutritive growth profile of bacterial isolates confirmed that media containing corn steep liquor (CSL) supported fast growth of *Bacillus* species and maximum urease production. In these terms, this medium was more successful in initiating growth and concrete production by selected isolates than the standard nutrient medium used. Moreover, reaching information about nutritional requirements of these bacteria could help to plan strategies for bioremediation of defects in building materials.

Lactose mother liquor and corn steep liquor has traditionally been regarded as an unwanted by-product of dairy or starch production. While in some countries, these by-products are used as feed to animals, in other countries, it has simply been disposed off in the dairy or starch plant waste water, thereby causing significant environmental problems (Gonzalez, 1996; Friend *et al*., 2004). The results obtained in this work demonstrated that these by-products can be used as a good and inexpensive growth media for *Bacillus* cells, allowing the cells to continuous urease and calcite production. Based on these properties, lactose mother liquor and corn steep liquor provides an interesting alternative nutritive agent suitable for use in microbial concrete production.

Since ". . .growth is the core of bacterial physiology. . ." (Ingraham *et al*., 1983) and since all experiments in cellular regulation are fundamentally physiological, it is essential to understand the growth physiology of the organism under study. The better growth in CSL might be due to the presence of certain nutrients or extra

carbon/nitrogen sources, vitamins and amino acids that were absent in the other formulations (Akhtar *et al*., 1997; Atkinson and Mavutuna, 1983). Amino acids such as isoleucine, arginine, and cystine; and vitamins such as inositol and choline stimulate the growth of bacterial cells and mixtures of these substances have a significant pronounced effect on bacterial growth (Bridson and Brecker, 1970). Such being the case, it is not surprising that there was better growth of bacterial isolates in case of CSL medium. Growth rate of bacteria differed significantly among media. Similarly, there was a significant interaction effect involving bacterial strains and media types. The measurement of the optical density allowed the determination of cell concentration in the media. The growth curve showed that cells started to grow after around three hours of inoculation and exponential phase continued up to 30 hours.

Calcite precipitation occurred predominantly in the areas close to the surface of the sand column. It is mainly due to the fact that facultatively anaerobic *Bacillus* cells grows at a higher rate in the presence of oxygen and consequently induces active precipitation of $CaCO_3$ around the surface area. Bacterial induced calcite precipitation in CSL media was significantly higher compared to standard nutrient media. Besides various nutritional sources, CSL also provides a variety of minerals such as Ca, Mg, P, Na, K, Zn *etc*. (Amartey and Jeffries, 1994) which could nonspecifically induce mineral deposition by providing a nucleation site. Urea and calcium chloride in the media supported microbial growth. Especially, Ca^{2+} is not likely to be utilized by microbial metabolic processes; it rather accumulates outside the cell (Silver *et al*., 1975). The solubility of $CaCO_3$ is a function of pH and affected by ionic strength in the aqueous medium. The growth of isolates appeared to be correlated with pH. The rise in pH causes a precipitation of calcium carbonate which occurs during urea degradation (Dick *et al*., 2006). In medium containing urea and $CaCl_2$, that supports microbial growth, NH_4^+ and Cl^- react with OH^- and H^+. Microbiologically-induced $CaCO_3$ precipitation occurs via far more complicated processes than chemically-induced precipitation. Possible biochemical reactions in

medium containing urea and $CaCl_2$ to precipitate $CaCO_3$ at the cell surface can be summarized as follows (Stocks-Fischer *et al*., 1999):

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

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

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

De Muynck *et al*. (2008) indicated that the type of bacterial culture and medium composition had a profound impact on calcite crystal morphology. Crystal growth can be inhibited or altered by the adsorption of proteins, organic matter or inorganic components to specify crystallographic planes of the growing crystals (Kawaguchi and Decho, 2002; Hammes *et al*., 2003; De Muynck *et al*., 2008). Differences in size and morphology between the different types of calcium source and different types of bacterial culture can also be attributed to the presence of organic matter. Hammes *et al*. (2003) suggested that differences in crystal morphology which are obtained with different bacterial cultures could be due to the level of the actual urease activity. Warren and Ferris (1998) demonstrated that the surfaces of bacteria can be particularly well-suited to act as mineral nucleation templates, providing a mechanism by which the activation energy required for precipitation to occur is lowered. As they demonstrate, under identical system conditions, authigenic precipitation can occur via nucleation and precipitation at the bacteria surface, yet no precipitation occur when no bacteria are present, presumably because the activation energy for bulk or homogeneous precipitation presents a greater barrier for the initiation of the precipitation process. As CSL is rich in ions, they might help bacteria to get nucleation template and calcite precipitation could be enhanced.

5.10 Conclusion

Based on higher urease productions, calcite precipitations, abundant EPS productions and mature biofilm production by bacterial isolates after utilization of CSL media, it can be concluded that such industrial by-product can be successfully used as a nutrient source to produce microbial concrete and expensive laboratory grade

medium can be replaced using these industrial economic medium. LML also served as a good nutrient source for bacterial isolates. This is the first documentation of urease producing isolates living on medium containing LML and CSL. These byproducts are potential environmental hazards. Recycling them as nutrient source has the double benefit of preventing environmental pollution and reduction of the cost of the process. Thus, production of microbial concrete using LML and CSL medium presents an ecological, environmentally friendly alternative and recycling of such industrial effluents provides greener option.

Chapter 6

Evaluation of microbes for remediation of defects in building materials and structures

6.1 Selection of strains

Biomineralization is based on the ability of bacteria to promote the precipitation of carbonates. Promising results of an innovative biotechnology based on microbial carbonate precipitation have lead to research concerning the use of bacteria in or on concrete. A suitable microbial source of urease has been identified and the capacity of the efficient bacteria to produce a high level of urease at an economical cost has been established. It was now necessary to investigate how urease and calcite precipitation performed under biocementation conditions in different building materials such as mortars, concretes and bricks on which structure of a building depends. Due to economic considerations, it was preferable to use the microorganism intact with no processing from cultivation step, preventing the cost of enzyme extraction and maintaining simplicity of the overall process. In the present work, first the criteria for the selection of calcite precipitating *Bacillus* strains were established. *Bacillus* sp. (CT-5) strain isolated from cement, capable of precipitating a dense and coherent calcium carbonate precipitate and concomitantly inducing a reduction of capillary water absorption in sand column, was characterized by a high urease activity, abundant EPS-production and a good biofilm production. Selection of this strain was based upon these criteria and that's why *Bacillus* sp. (CT-5) was further used to study durability aspects of building materials. The efficacy of this strain was also compared with *S. pasteurii* (Bp W) in terms of durability performance of building materials. To make the biocementation process economic, CSL medium was used to grow the cells and compared their performance with nutrient broth media (NB).

6.2 Compressive strength based on MICP

Based upon higher calcite and urease production, *Bacillus* sp. (CT-5) strain was chosen to study compressive strength test of mortar and its efficacy was also compared with *S. pasteurii* (Bp W). Biocementation was clearly visualized on mortars treated with bacterial cells (Fig. 6.1). Figure 6.2 summarizes the 3, 7 and 28 day compressive strength of cement mortar cubes containing *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5). The compressive strength had significantly increased for the mortar cubes that contained microbial cells irrespective of the media used to grow the cells. The highest compressive strength was obtained with mortar cubes that were incubated for long periods (28 days) regardless of media used to incubate or curing. The control mortar specimens were prepared using water instead of bacterial culture. The maximum compressive strength was obtained when mortars along with bacterial cells were cured in CSL media. Mixing of *S. pasteurii* (Bp W) in mortar cubes with CSL media showed 31% improvement in compressive strength at 28 days (30.4 MPa) with respect to control (23.2 MPa); whereas in case of nutrient media, it was 28 MPa. Similarly, mortar cubes prepared with *Bacillus* sp. (CT-5) when grown and cured in CSL media showed 44% improvement in compressive strength at 28 days (33 MPa) with respect to control; whereas in case of nutrient media, it was 31.4 MPa. There was a significant improvement in the strength when bacterial cells were grown in CSL media and also mortars were cured in the same media (Table 6.1).

To see the effects of media on the compressive strength, mortar cubes were prepared with nutrient and CSL media (without addition of bacterial cells). Results were also compared with control, where mortars were prepared with water. No visible calcification was observed when cubes were prepared and cured in any of media. There was not significant difference in the compressive strength values at 3-, 7- and 28-days when mortar cubes were prepared and cured with water, nutrient media or CSL media (Table 6.2 and Fig. 6.3). Media did not attribute towards enhancement of the strength. These results draw a conclusion that it's not media which is enhancing the compressive strength but concrete producing bacteria enhanced the strength.

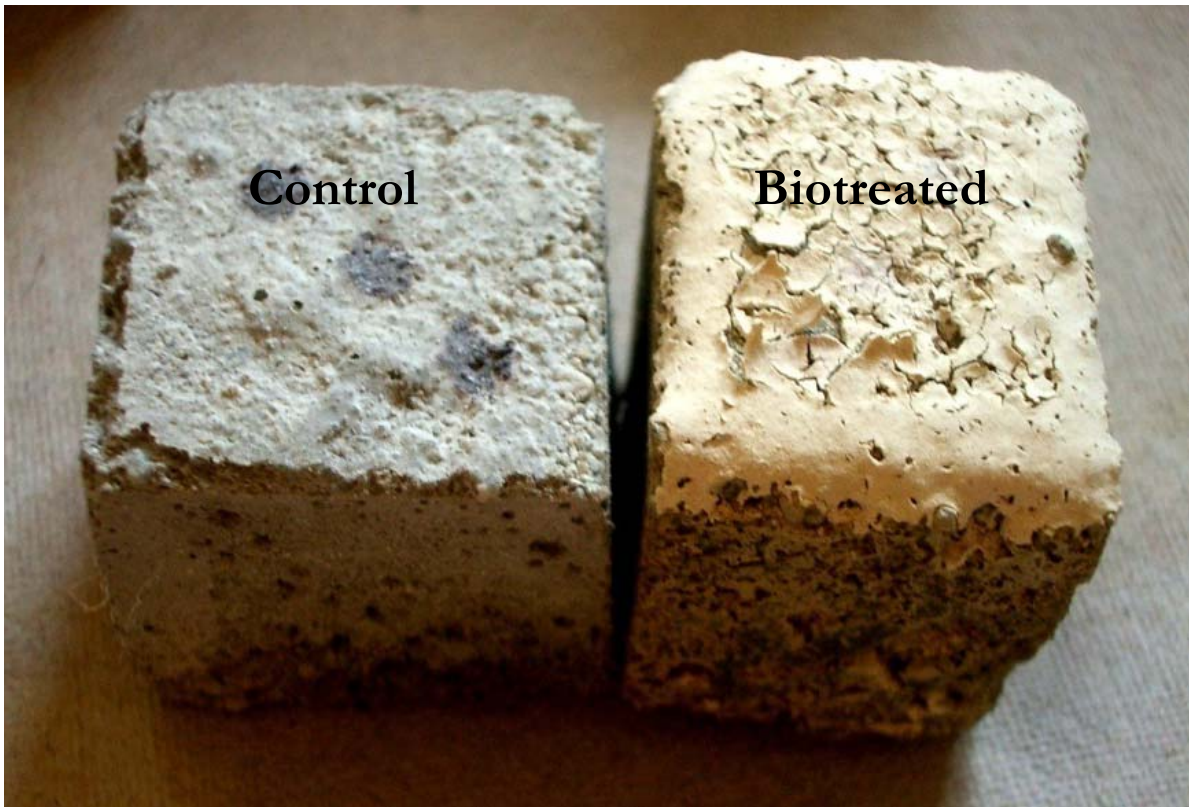

Figure 6.1 Microbial precipitation on the biotreated mortar cube

Table 6.1 Effect of bacterial isolates on the compressive strength of cement mortars at 3-, 7- and 28 days

Media	Compressive Strength (MPa)					
	***S. pasteurii* (Bp W)**			***Bacillus* sp. (CT-5)**		
	3 days	7 days	28 days	3 days	7 days	28 days
Control	8.6±0.14c	12.5±0.06c	23.2±0.17c	8.6±0.14c	12.5±0.05c	23.1±0.13c
NB	8.8±0.07b	14.0±0.20b	27.9±0.29b	9.5±0.08b	15.1±0.18b	31.4±1.30b
CSL	9.5±0.13a	17.0±0.14a	30.4±0.71a	9.8±0.05a	17.4±0.10a	33.4±0.60a

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

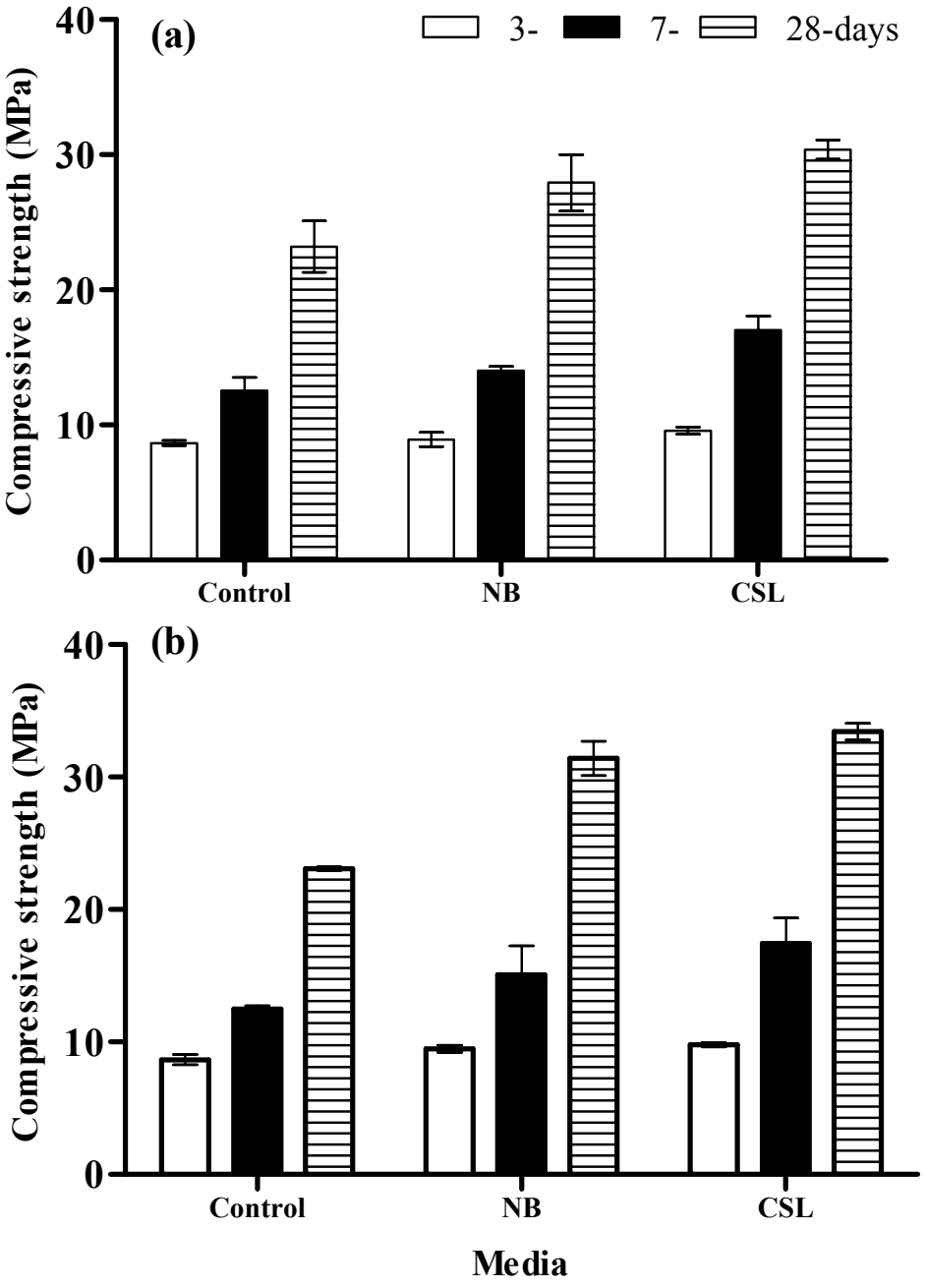

Figure 6.2 Effect of (a) *S. pasteurii* (Bp W) and (b) *Bacillus* sp. (CT-5) grown in nutrient media (NB) and CSL media on the compressive strength of cement mortar cubes cured for 3, 7 and 28 days

Table 6.2 Effect of CSL and NB media on the compressive strength of cement mortars cured for 3-, 7- and 28 days

Media	Compressive Strength (MPa)		
	3 days	7 days	28 days
Control	8.6±0.06a	12.5±0.03a	23.2±0.17a
NB	8.7±0.33a	12.7±0.16a	23.5±0.30a
CSL	8.6±0.32a	12.6±0.30a	23.4±0.36a

Values bearing same letters in the same column are not significant at $P<0.05$. Values are Mean ± SD (n=3).

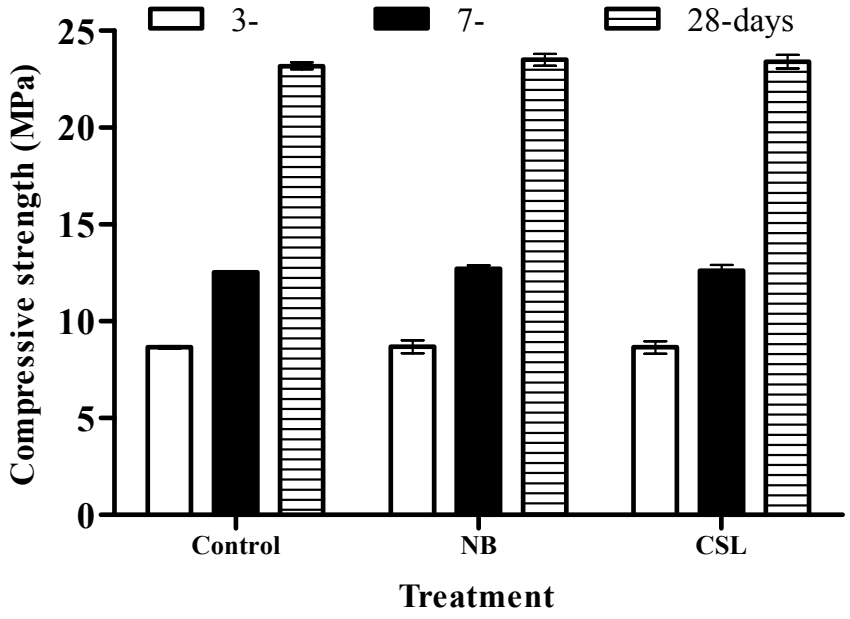

Figure 6.3 Effect of nutrient (NB) and CSL media on the compressive strength of mortar cubes at 3-, 7- and 28 days

6.3 Effects of different concentrations of bacteria on the compressive strength of mortars

This study was aimed at investigating the effect of the variation in the bacterial concentration on the compressive strength and also obtaining an optimum concentration of the bacterial cells for future studies. From the tested microbial concentrations, a concentration of 5×10^7 cells per cm^3 of both *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) has been found to induce the maximum compressive strength when grown and cured in CSL media (Fig. 6.4). The similar concentration of 5×10^7 cells per cm^3 was found to be most effective to increase compressive strength of mortars when cells were grown and mortars were cured in nutrient media (Fig. 6.5).

Changes of the compressive strength values between 3- and 28-day tests of mortar specimens by different concentrations of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) are presented in Tables 6.3 and 6.4. It is clear that live bacterial cells, at lower concentrations, increased the compressive strength of cement mortar with a longer incubation period. The results indicate, however, that the concentration of 5×10^3 cells per cm^3 in strengthening the cement matrix is not significant; although at lower concentrations, bacterial isolates continue to metabolize to induce calcite precipitation during the incubation period. As shown in Figs. 6.4, 6.5 and Tables 6.3, 6.4; the increase of the strength in cubes with both *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) appears to depend on the concentration of bacteria and compressive strength was significantly higher at concentration of 5×10^7 cells per cm^3.

Table 6.3 Compressive strength values of 3-, 7-, 14- and 28- day tests with cement mortars mixed with different concentrations of *S. pasteurii* (Bp W)

Concentration (cells/mm^3)	***S. pasteurii* (Bp W)**							
	Nutrient medium				**CSL medium**			
	Compressive strength (MPa)				**Compressive strength (MPa)**			
	3-day	7-day	14-day	28-day	3-day	7-day	14-day	28-day
0	8.5±0.2c	12.5±0.2d	15.9±0.43d	22.6±0.6e	8.6±0.06c	12.6±0.06d	16.1±0.08d	23.2±0.1d
5×10^3	8.7±0.1b	12.8±0.2c	16.8±0.34c	24.6±0.6b	8.8±0.04c	12.9±0.14c	16.9±0.17c	24.7±0.1c
5×10^5	8.8±0.1b	13.1±0.1b	17.4±0.18b	24.8±1.0b	9.0±0.06b	13.4±0.19b	17.9±0.12b	25.5±0.5b
5×10^7	9.3±0.2a	14.2±0.4a	19.2±0.92a	28.6±0.7a	9.5±0.14a	14.7±0.09a	20.2±0.29a	29.2±0.2a
5×10^8	8.5±0.1c	12.7±0.3c	16.1±0.4d	23.9±0.5d	8.6±0.1c	12.8±0.3c	16.6±0.5d	23.6±1.6d

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

Table 6.4 Compressive strength values of 3-, 7-, 14- and 28- day tests with cement mortars mixed with different concentrations of *Bacillus* sp. (CT-5)

Concentration (cells/mm^3)	***Bacillus* sp. (CT-5)**							
	Nutrient medium				**CSL medium**			
	Compressive strength (MPa)				**Compressive strength (MPa)**			
	3-day	7-day	14-day	28-day	3-day	7-day	14-day	28-day
0	8.5±0.12c	12.4±0.17d	15.9±0.4d	22.6±0.6d	8.6±0.1d	12.6±0.1d	16.2±0.1d	23.2±0.1d
5×10^3	8.9±0.19b	13.3±0.20c	16.9±0.5c	24.7±1.0bc	9.0±0.2c	13.6±0.1c	17.1±0.1c	24.9±0.9c
5×10^5	9.1±0.20b	15.1±0.61b	18.8±0.5b	25.0±0.4b	9.3±0.1b	15.5±0.5b	19.2±0.2b	27.8±0.8b
5×10^7	9.5±0.08a	15.8±0.50a	21.6±1.6a	32.1±0.5a	9.7±0.1a	17.4±0.1a	22.5±0.5a	33.8±0.3a
5×10^8	8.5±0.30c	12.8±0.3d	16.6±0.5c	24.2±1.1c	8.8±0.4d	13.1±0.6de	16.8±0.6d	24.3±1.4cd

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

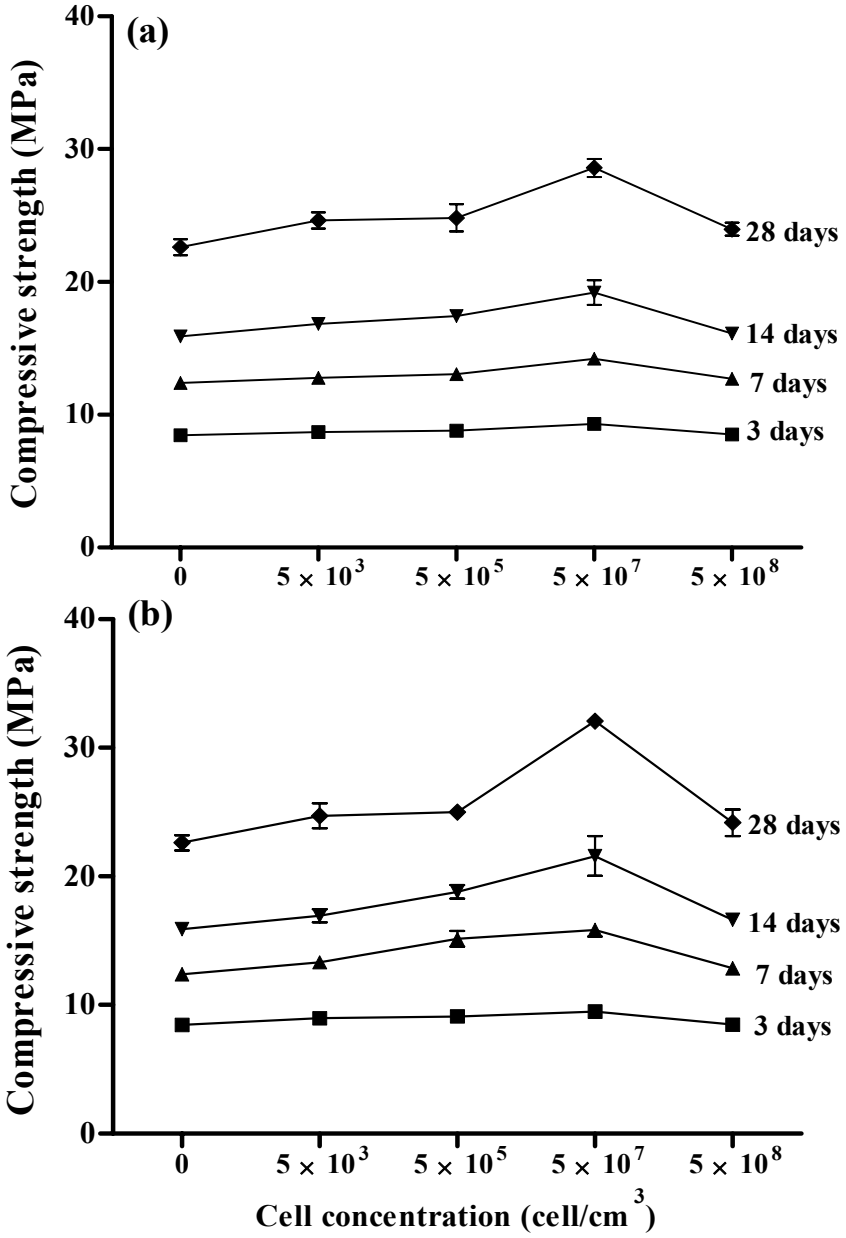

Figure 6.4 Effect of different concentrations of (a) *S. pasteurii* (Bp W) and (b) *Bacillus* sp. (CT-5) grown and cured in nutrient media, on the compressive strength of mortar cubes

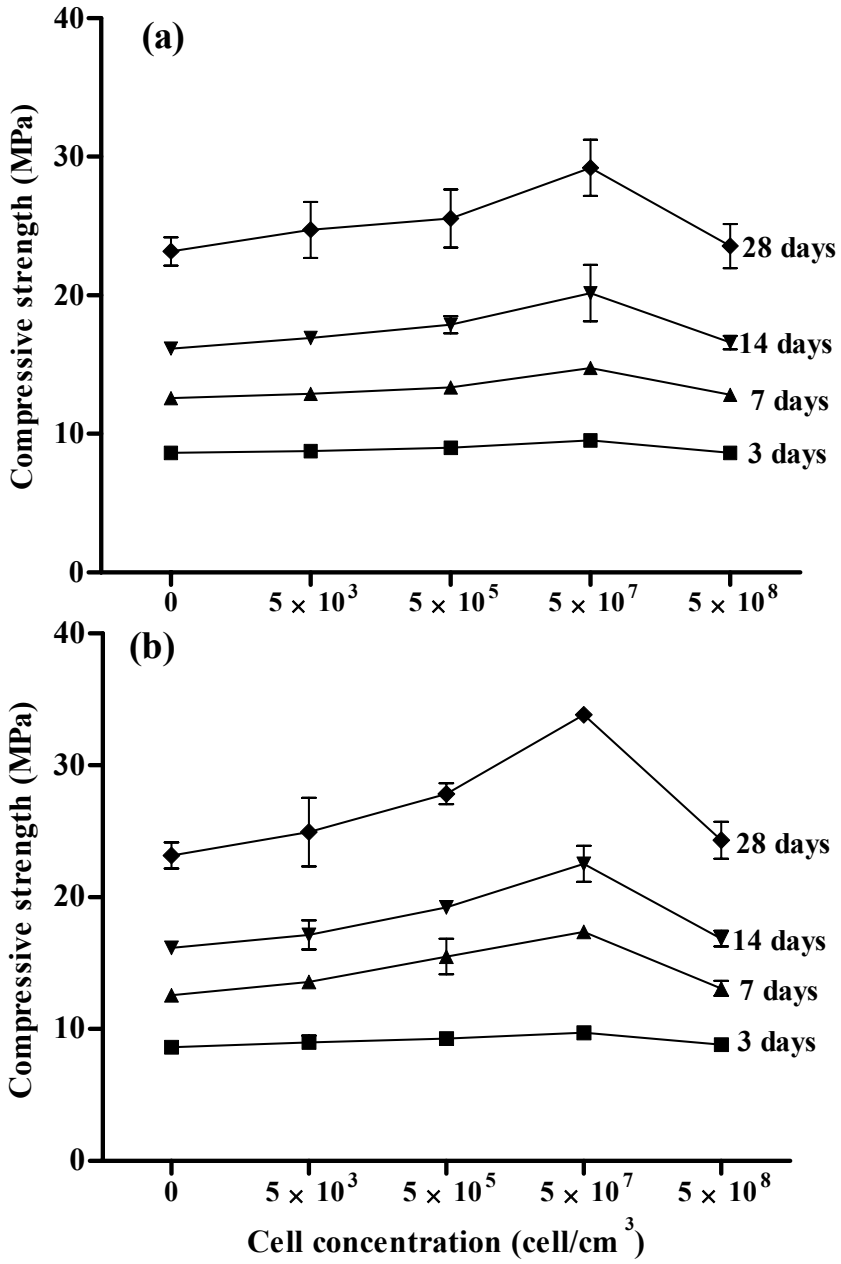

Figure 6.5 Effect of different concentrations of (a) *S. pasteurii* (Bp W) and (b) *Bacillus* sp. (CT-5) grown and cured in CSL media, on the compressive strength of mortar cubes

6.4 Effects of different concentrations of bacteria used for crack remediation on compressive strength of Portland cement mortar cubes

Compressive strengths of simulated cement mortar cubes with cracks of different depths were tested to determine the efficiency of improvement by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5). CSL medium was used to grow the cells and as curing media. Cracks were repaired with sand + bacteria. The compressive strength had significantly increased for the mortar cubes that contained microbial cells irrespective of the concentrations of cells used but a concentration of 5×10^7 cells per mm^3 was found to induce the maximum compressive strength. As expected, the overall compressive strength of Portland cement mortar cubes decreased with an increase in the crack depth, whether micro-organisms were present or not. Similar kinds of observations were reported by Ramachandran *et al*. (2001).

Differences of the test values between controls containing no cells and cell-laden crack specimens are depicted in Figures 6.6 and 6.7 by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) respectively. An increase of the strength in the presence of *Bacillus* sp. (CT-5) was most significant in the cubes prepared with 13.4 mm crack, whose microbial remediation increased the compressive strength by 40% of that of the control. Microbial remediation increased the compressive strength of deepest crack (27.2 mm) by 37% at 28 days. Irrespective of crack width, compressive strength significantly improved at 7- and 28-days when bioremediated with 5×10^7 cells per mm^3 of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) (Tables 6.5 and 6.6).

To determine whether the increase in compressive strength of the specimens with bacteria and sand in their cracks could be attributed to the microbial calcite precipitation, the crack

samples with the highest strength values were examined under microscopy. A clear calcite precipitation was found on the crack remediated area (Fig. 6.8). SEM examinations were made on the broken samples collected from the mortar cubes tested at 28 days that showed calcite precipitation on crack remediated specimens along with bacterial cells (Fig. 6.9). The sample taken from the area close to the surface showed calcite crystals grown all over the sand particles and precipitated with rod shaped bacteria. SEM indicated that $CaCO_3$ crystals were precipitated on crack surface, where sands were consolidated and cemented by $CaCO_3$ crystals resulting in to increase in the compressive strength. On closer observation of the area containing dense calcite precipitation, it was found that the calcium carbonate crystals were well developed near the surface of the crack. They had distinct and sharp edges, indicating a full growth of the crystals. Interior area of cracks showed lesser calcite precipitation and bacterial presence was also scarce. As noticed in the Figure 6.9, calcite crystals are seen with rod-shaped holes, which are presumably the space occupied by the bacteria inside the most interior area. It was expected that there will be little calcite precipitation inside the interior part of cracks as these bacteria are facultative anaerobic bacteria, grows better in the presence of oxygen which was available near the top surface.

Table 6.5 Compressive strength values of cement mortar cubes with cracks remediated with various concentrations (cells/cm^3) of *S. pasteurii* (Bp W)

Crack depth (mm)	7 Days				28 Days			
	0	5×10^6 cells	5×10^7 cells	5×10^8 cells	0	5×10^6 cells	5×10^7 cells	5×10^8 cells
13.4	12.3±1.31c	12.8±1.46c	13.9±1.20a	13.4±0.44ab	22.1±1.4c	25.1±0.15b	28.6±0.84a	27.1±0.71ab
18.8	12.0±1.23d	12.5±0.17bc	13.1±0.36a	12.7±0.25b	21.5±0.50c	23.5±0.62b	27.6±0.57a	26.8±0.73ab
27.2	11.9±1.04b	11.9±0.18b	12.4±0.42a	12.3±0.53a	20.4±0.40b	20.2±1.40b	26.7±0.74a	25.9±0.61ab

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

Table 6.6 Compressive strength values of cement mortar cubes with cracks remediated with various concentrations (cells/cm^3) of *Bacillus* sp. (CT-5)

Crack depth (mm)	7 Days				28 Days			
	0	5×10^6 cells	5×10^7 cells	5×10^8 cells	0	5×10^6 cells	5×10^7 cells	5×10^8 cells
13.4	12.4±0.31c	13.1±0.30b	14.7±0.39a	13.7±0.30b	22.1±0.95d	26.4±1.25c	30.9±0.75a	29.2±0.32b
18.8	12.0±0.2bc	12.6±0.16b	13.7±0.25a	13.0±0.20ab	21.5±0.50d	24.3±0.70c	30.1±0.31a	28.6±0.35b
27.2	11.9±2.04b	12.2±1.1c	13.0±0.20a	12.6±0.13ab	20.4±0.40d	22.8±0.75c	28.3±0.35a	26.7±0.42b

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

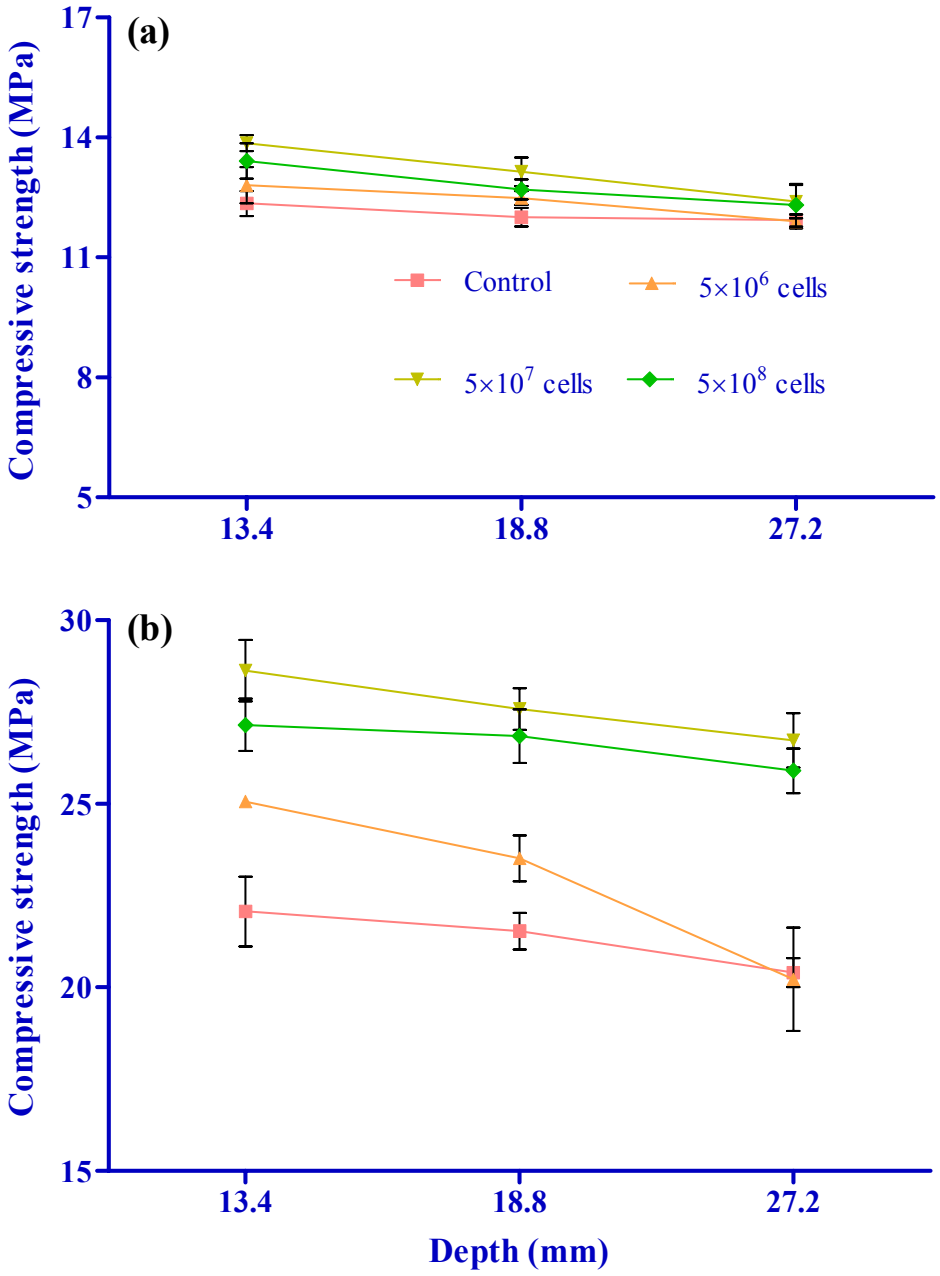

Figure 6.6 Compressive strengths of mortar cubes at (a) 7 days and (b) 28 days with cracks filled with various concentrations of *S. pasteurii* (Bp W)

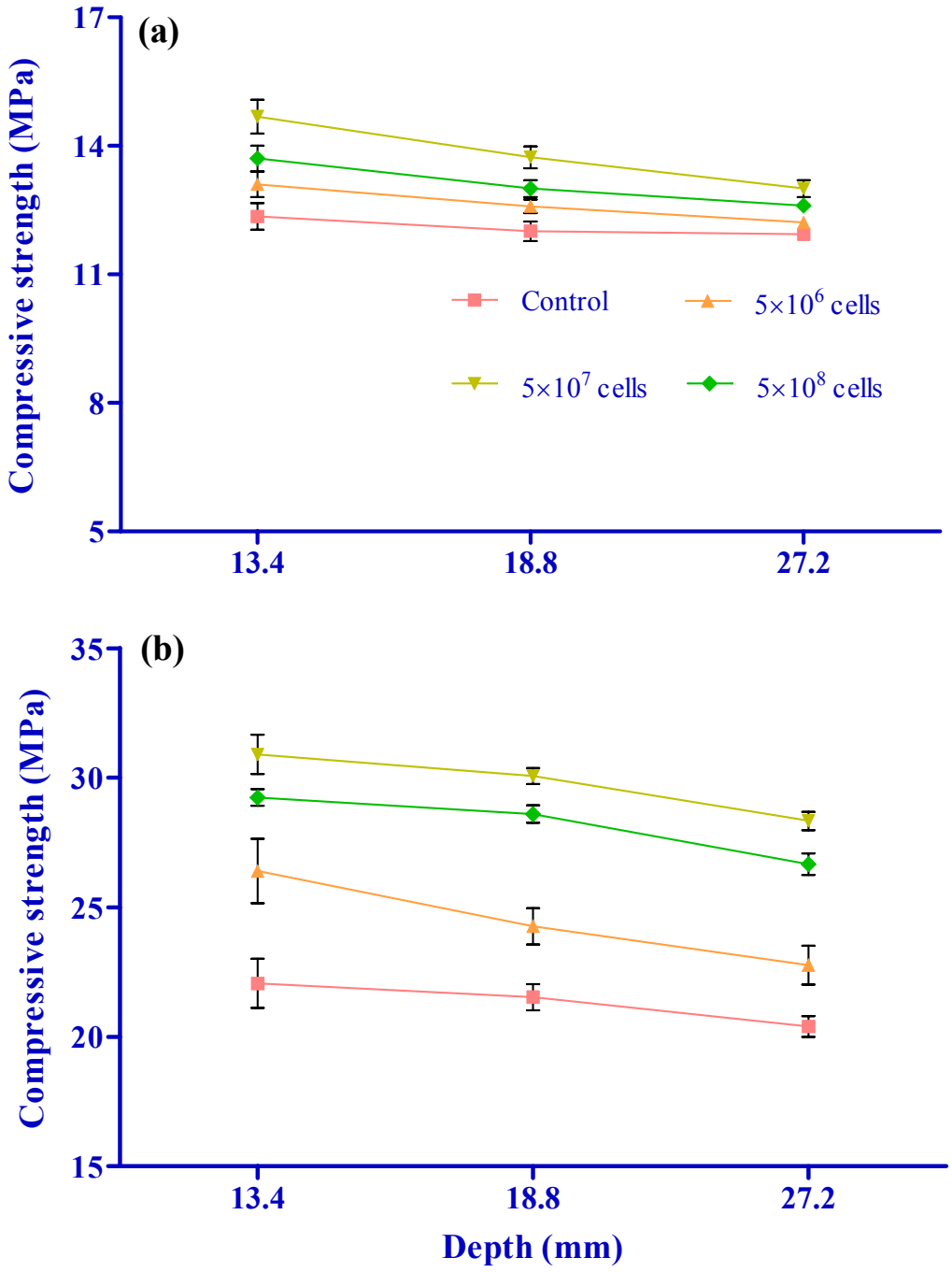

Figure 6.7 Compressive strengths of mortar cubes at (a) 7 days and (b) 28 days with cracks filled with various concentrations of *Bacillus* sp. (CT-5)

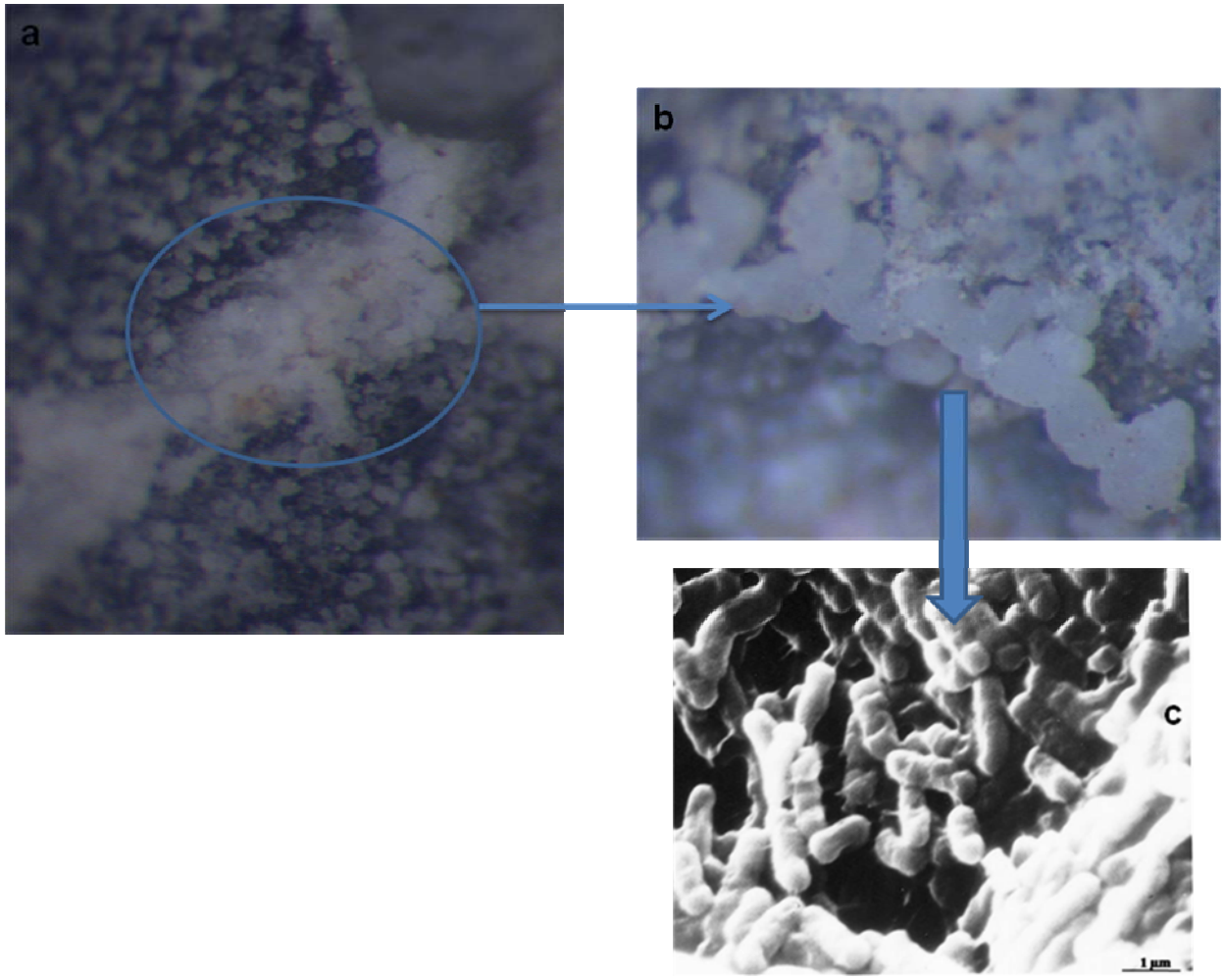

Figure 6.8 (a) Microscopic image of crack remediated area, **(b)** enlarged portion of crack remediated area showing calcite precipitation and **(c)** electron micrograph showing rod shaped bacteria embedded in crack remediated area

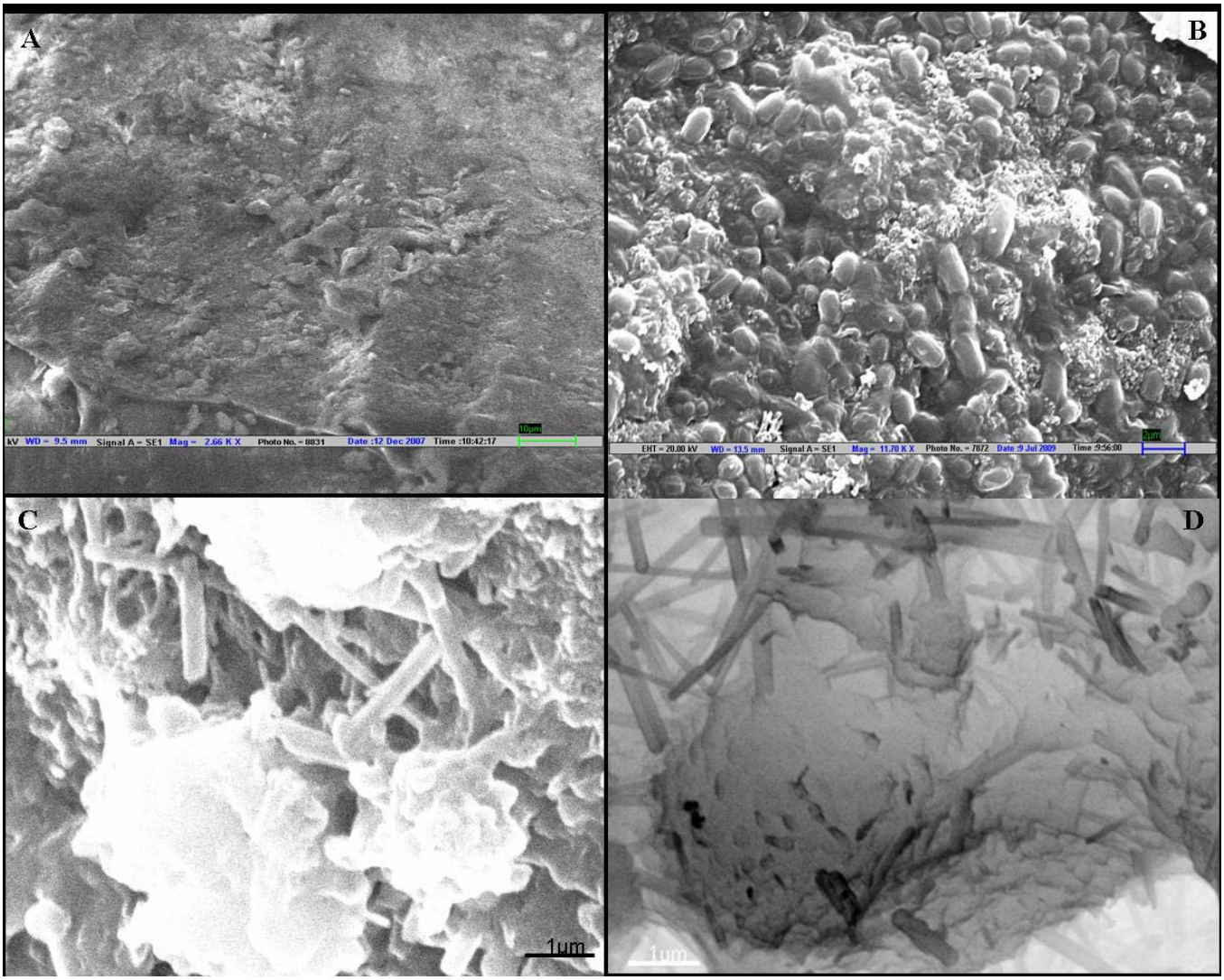

Figure 6.9 Scanning electron micrographs showing microbiological calcite precipitation in concrete cracks: (A) matrix of cement mortar prepared without bacteria; (B) sample taken near surface area of remediated crack, showing dense calcite precipitation between and on surface of sand particles showing calcite crystals with rod shaped *Bacillus* sp. (CT-5); (C) sample taken from interior area of concrete cracks showing calcite precipitation; and (D) sample taken from most interior of crack showing calcite precipitation with few rod empty holes housed by bacterial cells

6.5 Water absorption and total porosity

The decrease in permeability of mortar specimens treated with bacteria could be seen from the water absorption test. Fig. 6.10 shows the influence of the calcite precipitation on the mortar surface and the water absorption rate for mortar cubes. Over a period of 168 hours the cubes treated with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) in CSL media, absorbed nearly six times less water than the control cubes. The presence of bacteria resulted in a significant decrease of the water uptake compared to untreated specimens (control). As a result of the biomineralization, specimens showed significantly less water absorption than untreated specimens (Table 6.7).

Figure 6.11 presents the total porosity measurements for all mortar specimens. There was significant difference with specimens prepared with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) compared with control although *Bacillus* sp. (CT-5) was able to develop more cement-pore matrix. More than 50% reduction in the porosity was found when mortar specimens prepared using *Bacillus* sp. (CT-5). The decrease in permeability of mortar specimens treated with bacteria could be seen from the water absorption and porosity experiment. The deposition of a layer of calcium carbonate crystals on the surface resulted in a decrease of the permeation properties. Also the ability of *Bacillus* sp. (CT-5) to ferment maximum numbers of carbon substrates might be a reason to survive well on the top surface.

Table 6.7 Effect of bacterial [*S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5)] treatment on the rate of water absorption by cement mortars at different time intervals

Treatment	Time (hrs)								
	1	4	8	24	36	48	120	144	168
Control	0.4±1.1a	1.7±0.8a	2.6±1.6a	3.5±1.3a	3.8±0.9a	4.0±0.5a	4.4±0.3a	4.8±1.2a	4.9±0.1a
Bp W	0.3±0.2b	0.4±0.2b	0.5±0.1b	0.6±0.2b	0.6±0.1b	0.7±0.2b	0.8±0.1b	0.8±0.2b	0.9±0.2b
CT-5	0.2±0.4c	0.3±0.1c	0.5±0.1b	0.5±0.1c	0.6±0.1b	0.6±0.2b	0.7±0.1c	0.7±0.2c	0.8±0.1b

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

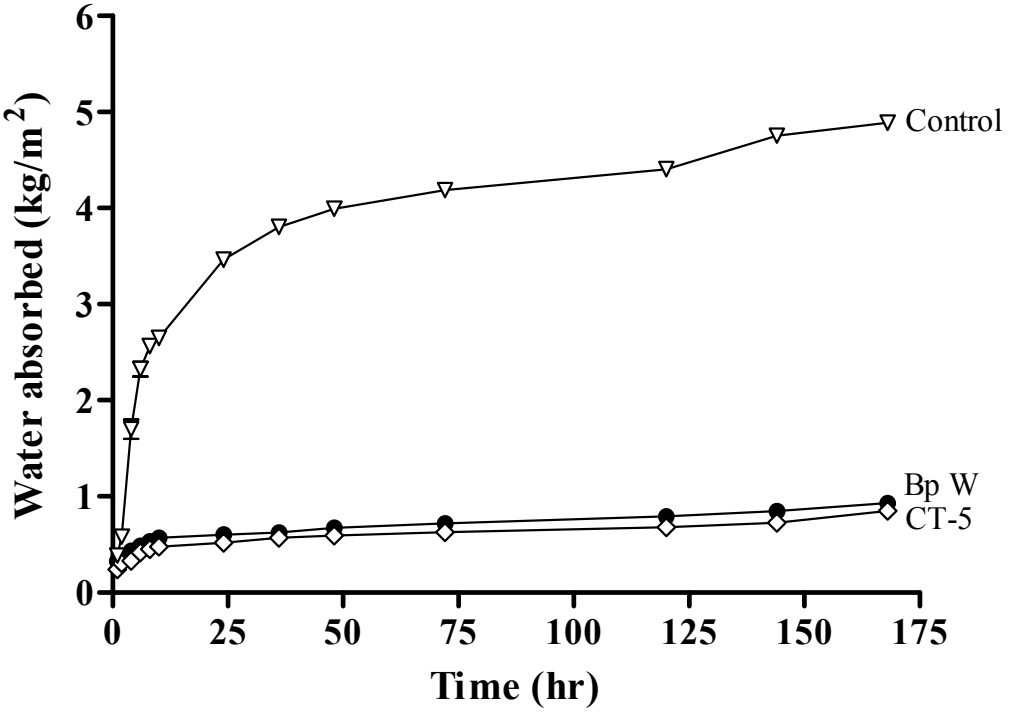

Figure 6.10 The influence of MICP on the rate of water absorption versus time for mortar cubes prepared with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5)

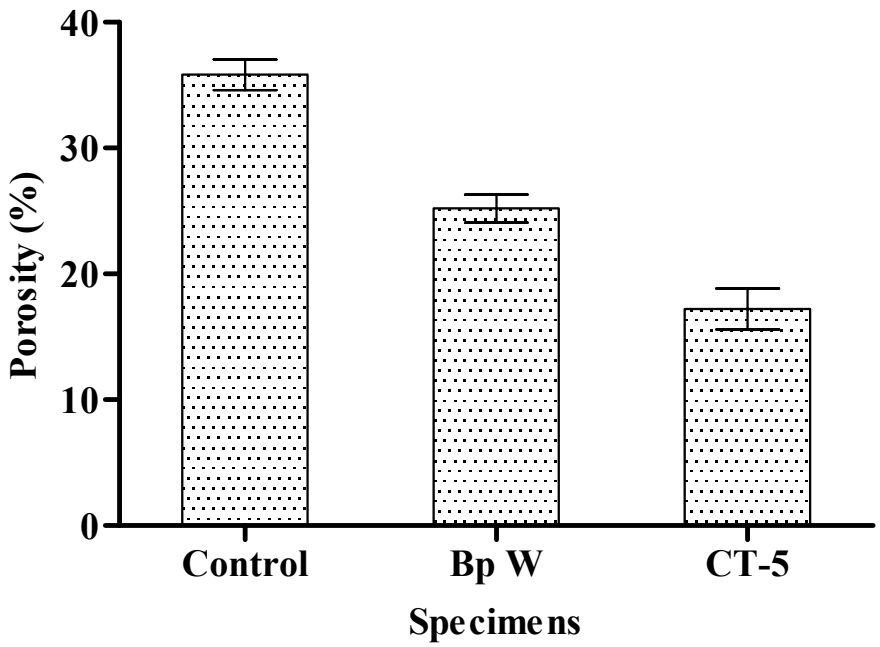

Figure 6.11 Total porosity measurement of control mortar specimens and their comparison with those prepared with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5)

6.6 Water Impermeability Test

6.6.1 Impermeability Evaluation of Control Specimen and Concrete prepared with bacterial cells in nutrient and CSL media

The concrete cubes of dimension 150 mm^3 were cast in water for control specimen. The test was carried out as per DIN 1048 standard. The average penetration and side penetration for control sample was 32.67 mm and 40 mm respectively. To study the microbial induced calcite precipitation, concrete cubes were cast and cured in nutrient and CSL media with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5). There was reduction in depth of penetration of water in the cube when casted and cured in nutrient media with bacterial cells. The average

water penetration was 12.33 mm and average side penetration was 27.33 mm when specimens were prepared with *S. pasteurii* (Bp W), both grown and cured in nutrient media; while average water penetration and side penetration was 11 mm and 25.33 mm respectively with same cells, both grown and cured in CSL media (Table 6.8). When concrete specimens were prepared with *Bacillus* sp. (CT-5), it provided more resistance to water permeability compared to *S. pasteurii* (Bp W) irrespective to media used. The average water penetration was 9 mm and average side penetration was 22.67 mm when specimens were prepared with *Bacillus* sp. (CT-5), both grown and cured in nutrient media; while average water penetration and side penetration was 5.67 mm and 13.67 mm respectively with same cells, both grown and cured in CSL media (Table 6.8).

The side penetration of water was significantly more than vertical penetration in all specimens prepared using bacterial cells. There was more calcite precipitation on the top surface of concrete cubes compared to side wall, when prepared using *S. pasteurii* (Bp W) or *Bacillus* sp. (CT-5), might be reason of low vertical water penetration. When cubes were casted in CSL medium, it showed better water impermeability as compared to cubes casted in nutrient medium irrespective of cells used to prepare specimens (Fig. 6.12). These results clearly demonstrate more calcite precipitation by *Bacillus* sp. (CT-5) when grown in CSL medium.

Table 6.8 Permeability results for samples prepared with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) in nutrient (NB) and CSL media

Sample	Penetration (mm)	Side penetration (mm)
Control	32.7±2.52a	40.0±3.00a
Bp W (NB)	12.3±2.08b	27.3±3.51b
Bp W (CSL)	11.0±2.00bc	25.3±3.06b
CT-5 (NB)	9.0±2.00c	22.7±3.06c
CT-5 (CSL)	5.7±1.53d	13.7±2.08d

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

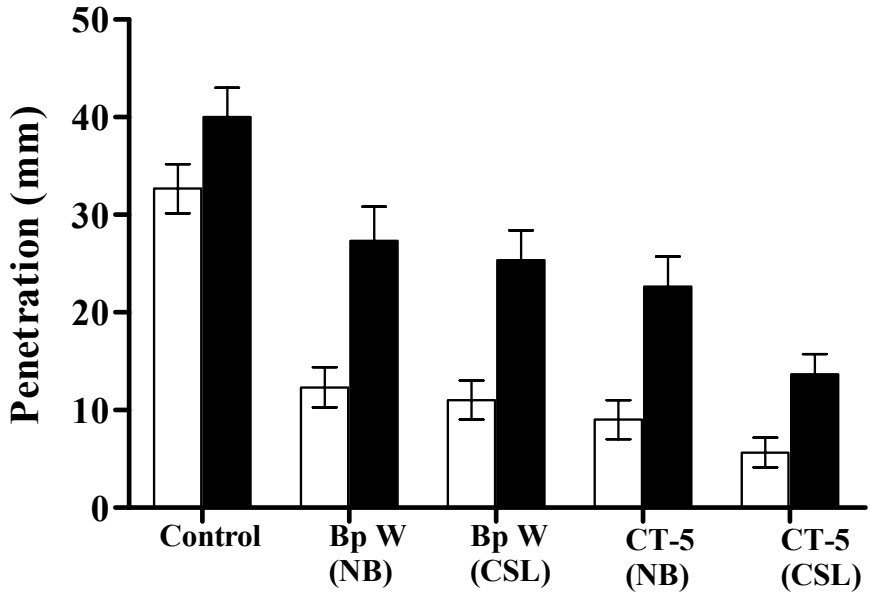

Figure 6.12 Water permeability in terms of side penetration (filled bar) and vertical penetration (open bar) shown by concrete specimens prepared with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) in nutrient (NB) and CSL media and comparison with control specimen

From these results it was clear that the permeability reduces substantially as compared to that in control samples. The average penetration in the bacterial samples was nearly six times lower compared to control, due to microbially induced calcite precipitation (MICP). Thus it is established that MICP effectively impedes percolation of water in concrete. On visual inspection it was observed that the amount of precipitation was maximum in CSL medium, a thick deposit was formed on top of the cubes. This might be due to high nutrient content in CSL that leads to higher growth of microbes. Same extent of reduction was not observed when the nutrient medium was used. However, such a trend was not observed in permeability in lateral direction. It was obvious that deposition on side was much less compared to top and bottom surface of the cubes.

Reduction of penetration was observed due to microbial deposition in concrete although the extent of reduction depends on medium used also. Here media containing CSL was more efficient than nutrient media thus provides an economic option towards impermeability evaluation by bacterial cells. As the calcite deposition was taking place in the pores of the concrete, naturally it sealed the path of water ingress. MICP lead to reducing the voids near the surface and ultimately, the properties of concrete improved.

6.7 Microbial calcite deposition in bricks

As the bricks are one of the most commonly used building materials, an attempt was also made to check calcite deposition and to draw a conclusion whether MICP lead to increase in compressive strength and decrease in water absorption of bricks. Microbial metabolic activities often contribute to selective cementation by producing relatively insoluble organic and inorganic compounds. Calcite precipitation is one such process that is induced due to microbial metabolic activities. Urease plays a key role in the deposition of the calcite in the form of precipitate. A layer of white deposit was clearly observed on the surface of the bricks due to microbial precipitation but there was no such deposition on control brick (Fig. 6.13). Brick samples were analyzed for their water absorption capacity and compressive strength, and the results are depicted in Table 6.9. When bricks were incubated with *Bacillus* sp. (CT-5) grown and cured in CSL medium, it resulted in 46.5% decrease in water

absorption. It is clear that more reduction in water absorption was found in CSL medium compared to nutrient medium. Maximum compressive strength was shown by *Bacillus* sp. (CT-5) grown in CSL medium (13.61 MPa). Microbiologically induced calcite precipitation by *Bacillus* sp. (CT-5) in CSL medium was found effective in increasing brick strength by reducing water absorption up to 46.5% and increased compressive strength by 39.5%. Also *Bacillus* sp. (CT-5) was more effective in calcite deposition compared to *S. pasteurii* (Bp W). It can be seen that the absorption of water by bricks when cured in media with grown bacterial cells was lower than that by a control sample, indicating that pores on the surface are blocked because of calcite deposition, thus preventing water and other pollutants from penetrating into the body of bricks ultimately increased the compressive strength. Thus, this process of bio-mineralization retarding the water absorption and increasing compressive strength in bricks may be effective in enhancing the durability and longevity of such civil structures, and it may be of immense help in the preservation of monumental structures constructed with bricks (Tiano *et al*., 1999).

Figure 6.13 Microbial induced calcite deposition on bricks. (a) Control, (b) MICP by *S. pasteurii* (Bp W) and (c) MICP by *Bacillus* sp. (CT-5)

Table 6.9 Effect of MICP on permeation properties and compressive strength test of bricks

Brick Samples Treatment	**% Water Absorption**	**% Reduction in Water Absorption**	**Compressive Strength (MPa)**	**% Increase in Compressive Strength**
Control	26.5±0.90	--	9.8±0.54	--
CT-5 (in NB)	19.0±1.24	28.4±1.5b	12.3±0.65	26.5±1.24b
CT-5 (in CSL)	14.2±0.91	46.5±1.6a	13.6±0.72	39.5±1.24a
Bp W (in NB)	21.6±1.12	18.6±1.8c	11.2±0.31	14.5±1.24d
Bp W (in CSL)	19.1±1.25	28.1±1.9b	11.6±0.29	19.3±1.24c

Values bearing different letters in the same column (in case of % Reduction in Water Absorption and % Increase in Compressive Strength) are significant at $P<0.05$. Values are Mean ± SD (n=3).

6.8 Rapid Chloride Permeability Test

Chlorides penetrate crack-free concrete by a variety of mechanisms: capillary absorption, hydrostatic pressure, diffusion, and evaporative transport. Of these, diffusion is predominant. Diffusion occurs when the concentration of chloride on the outside of the concrete member is greater than on the inside. This results in chloride ions moving through the concrete to the level of the rebar. When this occurs in combination with wetting and drying cycles and in the presence of oxygen, conditions are right for reinforcement corrosion. A second mechanism for chloride ingress is permeation, driven by pressure gradients. If there is an applied hydraulic head on one face of the concrete and chlorides are present, they may permeate into the concrete through a more common transport method *i.e*, absorption. As a concrete surface is exposed to the environment, it will undergo wetting and drying cycles. When water (possibly containing chlorides) encounters a dry surface, it will be drawn into the pore structure though capillary suction. Absorption is driven by moisture gradients. Typically, the depth of drying is small, however, and this transport mechanism will not, by itself, bring chlorides to the level of the reinforcing steel unless the concrete is of extremely poor quality and the reinforcing steel is shallow. The rate of chloride ion ingress into concrete is primarily dependent on the internal pore structure. The pore structure depends on other factors such as the mix design, degree of hydration, curing conditions, use of supplementary cementitious materials, and construction practices.

Therefore, wherever there is a potential risk of chloride-induced corrosion, the concrete should be evaluated for chloride permeability. For specification and quality-control purposes, such test is preferred that is simple to conduct and that can be performed in a short

time. The rapid chloride permeability test meets these goals. This test, originally developed by Whiting (1981), is commonly referred to as the "Rapid Chloride Permeability Test" (RCPT). This test is based on the electrical conductivity of concrete. The concrete sample is subjected to a potential difference of 60 V and the total charge passing through sample at the end of 6 hrs is measured and expressed in terms of Coulombs. A reduction in this total charge value indicates the better resistance to chloride ion penetration and lower permeability.

The permeability of concrete depends on the pore structure of concrete, while electrical conductivity or resistivity of concrete is determined by both pore structure and the chemistry of pore solution. The most efficient bacterial isolate, based on highest urease and calcite production, *i.e.*, *Bacillus* sp. (CT-5) was selected for RCPT analysis. The ASTM C1202-05 (ASTM, 2005) RCPT results of cylindrical concrete samples prepared without using bacterial cells are shown in Table 6.10 and compared with that prepared with *Bacillus* sp. (CT-5) grown in nutrient and CSL media (Tables 6.11 and 6.12). Similar kind of current profile was found when cylindrical concrete samples were prepared with *S. pasteurii* (Bp W) grown in nutrient and CSL media (data not shown). For each RCPT experiment, charges were passed through three specimens and measured the current.

Table 6.10 Charge passed (mA) through control cylindrical concrete specimens based on RCPT results, in triplicate

Time	Charge passed (mA)			Time	Charge passed (mA)			Time	Charge passed (mA)			Time	Charge passed (mA)		
(hr)	1	2	3	(hr)	1	2	3	(hr)	1	2	3	(hr)	1	2	3
00:05	106.7	151.8	168.1	**01:35**	143.2	160.4	169.0	**03:05**	152.6	168.3	180.3	**04:35**	154.9	168.9	173.7
00:10	111.3	154.6	170.9	**01:40**	143.7	161.4	170.3	**03:10**	152.5	168.5	180.7	**04:40**	155.2	168.2	174.1
00:15	109.5	145.8	158.5	**01:45**	144.2	162.0	171.2	**03:15**	152.7	168.5	180.5	**04:45**	155.5	168.0	173.4
00:20	108.7	143.3	153.5	**01:50**	144.5	162.9	172.4	**03:20**	153.3	168.4	177.2	**04:50**	155.5	168.3	174.5
00:25	109.5	142.2	154.5	**01:55**	144.8	163.7	173.0	**03:25**	153.5	168.4	176.4	**04:55**	155.5	168.1	171.9
00:30	112.1	140.3	149.9	**02:00**	145.3	164.3	173.9	**03:30**	153.6	168.4	177.4	**05:00**	155.5	168.4	174.3
00:35	114.6	141.6	147.1	**02:05**	146.4	165.2	174.3	**03:35**	153.5	168.5	175.2	**05:05**	155.4	168.1	173.5
00:40	117.4	143.3	149.9	**02:10**	147.3	165.7	175.4	**03:40**	153.9	168.4	176.0	**05:10**	155.8	167.5	172.6
00:45	120.6	145.4	152.0	**02:15**	148.3	165.9	176.3	**03:45**	153.8	168.3	175.3	**05:15**	156.3	169.0	172.8
00:50	123.7	147.0	153.6	**02:20**	148.8	166.3	177.1	**03:50**	153.8	168.2	174.6	**05:20**	156.5	170.4	172.4
00:55	127.0	148.6	156.0	**02:25**	149.3	166.5	177.8	**03:55**	153.9	168.2	174.6	**05:25**	156.6	170.4	173.2
01:00	130.3	150.7	157.9	**02:30**	150.1	167.1	178.8	**04:00**	154.0	167.7	174.9	**05:30**	156.8	170.5	172.6
01:05	133.1	152.1	159.7	**02:35**	150.3	167.5	178.8	**04:05**	154.1	168.1	173.8	**05:35**	156.8	170.6	171.4
01:10	135.7	153.6	161.0	**02:40**	150.7	167.7	178.8	**04:10**	154.4	168.0	174.1	**05:40**	156.8	170.7	175.2
01:15	137.9	154.8	162.6	**02:45**	150.5	167.9	178.6	**04:15**	154.6	168.2	173.6	**05:45**	156.9	170.8	173.4
01:20	139.8	156.4	163.7	**02:50**	150.6	168.1	179.8	**04:20**	154.7	168.3	173.1	**05:50**	156.9	170.5	173.9
01:25	141.4	157.7	162.8	**02:55**	151.0	168.3	179.5	**04:25**	154.9	168.3	175.5	**05:55**	157.0	170.2	171.0
01:30	142.7	159.0	167.0	**03:00**	152.0	168.2	179.2	**04:30**	154.6	168.4	172.5	**06:00**	157.3	170.4	172.1

Table 6.11 Charge passed (mA) through cylindrical concrete specimens prepared with *Bacillus* sp. (CT-5) in Nutrient medium based on RCPT results, in triplicate

Time	Charge passed (mA)			Time	Charge passed (mA)			Time	Charge passed (mA)			Time	Charge passed (mA)		
(hr)	1	2	3	(hr)	1	2	3	(hr)	1	2	3	(hr)	1	2	3
00:05	41.0	44.7	12.7	**01:35**	46.8	41.1	54.4	**03:05**	54.8	42.0	54.2	**04:35**	55.7	42.4	55.3
00:10	40.9	42.1	58.7	**01:40**	47.1	41.1	53.9	**03:10**	54.9	41.9	54.0	**04:40**	55.8	42.4	55.2
00:15	41.1	41.2	58.2	**01:45**	47.5	41.1	55.0	**03:15**	54.9	42.0	54.0	**04:45**	55.8	42.5	55.3
00:20	41.4	41.2	58.6	**01:50**	48.0	41.2	54.9	**03:20**	55.1	42.3	54.0	**04:50**	55.8	42.5	52.3
00:25	41.5	41.2	57.3	**01:55**	48.4	41.3	55.1	**03:25**	55.2	42.2	54.2	**04:55**	55.8	42.6	50.9
00:30	41.9	41.1	56.0	**02:00**	49.3	41.3	55.2	**03:30**	55.4	42.3	54.3	**05:00**	55.7	42.5	51.0
00:35	42.3	41.1	55.4	**02:05**	50.0	41.4	55.3	**03:35**	55.5	42.2	54.4	**05:05**	55.7	42.6	50.9
00:40	42.8	41.1	54.6	**02:10**	50.5	41.5	55.0	**03:40**	55.5	42.2	54.5	**05:10**	55.7	42.7	51.5
00:45	43.2	41.0	54.3	**02:15**	51.1	41.7	52.8	**03:45**	55.5	42.2	54.6	**05:15**	55.7	42.7	50.6
00:50	43.6	40.9	54.3	**02:20**	51.7	41.7	53.3	**03:50**	55.6	42.3	54.8	**05:20**	55.8	42.7	51.6
00:55	43.9	40.9	54.4	**02:25**	52.3	41.7	53.8	**03:55**	55.7	42.3	54.9	**05:25**	55.8	42.8	52.9
01:00	44.3	40.9	54.8	**02:30**	52.6	41.7	54.0	**04:00**	55.7	42.3	55.0	**05:30**	55.8	42.9	50.6
01:05	45.0	40.8	54.4	**02:35**	53.1	41.7	54.3	**04:05**	55.7	42.2	55.2	**05:35**	55.7	43.0	50.8
01:10	45.3	40.9	54.0	**02:40**	53.6	41.9	54.4	**04:10**	55.8	42.1	55.2	**05:40**	55.6	42.9	51.7
01:15	45.6	40.9	53.8	**02:45**	54.1	41.8	54.6	**04:15**	55.8	42.2	55.0	**05:45**	55.6	42.8	50.5
01:20	45.8	41.1	53.8	**02:50**	54.2	41.8	54.5	**04:20**	55.8	42.4	55.1	**05:50**	55.5	42.9	50.2
01:25	46.1	41.0	54.0	**02:55**	54.4	41.9	54.6	**04:25**	55.7	42.5	55.3	**05:55**	55.6	42.9	51.2
01:30	46.4	41.0	54.1	**03:00**	54.6	42.0	54.5	**04:30**	55.7	42.4	55.4	**06:00**	55.6	42.8	50.8

Table 6.12 Charge passed (mA) through cylindrical concrete specimens prepared with *Bacillus* sp. (CT-5) in CSL medium based on RCPT results, in triplicate

Time	Charge passed (mA)			Time	Charge passed (mA)			Time	Charge passed (mA)			Time	Charge passed (mA)		
(hr)	1	2	3	(hr)	1	2	3	(hr)	1	2	3	(hr)	1	2	3
00:05	63.1	63.5	64.7	**01:35**	57.9	63.1	63.8	**03:05**	62.1	65.9	66.4	**04:35**	62.8	66.8	67.2
00:10	63.3	64.0	65.2	**01:40**	59.4	63.3	64.4	**03:10**	62.1	66.0	66.6	**04:40**	62.8	66.9	67.1
00:15	62.9	63.7	65.1	**01:45**	59.7	63.8	64.7	**03:15**	62.1	66.1	66.7	**04:45**	62.8	66.8	67.0
00:20	62.2	62.7	64.8	**01:50**	59.8	64.0	64.9	**03:20**	62.1	66.3	66.8	**04:50**	62.8	66.9	67.0
00:25	61.4	61.3	65.0	**01:55**	60.2	64.2	65.0	**03:25**	62.2	66.3	66.9	**04:55**	62.8	66.9	67.1
00:30	60.4	60.9	65.0	**02:00**	60.1	64.4	65.3	**03:30**	62.3	66.4	66.9	**05:00**	62.8	66.9	67.0
00:35	59.5	61.0	65.1	**02:05**	60.3	64.6	65.4	**03:35**	62.4	66.5	66.8	**05:05**	62.8	67.2	67.1
00:40	58.7	61.0	65.0	**02:10**	60.7	64.7	65.9	**03:40**	62.5	66.4	66.7	**05:10**	62.8	67.2	67.0
00:45	58.3	61.0	65.2	**02:15**	60.6	64.9	66.0	**03:45**	62.5	66.4	66.6	**05:15**	62.7	67.4	66.9
00:50	58.6	61.1	65.6	**02:20**	60.9	64.9	66.2	**03:50**	62.6	66.3	66.7	**05:20**	62.6	67.3	67.0
00:55	58.4	61.0	65.3	**02:25**	61.2	64.9	66.2	**03:55**	62.6	66.5	66.8	**05:25**	62.6	67.3	66.9
01:00	58.1	61.0	64.9	**02:30**	61.4	65.2	66.3	**04:00**	62.6	66.6	66.8	**05:30**	62.4	67.3	66.8
01:05	57.7	61.0	64.2	**02:35**	61.4	65.4	66.4	**04:05**	62.6	66.6	66.9	**05:35**	62.3	67.3	66.9
01:10	57.4	61.1	63.9	**02:40**	61.6	65.6	66.4	**04:10**	62.7	66.4	66.9	**05:40**	62.2	67.2	66.8
01:15	57.2	61.3	63.8	**02:45**	61.6	65.7	66.5	**04:15**	62.7	66.5	66.8	**05:45**	62.1	67.1	66.6
01:20	57.2	61.4	63.5	**02:50**	61.7	65.7	66.5	**04:20**	62.7	66.8	67.0	**05:50**	62.0	66.9	66.7
01:25	57.1	62.3	63.4	**02:55**	61.8	65.8	66.4	**04:25**	62.8	66.8	67.2	**05:55**	61.9	66.9	66.8
01:30	57.3	62.9	63.7	**03:00**	62.0	65.8	66.6	**04:30**	62.8	66.7	67.2	**06:00**	61.8	66.9	66.6

The charge passed after getting current values from the table was found by using Simpson's one third rule. As per this if I_0, and I_n are initial and final current values and I_1, I_2, I_3 ……..I_{n-1} are values of current after successive intervals of 15 mins. Then the value of charge (Q) passed was given by

$$Q = \frac{h}{3}\left[\left(I_0 + I_n\right) + 2\left(I_1 + I_3 + ----\right) + 4\left(I_2 + I_4 + ----\right)\right]$$

Where $h = \frac{b-a}{n}$

b = Time of last reading = 360 mins = 21600 seconds

a = Time of first reading = 0, n = Numbers of intervals = 24

Table 6.13 summarizes the average results of RCPT experiments. The permeability class type was moderate for control concrete specimens whereas when specimens were treated with CT-5 cells in nutrient and CSL media, it was "very low" and "low" type respectively. For control sample, the average charge passed was 3177 Coulombs, whereas for sample prepared with CT-5 cells (grown in nutrient media) the charge passed was 975.3 Coulombs and when grown and cured in CSL media, it was 1300.7 Coulombs. When specimens were treated with Bp W cells, the class changed to "low". The average charge passed was 1054 and 1303 Coulombs in nutrient and CSL media respectively.

A reduction in this total charge value indicates the better resistance to chloride ion penetration and lower permeability. The lower chloride permeability of the samples containing bacterial cells is a result of a denser microstructure due to microbial induced calcite precipitation (MICP). The bacterial reaction may cause lower amount of capillary pores and clogging of the pores, which reduces chloride ion transport in concrete. Improvement of the aggregate cement paste interface by the MICP may also play a role in decreasing the chloride ion permeability.

Table 6.13 Permeability class distribution based on RCPT report of different concrete specimens and evaluation of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) in nutrient and CSL media

Treatment	Charge passed (Coulomb) (in triplicate)			Average charge passed (Coulomb)	Permeability Class as per ASTM C 1202
	1	2	3		
Control	2890	3245	3396	3177.00	Moderate
CT-5 (in NB)	1023	818	1085	975.33	**Very Low**
CT-5 (in CSL)	1243	1319	1340	1300.67	Low
Bp W (in NB)	1077	1054	1031	1054	Low
Bp W (in CSL)	1324	1270	1315	1303	Low

It was also observed that the current after 1 min in case of control samples were 70.67 milliamps. The current after 1 min in case of samples prepared with *Bacillus* sp. (CT-5) (grown in nutrient media) current was 33.67 milliamps whereas when grown and cured in CSL media, it was 37.67 milliamps (Fig. 6.14). Concrete specimens treated with *S. pasteurii* (Bp W) allowed 46.7 and 50 milliamps current after one min when cells were grown in nutrient and CSL media respectively. There was significantly higher current passed after one min in those specimens that were prepared without bacterial cells (Table 6.14), ultimately allowed more chloride ions to passed through concrete. So it can be concluded that the average current, after 1 min in case of samples prepared with *Bacillus* sp. (CT-5), was less than the control samples irrespective of media used and cured to grow bacterial isolate. Although nutrient medium was found to better in resisting chloride ion permeability compared to CSL but that was not significantly different. Presence of various ions such as Ca, Mg, P, Na, K, Zn and other elements (Amartey and Jeffries, 1994) present in CSL might attribute to chloride ion permeability more than nutrient broth.

Table 6.14 Rapid chloride penetration test results showing current passed in concrete specimens after 1 min, prepared with different treatments

Sample	Current passed after 1 min (mA)
Control	70.7 ± 13.32a
CT-5 (in NB)	33.7 ± 3.21d
CT-5 (in CSL)	37.7 ± 1.53c
Bp W (in NB)	46.7 ± 4.5bc
CT-5 (in CSL)	50.0 ± 4.0b

Values bearing different letters are significant at $P<0.05$. Values are Mean ± SD (n=3).

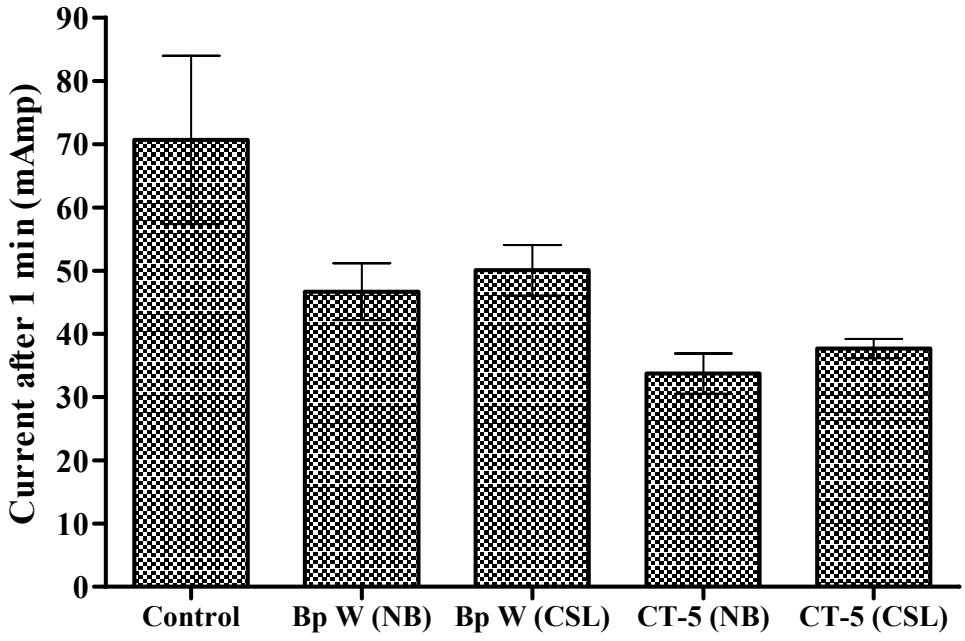

Figure 6.14 Comparison of Current after 1 min in concrete samples prepared with different treatments

6.9 Corrosion Analysis

Reinforced concrete (RC) structures are exposed to harsh environments yet are often expected to last with little or no repair or maintenance for long periods of time (often 100 years or more). To do this, a durable structure needs to be produced. For such structures like reinforced concrete bridges, one of the major forms of environmental attack is chloride ingress, which leads to corrosion of the reinforcing steel and a subsequent reduction in the strength, serviceability, and aesthetics of the structure. This may lead to early repair or premature replacement of the structure. A common method of preventing such deterioration is to prevent chlorides from penetrating the structure to the level of the reinforcing steel bar by using relatively impenetrable concrete. Here, the importance of MICP has been studied to control corrosion analysis.

Visible calcite precipitation was observed on RC specimen prepared with bacterial cells (Fig. 6.15). Corrosion was induced by current for 7 days with all the specimens. This gave the trend of reinforcement corrosion in the concrete samples with MICP. It was noticed that in control samples the crack continued to grow both in length and width. Corrosion products oozed out of the crack throughout the exposure. During accelerated corrosion, it is assumed that the electrical potential applied to the reinforcement attracted negatively charged chloride ions from the solution into the concrete and toward the positively charged reinforced bars. As the chloride ions reached the steel-concrete interface above threshold concentration, the steel surface began to corrode. The expansive products of corrosion-imposed tensile stresses on the concrete cover resulted in cracking when the tensile stresses exceeded the tensile strength of the cover material. Cracking, especially large cracks, would allow the conductive chloride solution to come into direct contact with the steel surface, thus providing a direct current path between the reinforcement and the electrodes in solution (So and Millard, 2007). The current response as a function of time under the fixed potential is shown in Fig. 6.16. The current-time curves were used to determine the initiation time of reinforcement corrosion by observing any instantaneous rise in

the current recorded. The considerable change in current values can be divided into three stages. The first stage was characterized by lower current values and this can be attributed to the formation of iron oxide layer, which protects steel temporarily. The second stage was characterized by an increase in current due to the intimation of micro-crack which resulted from steel corrosion. In this stage resistively decreased and ions penetrate into the steel. The third stage was characterized by relatively small current variation since resistively reached constant values.

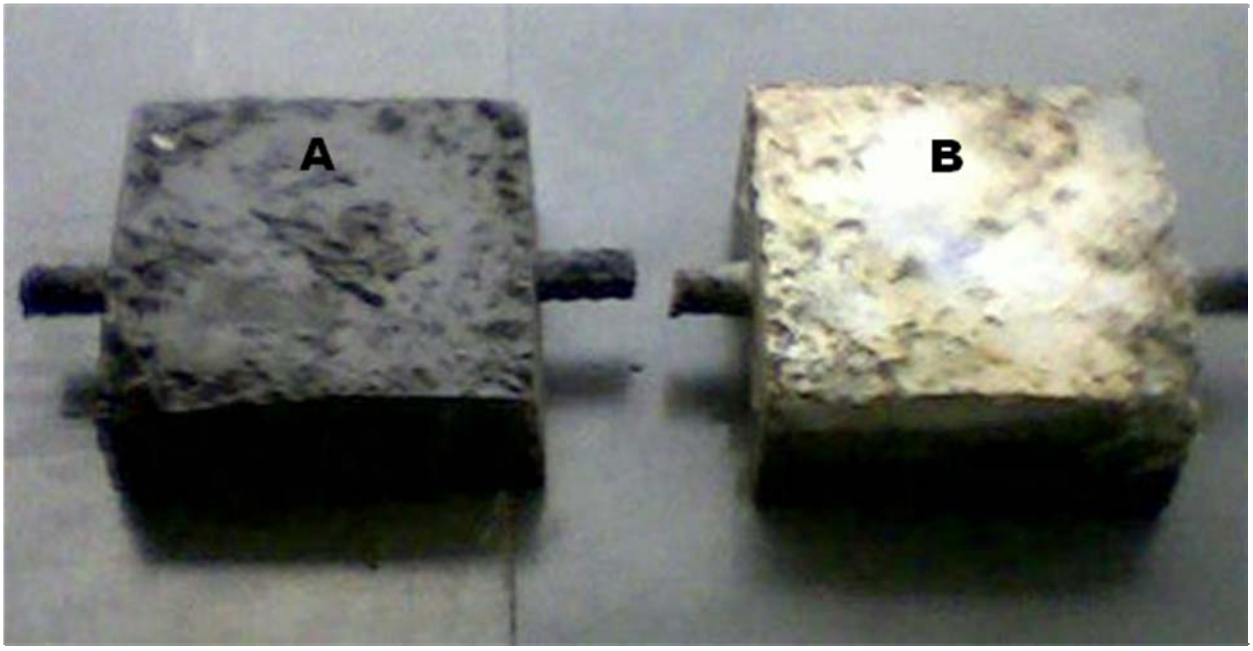

Figure 6.15 Visible calcite precipitation on the surface of RC specimen prepared with bacterial cells (B) and without bacterial cells (A)

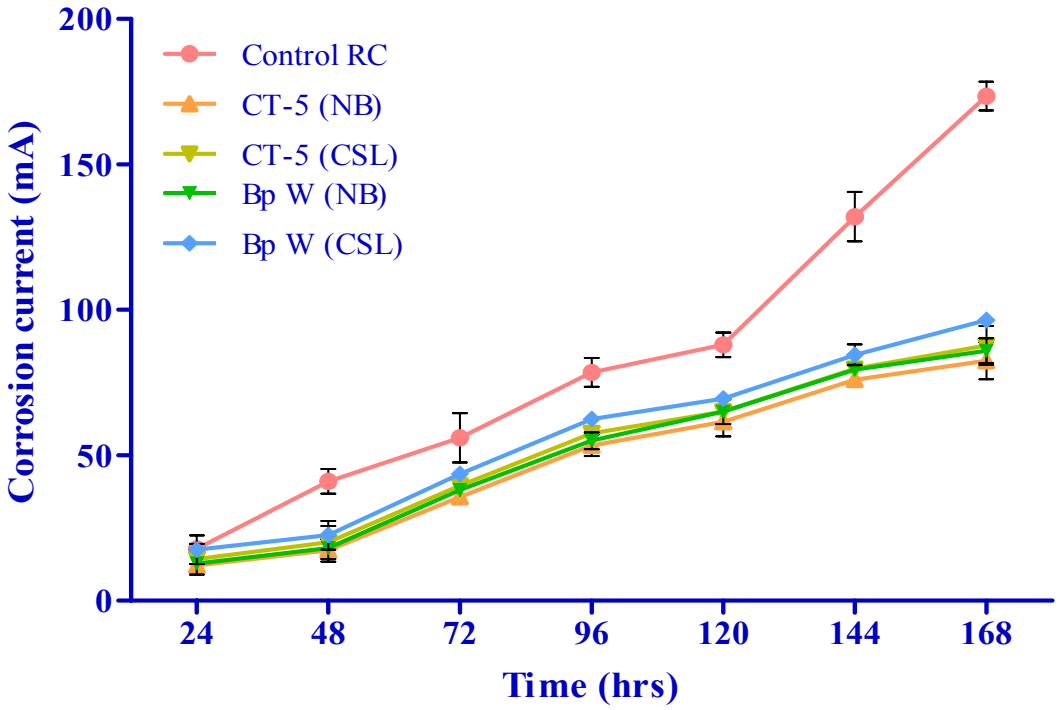

Figure 6.16 Current-time relationship at constant voltage of reinforcing bar (control and *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) treated specimens in nutrient (NB) and CSL media)

As seen from Fig. 6.16, the rapid increase in current (from 17.5 to 40 mA) was recorded for the control specimens during first two days. At the end of 7 days, a high current intensity (nearly 180 mA) was detected. The sudden rise of the current intensity coincided with the observation of a wider crack (explained later). The current recorded for the *Bacillus* sp. (CT-5) treated specimens was much lower, at approximately 55 mA until 4 days in both nutrient and CSL media while for *S. pasteurii* (Bp W) treated specimens, it was 57 mA and 63 mA in nutrient and CSL media respectively. At the end of 7 days, approximately 85 mA and 92 mA current was induced in *Bacillus* sp. (CT-5) and *S. pasteurii* (Bp W) treated specimens respectively in both nutrient and CSL media. The lower initial current recorded in those specimens compared with the control may reflect the higher electrical resistivity by MICP.

After inducing current in all the specimens including control, following observations were recorded:

1. The concrete was found to develop cracks within two days in case of control specimens, although no visible cracks were found in specimens prepared with bacterial (*S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5)) cells.
2. In 4 and 7 days the crack became wider and corrosion products oozed out in larger volumes in case of control specimens.
3. There were red and brown stains of rust on control concrete specimens (Figs. 6.17 and 6.18).
4. The cracks initiated at the surface of the reinforcement and ran along the direction of the reinforcement on the sides of the specimen.
5. Voltage between the reinforcing steel anode and cathode decreased with time indicating that the resistance has gone down with the progression of the crack.
6. In case of specimen prepared with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) brown stains of rust was visible after prolonged duration, at the end of 7 days.

Visible calcite precipitation was observed on bacterially treated RC specimens. Due to accelerated corrosion, the control RC specimens were found to develop cracks within two days. Corrosion products oozed out of the crack throughout the exposure period. At 4 and 7 days, the crack became wider and corrosion products oozed out in larger volumes. As a result, red and brown stains of rust on concrete were observed. The cracks initiated at the surface of the specimen and ran along the direction of the reinforcement on the sides of the specimens. They branched at a later stage leading to splitting of the specimens. The number of cracks on the surface of control specimens increased as corrosion progressed (Sahmaran *et al*., 2008). After 7 days (168 hrs) of accelerated corrosion, numerous (at least 7) microcracks with widths nearly 0.2 mm [0.008 in.] were observed on each surface. The maximum corrosion crack width was recorded at the end of the 7 days, at nearly 1.0 mm [0.04 in.]. In addition to longitudinal cracks, cover spalling was also observed.

In contrast to control samples, no profound effect of corrosion induction was observed on bacterial treated RC specimens. Microcracking on the surface of these specimens was first noted after 2 days. The fully grown calcite layers with distinct and sharp edges were found all over the surface of the cracked section thus acting as a corrosion inhibitor. At the end of 7 days (168 hrs) accelerated corrosion test, in addition to microcracks, one longitudinal localized crack of width of nearly 0.3 mm [0.012 in.] was observed. If a crack width of 0.3 mm [0.012 in.] is defined as a failure limit for RC specimens, as, for example, suggested by an earlier version of the ACI Building Code 28 or AASHTO 29 for crack width limit for outdoor exposures, the service life duration of bacterial specimens will be nearly five times more that of the control specimens (168 hrs for bacterially treated samples versus 36 hrs for control specimens under accelerated corrosion conditions). This indicates that MICP significantly extends service life (time to corrosion initiation plus time to corrosion propagation) under accelerated test conditions.

Figure 6.17 (A) RC specimen after corrosion and (B) Visible cracks and red and brown stains of rust on its surface

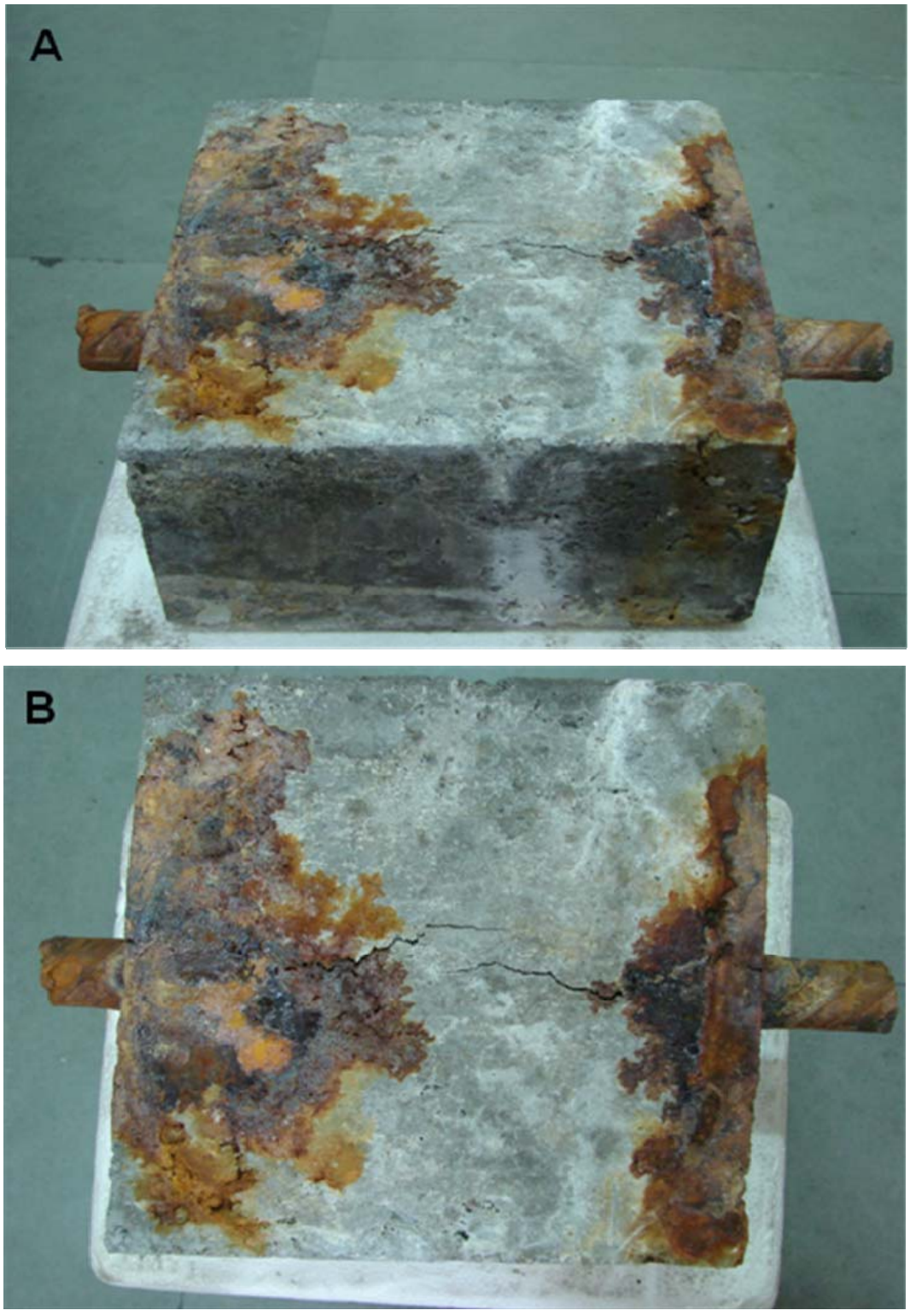

Figure 6.18 Visual inspections (A. horizontal view and B. vertical view) of cracks and rust developed after current induced corrosion on the control RC specimen

6.10 Corrosion Monitoring

One of the most widely used, practical, and standardized nondestructive method for monitoring steel corrosion in concrete structures is half-cell potential mapping (ASTM, 1999; Elsener *et al*., 2003). Despite its widespread application, half-cell potential mapping is usually associated with a number of practical difficulties which can be summarized as follows (Berkeley and Pathmanaban, 1990): (i) establishing a solid connection to the reinforcement, particularly in the case of densely reinforced members, such as bridge decks, can be a difficult task; (ii) establishing a proper wet connection between the external electrode and the reinforcement through the concrete cover can be problematic since a moisture (hence, resistivity) gradient always exists in the bulk of concrete, affecting the half-cell potential readings; (iii) the time required to establish an equilibrium condition between the external electrode and concrete is a function of a number of parameters including the thickness and the composition of the concrete cover (Elsener *et al*., 2003). Corrosion of the reinforcement steel used in concrete leads to formation of rust. As the steel corrodes, the volume of the rust also increases and at one stage the force induced by the corrosion products may exceed the tensile strength of the concrete and because of this, cracking of concrete will occur. These corrosion products would exert enormous stress on the surrounding concrete promoting the deterioration of concrete structures (Hoke *et al*., 1980). Methods of investigating metal loss are based on the premise that the corrosion of reinforcement steel is an electrochemical process. Among the various electrochemical methods, the increasingly used field technique for detecting corrosion activity in embedded steel is that of potential measurement. To analyze the corrosion behavior of steel in concrete, potential must be measured on the structures at different locations. Manually measuring the potential at different points on a large structure is a cumbersome process (Parthiban *et al*., 2000; Makar and Desnoyers, 2001). To acquire large number of data automatically at specified time intervals, automated measurement system is essential (Andrade and Alonso, 1996). Automated simultaneous measurement system is a reliable, more efficient and less time consuming method. Field Machine (ACM Corrosion Analyzer System, UK) is one of

the automated systems used to monitor corrosion in any RC specimen and was used to study corrosion in the RC specimens in the present study. Figure 6.19 schematically illustrates the test setup for a typical corrosion test in reinforced concrete structures by Field Machine.

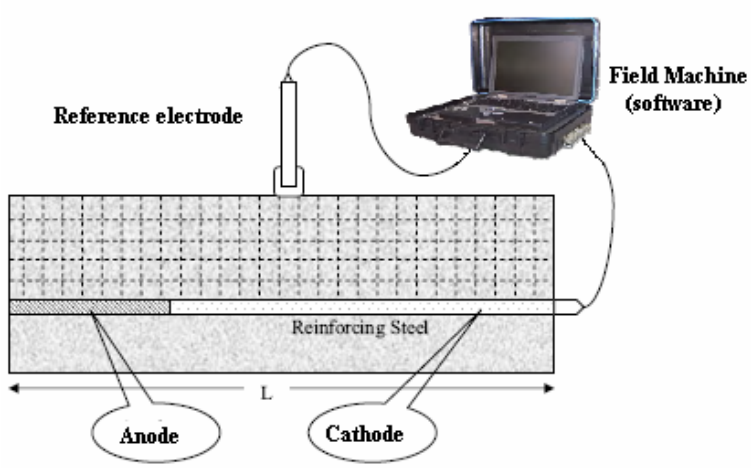

Figure 6.19 Schematic illustration of the corrosion measurement setup by Field Machine that is simulated in the present study

The simplest electrochemical test is potential mapping; this gives an idea of corrosion activity but does not give corrosion rate data. More sophisticated tests use the Field Machine to determine corrosion current density from cyclic sweeps and LPR (Linear Polarization Resistance). Cyclic sweeps are used to measure corrosion that proceeds at about the same rate all over the metals surface (uniform) and corrosion at discrete sites on the surface *e.g.* pitting crevice and stress corrosion cracking (localized). A typical experiment comprises of an electrochemical cell containing the electrolyte, a reference electrode, a platinum auxiliary and a working electrode of the metal under test. The results can be interpreted in the Analysis package.

LPR monitoring has been developed to determine the instantaneous corrosion current density measurement of reinforcing steel in concrete. The technique is rapid and non-intrusive, requiring only a connection to the reinforcing steel. The data provides a

valuable insight into the instantaneous corrosion current density of the steel reinforcement, giving more detailed information than a simple potential survey. The LPR data enables a more detailed assessment of the structural condition and is a major tool in deciding upon the optimum remedial strategy to be adopted. It is thus imperative that the LPR measurements obtained are accurate. The LPR technique is the most frequently used being both quick and easy. A small sweep typically from -10 mV to +10 mV at 10 mV/min around the rest potential is performed. The resulting current/voltage plot usually exhibits a straight line the inverse slope of which is proportional to the corrosion rate. The field machine enables laboratory precision to be taken outdoors to oilrigs, pipelines, concrete walls and just about anything that needs corrosion monitoring *in-situ* (Fig. 6.20).

The Tafel Plot (TP) extrapolation technique is another electrochemical method for calculating corrosion rate based on the intensity of the corrosion current density (I_{corr}) and the Tafel slopes. Tafel slopes also could be used to calculate corrosion current density with LPR. To measure uniform corrosion the method of Tafel extrapolation is used. Tafel slope is the slope of the straight line portion of the semi-logarithmic polarization curve (Fig. 6.21). To determine the degree of localized corrosion the amount of hysteresis between the positive going sweep and the negative going sweep is calculated. At large perturbations away from E_{corr} the reaction measured becomes almost totally oxidized (when going positive) or almost totally reduced (when going negative). The equations that describe the reactions at large overpotentials can be simplified to a linear relationship *i.e.*

Anodic overpotential = $b_a.\log (I_{app}/I_{corr})$; Cathodic overpotential = $(-b_c.\log (I_{app}/I_{corr}))$.

This allows an extrapolation of I_{app} from either the anodic or cathodic Tafel region to the open circuit potential and hence to obtain the corrosion current.

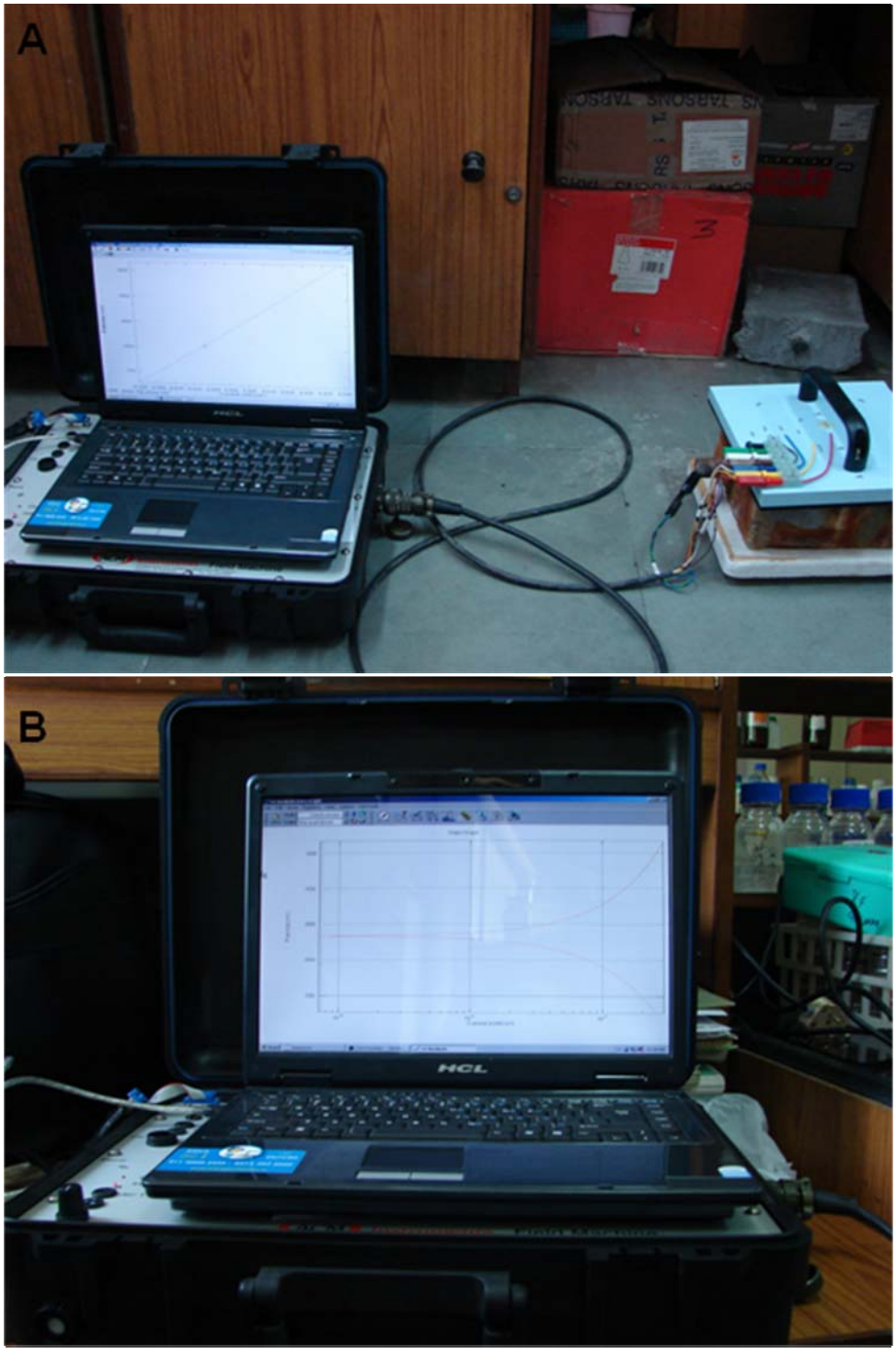

Figure 6.20 (A) and (B) Field Machine showing arrangement of test and set-up

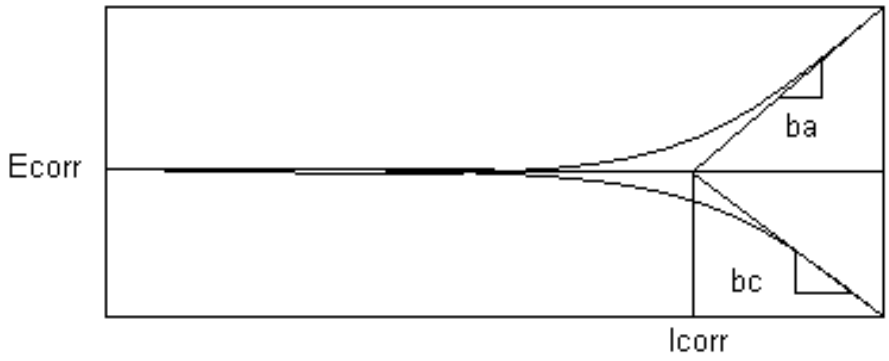

Figure 6.21 Schematic representation of Tafel plot

Both LPR and TP techniques are based upon application of either steady fixed levels of current, followed by monitoring of the potential (galvanostatic) or application of specific potential followed by monitoring of the current (potentiostatic).

In TP, corrosion current density can be calculated using straightforward substitution of Tafel slope values (b_a and b_c) to get the corrosion current and examined in Eq. (6.1) then, by calculating corrosion current density using Eq. (6.2)

$$I=I_{corr}\{\exp[S1(E-E_{corr})]-\exp[-S2(E-E_{corr})]\} \quad (6.1)$$

where $S1$= slope of the anodic branch=2.303/ b_a, $S2$ = slope of the cathodic branch = 2.303/ b_c, b_a = anodic Tafel constant, b_c = cathodic Tafel constant, E_{corr} = the corrosion potential, I_{corr} = the corrosion current in Ampere, E = the potential at any time, and I = the current at any time.

$$\text{Corrosion current density } (\mu m/yr) = 0.129\, I_{corr}\, \text{E.W.}\, /dA \quad (6.2)$$

where I_{corr} = the corrosion current intensity, in $\mu A/cm^2$; A = exposed surface area of the reinforcing steel, in cm; E.W. = the equivalent weight of steel, which is the atomic weight of an element that has the same combining capacity as a given weight of another element, where the standard is 8 for oxygen; and d = the density of the reinforcing steel, in g/cm^3.

To calculate the corrosion current density using the LPR method, I_{corr} is first calculated with Eq. (6.3) which is based on the Stern–Geary relationship. And then, using Eq. (6.2) the corrosion rate can be calculated (Mansfeld, 1977)

$$I_{corr} = b_a.b_c / 2.3R_p (b_a + b_c) \quad (6.3)$$

where R_p is the polarization resistance, in $k\Omega cm^2$, and b_a and b_c are constants, which could be obtained from a Tafel Plot.

The effectiveness of bacterial cells based on microbially induced calcite precipitation as a protective agent was determined in both control and bacterial RC specimens by measuring corrosion current density. Tafel plots of both control and bacterial RC (in nutrient and CSL media) specimens were made which permitted the determination of the corrosion rate (Figs. 6.22, 6.23 and 6.24). The electrochemical corrosion parameters obtained from the Tafel polarization curves are listed in Table 6.15. The rate of corrosion is considered proportional to the corrosion current density (I_{corr}) for the specimen. All the samples corroded at varying rates. The control RC specimens had the highest I_{corr}. In comparison all the bacterial RC specimens had significantly lower I_{corr}. Therefore, it is concluded that impediment to corrosion is effected by microbially induced calcite precipitation at any stage of corrosion. Around four-fold reduction in I_{corr} by *Bacillus* sp. (CT-5) suggests that the calcite precipitation has the effect of greatly reducing the diffusion of oxygen to the steel bar surface. In case of RC specimens with *S. pasteurii* (Bp W), I_{corr} was 22.6 mA/m^2 (in CSL medium) and 20 mA/m^2 (in nutrient medium); the corrosion current density were highly reduced compared with that of control RC specimens (60.8 mA/m^2).

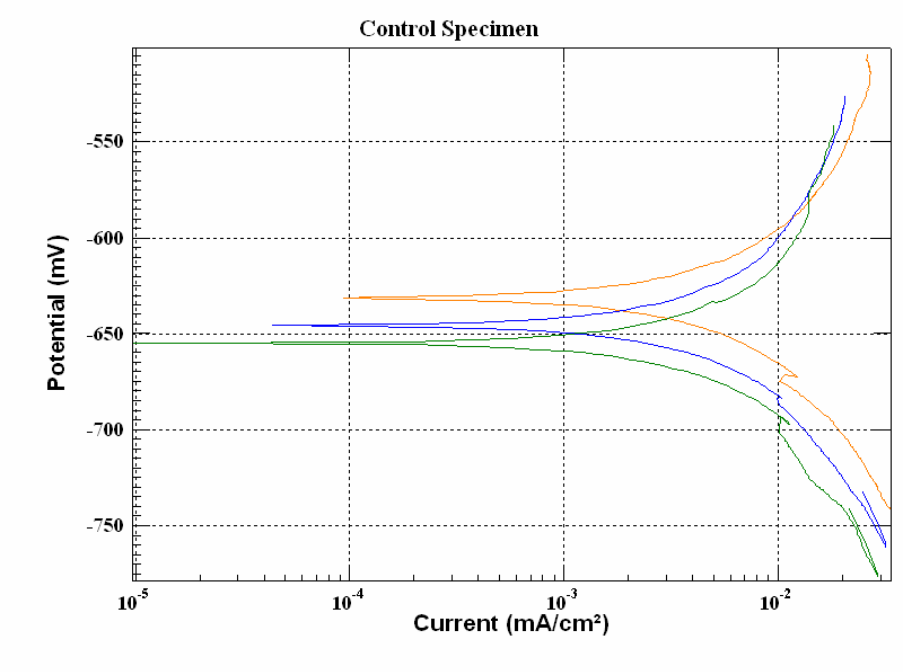

Figure 6.22 Tafel plot of control reinforced concrete specimen

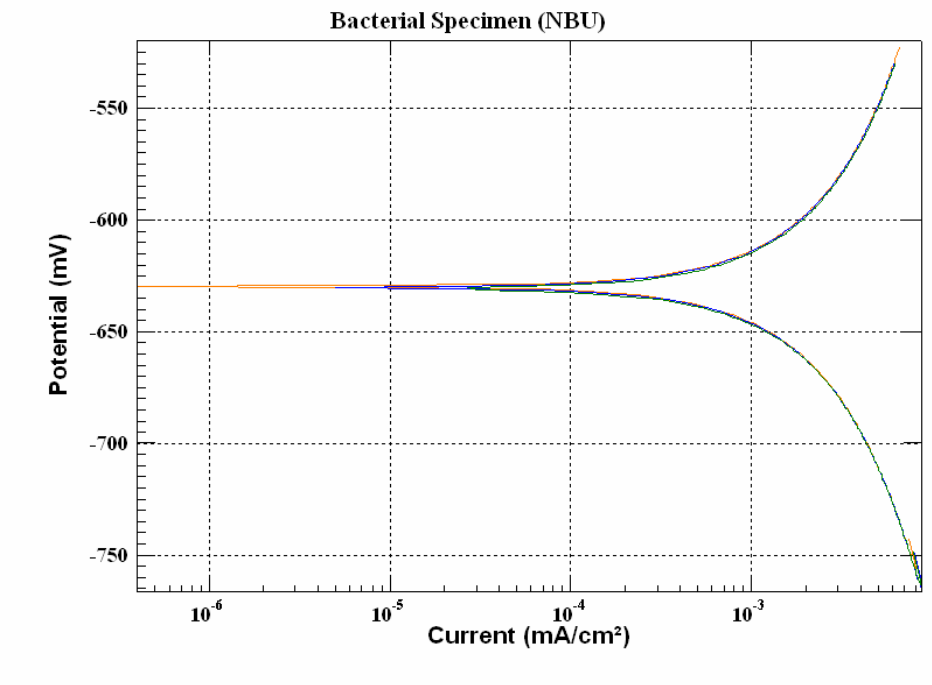

Figure 6.23 Tafel plot of bacterial [*Bacillus* sp. (CT-5)] reinforced concrete specimen, grown and cured in nutrient media

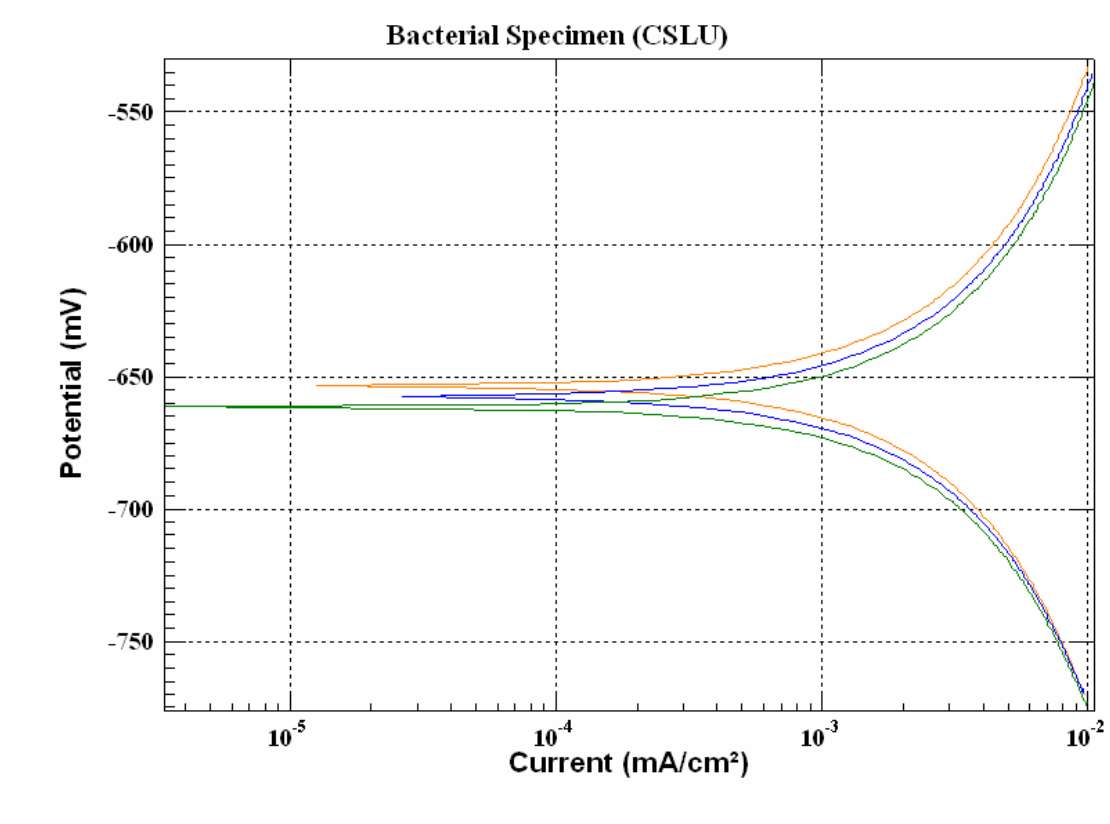

Figure 6.24 Tafel plot of bacterial [*Bacillus* sp. (CT-5)] reinforced concrete specimen, grown and cured in CSL media

The RC specimens treated with *Bacillus* sp. (CT-5) showed more resistance to current as LPR was found to be 16102.33 Ohm·cm^2 and 11890.33 Ohm·cm^2 (in nutrient and CSL media respectively) while LPR was 3942.23 Ohm·cm^2 in case of control specimens. LPR rate was also higher in case of *S. pasteurii* (Bp W) treated RC structures (11872 Ohm·cm^2 and 10742 Ohm·cm^2 in nutrient and CSL media respectively). These values also explain the behavior of bacterial cells to achieve low corrosion rate. The experiment shows that MICP dramatically slows down the rate of corrosion. One should expect increase in pullout strength, decrease in mass loss and increase in LPR rate due to MICP.

Table 6.15 Corrosion current density (Icorr) of control and bacterial treated RC specimens obtained from Field Machine.

RC Specimens	Icorr (mA/m^2)	Icorr (mm/year)	LPR ($Ohm.cm^2$)
Control RC	60.8 ± 0.001a	0.071 ± 0.006a	3943 ± 374d
RC with *Bacillus* sp. (CT-5) (in NB)	14.8 ± 0.001d	0.017 ± 0.001d	16102 ± 63a
RC with *Bacillus* sp. (CT-5) (in CSL)	20.0 ± 0.002c	0.023 ± 0.001c	11890 ± 226b
RC with *S. pasteurii* (Bp W) (in NB)	21.6 ± 0.002b	0.026 ± 0.003b	11872 ± 106b
RC with *S. pasteurii* (Bp W) (in CSL)	22.6 ± 0.002b	0.027 ± 0.002b	10742 ± 214c

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

6.11 Pullout strength and mass loss

A pullout test, by using a dynamometer and a reaction bearing ring, measures the force required to pullout from concrete a specially shaped insert whose enlarged end has been cast into the concrete. Because of its shape, the insert is pulled out with a cone of the concrete. The concrete is simultaneously in tension and in shear; the generating lines of the cones are defined by the key dimensions of the insert and the bearing ring. The main advantage of the pullout test is that it provides a direct measure of the *in situ* strength of concrete.

There is growing concern for corrosion damage in reinforced concrete structures with several decades service. Pullout tests are carried out to study the effect of reinforcement corrosion on the bond behavior and bending strength of reinforced concrete beams as per ASTM C234. The bond strength of plain bars and concrete initially increases with increasing corrosion, and then declines (Wei-liang and Yu-xi, 2001). The turning point depends on the cracking of the concrete cover. The bond strength of deformed bars and concrete increases with corrosion up to a certain amount, but with progressive increase in corrosion, the bond strength decreases, and the cracking of the concrete cover seems to have no effect on the bond strength.

Pullout tests were carried out just after corrosion monitoring. This was done by securing the concrete slab in a universal testing machine (UTM) and attaching the grip on to the protruding portion of the reinforcing bar (Fig. 6.25). In case of the control specimen, the bar pulled out of the specimen causing splitting of the slab. The pullout force was much larger in the case of specimens prepared with bacterial cells. It may be recalled that there was considerable corrosion and consequent loss of metal at the top interface. As a result, the reinforcing bar of all control specimens broke off at the interface. After splitting the slab completely with all the specimens there were more visible rust found on control samples as compared to bacterial specimens. A good correlation exists between the pullout force and percent mass loss. As the mass loss increases the pullout force reduces. Understandably, pullout

strength varies inversely with the mass loss *i.e.* corrosion reduces bond strength. The rate of loss of pullout strength in RC specimens with bacterial cells is the lowest. Mass loss percentage was very high in case of control RC specimen (5.94%) and in case of specimen prepared with *Bacillus* sp. (CT-5) in nutrient and CSL media, the mass loss percentage was 2.7% and 3.65% respectively while with *S. pasteurii* (Bp W) mass loss was 3% and 4% in nutrient and CSL media respectively (Fig. 6.26). The mass loss affects the bond between the rebar and concrete and thus adversely affects the pullout strength.

Figure 6.25 Pullout tests of RC structures

Figure 6.26 shows variation of mass loss with respect to pullout strength which indicates that the control sample has least pullout strength and highest percentage mass loss as compared to the bacterial specimens. The highest pullout load was shown by RC specimen prepared with *Bacillus* sp. (CT-5) grown and cured in nutrient media (34.97 kN) and when these cells were grown and cured in CSL media, pullout load was 30.89 kN. *S. pasteurii* (Bp W) treated RC specimens showed relatively lower pullout load (32.68 and 29.5 kN in nutrient and CSL media respectively). The control RC specimen showed pullout load of 26.32 kN only (Table 6.16). As the reinforcement loses mass the bar becomes thinner and the bond is also lost.

Table 6.16 Pullout strength and mass loss of reinforced bar from different concrete specimens

Sample	Pull out load (kN)	Weight loss (%)
Control	26.3 ± 0.75d	5.9 ± 0.08a
CT-5 (in NB)	35.0 ± 1.58a	2.7 ± 0.26d
CT-5 (in CSL)	30.7 ± 1.24bc	3.6 ± 0.36c
Bp W (in NB)	32.7 ± 1.3b	3.0 ± 0.3d
Bp W (in CSL)	29.5 ± 0.5c	4.1 ± 0.2b

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

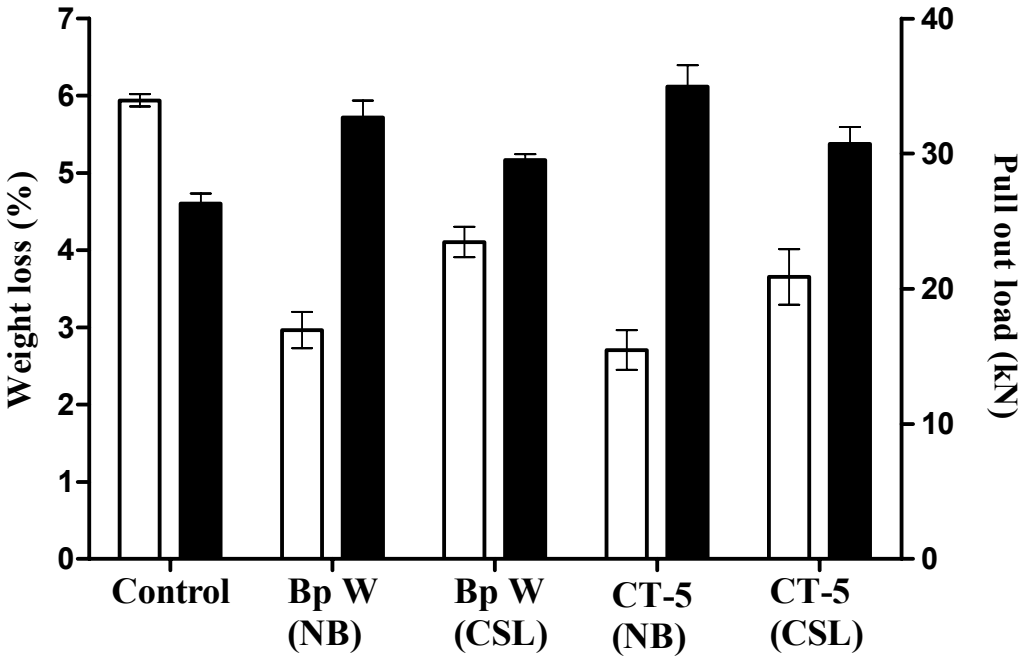

Figure 6.26 Variation of pullout load (filled bars)/ % mass loss (open bars) of control specimens and specimens treated with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) in nutrient (NB) and CSL media

Split surface of specimen after pullout test showed least rust patches in case of samples prepared with *Bacillus* sp. (CT-5) whereas in case of control specimen visible higher rust patches were observed (Fig. 6.27). A comparison of percent mass loss due to reinforcement corrosion reveals that the *Bacillus* sp. (CT-5) induced microbial calcite precipitation has effectively reduced the loss of mass by around 53%.

To check the presence of bacterial cells in the crack, specimens were subjected to SEM analysis. After the pullout test, the broken concrete samples from the cracked area were collected and dried at 60°C for 12 hrs. The samples were gold coated with a sputter coating Emitech K575 and examined in SEM (Zeiss EVO50) at accelerating voltages ranging from 30 to 35 kV.

The bacterial treatment resulted in the prominent presence of crystalline deposits on the surface of RC sample and also on cracked surface (Fig. 6.28A). On closer observations, many rod shaped bacteria associated with calcite crystals were found (Fig. 6.28B). It is mainly due to the fact that facultatively anaerobic bacteria of present study grows at a higher rate in the presence of oxygen and consequently induces active precipitation of calcite around the surface area. It is therefore expected to have more calcite precipitation in areas close to the surface in crack than the interior portion which can serve as a barrier to harmful substances to enter inside the sample and thus will improve its impermeability. The presence of crystalline calcite associated with bacteria indicates that bacteria served as nucleation sites during the mineralization process which played major role in getting reduced corrosion.

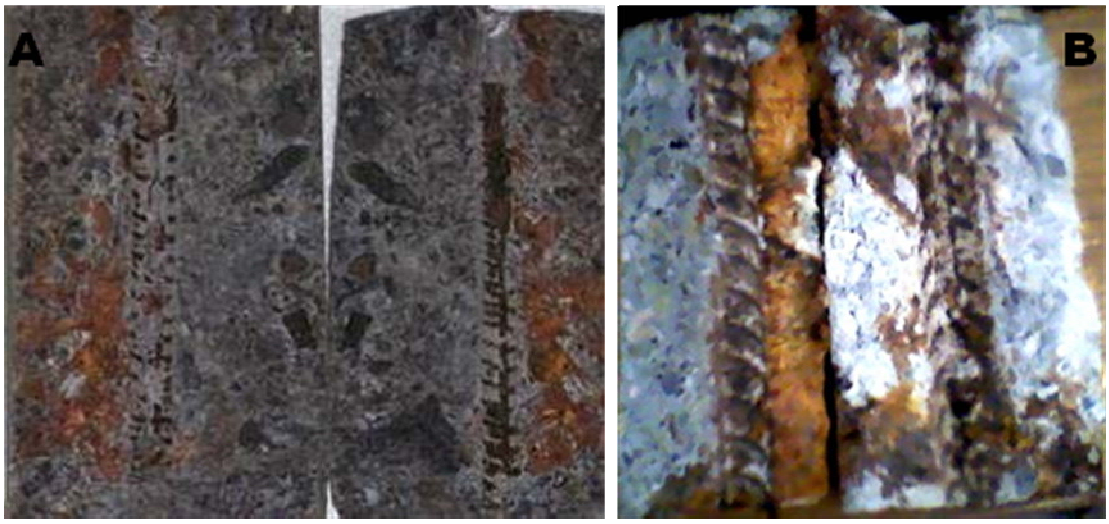

Figure 6.27 Split surfaces of (A) specimen prepared with CT-5 isolate and (B) control RC specimens after pullout test

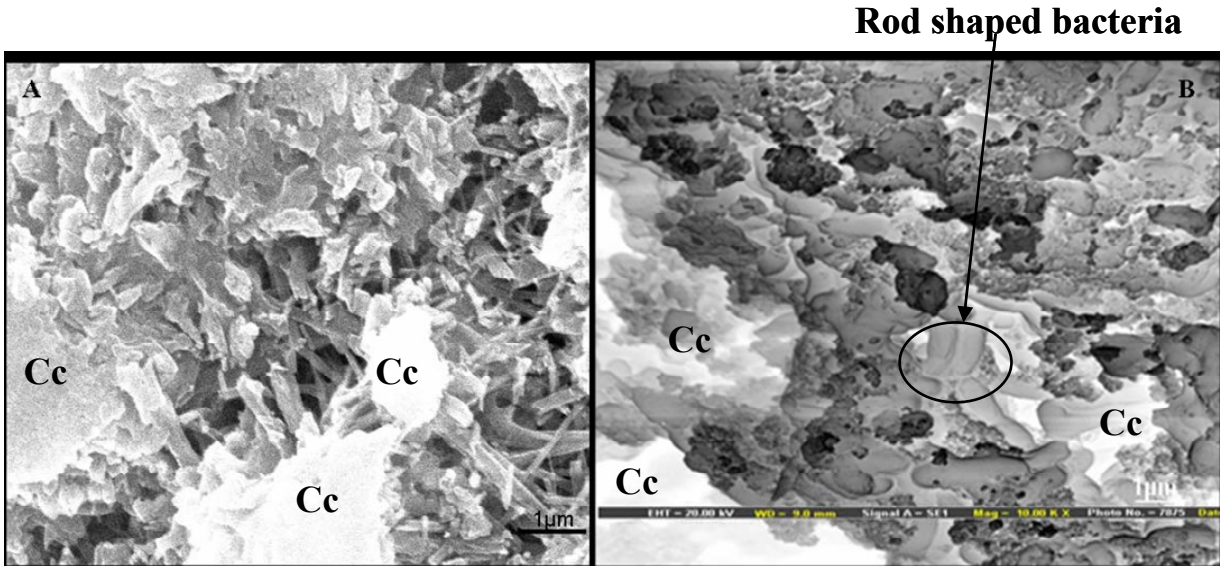

Figure 6.28 (A) Presence of calcite crystals formed by bacterial cells inside the bacterial treated RC specimen and (B) closer view of rod-shaped bacterial cells present in A

6.12 Tensile strength

Tensile strength is the resistance of a reinforced bar to a force tending to tear it apart, measured as the maximum tension the bar can withstand without tearing. Rebar is a structural member that is cast into concrete and masonry to provide strength and reinforcement. A reinforcing bar, rebar is used to compensate for the relatively weak tensile strength of these popular building materials. It is easily understood that maximum tensile strength will be shown by such bars those were less corroded. As corrosion lead to mass loss and ultimately less tensile strength will be shown by such bar.

Presently reinforced concrete (RC) has been developed and applied extensively. It combines the good compressive strength of concrete with the tensile strength of steel and has proven to be successful in terms of both structural performance and durability. One major flaw, namely its susceptibility to environmental attack, can severely reduce the strength and life of these structures. In humid conditions, atmospheric pollutants percolate through the concrete cover and cause corrosion of steel reinforcements. The resulting corrosion products occupy volumes several times that of the steel. The increased volume induces tensile stresses in the concrete that result in cracking, delamination and spalling. As a result, the reinforcements get exposed to direct environmental attack and the corrosion is accelerated. Due to corrosion the bars exerts bursting pressure on concrete. In absence of the MICP, tensile stresses develop in concrete that result in its cracking and spalling. The RC specimens containing bacterial cells resist the expansion pressure by $CaCO_3$ crystals and develop a hoop stress. As a result, concrete goes in compression. This results in better grip of concrete on steel.

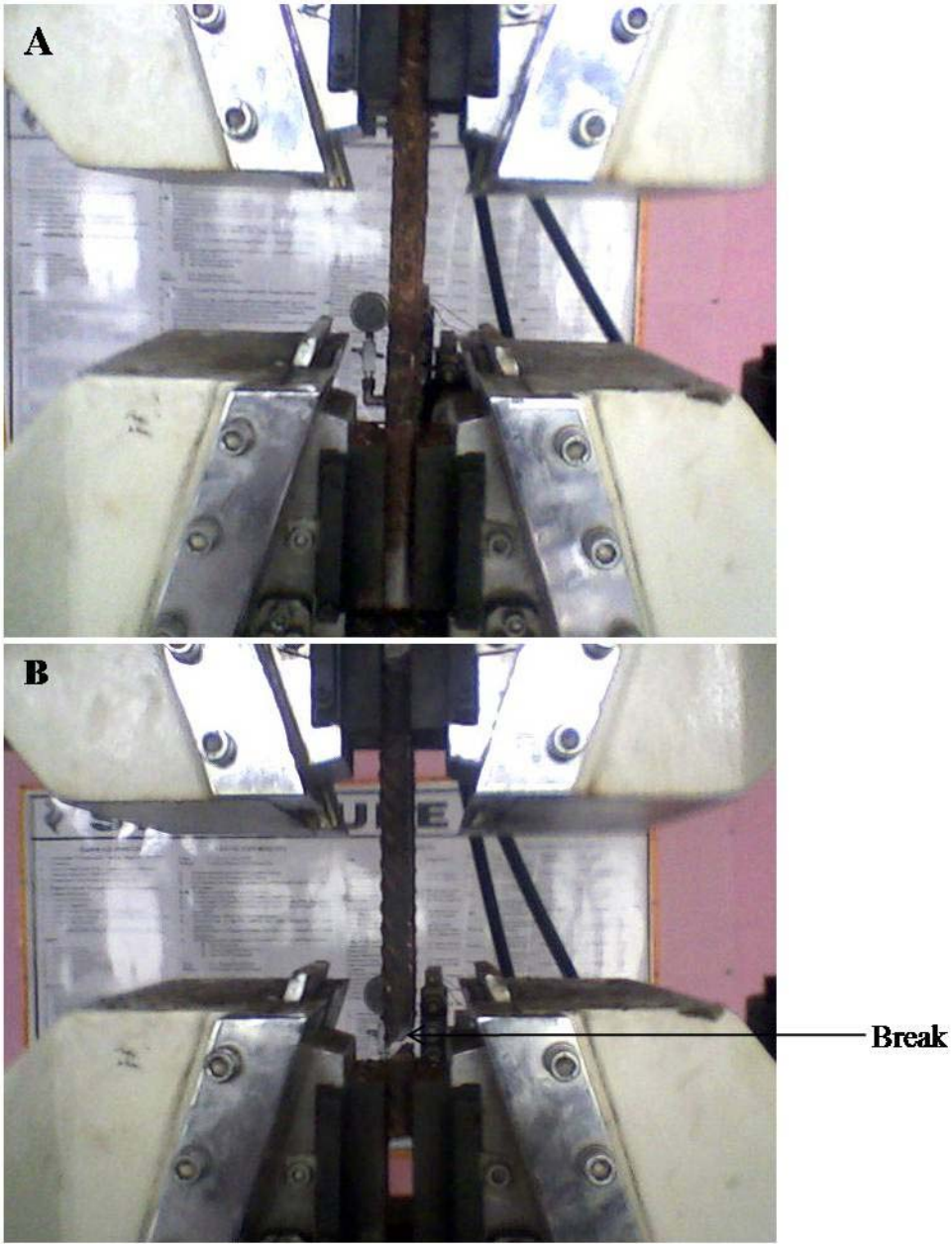

Figure 6.29 Tensile strength test of reinforced bar (A) before and (B) after break

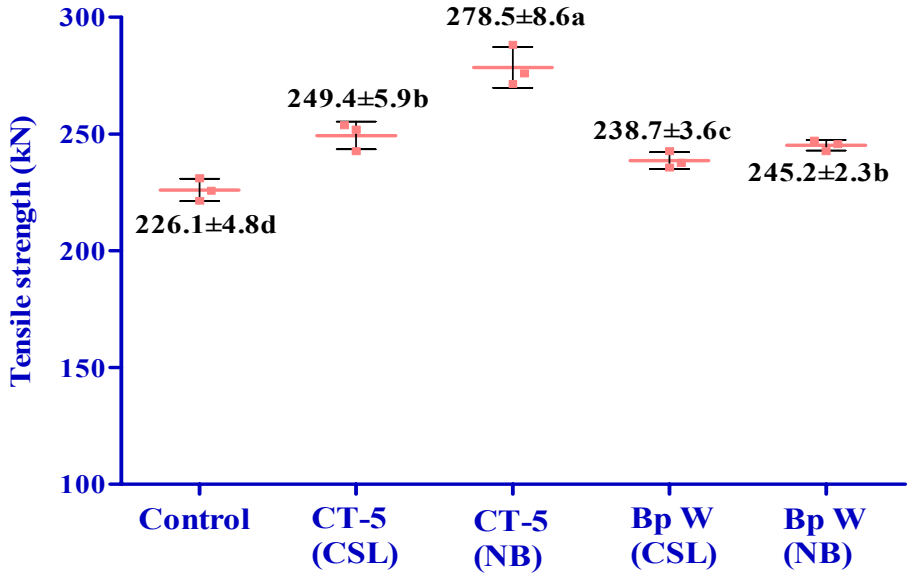

Figure 6.30 Tensile strength of rebar and their comparison between control and bacterial treated reinforced concrete (RC) specimens. Values bearing different letters are significant at $P<0.05$. Values shown on the figure are Mean ± SD (n=3).

Steel rebar is usually made of carbon steel and features ridges for better anchoring. Steel rebar is used to extend the lifetime of concrete structures such as highway bridge decks and parking garages. They are also used to strengthen and support outdoor walls made of concrete blocks and masonry. That's why it is important to protect rebars from getting corroded. Results of the present study clearly showed that *Bacillus* sp. (CT-5) provided a way to reduce corrosion of rebar by precipitating a calcite layer on concrete as well as rebar surface. The arrangement of rebar to perform tensile strength test is shown in Figure 6.29. Reduced rate of corrosion by reinforced concrete specimens treated with bacterial isolates lead to an increase in tensile strength of rebar irrespective of media used to grow the cells (Fig. 6.30). Tensile strength of rebars was significantly higher when reinforced concretes were prepared using *Bacillus* sp. (CT-5) and *S. pasteurii* (Bp W). This is the first report of bacterial treatment towards increment in the tensile strength of reinforced bar in concretes.

Discussion

6.13 Improvement in the durable properties of building materials based on MICP

The aim of this experimental study was to develop a methodology towards the enhancement in the durability of building structures and for protection of corrosion affected structures that had been treated with bacterial cells for their structural rehabilitation. Biomineralization is based on the ability of bacteria to promote the precipitation of carbonates. *Bacillus* sp. (CT-5) strain isolated from cement, capable of precipitating a dense and coherent calcium carbonate precipitate, higher urease production, abundant EPS and biofilm production was selected to study durability aspects of building materials and its efficacy was also compared with *Sporosarcina pasteurii* (Bp W). Addition of bacterial isolates showed a positive effect on the compressive strength of mortars. Improvement in compressive strength reaches a maximum at about 5×10^7cells/ml cell concentration. SEM examination reveals the growth of fibrous filler material within the pores due to the presence of such microorganisms. This growth is beneficial by the modification of the porosity and pore size distribution of cement mortar which it generates. Further at higher concentration of bacterial cells, a decrease in the compressive strength was noted. At higher cell concentration, the matrix integrity may disrupt due to excessive bacterial activity resulting in the decrease in compressive strength of mortar (Ghosh *et al*., 2009).

Filling cracks with bacteria and sand demonstrated a significant increase in compressive strength values of the cubes when compared with those without bacterial cells. Scanning electron micrographs identified that microbiological calcite precipitation occurred mainly close to the surface areas of the crack, where a dense growth of calcite crystals embedded with cells was observed. Experiments showed that the biomineralization technology can be applied on mortar surfaces to enhance the durability. In general, microbially induced calcite precipitation is effective in surface consolidation in porous media and further remediation of surface cracks. It is

mainly due to the fact that bacterial cells grow more actively in the presence of oxygen and subsequently induce calcite precipitation heavily close to the surface area of the crack (Ramachandran *et al*., 2001). The overall trend of an increase in compressive strength up to 28 days might be attributed to the behavior of microbial cells within the cement mortar matrix. During the initial curing period, microbial cells obtained good nourishment, because the cement mortar was still porous; but growth was not proper as there was completely new environment for microbes. It is also possible that as the pH of the cement remained high, cells were in inactive condition and as curing period was increased, it started growing slowly. Upon cell growth, calcite would precipitate on the cell surface as well as within the cement mortar matrix which might be also attributed by the various ions present in media, especially in media containing CSL. Thus, the cement mortar became less porous and permeable. Once many of the pores in the matrix were plugged, the flow of the nutrients and oxygen to the bacterial cells stopped, eventually the cells either died or turned into endospores and acted as an organic fiber, increasing the compressive strength of the mortar cubes. This explains the behavior of the increased compressive strength at the age of 28 days in cement mortar cubes prepared with microbial cells. There was significant improvement in the compressive strength of building materials based on MICP produced by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5), supported by previous studies (Ramachandran *et al*., 2001; Bang *et al*., 2001; Ghosh *et al*., 2005). This improvement in compressive strength is probably due to deposition of $CaCO_3$ on the microorganism cell surfaces and within the pores of cement–sand matrix, which plug the pores within the mortar (Ramakrishnan *et al*., 1998; Ramachandran *et al*., 2001; De Muynck *et al*., 2005). This increase in the matrix strength (for concrete made with bacterial cells) would have resulted in lesser mean expansion and would have eventually increased the overall durability performance of the concrete (Ramakrishnan *et al*., 2001). Thus it was concluded that the increase in compressive strengths is mainly due to consolidation of the pores inside the cement mortar cubes with microbiologically induced calcium carbonate precipitation.

6.14 Impermeability based on MICP

Concrete is an aggregate structure and consists of cement, coarse aggregates, sand, mineral material and pores. Pore volume and size determine the capacity for fluid storage and salt accumulation. Both factors are important for the further erosion and the durability of the concrete structure. Weathering and ingression of chemicals are the main cause for the deterioration of concrete structures (Akman and Ozyurt, 2002). A decrease in the ability to absorb water will result in a deceleration of the weathering process. A thin restoring layer of calcite can act as an alternative for the natural calcite skin on the concrete surface. Biologically produced by ureolytic activity, the calcite layer clogged the pores at the surface of the concrete to decrease the water absorption rate. The growth related microbiological urea degradation is the main source of inorganic carbon dioxide in calcium and carbonate oversaturated medium and the cause of calcium carbonate precipitation (Merz-Preiss and Riding, 1999; Yates and Robbins, 1999, Warren *et al*., 2001).

Water absorption is the process whereby fluid is drawn into a porous unsaturated material under the action of capillary forces. The capillary suction depends on the pore volume and geometry, and the saturation level of the concrete structure. Such tests also measure porosity which may be insensitive to the transport mechanisms influencing durability (Beushausen *et al*., 2003; Cultrone *et al*., 2004; Prikryl *et al*., 2003). Microbial treatments by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) were effective in reducing water absorption. The higher water absorption coefficient of the most porous specimens without the biological treatment could be due to absence of MICP reactions on the surface. Repeated application of bacteria and a calcium source could result in an additional decrease of the water absorption (De Muynck *et al*., 2007). Nemati and Voordouw (2003) noticed an additional decrease of the permeability of sandstone cores after injecting $CaCO_3$ forming reactants for a second time. To see the effect of biodeposition on mortars, some group of researchers immersed the specimens in grown bacterial culture and observed impermeability to aggrasive substances (Dick *et al*., 2006; De Muynck *et al*., 2008). However, to larger

specimens these experiments are not feasible and to consider this, in the present study a successful attempt was made to improve impermeability by addition of bacterial culture during construction. It was also established that microbially induced calcite precipitation (MICP) effectively impedes percolation of water in concrete.

Along with water absorption, chloride ingress is also a common factor that damage building structures. The rapid chloride permeability test (RCPT) - American Society of Testing and Materials (ASTM) test method C1202 or American Association of States Highway and Transportation Officials (AASHTO) Test Method T277, is virtually a measurement of electrical conductivity of concrete, which depends on both the pore structure characteristics and pore solution chemistry of concrete. RCPT has been used to evaluate the chloride permeability of hardened cement mortars and concretes made with special cements or supplementary cementing materials (Douglas *et al*., 1992; Shi, 1996; Shah *et al*., 2000). Chlorides ingress is probably the most dominant factor that adversely affects the corrosion process of steel in concrete, and several case studies have been reported (Weyers and Cady, 1987; Amleh and Mirza, 2002). The steel reinforcement in concrete is protected by several methods. One is by using ordinary Portland cement (OPC) with very low water-binder ratio. However, this can adversely impact on workability, heat of hydration, and shrinkage. MICP provides a better option without compromising w/b ratio and improve durability of concrete structures. Also, the rate of chloride penetration into concrete is affected by the chloride binding capacity of the concrete. A portion of the chloride ions reacts with the concrete matrix formed may be due to MICP becoming either chemically or physically bound, and this binding reduces the rate of diffusion. The chloride binding capacity is controlled by the cementing materials used in the concrete (Midgley and Illston, 1984). All the samples were conditioned as per the procedure described in the ASTM C 1202-05. The moderate to high chloride ion penetrability observed in control samples has been reduced to low or very low by MICP produced by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5). The reduced charge at in the bacterial specimens can be attributed to the finer pore-size distribution with increased period of curing (Mehta and Manmohan, 1980). From the results of

chloride penetration test it was concluded that MICP helps to reduce the charge passed which is a good indicator of chloride ion migration.

6.15 Corrosion analysis

All the reinforced concrete specimens prepared with bacterial cells indicated that the resistance to corrosion increases enormously due to microbially induced calcite precipitation. The control specimens showed loss of resistance and that might be due to the diffusion of moisture in the samples and oozing of the water soluble corroded products. Herholdt *et al*. (1985) presented the sequence of corrosion products that form on steel according to the availability of water and oxygen present in the system, shown in Figure 6.31.

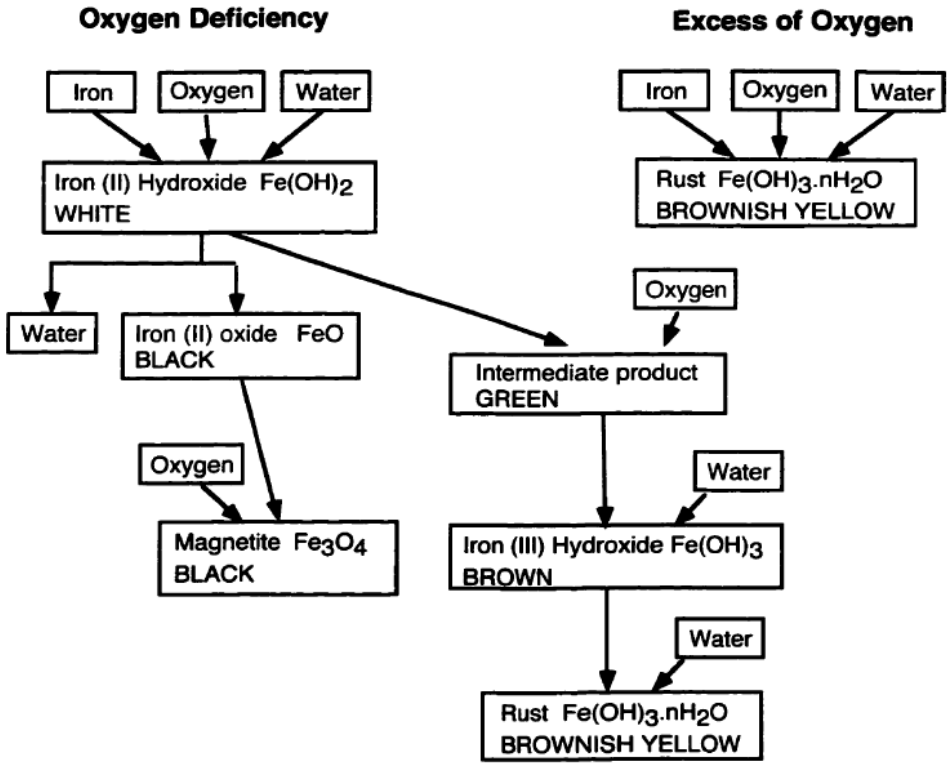

Figure 6.31 Flowchart of Product formed during the Corrosion Process depending upon the availability of Water and Oxygen (Herholdt *et al*., 1985).

It is worthwhile to mention that in this experiment all samples were subjected to the same rate of corrosion by applying constant voltage through them. However, to enforce same rate of corrosion much higher energy was spent for bacterial samples. It can be concluded that RC specimens treated with *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) with MICP abilities impede further corrosion in addition to strength enhancement of the structure.

The bars used in these experiments are the standard reinforcing bars that have helical ribs on their surface to enhance bond. A close examination of the control specimen bars revealed that the bars had very high corrosion products on their surface and ribs were almost lost (this may be one of the reasons for the reduced bond strength); whereas in case of protected bars [using *Bacillus* sp. (CT-5)] very little corrosion products on their surface were found and the ribs in these bars were much less pronounced. MICP samples had a smaller patch of rust than the control samples and they had higher pullout strength. A note should also be made that the high pH of concrete makes the concrete more reactive than the reinforcing steel. As corrosion agents permeate into the concrete, they often react with the concrete before ever reach the embedded steel. The concrete therefore, protects the steel from corrosion. If the permeability is reduced, the rate at which the corrosion process occurs is reduced by two mechanisms. First, the amount of time required for the corrosion agent to reach the embedded steel is increased due to barrier formed by biofilm and MICP. Both *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) were able to produce enormous amounts of EPS and biofilm along with urease and calcite, as discussed previously. Biofilm and EPS production by bacteria also played a major role towards enhancement of compressive strength, reducing permeability and corrosion rate of any reinforcement. When the biofilm produced from *Bacillus subtilis*, was genetically engineered to secrete polyglutamate or polyaspartate, an additional small increase in corrosion inhibition on aluminium occurred (Ornek *et al*., 2001). Biofilms probably comprise the normal environment for most microbial cells in many natural and artificial habitats such as concrete surfaces, and as such are complex associations of cells, extracellular products and detritus either trapped

within the biofilm or released from cells which have lysed as the biofilm ages (Christensen, 1989). The main 'cement' for all these cells and products is the mixture of polysaccharides secreted by the cells established within the biofilm. The attachment of microorganisms to the concrete surface is a complex process, with many variables affecting the outcome (Pratt and Kolter, 1998). Further, growth requires a complex developmental pathway involving a series of events that are regulated in response to environmental- and bacterial-derived signals. The solid–liquid interface between concrete surface and nutrient sources or water provides an ideal environment for the attachment and growth of microorganisms (Costerton *et al*., 1999).

Secondly, as the amount of time required for the corrosion agents to reach the embedded steel increases, the amount of reaction that occurs between the corrosion agents and the concrete also increases. This increase results in a significant reduction in the amount of corrosion agents, if any, that permeates into the concrete to the depth of the embedded steel. Significant reduction in the corrosion rate of rebar inside the concretes was obtained based on MICP produced by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5). Field Machine (ACM Corrosion Analyzer System, UK) was used to measure the corrosion rate. It measures the corrosion rate by calculation LPR (Linear Polarization Resistance) rate and Tafel plot. The LPR technique has become a well-established method of determining the instantaneous corrosion rate measurement of reinforcing steel in concrete (Broomfield, 1996). Due to the widespread corrosion of reinforcing steel in concrete structures there has been a concerted demand for the development of non-destructive techniques to enable accurate assessment of the condition of reinforced concrete structures. Tafel plots provide a direct measure of the corrosion current, which can be related to corrosion rate (Song and Saraswathy, 2007).

6.16 Conclusion

In conclusion, the positive potential of microbially induced calcite precipitation that has been demonstrated in present study offers an interesting concept of the enhancement of durable properties such as compressive strength, crack remediation, impermeability to aggressive substances and corrosion of building materials in various applications. The microbial treatment resulted in an increased resistance of mortar specimens towards degradation processes. The results from this research tend to confirm the interrelationship that exists between transport and degradation mechanisms occurring in concrete. The transport mechanisms in concrete are influenced by the calcite precipitation by ureolytic bacteria and a calcium salt. The presence of a layer of calcium carbonate due to MICP produced by *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) resulted in a decrease of the permeation properties of cementitious materials, increased compressive strength and reduced corrosion rate of RC structures. The ASTM C 1202-05 classifies concretes in terms of chloride ion penetrability and based on this classification, the concretes containing *Bacillus* sp. (CT-5) can be considered as concretes of very low permeability. The lower chloride permeability of the concretes with bacterial cells is a result of a denser microstructure. The microbial induced calcite precipitation reaction may cause lower amount of capillary pores and clogging of the pores, which reduces chloride ion transport in concrete. The use of bacterial cells has thus become a viable solution not only to some durability problems but also as an environmentally responsible course of action.

Calcium carbonate precipitation has efficiency to reduce the corrosion rate. The effectiveness of calcium carbonate precipitation as a protective film in reducing the corrosion rate of the underlying mild steel was determined by measuring the corrosion rate. Compared with the electrochemical corrosion parameters of specimens, the corrosion current is much lower with *Bacillus* sp. (CT-5) irrespective of CSL or standard nutrient media used to grow the cells, indicating the specimens with bacterial cells have better corrosion resistance than control samples. It is known

that the corrosion resistance of any reinforcement depends largely on the ability to form a surface protective film such as calcite deposition and biopolymer/biofilm production by bacteria. The corrosion of the reinforcing steel, because of its importance to the strength of the overall member, is an issue of particular importance. To meet the performance criteria of concrete throughout its service life, the corrosion of the reinforcing steel must be significantly reduced by employing the MICP technique. MICP plays an important role and effective in filling corrosion cracks and impeding for the corrosion of reverse and enhancement in the durability of building materials and structures. Finally, the present study indicates that corrosion protection induced from MICP that are applied to reinforced structures for strengthening is a bonus. With ever increasing number of RC structures that are deteriorating, the proposed technique should be a very attractive solution for their protection. In addition, industrial by-product corn steep liquor was successfully utilized as a nutrient source to grow bacterial cells and a curing medium; towards development of microbial concrete that provides a greener, ecological and eco-friendly option.

Chapter 7

Enhancement of cementing activity of bacteria by strain improvement

---

7.1 Mutagenesis

Ultraviolet (UV) irradiation as a physical mutagenic agent is widely used for strain improvement of bacterial species and was used in the present study for over production of urease and calcite. Mutagenesis of bacterial isolates was carried out using different exposure times from irradiation source. Two bacterial isolates *viz*. *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) based on their performance towards urease and calcite precipitation were subjected to UV irradiation to enhance the cementing activity of bacteria. The concentration of viable cells present in aqueous suspensions was measured by spread plating after UV exposure. Effect of UV exposure of selected bacterial isolates for different time intervals (5-30 min) was evaluated through constructing a survival curve plot (Fig. 7.1). The results revealed a gradual decline in percentage of survivors with increase in UV exposure time. Few colonies were selected randomly from the plates, which were irradiated to UV for 15 min and 20 min, as less than 10 % survival rate was recorded from these plates. It is obvious from the data that in all the cases irradiation had lethal and mutagenic effects on bacteria. Curves with maximum mutation frequencies are well known in the case of cell irradiation with UV or ionizing radiation (Bresller, 1976). It is assumed to be connected with the great probability of mutated cell destruction. Results in Table 7.1

showed that the survival percentages in case of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) were decreased by increasing the time of exposure whereas the survival percentage was very low (<0.1 %) at 20 min exposure time. These colonies were transferred on Urea Agar base medium (HiMedia, India) to check the production of urease based on the intensity of pink color. Based on these qualitative method, a total of four colonies from *S. pasteurii* (Bp W) (designated as Bp M-1, Bp M-2, Bp M-3 and Bp M-4) and three colonies from *Bacillus* sp. (CT-5) (designated as CT5 M-1, CT5 M-2 and CT5 M-3) were selected which showed better pink color intensity. These isolates were cultured at least five times to assure incapability of reverse mutation and qualitative urease production. These were compared with their wild type strain for quantitative urease production and calcite precipitation. Nutrient and CSL media were used to study urease production and calcite precipitation by these mutants.

7.2 Urease production by mutants

Few mutants developed from UV irradiation showed significantly higher urease production compared to wild type strains. Even in case of all the mutants, maximum urease production was observed at the end of 120 hours as described previously. One of the mutants of *S. pasteurii* (Bp W), Bp M-3, produced higher amount of urease compared to other isolates including its wild type irrespective of media used to grow the cells, although higher production was obtained when mutants were grown in media containing CSL. The urease production by Bp M-3 was 570.12 and 560 U/ml while its parent strain produced 441.5 and 427.3 U/ml of urease in CSL and nutrient

media respectively (Fig. 7.2). Maximum urease was recorded by mutant Bp M-3 (Tables 7.2 and 7.3).

All the mutants developed from *Bacillus* sp. (CT-5) showed better urease production from their wild type strain. CT5 M-3 showed significantly higher urease production (705.84 and 694.12 U/ml urease in CSL and nutrient media respectively) compared with other mutants (Fig. 7.3). Its parental strain, *Bacillus* sp. (CT-5), produced 675 and 665 U/ml of urease in CSL and nutrient media respectively. Significant differences in urease activity were found between the different mutants of *Bacillus* sp. (CT-5) (Tables 7.4 and 7.5). Also, there was a significant difference in urease production among different media by mutants and CSL medium was preferred nutrient source even for mutants.

Improvement of microbial strains for the overproduction of enzymes has been the hallmark of all commercial processes. Such improved strains can reduce the cost of the process with increased productivity and may also possess some specialized desirable characteristics. Effectiveness of physical mutagenesis by UV radiation in strain improvement for enhanced urease productivity was demonstrated in the present investigation. It is hoped that the high yielding mutant strain of the wild type isolates can be exploited commercially for large scale industrial production of urease.

Table 7.1 Survival of bacterial isolates, *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5), after UV exposure for different time intervals

Time	**Bp W**		**CT-5**	
(min)	Cfu/ml	Survival (%)	Cfu/ml	Survival (%)
0	50×10^6	100	50×10^6	100
5	38×10^6	76	22×10^6	44
10	62×10^5	12.4	33×10^5	6.6
15	27×10^4	0.54	7400	0.0148
20	6×10^3	0.012	200	0.0004
30	0	0	0	0

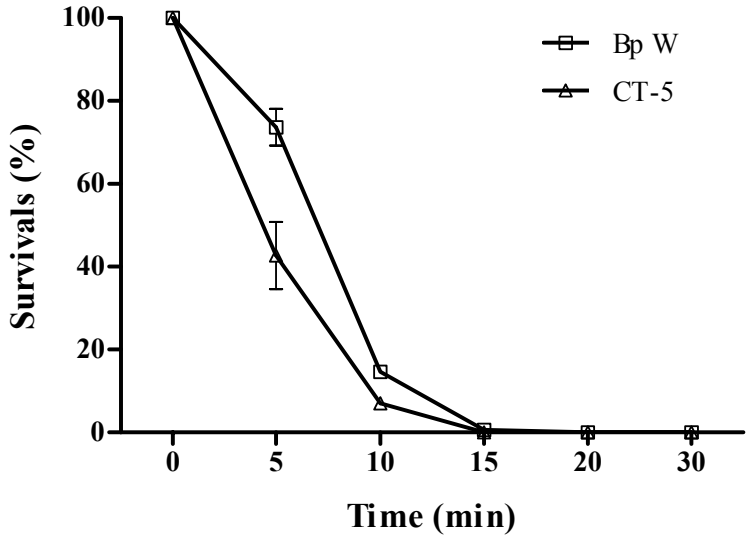

Figure 7.1 Survival curve of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) after UV irradiation for different time intervals

Table 7.2 Comparison of urease activities (U/ml) by wild type and phenotypic mutants of *S. pasteurii* (Bp W) in CSL media

Isolates	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Bp W	285.6±6.2c	385.6±3.2b	407.8±3.4c	420.5±2.1c	441.4±7.3d	384.3±2.7c	360.4±3.9c
Bp M-1	378.7±1.1b	384.7±1.2b	387.8±0.3d	395.2±0.9e	443.1±0.8d	372.6±0.5d	351.8±1.5d
Bp M-2	387.5±0.4ab	402.9±0.9ab	410.6±0.9c	419.1±0.7d	451.3±1.2c	382.7±2.3c	350.4±8.3d
Bp M-3	388.6±1.2ab	414.1±1.0a	430.2±7.3a	480.4±3.1a	565.2±4.3a	539.2±0.7a	446.2±3.3a
Bp M-4	392.0±7.0a	409.3±0.8ab	424.5±5.5b	447.7±4.4b	471.6±0.8b	402.8±2.5b	377.5±2.2b

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

Table 7.3 Comparison of urease activities (U/ml) by wild type and phenotypic mutants of *S. pasteurii* (Bp W) in nutrient media

Isolates	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Bp W	276.7±3.0d	377.3±6.7b	400.7±0.7bc	412.7±10c	427.3±0.7d	371.3±0.7c	334.7±1.3d
Bp M-1	376.7±3.3c	380.7±3.3b	382.0±0.7c	392.7±8.7d	438.0±2.0c	368.0±1.3c	343.3±3.3c
Bp M-2	385.3±1.3b	398.7±1.3ab	406.7±6.7b	411.9±10c	446.7±6.7b	370.3±0.3c	343.3±10c
Bp M-3	383.3±2.0b	406.7±6.7a	420.7±0.7a	473.3±6.7a	550.0±10a	534.0±0.7a	439.3±2.0a
Bp M-4	401.3±25a	407.3±6.0a	420.7±4.7a	425.3±1.3b	450.0±3.3b	392.0±9.3b	356.7±3.3b

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

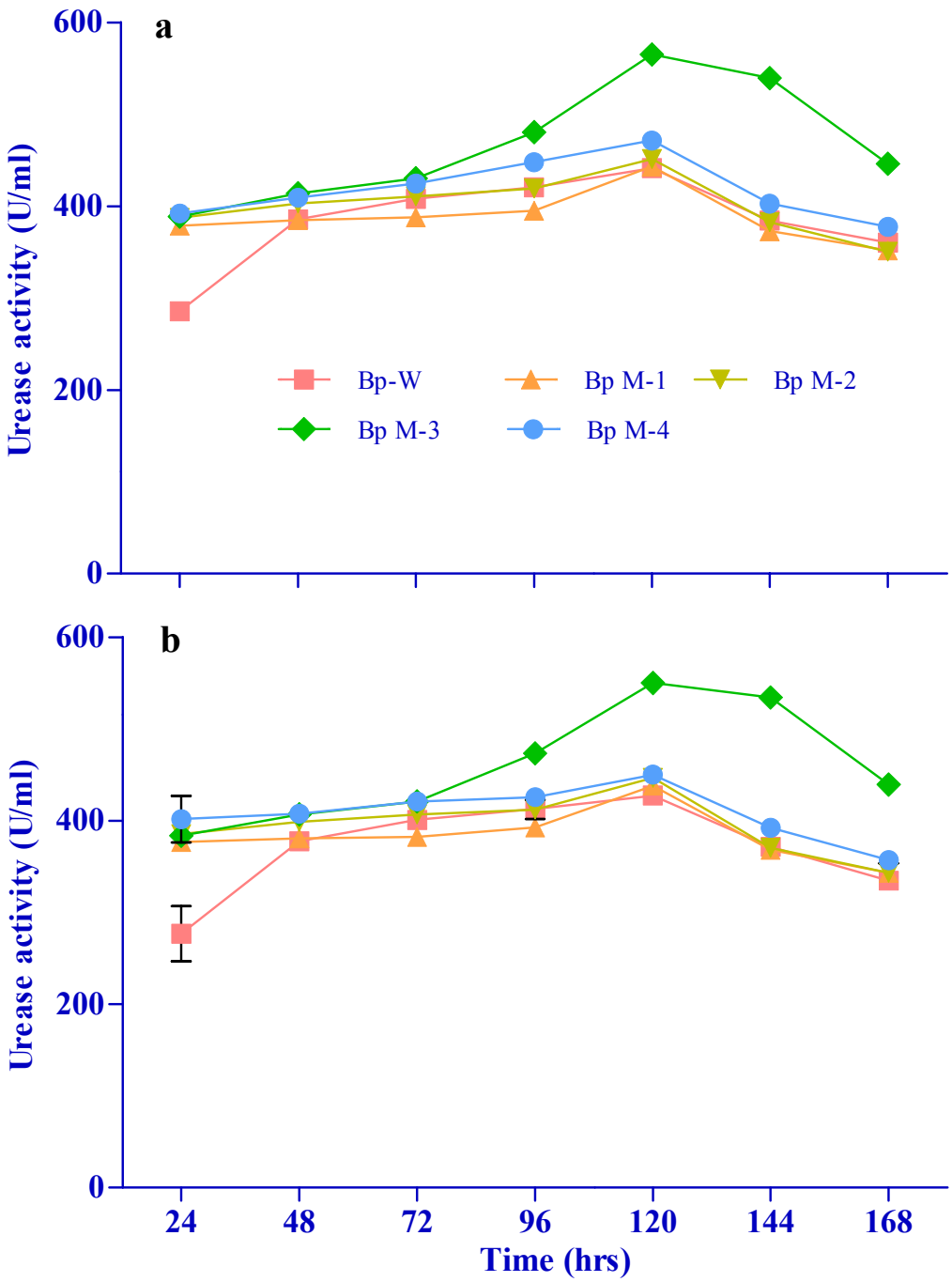

Figure 7.2 Urease activity by phenotypic mutants of *S. pasteurii* (Bp W) in (a) CSL and (b) nutrient media

Table 7.4 Comparison of urease activities produced by wild type and phenotypic mutants of *Bacillus* sp. (CT-5) in CSL media

Isolates	Urease Activity (U/ml)						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
CT-5	360.7±4.5c	441.1±1.1c	554.4±2.5c	619.9±1.3c	675.5±0.8c	620.4±1.9c	577.0±4.1c
CT5 M-1	381.9±9.7b	470.3±6.6b	568.8±6.4b	637.8±6.7b	682.1±2.6b	637.3±6.1b	606.4±6.2b
CT5 M-2	341.1±3.9d	421.5±1.6d	534.8±1.1d	600.3±0.9d	655.9±9.8d	600.8±0.7d	557.4±5.6d
CT5 M-3	405.0±8.9a	493.4±6.2a	591.9±5.8a	660.8±6.4a	705.2±1.9a	660.4±5.7a	629.5±6.2a

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

Table 7.5 Comparison of urease activities produced by wild type and phenotypic mutants of *Bacillus* sp. (CT-5) in nutrient media

Isolates	Urease Activity (U/ml)						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
CT-5	344.0±1.7c	431.5±4.8c	537.3±2.7c	609.8±1.3c	665.2±2.9c	613.8±1.5c	567.6±3.2c
CT5 M-1	356.8±4.4b	445.8±5.3b	555.9±4.9b	627.9±4.9b	673.5±2.4b	625.7±4.8b	583.9±5.5b
CT5 M-2	324.4±1.8d	411.9±5.6d	517.7±3.5d	590.2±2.2d	645.6±3.8d	594.2±2.5d	548.1±2.9d
CT5 M-3	378.3±6.5a	467.3±7.2a	577.4±5.1a	649.4±5.1a	695.0±4.4a	647.2±6.8a	605.4±3.4a

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

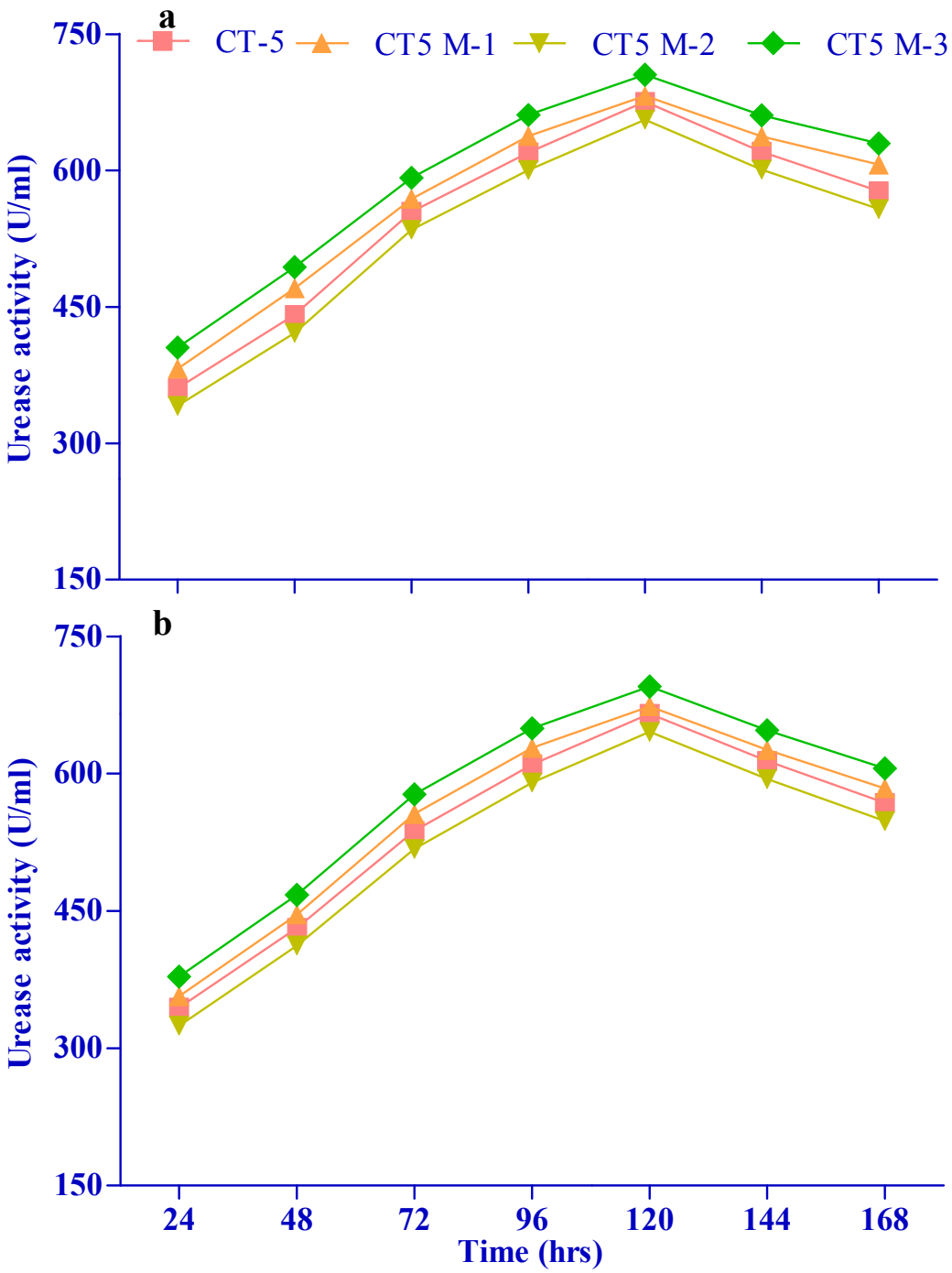

Figure 7.3 Urease activity by phenotypic mutants of *Bacillus* sp. (CT-5) in (a) CSL and (b) nutrient media

7.3 Calcite precipitation by mutants

Among the *S. pasteurii* (Bp W) mutants, Bp M-3 precipitated maximum calcite compared to other mutants including its wild type irrespective of media used to grow the cells. The microbially induced calcite precipitation (MICP) in the upper layer of sand column by Bp M-3 in CSL and nutrient media was 31.76 and 30.09% respectively (Fig. 7.4). In case of *Bacillus* sp. (CT-5) mutants, CT5 M-3 showed maximum calcite precipitation. CT5 M-3 precipitated 35.15 and 33.23% calcite in CSL and nutrient media respectively (Fig. 7.5). Significantly higher amount of calcite was precipitated in the sand column consolidated with mutants Bp M-3 and CT5 M-3 (Table 7.6). CSL media helped mutants to precipitate higher calcite compared to nutrient media. When mutants were grown in CSL media, it produced significantly higher calcite compared to nutrient media.

Calcite precipitation by mutants developed from all the isolates followed similar trend of urease production. Calcite precipitation was directly proportional to urease production which is very well known. The results indicated that urease activity was affected and improved by exposure to UV irradiation. Ultraviolet radiation is one of the well-known and most commonly used mutagen. It is universally used to induce genetically improved strains. Aly *et al*. (2001) indicated that UV treatment was more effective as a mutagenic agent than some chemical mutagens such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) in isolation of mutants in *Bacillus sphaericus*.

Table 7.6 Comparison of calcite contents in different layers of sand columns prepared by wild type and phenotypic mutants of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) in different media

Isolates	Calcite content (%) in different layers					
	CSL media			Nutrient media		
	Upper	Middle	Lower	Upper	Middle	Lower
Bp W	29.7±0.5c	20.1±0.1cd	9.5±0.2c	28.4±0.5c	18.7±0.3d	8.1±0.2e
Bp M-1	29.9±0.6c	20.3±0.1c	9.8±0.2c	28.6±0.4b	18.9±0.3c	8.3±0.1d
Bp M-2	30.6±1.0b	20.9±0.6b	10.4±0.6b	29.2±0.4ab	19.5±0.4b	8.9±0.3c
Bp M-3	31.4±0.6a	21.7±0.1a	11.1±0.2a	30.0±0.5a	20.3±0.3a	9.7±0.4a
Bp M-4	30.9±0.9b	21.2±0.4ab	10.6±0.5b	29.5±0.3ab	19.8±0.2b	9.2±0.2b
CT-5	32.2±0.3b	21.6±0.2b	9.7±0.2b	30.9±0.2b	19.5±0.2b	8.9±0.3c
CT5 M-1	32.5±0.3b	21.9±0.2b	10.0±0.2b	31.2±0.2b	19.8±0.2b	9.2±0.2b
CT5 M-2	29.4±0.2c	18.9±0.4c	6.9±0.1c	28.2±0.3c	16.7±0.3c	6.1±0.2d
CT5 M-3	34.9±0.2a	24.4±0.2a	12.4±0.1a	33.6±0.4a	22.2±0.4a	11.6±0.3a

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

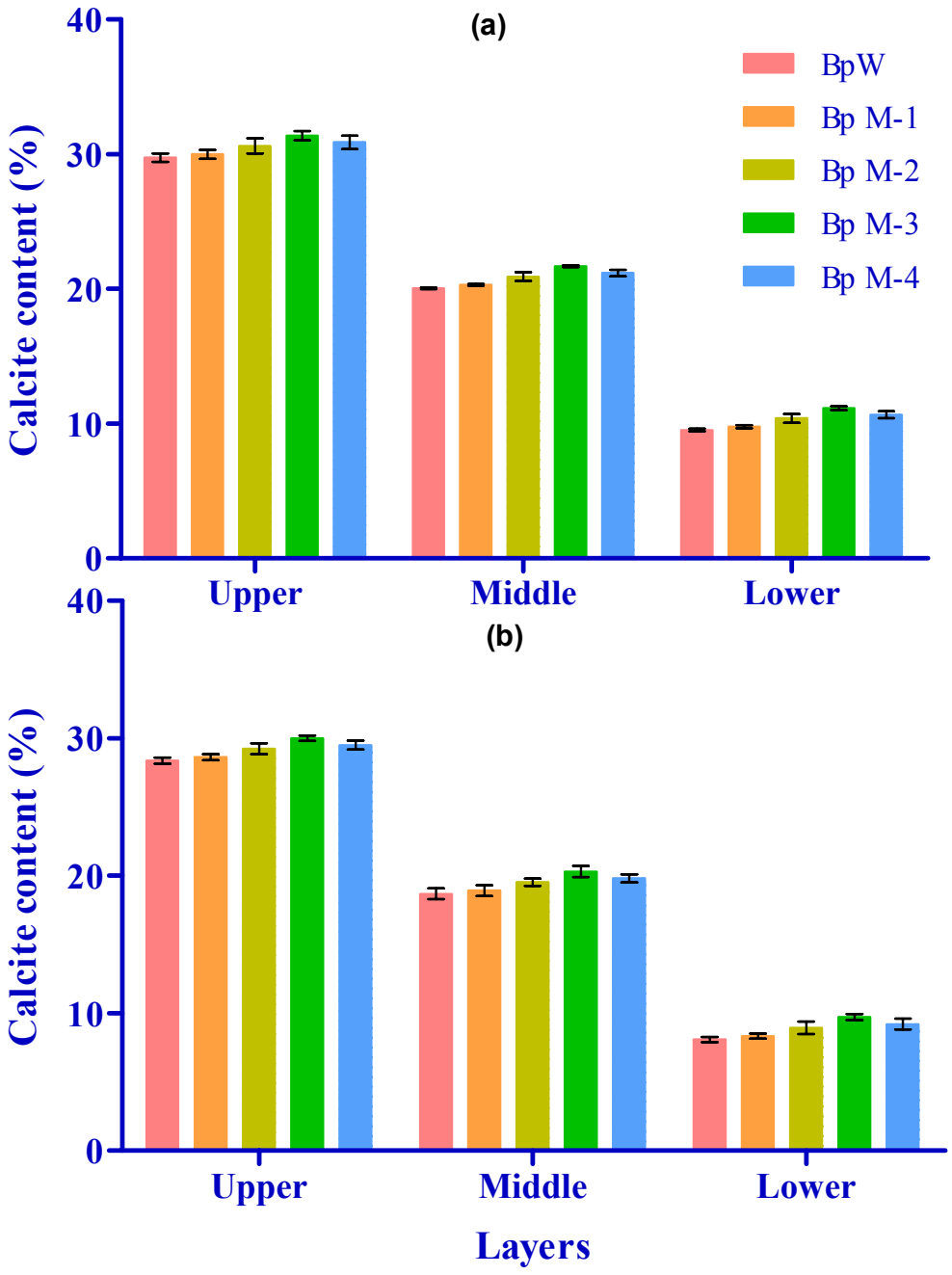

Figure 7.4 Calcite content by wild type and phenotypic mutants of *S. pasteurii* (Bp W) in (a) CSL and (b) nutrient media

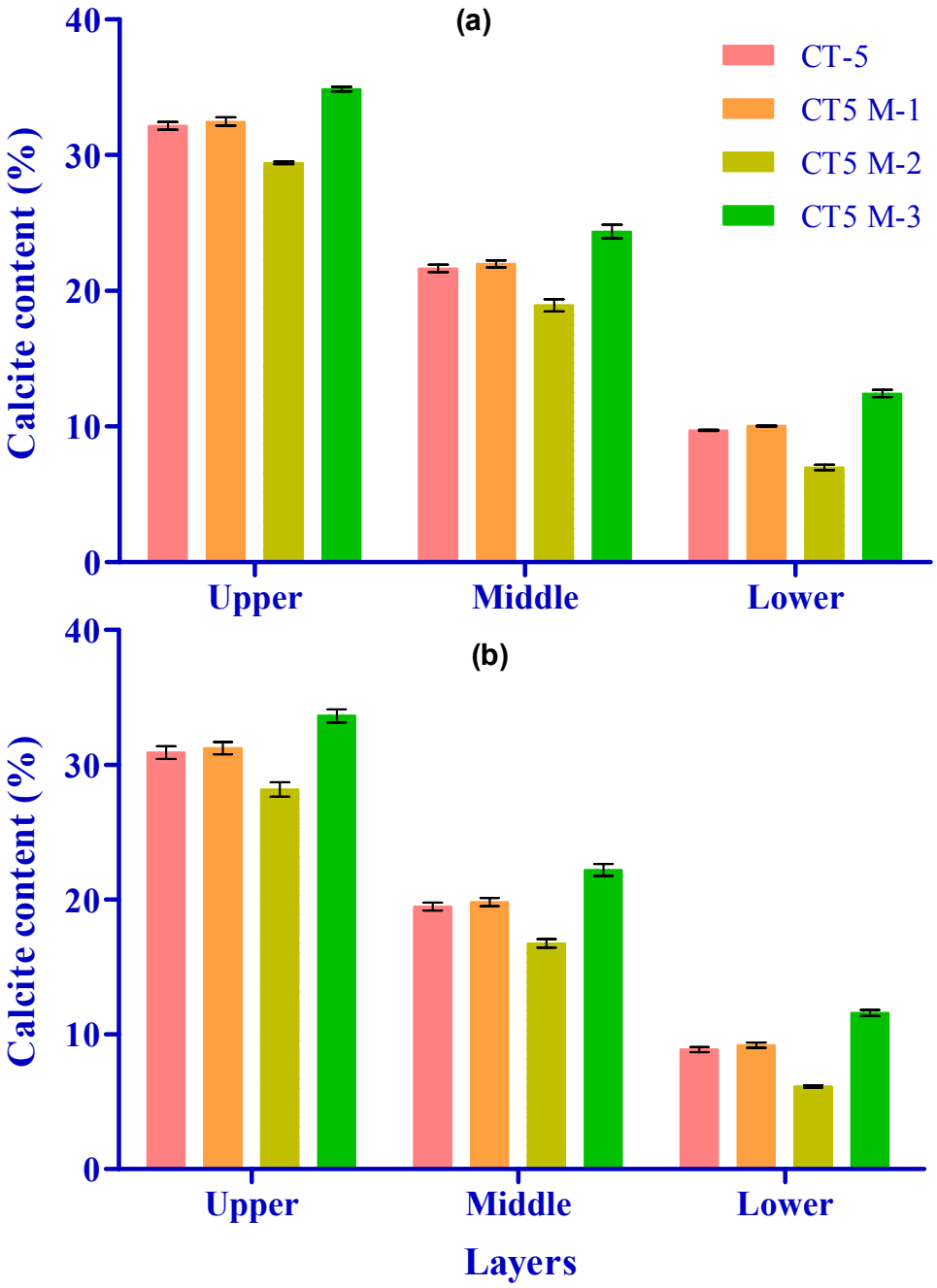

Figure 7.5 Calcite content by wild type and phenotypic mutants of *Bacillus* sp. (CT-5) in (a) CSL and (b) nutrient media

The cementing activity of *S. pasteurii* (Bp W) was improved by mutagenesis in the present study. Among *S. pasteurii* (Bp W) mutants, Bp M-3 produced significantly higher urease and also precipitated maximum amount of calcite when consolidated in sand. To determine the presence of microbial calcite precipitation, samples collected using this mutant strain were subjected to SEM-EDX and XRD analysis. SEM analysis confirmed calcite crystals embedded with bacteria between and on the surface of sand grains (Fig. 7.6). Further, EDX and XRD confirmed majority of carbonate deposits as calcite crystals (Fig. 7.7). The present study established the improvement in bacterial species for hyper activity of cementing enzyme.

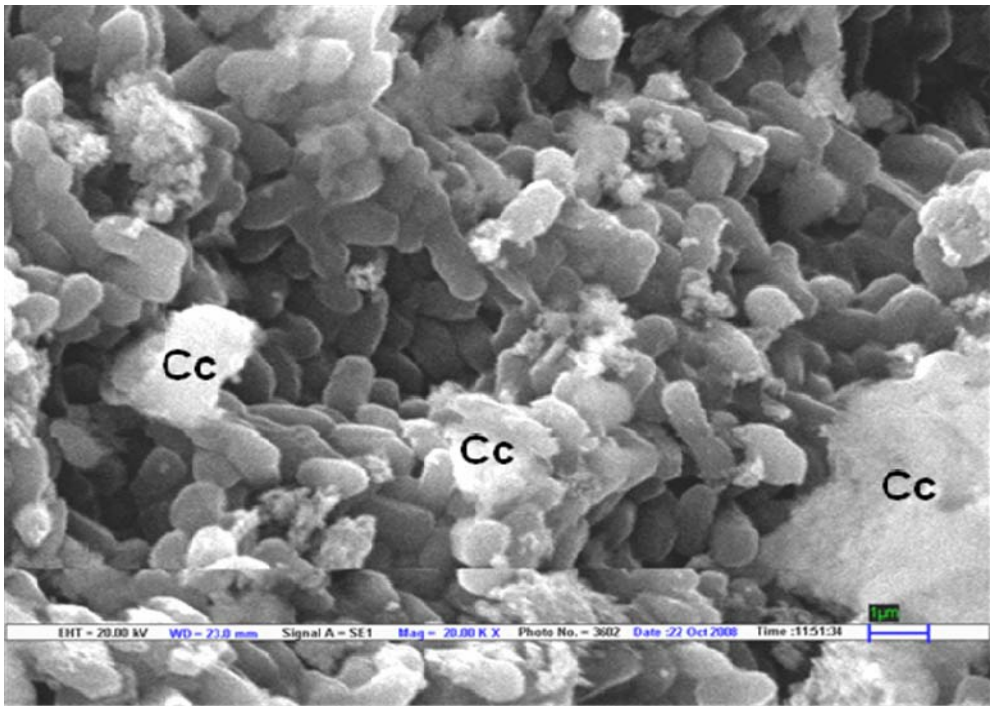

Figure 7.6 Scanning electron micrograph of microbially induced calcite precipitation by mutant Bp M-3 in sand consolidation (Cc-calcite crystals)

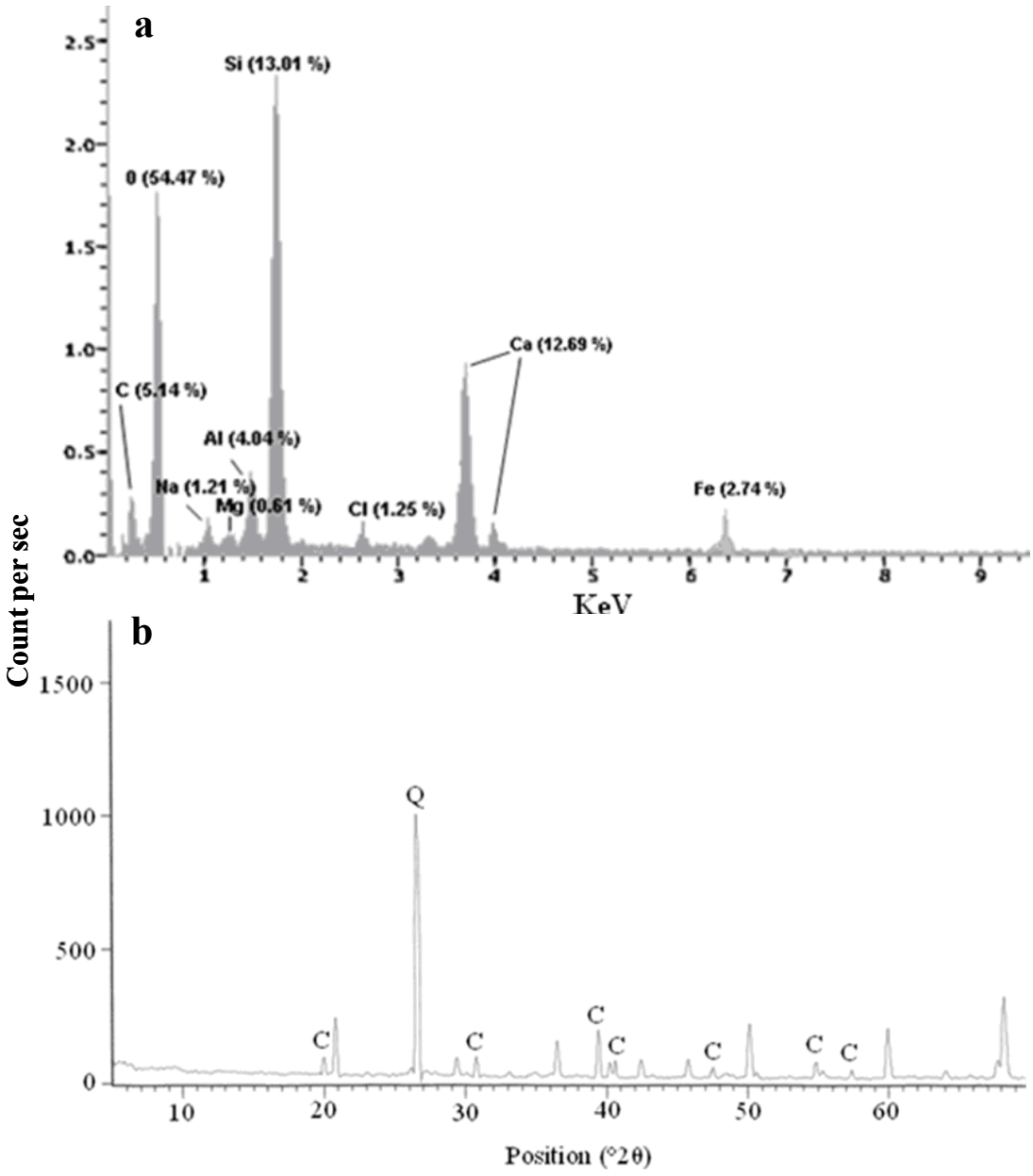

Figure 7.7 (a) Energy-dispersive X-ray spectrum of the microbial precipitation with Bp M-3 in sand. The abundant presence of Ca was evident, and the precipitation was inferred to as calcite ($CaCO_3$) crystals. **(b)** XRD analysis of the sand column treated with Bp M-3 (C-calcite, Q-quartz)

7.4 Discussion

The aim of strain improvement programme was to deregulate the regulatory mechanisms of an organism such that maximum metabolic energy is devoted to a single product, either a particular protein or a product from a metabolic pathway. General approach of strain improvement is mutation (random mutagenesis). Random mutagenesis is based on mutation and screening to improve the strain. The approach of random mutagenesis using UV irradiation, a mutagenic substance with a higher degree of specificity, was introduced in strain improvement programme in this study. Mutagens lead to mutation by inducing lesion or modification in base sequence of DNA that remains un-repaired. Thus, an attempt was made to develop bacterial isolates of enhanced ability to precipitate calcite by mutagenesis by selection into efficient strain for production of urease. Effectiveness of physical mutagenesis by UV radiation in strain improvement for enhanced urease productivity and calcite precipitation was demonstrated. UV irradiation exerted its effect on growth and survivability of *Bacillus* sp. With the increase of mutagen time duration, viable count was found to decline proportionally that suggested the high dose of UV irradiation might be lethal to the organisms. It is hoped that the high yielding mutant strain of bacterial isolates can be exploited commercially for large scale industrial production of urease. The parent test strains were treated with UV irradiations. This study presents the improved calcite precipitation and urease production, using mutants of *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) obtained by random mutagenesis of wild isolates using UV irradiation. The mutants obtained were screened based on their ureolytic activity and best mutants were selected for further studies. Mutant strains offered increased urease and calcite production compared to wild strain.

Mutants among all the isolates showed different responses to UV radiation for urease production. These variations are more probably due to the differences induced in their genetic background (Ben, 2003). Nagami and Tanaka (1986) proved that mutation of genes controlling cell membrane composition led to the hyper production of enzymes such as proteases. They also pointed to the effect of

mutations on regulatory genes which are associated with the structural genes. However, the application of UV irradiation, whatever the mutation(s) include either modifying or structural genes, led to the isolation of hyper enzyme producing cultures (Qadeer *et al.,* 1980). There are evidences that indicate the implementation of mutagenesis through UV for strain improvement (Wu *et al*., 2006; Besoain *et al*., 2007).

7.5 Conclusion

It was inferred from the results that the difference in gene expression of some positive mutants might be a cause of hyperactivity of enzyme in mutants than parent strain. In conclusion, this work represented the first report of strain improvement in bacterial species such as *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) for hyper-cementing activity and microbial concrete producing enzyme and suggested that these mutants could be used further for various applications in bioremediation.

Chapter 8

Summary

Cementing enzyme production via microbiologically induced calcite precipitation (MICP) by way of urea hydrolysis, presents a promising novel biotechnology for the enhancement of durability of building materials and structures. Concrete is most widely used building material throughout the world. Concrete can be considered as a kind of artificial rock with properties more or less similar to certain natural rocks. As it is strong, durable, and relatively cheap, concrete is the most used construction material worldwide, which can easily be recognized as it has changed the physiognomy of rural areas. The objective of the present research work was to develop an effective, economic and eco-friendly microbial process, based on microbially induced calcite precipitation, to remediate the building materials and structures with self-healing ability and also to enhance their durability. Natural processes, such as weathering, faults, land subsidence, earthquakes, and human activities create fractures and fissures in concrete structures. These fractures and fissures are detrimental since they can reduce the service life of the structure. Weathering induces an increased porosity, structural weakening of surface layers and unattractive appearance. Synthetic agents such as epoxies and surface treatments with water repellents such as silanes or siloxanes, or with pore blockers are applied for remediation of these structures. However these and other treatments with organic and anorganic products involve some disadvantages, such as different thermal expansion, degradation with age and the need for constant maintenance. Appearance of cracks and fissures is an inevitable phenomenon during the aging process of concrete structures when exposed to weather changes. Such cracking leads to easy passage for aggressive environment to reach the reinforcement and initiate corrosion. Moreover, sometimes repair is carried out in the areas where it is not possible to shut down the plant or it is hazardous for human beings. Hence, in such situations a way should be found out to self healing materials that seal the cracks automatically which can be achieved through biomineralization. Biologically induced mineralization

refers to processes by which an organism modifies its local microenvironment creating conditions suitable for the chemical precipitation of extracellular mineral phases. Mineral formation in aqueous environments is achieved by introducing small perturbations, as biologically produced metabolic end-products, or the release of particular ions by the cells, that will induce the mineral to precipitate. A novel technique towards enhancement in the durability of building materials or structures by utilizing microbiologically induced calcite precipitation is discussed. Microbiologically induced calcite precipitation (MICP) is a technique that comes under a broader category of science called biomineralization.

The strategy of physiological characterization, total DNA extraction, PCR-amplification, screening of clone libraries and sequence determination of cloned 16S rRNA genes enabled the detection and recognition of bacterial species from samples of the extreme environment. Bacteria were isolated from extreme alkaline environments such as calcareous sludge, cement, alkaline soil and limestone area. Phylogenetic analysis revealed all the bacterial isolates belonging to genera *Bacillus*. The isolates [*Bacillus* sp. (CT-5), *Bacillus* sp. (LS-2), *Bacillus* sp. (CT-2), *B. subtilis* (AS-4), *Bacillus* sp. (LS-4) and *B. megaterium* (SN-3)] of the present work also showed the ability to tolerate a wide range of salinity, pH, temperatures and produced higher amounts of EPS and biofilm. The efficiency of these isolates was checked for urease production. A rapid, simple and economical zymogram method to detect urease activity in the isolates was established. One of the cement isolates *Bacillus* sp. (CT-5) was able to produce maximum amount of urease and calcite.

Urease was the most expensive component of the microbial concrete production, so cost-effective production was desired, thus an economic growth procedure was developed for large scale cultivation of bacterial isolates. The cultivation medium was economized and expensive components were replaced with industrial by-products such as lactose mother liquor (LML) and corn steep liquor (CSL). The nutritive growth profile of bacterial isolates confirmed that media containing corn steep liquor (CSL) supported fast growth of *Bacillus* species and maximum urease production. Based on higher urease productions, calcite precipitations, abundant EPS

productions and mature biofilm production by bacterial isolates after utilization of CSL media, it can be concluded that such industrial by-product can be successfully used as a nutrient source to produce microbial concrete and expensive laboratory grade medium can be replaced using these industrial economic medium.

Microbial urease is able to produce concrete by precipitating calcium carbonate (calcite) in environment. The hydrolysis of urea by the widely distributed enzyme urease is special in that it is one of the few biologically occurring reactions that can generate carbonate ions without an associated production of protons. When this hydrolysis occurs in a calcium-rich environment, calcite precipitates from solution forming a solid-crystalline material. Calcium carbonate is a major constituent of concrete which makes any building materials or structure stronger. Due to the ability to precipitate calcite, urease is regarded as "cementing enzyme". The bacterial isolates in the present study were able to use urea as an energy source, resulting in the production of ammonia and carbonate. This increases the pH and the carbonate concentration near the cells, triggering Ca^{2+} and CO_3^{2-} to precipitate as $CaCO_3$. The ability of bacteria isolated from the cement samples to precipitate carbonate has been demonstrated for the first time. The biocementation capability of these strains was better than that of *Sporosarcina pasteurii*, a known urease producer. As a microbial sealant, $CaCO_3$ exhibited its positive potential in consolidation of sand. Out of all the isolates of present study, *Bacillus* sp. (CT-5) produced 1.59 mg calcite/cell dry mass (mg) at flask level experiment while calcite constituted 30.9 %, 19.59 % and 8.9 % in the upper, middle and lower layer respectively, of the total weight of the sand column plugged by this strain. The precipitated minerals were studied and confirmed in microbial sand column by X-ray diffraction, scanning electron microscopy and EDX.

MICP is highly desirable because the calcite precipitation induced as a result of microbial activities, is pollution free and natural. The technique can be used to improve the compressive strength and stiffness of cracked concrete specimens. The effect of different concentrations of bacteria on the strength and durability of concrete was also studied. It was found that all the concrete specimens with bacteria

(at concentrations of 5×10^7 cfu/ml) performed better than the control samples (without bacteria). The study showed that there was a significant increase in compressive strength of the cement mortar cubes containing bacterial cells. Findings of microbiologically enhanced crack remediation from this study offer an interesting and unique concept in crack remediation technique for a wide range of potential applications. Microbial [*Bacillus* sp. (CT-5)] remediation increased the compressive strength by 40% of that of the control. For instance, biological metabolic products may be utilized as sealing or caulking material for filling the gaps in building structures. Furthermore, multipurpose application of this remediation technique may become feasible by utilizing the bacterial cells. The calcite layer formed by bacterial isolates on the concrete surface improved the impermeability of the specimen, thus increasing its resistance to adverse conditions (such as alkaline, sulfate and freeze-thaw attack). The presence of a layer of calcium carbonate and microbial biomass resulted in a decrease of the permeation properties of cementitious materials. As a result, an increased resistance towards water and chloride migration was noticed. When concrete specimens were treated with *Bacillus* sp. (CT-5), the permeability class changed to very low type.

Most of the studies based on MICP were carried out to evaluate compressive strength, water absorption and crack remediation of mortars and concretes. The role of MICP in reducing the corrosion rate of reinforced concretes has not been studied in detail. One of the most important concerns in case of the reinforced concrete structures is the corrosion of the embedded rebars. The effectiveness of calcium carbonate precipitation as a protective film in reducing the corrosion rate of the underlying mild steel was determined by measuring the corrosion rate. Bacterial isolates used in the present study successfully reduced the corrosion rate and MICP favorably intervened the deterioration of bond between concrete and rebars by impeding their corrosion. Overall, this study demonstrated that biomineralization induced by bacterial activity results in significant protection and enhancement in the durability of building materials or structures. Corrosion rate measurement showed that MICP by *Bacillus* sp. (CT-5) lead to around four-fold reduction in the corrosion

rate of RC (reinforced concrete) specimens. To the best of our knowledge this is the first report of getting enhancement in the durability aspects of building materials by a cement isolate, *Bacillus* sp. (CT-5).

Microbiologically induced calcite precipitation was found effective in increasing brick strength by reducing water absorption up to 46.5%. Among different microbial cultures, *Bacillus* sp. (CT-5) was found to be the highest producer of urease and was an effective inducer of calcite deposition on the surface of the bricks. The reduction of water absorption was attributed to biocalcification on the surface, leading to a reduction in permeability and a subsequent decrease in the diffusion of water and other corrosive ions that also increased the compressive strength of bricks. In conclusion, biocalcification would be an effective method for increasing the durability of bricks.

Finally a successful attempt was made to develop bacterial isolates of enhanced ability to precipitate calcite by mutagenesis by selection into efficient strain for production of urease. Effectiveness of physical mutagenesis by UV radiation in strain improvement for enhanced urease productivity and calcite precipitation was demonstrated. Mutant strains offered increased urease and calcite production compared to wild strain. It is hoped that the high yielding mutant strain of bacterial isolates can be exploited commercially for large scale industrial production of urease. The present work represented the first report of strain improvement in bacterial species such as *S. pasteurii* (Bp W) and *Bacillus* sp. (CT-5) for hyper-cementing activity and microbial concrete producing enzyme and suggested that these mutants could be used further for various applications in bioremediation.

The introduction of MICP offers higher quality concretes with adequate impermeability, compressive strength and reduced reinforced corrosion. This study has conclusively established that high strength economical cementation can be achieved using MICP via urea hydrolysis to “proof-of-concept” stage. This is the first published study to achieve enhancement in the durable properties of concrete structures via the application of indigenous bacterial isolates of cement. The

bioremediation technology has many advantages of over existing conventional technologies, including self-healing ability and *in-situ* application. Cementation can be readily achieved without any additional processing of the bacterial culture liquid (*e.g.* concentration, lysis, removal of medium), by directly mixing the culture with the cementation components and direct application into the construction material. A cost-efficient and eco-friendly greener cementation procedure to remediate cracks and defects in building materials and structures has been developed.

References

Abou-Zeid MN, Meggers D, McCabe SL (2003). Parameters affecting the rapid chloride permeability test. *Concr Inter* 25 (11): 61-66.

Adolphe JP, Loubière JF, Paradas J, Soleilhavoup F (1990). Procédé de traitement biologique d'une surface artificielle. European patent 90400G97.0. (after French patent 8903517, 1989).

Akhtar M, Lentz MJ, Blanchette RA, Kirk TK (1997). Corn steep liquor lowers the amount of inoculum for biopulping. *Tappi J* 80 (6): 161-164.

Akman MS, Özyurt N (2002). Effects of sea water on concrete impermeability of shore structures. 4th National Symposium of Shore Engineering, October 24-27, 2002, Antalya, Turkey, Vol. 1. pp. 3-18.

Altalo K, Gashe BA (1993). Protease production by a thermophilic *Bacillus* species (P-001A) which degrade various kinds of fibrous protein. *Biotechnol Lett* 15: 1151-1156.

Altschul SF, Madden TL, Schaffer AA, Zhang S, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389-3402.

Aly AH, Farag A, El-Sayed (2001). Isolation and characterization of sporeless mutants in *Bacillus sphaericu*s. *Arab J Biotech* 4 (2): 207-216.

Al-Zahani MM, Al-Dulaijan SU, Ibrahim M, Saricimen H, Sharif FM (2002). Effect of waterproofing coating on steel reinforcement corrosion and physical properties of concrete. *Cem Concr Comp* 24 (1): 127-137.

Amann RI, Ludwig W, Schleifer KH (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Molecular Biol Rev* 59: 143-169.

Amarger N, Bours M, Revoy F, Allard MR, Laguerre G (1994). *Rhizobium tropici* nodulates field-grown *Phaseolus vulgaris* in France. *Plant Soil* 161: 147–156.

Amartey SA, Jeffries TW (1994). Comparison of corn steep liquor with other nutrients in the fermentation of D-xylose by *Pichia stipitis* CBS 6054. *Biotechnol Lett* 16 (2): 211-214.

Amleh L, Mirza MS (2002). Durability field investigation of an abandoned concrete bridge. Proceedings of the International Conference on Sustainable Concrete Construction, Univ. of Dundee, Scotland, UK, pp. 617-626.

Andrade C, Alonso C (1996). Corrosion rate monitoring in the laboratory and on-site. *Constr Build Mater* 10: 315-328.

Andrews WH, June GA, Sherrod PS, Hammack TS, Amaguana RM (1995). FDA Bacteriological analytical manual, 8th ed. AOAC International, Gaithersburg, MD.

Aono R, Ito M, Machida T (1999). Contribution of cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphile *Bacillus lentus* C-125. *J Bacteriol* 181: 6600-6606.

APHA (1989). American Public Health Association. Standard methods for the examination of water and wastewater, 17th ed., Washington, DC.

APHA (1992). American Public Health Association. Standards methods for the examination of water and wastewater. 18th ed., Washington, D.C.

ASTM (1999). Standard test method for half-cell potentials of uncoated reinforcing steel in concrete. *ASTM C 876-91*, ASTM, Philadelphia.

ASTM (2005). Standard test method for electrical indication of concrete's ability to resist chloride ion penetration. *ASTM C 1202-05*, ASTM, Philadelphia.

Atkinson B, Mavutuna F (1983). Biochemical Engineering and Biotechnology Handbook, The Nature Press, New York, pp. 57.

Bachmeier KL, Williams AE, Warmington JR, Bang SS (2002). Urease activity in microbiologically-induced calcite precipitation. *J Biotechnol* 93: 171-181.

Bang SS, Galinat JK, Ramakrishnan V (2001). Calcite precipitation induced by polyurethane-immobilized *Bacillus pasteurii*. *Enzyme Microbial Tech* 28: 404-409.

Bang SS, Ramakrishnan V (2001). Microbiologically-enhanced crack remediation (MECR). Proceedings of the International Symposium on Industrial Application of Microbial Genomes, Daegu, Korea, pp. 3-13.

Barabesi C, Galizzi, A, Mastromei G, Rossi M, Tamburini E, Perito B (2007). Bacillus subtilis gene cluster involved in calcium carbonate biomineralization. *J Bacteriol* 189 (1): 228-235.

Bazylinski DA, Frankel RB, Konhauser KO (2007). Modes of biomineralization of magnetite by microbes. *Geomicrobiol J* 24: 465-475.

Beijerinck M (1913). *De Infusies en de Ontdekking der Backterien, Jaarboek van de Koninklijke Akademie v. Wetenschappen*. Muller, Amsterdam.

Ben FK (2003). Microbial repair mechanisms after UV treatment. Technical Background Article, *Berson UV Techniek* 1: 1-4.

Benini S, Rypniewski W, Wilson KS, 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: 205-216.

Berg J, Tymoczko J, Stryer L (2002). Biochemistry. WH Freeman and Company, New York.

Berkeley KGC, Pathmanaban S (1990). Cathodic protection of reinforcement steel in concrete, Butterworth-Heinemann, London.

Besoain XA, Perez LM, Araya A, Lefever L, Sanguinetti M, Montealegre JR (2007). New strains obtained after UV treatment and protoplast fusion of native *Trichoderma harzianum*: their biocontrol activity on *Pyrenochaeta lycopersici*. *Electronic J Biotech* 10 (4): 604-617.

Beushausen HD, Alexander MG, Mackechnie J (2003). Aspekte der Dauerhaftigkeit des Betons in einem internationalen. *Vergleich Concrete Precast Plant Technol* 7: 22-33.

Beveridge TJ, Meloche JD, Fyfe WS, Murray RGE (1983). Diagenesis of metals chemically complexed to bacteria: laboratory formation of metal phosphates, sulfides, and organic condensates in artificial sediments. *Appl Environ Microbiol* 45: 1094-1108.

Bickley J, Hooton RD, Hover KC (2006). Preparation of a performance-based specification for cast-in-place concrete. RMC Research Foundation. pp. 1-36.

Bodek I, Lyman WJ, Reehl WF, Rosenblatt DH (1988). Environmental inorganic chemistry - properties, processes and estimation methods. Pergamon Press.

Boquet E, Boronat A, Ramos-Cormenzana A (1973). Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. *Nature* 246, 527-529.

Braissant O, Cailleau G, Dupraz C, Verrecchia EP (2003). Bacterially induced mineralization of calcium carbonate in terrestrial environments: the role of exopolysaccharides and amino acids. *J Sediment Res* 73: 485-490.

Bresller SE (1976). The mechanism and the kinetics of reparation mutagenesis. *Genetika* 12: 153-160.

Bridson EY, Brecker A (1970). Methods in microbiology, *In* Norris and Ribbons (ed), Vol. 3A. Academic Press, New York.

Broomfield JP (1996). Techniques to assess the corrosion activity of steel reinforced concrete structures. *In* Berke NS, Escalante E, Nmai CK, Whiting D (eds), *ASTM STP* 1276, pp. 91-106.

Burne RA, Chen RE (2001). Bacterial ureases in infectious diseases. *Microbes Infect* 2: 533-542.

Burne RA, Marquis RE (2000). Alkali production by oral bacteria and protection against dental caries. *FEMS Microbiol Lett* 193: 1-6.

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. *Geomicrobiol J* 20: 85-99.

Castanier S, Le Metayer-Levrel G, Perthuisot JP (1999). Ca-carbonates precipitation and limestone genesis - the microbiolgeologist point of view. *Sediment Geol* 126: 9-23.

Castanier S, LeM´etayer-Levrel G, Perthuisot JP (2000). Bacterial roles in the precipitation of carbonate minerals. *In* Riding RE, Awramik SM (eds), Microbial sediments. Heidelberg: Springer-Verlag, pp. 32-39.

Chaturvedi S, Chandra R, Rai V (2006). Isolation and characterization of *Phragmites australis* (L.) rhizosphere bacteria from contaminated site for bioremediation of colored distillery effluent. *Ecol Eng* 27 (3): 202-207.

Christensen BE (1989). The role of extracellular polysaccharides in biofilms. *J Biotechnol* 10: 181-202.

Christensen WB (1946). Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J Bacteriol* 52: 461.

Ciurli S, Marzadori C, Benini S, Deiana S, Gessa C (1996). Urease from the soil bacterium: *Bacillus pasteurii* immobilization on Ca-polygalacturonate. *Soil Biol Biochem* 28: 811-817.

Claus D, Berkeley RCW (1986). Genus *Bacillus* in Bergeys manual of systematic bacteriology. Sneath PH (ed), The Willams and Wikins company, Baltimore.

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA *et al*. (2003). The ribosomal database project (RDP-II): previewing a new auto aligner that allow a regular updates and the now prokaryotic taxonomy. *Nucleic Acids Res* 31: 442-443.

Costerton JW, Stewart PS and Greenberg EP (1999). Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318-1322.

Cultrone G, Sebastian E, Elert K, de la Torre MJ, Cazalla O, Rodriguez-Navarro C (2004). Influence of mineralogy and firing temperature on the porosity of bricks. *J Eur Cer Soc* 24: 547-564.

Davey ME, O'toole GA (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol Rev* 64 (4): 847-867.

Day JL, Ramakrishnan V, Bang SS (2003). Microbiologically induced sealant for concrete crack remediation. *In* Proceedings of 16th Engineering Mechanics Conference, Seattle.

De Belie N, De Graef B, De Muynck W, Verstraete W (2005). Bacteria as protagonists for concrete: bacterial cleaner and bacterial builder. *fib* Symposium "Keep Concrete Attractive", Budapest 2005.

De Belie N, De Muynck W (2008). Crack repair in concrete using biodeposition. *In* Proceedings of ICCRR Cape Town, South Africa.

De Jong JT, Fritzges MB, Nüsslein K (2006). Microbial induced cementation to control sand response to undrained shear. *ASCE J Geotech Geoenviron Eng* 132 (11): 1381-1392.

De Martin Llano JJ, Segura JMG, Gavilanes JG (1989). Selective silver staining of urease activity in polyacrylamide gels. *Anal Biochem* 177: 37-40.

De Muynck W, Cox K, De Belie N, Verstraete W (2008). Bacterial carbonate precipitation as an alternative surface treatment for concrete. *Constr Build Mater* 22 (5): 875-885.

De Muynck W, De Belie N, Verstraete W (2007). Improvement of concrete durability with the aid of bacteria. Proceedings of the First International Conference on Self Healing Materials. 18-20 April 2007, Noordwijk aan Zee, The Netherlands.

De Muynck W, De Belie N, Verstraete W (2010). Microbial carbonate precipitation in construction materials: A review. *Ecol Eng* 36 (2): 118-136.

De Muynck W, Debrouwer D, De Belie N, Verstraete W (2008). Bacterial carbonate precipitation improves the durability of cementitious materials. *Cem Concr Res* 38 (7): 1005-1014.

De Muynck W, Dick J, De Graef B, De Windt W, Verstraete W, De Belie N (2005). Microbial ureolytic calcium carbonate precipitation for remediation of concrete surfaces. *In* Alexander M, Beushausen H-D, Dehn F, Moyo P (eds), Proceedings of International conference on concrete repair, rehabilitation and retrofitting. South Africa, Cape Town, pp. 296-297.

De Siano T, Padhi S, Schaffner DW, Montville TJ (2006). Growth Characteristics of Virulent *Bacillus anthracis* and potential surrogate strains. *J Food Protec* 69 (7): 1720-1723.

Des Marais DJ (1997). Long-term evolution of the biogeochemical carbon cycle. *In* Geomicrobiology, Interactions between Microbes and Minerals, Banfield JE, Nealson KH (eds), Mineral. Soc. Am., Washington, DC, pp. 427-448.

Dick J, De Windt W, De Graef B, Saveyn H, Van der Meeren P, De Belie N, Verstraete W (2006). Bio-deposition of a calcium carbonate layer on degraded limestone by *Bacillus* species. *Biodegradation* 17 (4): 357-367.

Diercks M, Sand W, Bock E (1991). Microbial corrosion of concrete. *Experientia* 47: 514-516.

Dittrich M, Muller B, Mavrocordatos D, Wehrli B (2003). Induced calcite precipitation by cyanobacterium *Synechococcus*. *Acta Hydrochim Hydrobiol* 31: 162–169.

Dixon NE, Riddles PW, Gazzold C, Blakeley RL, Zerner B (1980). Jack bean urease (EC 3.5.1.5) V. on the mechanism of action of urease on urea, formamide, acetamide, N-methylurea and related compounds. *Canadian J Biochem* 58: 1335-1344.

Douglas E, Bilodeau A, Malhotra VM (1992). Properties and durability of alkali-activated slag concrete. *ACI Mater J* 89 (5): 509-516.

Douglas S, Beveridge TJ (1998). Mineral formation by bacteria in natural microbial communities. *FEMS Microbiol Ecol* 26: 79-88.

Drew GH (1910). The action of some denitrifying bacteria in tropical and temperate seas, and the bacterial precipitation of calcium carbonate in the sea. *J Marine Biol Assoc* 9: 142-155.

Dunn BE, Grütter MG (2001). *Helicobacter pylori* springs another surprise. *Nature Struc Biol* 8: 480-482.

Durham DR (1987). Utility of subtilisin GX as a detergent additive. *J Appl Bacteriol* 63: 381-386.

Ehrlich HL (1996). How microbes influence mineral growth and dissolution. *Chem Geol* 132 (1–4): 5-9.

Ehrlich HL (1998). Geomicrobiology: its significance for geology. *Earth Science Rev* 45: 45-60.

Elsener B, Andrade C, Gulikers J, Polder R, Raupach M (2003). Half-cell potential measurements—Potential mapping on reinforced concrete structures. *Mater Struct* 36: 461-471.

El-Shora HM (2001). Properties and immobilization of urease from *Chenopodium album* (C3). *Botanical Bull Academia Sinica* 42: 251-258.

Ercole C, Cacchio P, Botta AL, Centi V, Lepidi A (2007). Bacterially induced mineralization of calcium carbonate: the role of exopolysaccharides and capsular polysaccharides. *Microsc Microanal* 13: 42-50.

Ercole C, Cacchio P, Cappuccio G, Lepidi A (2001). Deposition of calcium carbonate in karst caves: role of bacteria in stiffe's cave. *Int J Speleol* 30A: 69-79.

Falkinham III JO, Hoffman PS (1984). Unique developmental characteristics of the swarm and short cells of *Proteus vulgaris* and *Proteus mirabilis*. *J Bacteriol* 158: 1037-1040.

Ferris FG, Stehmeier LG (1992). Bacteriogenic mineral plugging. USA patent US5143155.

Finnerty WR, Singer ME (1983). Microbial enhancement of oil recovery. *Biotechnol* 1: 47-54.

Flemming HC, Wingender J, Griegbe T, Mayer C (2000). Physico-chemical properties of biofilms. *In* Evans LV (ed) Biofilm: recent advances in their study and control. Harwood, Amsterdam, pp. 19-34.

Friedman LE, de Passerini Rossi BN, Messina MT, Franco MA (2001). Phenotype evaluation of *Bordetella bronchiseptica* cultures by urease activity and congo red affinity. *Lett Appl Microbiol* 33: 285-290.

Friedrich B, Magasanik B (1977). Urease of *Klebsiella aerogenes*: control of its synthesis by glutamine synthetase. *J Bacteriol* 28: 313-322.

Friend MA, Kaiser AG, Piltz JW, Sillence MN, Jolliffe SK (2004). Use of delactosed whey permeate as a supplement for cattle on a cereal straw based diet. *Australian J Exper Agricul* 44 (9): 833-840.

Fry J (2000). Bacterial diversity and unculturables. *Microbiol Today* 27: 186-188.

Fujita Y, Ferris FG, Lawson RD, Colwell FS, Smith RW (2000). Calcium carbonate precipitation by ureolytic subsurface bacteria. *Geomicrobiol J* 17: 305-318.

Fukumoto J, Yamamoto T, Tsuru D (1971). Process for producing detergent resisting alkaline protease. Canadian Patent 910: 214.

Gadve S, Mukherjee A, Malhotra SN (2009). Corrosion of steel reinforcements embedded in FRP wrapped concrete. *Constr Build Mater* 23: 153-161.

Gambhir ML (2006). Concrete Technology 3rd Edition, Tata McGraw Hill Publishing Company Ltd, India: New Delhi.

Gauckler LJ, Baader GF (1999). Ceramic forming using enzyme catalyzed reactions. *Mater Chem Phy* 2509: 1-25.

Ghiorse WC (1984). Biology of iron-and manganese-depositing bacteria. *Ann Rev Microbiol* 38: 515-550.

Ghosh S, Biswas M, Chattopadhyay BD, Mandal S (2009). Microbial activity on the microstructure of bacteria modified mortar. *Cem Concr Compos* 31: 93-98.

Ghosh P, Mandal S, Chattopadhyay BD, Pal S (2005). Use of microorganism to improve the strength of cement mortar. *Cem Concr Res* 35: 1980-1983.

Gillis PBM, De Leg J (1992). The genus *Aquaspirillum*. *In* The prokaryotes: A handbook on the biology of bacteriai ecophysiology, isolation, identification, and applications, Balows A *et al.* (eds), Springer-Verlag, New York, pp. 2569-2582.

Gollapudi UK, Knutson CL, Bang SS, Islam MR (1995). A new method for controlling leaching through permeable channels. *Chemosphere* 30: 695-705.

Gonzalez Siso MI (1996). The biotechnological utilization of cheese whey. *Biores Tech* 57: 1-11.

Gordon RE, Haynes WC, Pang CH (1973). The Genus *Bacillus*. US Dep Agric Handbook. pp. 427.

Gundersen K (1958). Influence of corn steep liquor on the oxidation of ammonia to nitrite by *Nitrosomonas europaea*. *J Gen Microbial* 19: 190-197.

Ha N, Oh ST, Sung JY, Cha KA, Lee MH, Oh BH (2001). Supramolecular assembly and acid resistance of *Helicobacter pylori* urease. *Nature Struc Biol* 8: 505-509.

Haltrich D, Preiss M, Steiner W (1993). Optimization of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune*. *Enzyme Microbial Tech* 15: 854-860.

Hamilton WA (2003). Microbially influenced corrosion as a model system for the

study of metal microbe interactions: a unifying electron transfer hypothesis. *Biofouling* 19 (1): 65-76.

Hammes F, Seka A, de Knijf S, Verstraete W (2003). A novel approach to calcium removal from calcium-rich industrial wastewater. *Water Res* 37: 699-704.

Hammes F, Verstraete W (2002). Key roles of pH and calcium metabolism in microbial carbonate precipitation. *Re/Views Environ Sci Biotechnol* 1: 3-7.

Herholdt AD, Justesen CFP *et al*. (eds) (1985). Beton-Boen (The Concrete Book – in Danish). Aalborg, Denmark, Aalborg Portland.

Hewlett PC (1990). Methods of protecting concrete – coatings and linings. Proceedings of international conference: Protection of concrete, *In* Dhir RK, Green JW (eds), Dundee, Scotland, pp. 105-134.

Heydorn A, Nielsen AT, Hentzer M, Sternberg C, Givskov M, Ersbøll B, Molin S (2000). Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* 146: 2395-2407.

Hirayama C, Sugimura M, Saito H, Nakamura M (2000). Purification and properties of urease from the leaf of mulberry, *Morus albus*. *Phytochemistry* 53: 352-330.

Hoch IE (1997). Avaliação da Substituição do Extrato de Levedura por Milhocina no Cultivo de *Zymomonas mobilis* Para a Produção da Enzima Glicose-Frutose Oxirredutase. Monografia apresentada ao Curso de Química Industrial UNIVILLE.

Hogg S (2005). Essential Microbiology. John Wiley & Sons, New York.

Hoke JH, Pickering HW, Rosengarth K (1980). Cracking of reinforced concrete, ILZRO project ZE271 progress report 3, Dept. of Mat. Sci. Eng., Penns State Univ., University Park, PA.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (2000). Bergey's Manual of Determinative Bacteriology, 9th edition, Baltimore, Maryland: Williams and Wilkins, pp. 787.

Hooton RD, Geiker MR, Bentz EC (2002). Effects of curing on chloride ingress and implications on service life. *ACI Mater J* 99 (2): 201-206.

Horner-Devine MC, Carney KM, Bohannan BJM (2004). An ecological perspective on bacterial biodiversity. *Proc R Soc Biol Sci B* 271: 113–122.

Ingraham JL, Maaløe O, Neidhardt FC (1983). Growth of the bacterial cell, p. ix, Sinauer Associates, Inc., Sunderland, Mass, pp. 227-265.

IS 4031 (1988). Determination of compressive strength of hydraulic cement, Bureau of Indian Standard, New Delhi.

IS 8112 (1989). Specification for 43-grade Ordinary Portland cement, Bureau of Indian Standard, New Delhi.

Ivey DM, Ito M, Gilmour R, Zemsky J, Guffanti AA, Sturr MG, Hicks DB, Krulwich TA (1998). Alkaliphile bioenergetics. *In* Horikoshi K, Grant WD (eds.), Extremophiles: Microbial life in extreme environments. Wiley-Liss, New York, pp. 181-210.

Jahns T (1996). Ammonium/urea-dependent generation of a proton electrochemical potential and synthesis of ATP in *Bacillus pasteurii*. *J Bacteriol* 178: 403-409.

Jenneman GE, Knapp RM, Mclnerney MJ, Menzie DE, Revus DE (1984). Experimental studies of *in situ* microbial enhanced oil recovery. *Soc Pet Eng J* 24: 33-37.

Jonkers HM, Schlangen E (2007). Crack repair by concrete-immobilized bacteria. *In* Schmets AJM, Van der Zwaag S, (eds), Proceedings of First International Conference on Self Healing Materials. Noordwijk, The Netherlands, pp. 7.

Jonkers HM, Thijssen A, Muyzer G, Copuroglu O, Schlangen E (2010). Application of bacteria as self-healing agent for the development of sustainable concrete. *Ecol Eng* 36 (2): 230-235.

Kaltwasser H, Kramer J, Conger WR (1972). Control of urease formation in certain aerobic bacteria. *Archiv fur Mikrobiologie* 81: 178-196.

Kantzas A, Ferris FG, Stehmeier L, Marentette DF, Jha KN, Mourits FM (1992). A novel method of sand consolidation through bacteriogenic mineral plugging (CIM 92-46). *In* Proceedings of the CIM Annual Technical Conference 1992, Vol. 2, pp. 1-15, Petroleum Society of CIM, Calgary, Canada.

Kawaguchi T, Decho AW (2002). A laboratory investigation of cyanobacterial extracellular polymeric secretions (EPS) in influencing $CaCO_3$ polymorphism. *J Cryst Growth* 240: 230-235.

Kennedy HE, Speck ML (1954). Studies on corn steep liquor in the nutrition of certain lactic acid bacteria. *J Dairy Sci* 87: 635.

Kerr PS, Blevins DG, Rapp BJ, Randall DD (1983). Soybean leaf urease: comparison with seed urease. *Physiologia plantarum* 57: 339-345.

Khan MI, Lynsdale CJ (2002). Strength, permeability, and carbonation of high-performance concrete. *Cem Concr Res* 32 (1): 123-131.

Klein C, Hurlbut CSJ (1999). Manual of Mineralogy, 21st edn. John Wiley & Sons, Inc., New York.

Klein J, Kluge M (1981). Immobilization of microbial cells in polyurethane matrices. *Biotechnol Lett* 3: 65-70.

Knoll AH (1985). The distribution and evolution of microbial life in the late proterozoic era. *Ann Rev Microbiol* 39: 391-417.

Knorre H, Krumbein W (2000). Bacterial calcification. *In* Riding RE, Awramik SM (eds.), Microbial Sediments. Springer-Verlag, Berlin, Germany, pp. 25–31.

Kristiansen B (2001). Process Economics. *In* Ratledge C, Kristiansen B (eds), Biotechnology, 2nd edn. Cambridge University Press, Cambridge.

Krulwich TA, Guffanti A (1989). The Na^+ cycle of extreme alkalophiles: A secondary Na^+/H^+ antiporter and Na^+/solute symporters. *J Bioenerget Biomembr* 21: 663-677.

Kumar J, Jana AK (2009). Cell growth prediction for *Bacillus licheniformis* through artificial neural network at simultaneous multiple variation in concentration of nutrients in media. *Kathmandu Univ J Sci Eng Tech* 5: 79-87.

Laemmli UK (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Laguerre G, Maringui P, Allard MR, Charnay MP, Houvrier P, Mazurier SI, Rigottier-Gois H, Amarger N (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl Environ Microbiol* 62: 2029–2036.

Lappin-Scott HM, Cusack F, Costerton JW (1988). Nutrients resuscitation and growth of starved cells in sandstone cores: a novel approach to enhanced oil recovery. *Appl Environ Microbiol* 54: 1373-1382.

Le Metayer-Levrel G, Castanier S, Orial G, Loubiere JF, Perthuisot JP (1999). Applications of bacterial carbonatogenesis to the protection and regeneration of limestones in buildings and historic patrimony. *Sediment Geol* 126 (1–4): 25–34.

Liggett RW, Koffler H (1948). Corn steep liquor in microbiology. *Bact Rev* 12: 297-311.

Lomborg B (2001). The Skeptical Environmentalist: Measuring the Real State of the World. Cambridge University Press, pp. 138.

Lopez-Garcia P, Kazmierczak J, Benzerara K, Kempe S, Guyot F, Moreira D (2005). Bacterial diversity and carbonate precipitation in the giant microbialites from the highly alkaline Lake Van, Turkey. *Extremophiles* 9: 263-274.

Louws FJ, Fulbright DW, Stephens CT, DeBruijn FJ (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl Environ Microbiol* 60: 2286-2295.

Lowenstan HA, Weiner S (1988). On Biomineralization. Oxford University Press, New York.

MacLeod A, Lappin-Scott HM, Costerton JW (1988). Plugging of a model rock system by using starved bacteria. *Appl Environ Microbiol* 54: 1365-1372.

Mahasenan N, Smith S, Humphreys K, Kaya Y (2003). The cement industry and global climate change: current and potential future cement industry CO_2 emissions. Greenhouse Gas Control Technologies-6th International Conference. Oxford: Pergamon. pp. 995-1000.

Maidak BL, Cole JR, Lilburn TG, Parker Jr CT, Saxman PR, Farris RJ, Garrity G M, Olsen GJ, Schmidt TM, Tiedje JM (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* 29: 173-174.

Mailvaganam NP (1997). Effective use of bonding agents. Construction Technology Update No. 11, National Research Council of Canada, December 1997.

Makar J, Desnoyers R (2001). Magnetic field techniques for the inspection of steel under concrete cover. *NDT E Int* 34: 445-456.

Malhotra VM, Mehta PK (1996). Pozzolanic and cementitious materials. Advanced Concrete Technology, Vol. 1. Canada. Gordon and Breach Publishers.

Manachini PL, Fortina MG (1998). Production in seawater of thermostable alkaline protease by a halotolerant strain of *Bacillus licheniformis*. *Biotechnol Lett* 20: 565-568.

Mann S (1988). Molecular recognition in biomineralization. *Nature* 332: 119-124.

Mansfeld F (1977). *In* Polarization Resistance Measurement, Electrochemical Techniques for Corrosion, National Association of Corrosion Engineers, Houston, pp. 18-26.

Marangoni C, Furigo Jr A, Aragao GMF (2001).The influence of substrate source on the growth of *ralstonia eutropha,* aiming at the production of polyhydroxyalkanoate. *Braz J Chem Eng* 18 (2): 175-180.

Marshall BJ, Barrett LJ, Prakash C, McCallun RW, Guerrant RL (1990). Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bacterial effect of acid. Gastroenterology 99: 697-702.

McConnaughey TA, Whelan FF (1997). Calcification generates protons for nutrient and bicarbonate uptake. *Earth Sci Rev* 42: 95-117.

Mehrotra S, Pandey PK, Gaur R, Darmwal NS (1998). The production of alkaline protease by a Bacillus species isolate. *Biores Tech* 67: 201-203.

Mehta PK, Manmohan D (1980). Pore size distribution and permeability of hardened cement pastes. Proceedings of 7th International Congress Chem. Cem., Vol. 3, Editions Septima, Paris, VII: pp. 1-5.

Merz-Preiss M, Riding R (1999). Cyanobacterial tufa calcification in two freshwater streams: ambient environment, chemical thresholds and biological processes. *Sediment Geol* 126 (1–4): 103-124.

Midgley HG, Illston JM (1984). The penetration of chlorides into hardened cement pastes. *Cem Con Res* 14 (4): 546-558.

Mobley HLT, Hausinger RP (1989). Microbial ureases: significance, regulation and molecular characterisation. *Microbiol Rev* 53: 85-108.

Mobley HLT, Island MD, Hausinger RP (1995). Molecular biology of microbial ureases. *Microbiol Rev* 59: 451-480.

Morikawa M, Kagihiro S, Haruki M, Takano K, Branda S, Kolter R, Kanaya S (2006). Biofilm formation by a *Bacillus subtilis* strain that produces γ-polyglutamate. *Microbiol* 152: 2801-2807.

Morita RY (1980). Calcite precipitation by marine bacteria. *Geomicrobiol J* 2: 63-82.

Mörsdorf G, Kaltwasser H (1989). Ammonium assimilation in *Proteus vulgaris, Bacillus pasteurii* and *Sporosarcina ureae*. *Arch Microbiol* 152: 125-131.

Morse JW (2003). Formation and Diagensis of Carbonate Sediments. *In* Treatise on GeoChemistry, Vol. 7, Sediments, Diagenesis and Sedimentary Rocks. Mackenzie FT (ed), Elsevier Press.

Moyer AJ, Coghill RD (1946). Penicillin. VIII. Production of penicillin in surface cultures. *J Bacteriol* 51: 57-78.

Moyer AJ, Umberger EJ, Stuibbs JJ (1940). Fermentation of concentrated solutions of glucose to gluconic acid - Improved process. *Ind Eng Chem* 32: 1379-1383.

Mulrooney S, Zakharian T, Schaller RA, Hausinger RP (2001). Dual effects of ionic strength on *Klebsiella aerogenes* urease: pH dependent activation and inhibition. *Arch Biochem Biophy* 394: 280-282.

Muyzer G, De Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturating gradient gel electrophoresis analysis of polymerase chain reaction-amplified gene coding for 16S rRNA. *Appl Environ Microbiol* 59: 695-700.

Nagami Y, Tanaka T (1986). Molecular cloning and nucleotide sequence of a DNA fragment from *Bacillus natto* that enhances production of extracellular proteases and levansucrase in *Bacillus subtilis*. *J Bacteriol* 166 (1): 20-28.

Natarajan KR (1995). Kinetic study of the enzyme urease from *Dolichos biflorus*. *J Chem Educ* 72: 556-557.

Neidhardt FC, Ingraham JL, Schaechter M (1990). Physiology of the bacterial cell: a molecular approach. Sinauer Associates, Sunderland, MA.

Nemati M, Greene EA, Voordouw G (2005). Permeability profile modification using bacterially formed calcium carbonate: comparison with enzymic option. *Process Biochem* 40 (2): 925-933.

Nemati M, Voordouw G (2003). Modification of porous media permeability, using calcium carbonate produced anzymatically *in situ*. *Enz Microbial Tech* 33: 635-642.

Neville AM (1973). Properties of concrete. A Halsted Press Book, John Wiley and Sons, New York.

Neville AM (1996). Properties of concrete, 4th ed., Pearson Higher Education, Prentice Hall, New Jersey.

Nokken MR, Hooton RD (2006). Electrical conductivity testing. *Concr Inter* 28 (10): 58-63.

Nolan E, Basheer PAM, Long AE (1995). Effects of three durability enhancing products on some physical properties of near surface concrete. *Constr Build*

Mater 9 (5): 267-272.

Obayori OS, Adebusoye SA, Ilori MA, Oyetibo GO, Omotayo AE, Amund OO (2010). Effects of corn steep liquor on growth rate and pyrene degradation by *Pseudomonas* strains. *Current Microbiol* 60 (6): 407-411.

Örnek D, Jayaraman A, Wood TK, Sun Z, Hsu CH, Mansfeld F (2001). Pitting corrosion control using regenerative biofilms on aluminium in artificial seawater. *Corrosion Sci* 43: 2121-2133.

Papke RT, Ward DM (2004). The importance of physical isolation to microbial diversification. *FEMS Microbiol Ecol* 48 (3): 293-303.

Parthiban GT, Saraswathy V, Rengaswamy NS (2000). Cathodic protection of concrete structures using magnesium alloy anode. *Bull Electrochem* 16: 253-257.

Picken JC, Bauriedel WR (1950). Comparative bioautography of vitamin B12 and related growth factors using *Euglena gracilis* and *Lactobacillus leichmannii*. *Proc Soc Exptl Biol Med* 75: 511-515.

Pratt LA, Kolter R (1998). Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* 30*:* 285-293.

Prescott LM, Harley JP, Klein DA (1993). Microbiology, 2nd edn. Wm. C. Brown Publishers, Dubuque.

Prikryl R, Lokajicek T, Svobodova J, Weishauptova Z, (2003). Experimental weathering of marlstone from Predni Kopanina (Czech Republic) – historical building stone of Prague. *Build Environ* 38: 1163-1171.

Purva Soni SK, Gupta LK, Gupta JK (1998). Thermostable alkaline protease from alkalophilic *Bacillus* sp. IS-3. *Indian J Microbiol* 38: 149-152.

Qadeer MA, Anjum JI, Akhtar R (1980). Biosynthesis of enzymes by solid substrate fermentation. *Pakistan J Sci Res* 23 (1): 25-29.

Raiders RA, Knapp RM, McInerney MJ (1989). Microbial selective plugging and enhanced oil recovery. *J Ind Microbiol* 4: 215-230.

Ramachandran SK, Ramakrishnan V, Bang SS (2001). Remediation of concrete using microorganisms. *American Con Inst Mat J* 98: 3-9.

Ramakrishnan V, Bang SS, Deo KS (1998). A novel technique for repairing cracks in high performance concrete using bacteria. Proceedings of International conference on high performance high strength concrete. Perth, Australia, pp. 597-618.

Randolph AD, Larson MA (1988). Theory of particulate processses analysis and techniques of continuous crystallization, 2nd edn. Academic Press Inc., California.

Reddy BVV, Jagadish KS (2003). Embodied energy of common and alternative building materials and technologies. *Energy Build* 35: 129-137.

Reid RP, Dupraz C, Visscher PT, Decho AW, Sumner DY (2003). Microbial processes forming modern marine stromatolites: microbe–mineral interactions with a three-billion-year rock record. *In* Fossil and Recent Biofilms – a Natural History of Life on Earth, Krumbein WE, Paterson DM, Zavarzin GA (eds), pp. 103–118. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Richards LA (1954). Diagnosis and improvement of saline and alkali soils. Hand Book 60. United States Department of Agriculture.

Riding RE (2000). Microbial carbonates: The geological record of calcified bacterial-algal mats and biofilms. *Sedimentology* 47 (1): 179-214.

Rivadeneyra M.A, Delgado R, Delgado G, Moral A, Ferrer MR, Ramos-Cormenzana A (1993). Precipitation of carbonate by *Bacillus* sp. isolated from saline soils. *Geomicrobiol J* 11: 175-184.

Rivadeneyra MA, Delgado G, Ramos-Cormenzana A, Delgado R (1997). Precipitation of carbonates by *Deleya halophila* in liquid media: pedological implications in saline soils. *Arid Soil Res Rehabil* 11: 35-49.

Rivadeneyra MA, Delgado G, Ramos-Cormenzana A, Delgado R (1998). Biomineralisation of carbonates by *Halomonas eurihalina* in solid and liquid media with different salinities: crystal formation sequence. *Res Microbiol* 149: 277-287.

Rivadeneyra MA, Delgado R, Moral A, Ferrer MR, Ramos-Cormenzana A (1994). Precipitation of calcium carbonate by *Vibrio* spp. from an inland saltern. *FEMS Microbiol Ecol* 13: 197-204.

Rivadeneyra MA, Delgado R, Quesada E, Ramos-Cormenzana A (1991). Precipitation of calcium carbonate by *Deleya halophila* in media containing NaCl as sole salt. *Curr Microbiol* 22: 185-190.

Robinson CB, Samocha TM, Fox JM, Gandy RL, McKee DA (2005). The use of inert artificial commercial food sources as replacements of traditional live food items in the culture of larval shrimp, *Farfantepenaeus aztecus*. *Aquaculture* 245: 135-147.

Roche (2001). Specialty enzymes for industry. Roche Diagnostics GmbH.

Rodriguez-Navarro C, Rodriguez-Gallego M, Ben Chekroun K, Gonzalez-Munoz MT (2003). Conservation of ornamental stone by *Myxococcus xanthus*-induced carbonate biomineralization. *Appl Environ Microbiol* 69: 2182-2193.

Ruiz C, Monteoliva-Sanches M, Huertas F, Ramos-Cormenzana A (1988). Calcium carbonate precipitation by several species of *Myxococcus*. *Chemosphere* 17: 835-838.

Sadowsky MJ, Kinkel LL, Bowers JH, Schottel JL (1996). Use of repetitive intergenic DNA sequences to classify pathogenic and disease suppressive *Streptomyces* strains. *Appl Environ Microbiol* 62: 3489-3493.

Sahmaran M, Li VC, Andrade C (2008). Corrosion resistance performance of steel-reinforced engineered cementitious composite beams. *ACI Mater J* 105 (3): 243-250.

Sanger F, Nicklen S, Chase AR (1977). DNA Sequencing with Chain Terminating Inhibitors. *Proc Natl Acad Sci* 74 (12): 5463-5468.

Satyanarayana T, Raghukumar C, Shivaji S (2005). Extremophilic microbes: Diversity and perspectives. *Current Sci* 89 (1): 78-90.

Sawada K (1998). Mechanisms of crystal growth of ionic crystals in solution. Formation, transformation, and growth inhibition of calcium carbonates, *In* Ohtaki H (ed), Crystallization Processes, Vol. 3. John Wiley & Sons, New York, pp. 39-68.

Schultze-Lam S, Fortin D, Davis BS, Beveridge TJ (1996). Mineralisation of bacterial surfaces. *Chem Geol* 132: 171-181.

Schultze-Lam S, Harsuz G, Beveridge TJ (1992). Participation of a cyanobacterial S-layer in fine-grain mineral formation. *J Bacterial* 174: 7971-7981.

Selenska-Pobell S, Gigova L, Petrova N (1995). Strain-specific fingerprints of *Rhizobium galegae* generated by PCR with arbitrary and repetitive primers. *J Appl Bacteriol* 79: 425-431.

Shah SP, Wang K, Weiss WJ (2000). Mixture proportioning for durable concrete. *Concr Inter* 22 (9): 73-78.

Sharma V, Chaudhary R, Khurana JM, Muralidhar K (2008). In-gel detection of urease activity by nitroprusside-thiol reaction. *Phytochem Anal* 19 (2): 99-103.

Sharp RJ, Munster MJ (1986). *In* Microbes in external environments, Herbert RA, Codd GA (eds), Academia press, London, pp. 233.

Shaw JC, Bramhill B, Wardlaw NC, Costerton JW (1985). Bacterial fouling in a model core system. *Appl Environ Microbiol* 49: 693-701.

Shi C (1996). Strength, pore structure and permeability of high performance alkali-activated slag mortars. *Cem Concr Res* 26 (12): 1789-1800.

Shreir LL, Han RL *et al*., (eds.) (1994). Corrosion. Butterworth-Heinemann Ltd, Toronto.

Silver S, Toth K, Scribner H (1975). Facilitated transport of calcium by cells and subcellular membranes of *Bacillus subtilis* and *Escherichia coli*. *J Bacteriol* 12: 880-885.

Simon MA, Bonner JS, Page CA, Townsend RT, Mueller DC, Fuller CB, Autenrieth RL (2004). Evaluation of two commercial bioaugmentation products for enhanced removal of petroleum from a wetland. *Ecol Eng* 22 (4–5): 263-277.

Smith NR, Gordon RE, Clark FE (1952). Aerobic Spore Forming Bacteria. US Dep Agric Monogr 16, Washington, DC.

Sneath PHA (1986). Bergey's Manual of Systematic Bacteriology. Vol. 2. Williams & Wilkins, Baltimore.

So HS, Millard SG (2007). On-site measurements on corrosion rate of steel in reinforced concrete. *ACI Mater J* 104 (6): 638-642.

Song HW, Saraswathy V (2007). Corrosion monitoring of reinforced concrete structures – a review. *Int J Electrochem Sci* 2: 1-28.

Stocks-Fischer S, Galinat JK, Bang SS (1999). Microbiological precipitation of $CaCO_3$. *Soil Biol Biochem* 31: 1563-1571.

Stoodley P, Lewandowski Z, Boyle JD, Lappin-Scott HM (1999). Structural deformation of bacterial biofilms caused by short-term fluctuations in fluid shear: an in situ investigation of biofilm rheology. *Biotechnol Bioeng* 65: 83-92.

Stoodley P, Sauer K, Davies DG, Costerton JW (2002). Biofilms as complex differentiated communities. *Ann Rev Micro* 56: 187-209.

Stubbs JJ, Lockwood LB, Roe ET, Tabenkin B, Ward GE (1940). Ketogluconic acid from glucose. Bacterial production. *Ind Eng Chem* 32: 1626-1631.

Sujjavanich S, Sida V, Suwanvitaya P (2005). Chloride permeability and corrosion risk of high-volume fly ash concrete with mild-range water reducer. *ACI Mater J* 102 (3): 177-182.

Szacilowski K, Wanat A, Barbieri A, Wasielewska E, Witko M, Stochel G, Stasicka Z (2002). Reactions of the $[Fe(CN)_5NO]^{2-}$ complex with biologically relevant thiols. *New J Chem* 26: 1495-1502.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24: 1596-1599.

Thompson JB, Ferris FG, Smith DA (1990). Geomicrobiology and sedimentology of the mixolimnion and chemocline in Fayetteville Green Lake, New York, USA. *Palaios* 5: 52-75.

Tiano P, Biagiotti L, Mastromei G (1999). Bacterial bio-mediated calcite precipitation for monumental stones conservation: methods of evaluation. *J Microbiol Methods* 36: 138-145.

Tolker-Nielsen T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS, Molin S (2000). Development and dynamics of *Pseudomonas* sp. biofilms. *J Bacteriol* 182: 6482-6489.

Torsvik V, Ovreas L, Thingstad TF (2002). Prokaryotic diversity-Magnitude, dynamics and controlling factors. *Science* 296: 1064-1067.

Torsvik V, Sorheim R, Gokoyr J (1996). Total bacterial diversity in soil and sediment communities – a review. *J Ind Microbiol* 17: 170-178.

Toy, S (1955). A History of Fortification. London: William Heinemann.

Trichet J, Défarge C (1995). Non-biologically supported organomineralization. *Bull Inst Océanogr Monaco Spec No* 14: 203-236.

Tsuneda S, Jung J, Hayashi H, Aikawa H, Hirata A, Sasaki H (2003). Influence of extracellular polymers on electrokinetic properties of heterotrophic bacterial cells examined by soft particle electrophoresis theory. *Colloid Surface B* 29: 181-188.

Tyler B (1978). Regulation of the assimilation of nitrogen compounds. *Annual Rev Biochem* 47: 1127-1162.

Udert KM, Larsen TA, Gujer W (2003). Estimating the precipitation potential in urine-collecting systems. *Water Res* 37: 2667-2677.

Uhlig HH, Revie RW (1985). Corrosion and Corrosion Control. John Wiley & Sons, Toronto.

Updegraff DM (1982). Plugging and penetration of petroleum reservoir rock by microorganisms. *In* Proceedings of 1982 International Conference on Microbial Enhancement of Oil Recovery, U.S. Dept. of Energy, Oklahoma, pp. 80-85.

Urzi C, Garcia-Valles M, Vendrell M, Pernice A (1999). Biomineralization processes on rock and monument surfaces observed in field and in laboratory conditions. *Geomicrobiol J* 16 (1): 39-54.

Vasala A, Panula J, Neubauer P (2005). Efficient lactic acid production from high salt containing dairy by-products by *Lactobacillus salivarius* ssp. *salicinius* with pre-treatment by proteolytic microorganisms. *J Biotechnol* 117 (4): 421-431.

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu DY, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304: 66-74.

Versalovic J, Koeuth T, Lupski JR (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting bacterial genomes. *Nucleic Acids Res* 19: 6823-6831.

Wakabayashi Y, Takamiya R, Mizuki A, Kyokane T, Goda N, Yamaguchi T, Takeoka S, Tsuchida E, Suematsu M, Ishimura Y (1999). Carbon monoxide overproduced by heme oxygenase-1 causes a reduction of vascular resistance in perfused rat liver. *Am J Physiol Gastrointest Liver Physiol* 277: G1088-G1096.

Wang X, Ruchenstein E (1993). Preparation of porous polyurethane particles and their use of enzyme immobilization. *Biotechnol Prog* 9: 661-665.

Ward OP (1983). Proteinases. *In* Fogarty WM (ed) Microbial enzymes and biotechnology. Applied Science Publishers, London, England, pp. 251-305.

Warren LA, Ferris FG (1998). Continuum between sorption and precipitation of Fe (III) on microbial surfaces. *Envir Sci Technol* 32: 2331-2337.

Warren LA, Maurice PA, Ferris FG (2001). Microbially mediated calcium carbonate precipitation: implications for interpreting calcite precipitation and for solid-phase capture of inorganic contaminants. *Geomicrobiol J* 18: 93-115.

Wei-liang J, Yu-xi Z (2001). Effect of corrosion on bond behavior and bending strength of reinforced concrete beams. *J Zhejiang Univ – Sci A* 2 (3): 298-308.

Weisberg WA, Barns SM, Pelletier DA, Lane DJ (1991). 16S rDNA amplification for phylogenetic study. *J Bacteriol* 173: 697-703.

Wells PA, Lockwood LB, Stubbs JJ, Roe ET, Porges N, Gastrocik EA (1939). Sorbose from sorbitol semiplantscale production by *Acetobacter suboxydans*. *Ind Eng Chem* 31: 1518-1521.

Welsh J, McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res* 18: 7213-7218.

Weyers RE, Philip Cady D (1987). Deterioration of concrete bridge decks from corrosion of reinforcing steel. *Concr Int Des Constr* 9: 15-20.

Whiffin VS (2004). Microbial $CaCO_3$ precipitation for the production of biocement. PhD thesis. School of Biological Sciences & Biotechnology. Murdoch University, Australia.

Whiffin VS, van Paassen L, Harkes MP (2007). Microbial carbonate precipitation as a soil improvement technique. *Geomicrobiol J* 24: 417-423.

Whiting D (1981). Rapid measurement of the chloride permeability of concrete. *Public Roads* 45 (3): 101-112.

Whitman WB, Coleman DC, Wiebe WJ (1998). Prokaryotes: The unseen majority. *Proc Natl Acad Sci* 95: 6578-6583.

Williamson GS, Weyers RE, Brown MC, Ramniceanu A, Sprinkel MM (2008). Validation of the probability-based chloride-induced corrosion service-life model. *ACI Mater J* 105 (4): 375-380.

Winsten WA, Eigen E (1949). Paper chromatography of vitamin B12 and related bacterial growth factors. *J Biol Chem* 181: 109-119.

Woese C, Kandler O, Wheelis M (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci* 87 (12): 4576-4579.

Wong HH, Lee SY (1998). Poly (3-Hydroxybutyrate) production from whey by high-density cultivation of recombinant *Escherichia coli. Appl Microbiol Biotechnol* 50: 30-33.

Wranglén G (1985). An Introduction to Corrosion and Protection of Metals. New York, NY, Chapman and Hall.

Wright DT (1999). The role of sulphate-reducting bacteria and cyanobacteria in dolomite formation in distal ephemeral lakes of the Coorong region, South Australia. *Sediment Geol* 126: 147-157.

Wu C, Teo VSJ, Farrell RL, Bergquist P.L, Nevalainen KMH (2006). Improvement of the secretion of extracellular proteins and isolation and characterization of the amylase I (amy1) gene from *Ophiostoma floccosum*. *Gene* 384: 96-103.

Yates KK, Robbins LL (1999). Radioisotope tracer studies of inorganic carbon and Ca in microbially derived $CaCO_3$. *Geochim Cosmochim Acta* 63 (1): 129-136.

Yoon JH, Lee KC, Weiss N, Kho YH, Kang KH, Park YH (2001). *Sporosarcina aquimarina* sp. nov., a bacterium isolated from seawater in Korea, and transfer of *Bacillus globisporus* (Larkin and Stokes 1967), *Bacillus psychrophilus* (Nakamura 1984) and *Bacillus pasteurii* (Chester 1898) to the genus *Sporosarcina* as *Sporosarcina globispora* comb. nov., *Sporosarcina psychrophila* comb. nov. and *Sporosarcina pasteurii* comb. nov., and emended description of the genus *Sporosarcina*. *Inter J System Evolution Microbiol* 51: 1079-1086.

Zhang Z, Ansari F, Vitillo N (2005). Automated determination of entrained air-void parameters in hardened concrete. *ACI Mater J* 102 (1): 42-48.

Zhong L, Islam MR (1995). A new microbial plugging process and its impact on fracture remediation. *In* Proceedings of Society of Petroleum Engineers. Annual Technical Conference, Dallas, Texas, pp. 703-715.

Zhong W, Yao W (2008). Influence of damage degree on self-healing of concrete. *Const Build Mater* 22 (6): 1137-1142.

Appendix I

TBE buffer (10×)

Tris-HCl	0.09 M (pH 8)
Boric acid	0.9 M
EDTA	0.02 M (pH 8)

Agarose gel loading dye (6×)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Glycerol in water	30.0%

Plasmid extraction solution I (10×)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na_2EDTA	10mM

Plasmid extraction solution II

NaOH	5M
SDS	10%

Plasmid extraction solution III

5.0 M K-acetate (pH 4.5)

Ligation reaction of amplicon in pTZ57R/T

Plasmid pTZ57R/T (55ng/µl)	3µl
Amplicon (75ng/µl)	4µl
Buffer (10×)	3µl
T4 Ligase	1µl
H_2O	19µl

Primers

M13 forward primer	5'-GTAAAACGACGGCCAGT-3'
M13 reverse primer	5'-CAGGAAACAGCTATGAC-3'
BOXA1R primer	5'-CTACGGCAAGGCGACGCTGACG-3'
16S rDNA forward primer	5'-AGAGTTTGATCCTGGCTCAG-3'
16S rDNA reverse primer	5'-ACGGGCGGTGTGTTC-3'

Recipes for SDS/PAGE gels and buffers

Acrylamide stock solution (30%)	
Acrylamide	29.2%
N, N-methyelenebisacrylamide	0.8%
Stored in amber colored bottle at 4^{o}C	
1.5M Tris-Cl (pH 8.8)	18.5 g/100 ml
0.5M Tris-Cl (pH 6.8)	3 g/50 ml
Stacking gel (4.5%)	**5 ml**
Acrylamide	800 µl
Milli-Q water	2.85 ml
Tris-HCl (0.5M and pH 6.8)	1.25 ml
SDS (10%, W/V)	50 µl
TEMED	2.5 µl
APS (10%, W/V)	50 µl
Resolving Gel (12 %)	**10 ml**
Acrylamide	4 ml
Milli-Q water	3.35 ml
Tris-HCl (1.5M and pH 8.8)	2.5 ml
SDS (10%, W/V)	70 µl
TEMED	5 µl
APS (10%, W/V)	70 µl
Tank Buffer (1 L)	
Tris-HCl	6.05 g
Glycine	28.8g
SDS (10%, W/V)	10 ml
2× Sample Buffer	
0.5M Tris-HCl (pH 6.8)	2.5 ml
2-mercaptoethanol	0.8 ml
SDS (10%, W/V)	2 ml
Glycerol	2 ml
Bromophenol blue	0.006 g
Final volume	10 ml

Appendix II

16S rRNA sequences of bacterial isolates

Bacillus sp. LS-2 16S ribosomal RNA gene, partial sequence

```
LOCUS       GU301923               1515 bp    DNA     linear   BCT 24-JAN-2010
DEFINITION  Bacillus sp. LS-2 16S ribosomal RNA gene, partial sequence.
ACCESSION   GU301923
VERSION     GU301923.1  GI:284018997
KEYWORDS    .
SOURCE      Bacillus sp. LS-2
  ORGANISM  Bacillus sp. LS-2
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1515)
  AUTHORS   Achal,V., Reddy,M.S. and Mukherjee,A.
  TITLE     Isolation and characterization of urease producing and calcifying
            bacteria from limestone area
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1515)
  AUTHORS   Achal,V., Reddy,M.S. and Mukherjee,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2009) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147001, India
FEATURES             Location/Qualifiers
     source          1..1515
                     /organism="Bacillus sp. LS-2"
                     /mol_type="genomic DNA"
                     /strain="LS-2"
                     /isolation_source="limestone area"
                     /db_xref="taxon:709444"
     rRNA            <1..>1515
                     /product="16S ribosomal RNA"
ORIGIN
        1 agagtttgat cctggctcag gacgaacgct ggcggcgtgc ctaatacatg caagtcgagc
       61 ggacttgagg gagcttgctc ccgatggtca gcggcggacg ggtgagtaac acgtgggcaa
      121 cctgcctgta agactgggat aactccggga aaccggggct aataccggat aattcattcc
      181 ctcacatgag ggaatgctga aagacggctt ttgctgtcac ttacagatgg gcccgcggcg
      241 cattagctag ttggtgaggt aacggctcac caaggccacg atgcgtagcc gacctgagag
      301 ggtgatcggc cacactggga ctgagacacg gcccagactc ctacgggagg cagcagtagg
      361 gaatcttccg caatggacga aagtctgacg gagcaacgcc gcgtgagcga agaaggcctt
      421 cgggtcgtaa agctctgttg tcagggaaga acaagtaccg gagtaactgc cggcaccttg
      481 acggtacctg accagaaagc cacggctaac tacgtgccag cagccgcggt aatacgtagg
      541 tggcaagcgt tgtccggaat tattgggcgt aaagcgcgcg caggcggtcc tttaagtctg
      601 atgtgaaagc ccacggctca accgtggagg gtcattggaa actgggggac ttgagtgcag
      661 aagaggagag cggaattcca cgtgtagcgg cgaaatgcgt agagatgtgg aggaacacca
      721 gtggcgaagg cggctctctg gtctgtaact gacgctgaag cgcgaaagcg tggggagcga
      781 acaggattag ataccctggt agtccacgcc gtaaacgatg agtgctaagt gttagagggt
      841 ttccgccctt tagtgctgca gcaaacgcat taagcactcc gcctggggag tacggccgca
      901 aggctgaaac tcaaaggaat tgacgggggc ccgcacaagc ggtggagcat gtggtttaat
      961 tcgaagcaac gcgaagaacc ttaccaggtc ttgacatcct ctgccactcc tggagacagg
     1021 acgttcccct tcgggggaca gagtgacagg tggtgcatgg ttgtcgtcag ctcgtgtcgt
     1081 gagatgttgg gttaagtccc gcaacgagcg caacccttgt tcttagttgc cagcattcag
     1141 ttgggcactc taaggagact gccggtgaca aaccggagga aggtggggat gacgtcaaat
     1201 catcatgccc cttatgacct gggctacaca cgtgctacaa tggatagaac aaagggcagc
     1261 gaagccgcga ggtgaagcca atcccataaa tctattctca gttcggattg caggctgcaa
     1321 ctcgcctgca tgaagccgga atcgctagta atcgcggatc agcatgccgc ggtgaatacg
     1381 ttcccgggcc ttgtacacac cgcccgtcac accacgagag tttgtaacac ccgaagtcgg
     1441 tggggtaacc ttttggagcc agccgcctaa ggtgggacag atgattgggg tgaagtcgta
     1501 acaaggtaac caatc
```

Bacillus megaterium strain SN-3 16S ribosomal RNA gene, partial sequence

```
LOCUS       GU301922                1509 bp    DNA     linear   BCT 24-JAN-2010
DEFINITION  Bacillus megaterium strain SN-3 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   GU301922
VERSION     GU301922.1  GI:284018996
KEYWORDS    .
SOURCE      Bacillus megaterium
  ORGANISM  Bacillus megaterium
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1509)
  AUTHORS   Achal,V., Reddy,M.S. and Mukherjee,A.
  TITLE     Isolation and characterization of urease producing and calcifying
            bacteria from calcareous sludge
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1509)
  AUTHORS   Achal,V., Reddy,M.S. and Mukherjee,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2009) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147001, India
FEATURES             Location/Qualifiers
     source          1..1509
                     /organism="Bacillus megaterium"
                     /mol_type="genomic DNA"
                     /strain="SN-3"
                     /isolation_source="calcareous sludge"
                     /db_xref="taxon:1404"
     rRNA            <1..>1509
                     /product="16S ribosomal RNA"
ORIGIN
        1 agagtttgat cctggctcag gatgaacgct ggcggcgtgc ctaatacatg caagtcgagc
       61 gaactgatta gaagcttgct tctatgactt tagcggcgga cgggttagta acacgtgggc
      121 aacctgcctg taagactggg ataacttcgg gaaaccgaag ctaataccgg ataggatctt
      181 ctccttcatg ggagatgatt gaaagatggt ttcggctatc acttacagat gggcccgcgg
      241 tgcattagct agttggtgag gtaacggctc accaaggcaa cgatgcatag ccgacctgag
      301 agggtgatcg gccacactgg gactgagaca cggcccagac tcctacggga ggcagcagta
      361 gggaatcttc cgcaatggac gaaagtctga cggagctacg ccgcgtgagt gatgaaggct
      421 ttcgggtcgt aaaactctgt tgttagggaa gaacaagtac gagagtaact gctcgtacct
      481 tgacggtacc taaccagaaa gccacggcta actacgtgcc agcagccgcg gtaatacgta
      541 ggtggcaagc gttatccgga attattgggc gtaaagcgcg cgcaggcggt ttcttaagtc
      601 tgatgtgaaa gcccacggct caaccgtgga gggtcattgg aaactgggga acttgagtgc
      661 agaagagaaa agcggaattc cacgtgtagc ggtgaaatgc gtagagatgt ggaggacacc
      721 agtggcgaag cggctttttg gtctgtaact gacgctgagg cgcgaagcgt ggggagcaaa
      781 caggattaga taccctggta gtcacgccgt aaacgatgag tgctaagtgt tagagggttt
      841 ccgcccttta gtgctgcagc taacgcatta agcactccgc ctggggagta cggtcgcaag
      901 actgaaactc aaaggaattg acgggggccc gcacaagcgg tggagcatgt ggtttaattc
      961 gaagcaacac gaagaacctt accaggtctt gacatcctct gacaactcta gagatagagc
     1021 gttccccttc gggggacaga gtgacaggtg gtgcatggtt gtcgtcagct cgtgtcgtga
     1081 gatgttgagt taagtcccgc aacgagcgca acccttgatc ttagttgcca gcatttagtt
     1141 gggcactcta aggtgactgc cggtgacaaa ccggaggaag gtggggatga cgtcaaatca
     1201 tcatgccccc tatgacctgg gctacacacg tgctacaatg gatggtacaa agggctgcaa
     1261 gaccgcgagg tcaagccaat cccataaaac cattctcagt gcggattgta ggctgcaact
     1321 cgcctacatg aagctggaat cgctagtaat cgcagatcag catgccgcgg tgaatacgtt
     1381 cccgggcctt gtacacaccg cccgtcacac cacgagagtt tgtaacaccc gaagtcggtg
     1441 gagtaaccgt aaggagctag ccgcctaagg tgggacagat gattggggtg aggtcgtaac
     1501 aaggtaacc
```

Bacillus sp. CT2 16S ribosomal RNA gene, partial sequence

```
LOCUS       FJ973471                1507 bp    DNA     linear   BCT 07-JUN-2009
DEFINITION  Bacillus sp. CT2 16S ribosomal RNA gene, partial sequence.
ACCESSION   FJ973471
VERSION     FJ973471.1  GI:238801104
KEYWORDS    .
SOURCE      Bacillus sp. CT2
  ORGANISM  Bacillus sp. CT2
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1507)
  AUTHORS   Reddy,M.S., Achal,V. and Mukherjee,A.
  TITLE     Isolation and characterization of urease producing and calcifying
            bacteria from cement
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1507)
  AUTHORS   Reddy,M.S., Achal,V. and Mukherjee,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-APR-2009) Biotechnology, Thapar University, Bhadson
            Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
     source          1..1507
                     /organism="Bacillus sp. CT2"
                     /mol_type="genomic DNA"
                     /strain="CT2"
                     /isolation_source="cement"
                     /db_xref="taxon:648192"
     rRNA            <1..>1507
                     /product="16S ribosomal RNA"
ORIGIN
        1 tcctggctca ggacgaacgc tggcggcgtg cctaatacat gcaagtcgag cgaacagaaa
       61 aggagcttgc tcctttgacg ttagcggcgg acgggtgagt aacacgtggg caacctaccc
      121 tatagtttgg gataactccg ggaaaccggg gctaataccg aataatctct tttgcttcat
      181 ggtaaaagac tgaaagacgg tttcggctgt cgctatagga tgggcccgcg gcgcattagc
      241 tagttggtga ggtaacggct caccaaggcg acgatgcgta gccgacctga gagggtgatc
      301 ggccacactg ggactgagac acggcccaga ctcctacggg aggcagcagt agggaatctt
      361 ccacaatggg cgaaagcctg atggagcaac gccgcgtgag tgaagaaggt tttcggatcg
      421 taaaactctg ttgtaaggga agaactagta cagtagtaac tggctgtacc ttgacggtac
      481 cttgttagaa agccacggct aactacgtgc cagcagccgc ggtaatacgt aggtggcaag
      541 cgttgtccgg aattattggg cgtaaagcgc gcgcaggtgg tttcttaagt ctgatgtgaa
      601 agcccacggc tcaaccgtgg agggtcattg gaaactggga gacttgagtg cagaagagga
      661 aagtggaatt ccatgtgtag cggtgaaatg cgtagagata tggaggaaca ccagtggcga
      721 aggcgacttt ctggtctgta actgacactg aggcgcgaaa gcgtggggag caaacaggat
      781 tagataccct ggtagtccac gccgtaaacg atgagtgcta agtgttagag ggtctccgcc
      841 ctttagtgct gaagttaacg cattaagcac tccgcctggg gagtacggcc gcaaggctga
      901 aactcaaagg aattgacggg ggcccgcaca agcggtggag catgtggttt aattcgaagc
      961 aacgcgaaga accttaccag gtcttgacat cctctgacaa ccctagagat agggcttctc
     1021 cttcgggagc agagtgacag gtggtgcatg gttgtcgtca gctcgtgtcg tgagatgttg
     1081 ggttaagtcc cgcaacgagc gcaacccttg atcttagttg ccatcattaa gttgggcact
     1141 ctaaggtgac tgccggtgac aaaccggagg aaggtgggga tgacgtcaaa tcatcatgcc
     1201 ccttatgacc tgggctacac acgtgctaca atggacggta caaagagctg caagaccgcg
     1261 aggtggagct aatctcataa aaccgttctc agttcggatt gtaggctgca actcgcctac
     1321 atgaagctgg aatcgctagt aatcgcggat cagcatgccg cggtgaatac gttcccgggc
     1381 cttgtacaca ccgcccgtca caccacgaga gtttgtaaca cccgaagacg gtggggtaac
     1441 ctttttggag ccagccgcct aaggtgggac agatgattgg ggtgaagtcg taacaaggta
     1501 accaatc
```

Bacillus sp. CT5 16S ribosomal RNA gene, partial sequence

```
LOCUS       FJ973470                1508 bp    DNA     linear   BCT 07-JUN-2009
DEFINITION  Bacillus sp. CT5 16S ribosomal RNA gene, partial sequence.
ACCESSION   FJ973470
VERSION     FJ973470.1  GI:238801103
KEYWORDS    .
SOURCE      Bacillus sp. CT5
  ORGANISM  Bacillus sp. CT5
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1508)
  AUTHORS   Reddy,M.S., Achal,V. and Mukherjee,A.
  TITLE     Isolation and characterization of urease producing and calcifying
            bacteria from cement
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1508)
  AUTHORS   Reddy,M.S., Achal,V. and Mukherjee,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-APR-2009) Biotechnology, Thapar University, Bhadson
            Road, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
     source          1..1508
                     /organism="Bacillus sp. CT5"
                     /mol_type="genomic DNA"
                     /strain="CT5"
                     /isolation_source="cement"
                     /db_xref="taxon:648193"
     rRNA            <1..>1508
                     /product="16S ribosomal RNA"
ORIGIN
        1 tcctggctca ggatgaacgc tggcggcgtg cctaatacat gcaagtcgag cgaatggatt
       61 aagagcttgc tcttatgaag ttagcggcgg acgggtgagt aacacgtggg taacctgccc
      121 ataagactgg gataactccg ggaaaccggg gctaataccg gataacattt tgaaccgcat
      181 ggttcgaaat tgaaaggcgg cttcggctgt cacttatgga tggacccgcg tcgcattagc
      241 tagttggtga ggtaacggct caccaaggca acgatgcgta gccgacctga gagggtgatc
      301 ggccacactg ggactgagac acggcccaga ctcctacggg aggcagcagt agggaatctt
      361 ccgcaatgga cgaaagtctg acggagcaac gccgcgtgag tgatgaaggc tttcgggtcg
      421 taaaactctg ttgttaggga agaacaagtg ctagttgaat aagctggcac cttgacggta
      481 cctaaccaga aagccacggc taactacgtg ccagcagccg cggtaatacg taggtggcaa
      541 gcgttatccg gaattattgg gcgtaaagcg cgcgcaggtg gtttcttaag tctgatgtga
      601 aagcccacgg ctcaaccgtg gagggtcatt ggaaactggg agacttgagt gcagaagagg
      661 aaagtggaat tccatgtgta gcggtgaaat gcgtagagat atggaggaac accagtggcg
      721 aaggcgactt tctggtctgt aactgacact gaggcgcgaa agcgtgggga gcaaacagga
      781 ttagataccc tggtagtcca cgccgtaaac gatgagtgct aagtgttaga gggtttccgc
      841 cctttagtgc tgaagttaac gcattaagca ctccgcctgg ggagtacggc cgcaaggctg
      901 aaactcaaag gaattgacgg gggcccgcac aagcggtgga gcatgtggtt taattcgaag
      961 caacgcgaag aaccttacca ggtcttgaca tcctctgaca accctagaga tagggcttct
     1021 ccttcgggag cagagtgaca ggtggtgcat ggttgtcgtc agctcgtgtc gtgagatgtt
     1081 gggttaagtc ccgcaacgag cgcaaccctt gatcttagtt gccatcattt agttgggcac
     1141 cctaaggtga ctgccggtga caaaccggag gaaggtgggg atgacgtcaa atcatcatgc
     1201 cccttatgac ctgggctaca cacgtgctac aatggacggt acaaagagct gcaagaccgc
     1261 gaggtggagc taatctcata aaaccgttct cagttcggat tgtaggctgc aactcgccta
     1321 catgaagctg gaatcgctag taatcgcgga tcagcatgcc gcggtgaata cgttcccggg
     1381 ccttgtacac accgcccgtc acaccacgag agtttgtaac acccgaagtc ggtggggtaa
     1441 cctttttgga gccagccgcc taaggtggga cagatgattg gggtgaagtc gtaacaaggt
     1501 aaccaatc
```

Bacillus sp. LS-4 16S ribosomal RNA gene, partial sequence

```
LOCUS       GU301924                1512 bp    DNA     linear   BCT 24-JAN-2010
DEFINITION  Bacillus sp. LS-4 16S ribosomal RNA gene, partial sequence.
ACCESSION   GU301924
VERSION     GU301924.1  GI:284018998
KEYWORDS    .
SOURCE      Bacillus sp. LS-4
  ORGANISM  Bacillus sp. LS-4
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1512)
  AUTHORS   Achal,V., Reddy,M.S. and Mukherjee,A.
  TITLE     Isolation and characterization of urease producing and calcifying
            bacteria from limestone area
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1512)
  AUTHORS   Achal,V., Reddy,M.S. and Mukherjee,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2009) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147001, India
FEATURES             Location/Qualifiers
     source          1..1512
                     /organism="Bacillus sp. LS-4"
                     /mol_type="genomic DNA"
                     /strain="LS-4"
                     /isolation_source="limestone area"
                     /db_xref="taxon:709445"
     rRNA            <1..>1512
                     /product="16S ribosomal RNA"
ORIGIN
        1 agagtttgat cctggctcat gacgaacgct ggcggcgtgc ctgatacatg caagtcgagc
       61 ggacagatgg gagcttgctc cctgatgtta gcggcggacg ggtgagtaac acgtgggtaa
      121 cctgcctgta agactgggat aactccggga aaccggggct aataccggat ggttgtttga
      181 accgcatggt tcaaacataa aaggtggctt cggctaccac ttacagatgg acccgcggcg
      241 cattagctag ttggtgaggt aacggctcac caaggcaacg atgcgtagcc gacctgagag
      301 ggtgatcggc gacactggga ctgagacacg gcccagactc ctacgggagg cagcagtagg
      361 gaatcttccg caatggacga aagtctgacg gagcaacgcc gcgtgagtga tgaaggtttt
      421 cggatcgtaa agctctgttg ttagggaaga acaagtaccg ttcgaatacg gcggcacctt
      481 gacggtacct aaccagaaag ccacggctaa ctacgtgcca gcagccgcgg taatacgtag
      541 gtggcaagcg ttgtccggaa ttattgggcg taaagggctc gcaggcggtt ccttaagtct
      601 gatgtgaaag cccccggctc aaccggggag ggtcattgga aactggggaa cttgagtgca
      661 gaagaggaga gtggaattcc acgtgtagcg gtgaaatgcg tagagatgtg gaggaacacc
      721 agtggcgaag gcgactctct ggtctgtaac tgacgctgaa gagcgaaagc gtggggagcg
      781 aacaggatta gataccctgg tagtccacgc cgtaaacgat gagtgctaag tgttaggggg
      841 tttccgcccc tttagtgctg cagctaacgc attaagcact ccgcctgggg agtacggtcg
      901 caagactgaa actcaaagga attgacggag gcccgcacaa gcggtggagc atgtggttta
      961 attcgaagca acgcgaagaa ccttaccagg tcttgacatc ctctgacaat cctagagata
     1021 ggacgtcccc ttcgggggca gagtgacagg tggtgcatgg ttgtcgtcag ctcgtgtcgt
     1081 gagatgttgg gttaagtccc gcaacgagcg caacccttga tcttagttgc cagcattcag
     1141 ttgggcactc taaggtgact gccggtgaca aaccggagga aggtggggat gacgtcaaat
     1201 catcatgccc cttatgacct gggctacaca cgtgctacaa tggacagaac aaagggcagc
     1261 gaaaccgcga ggttaagcca atcccacaaa tctgttctca gttcggatcg cagtctgcaa
     1321 ctcgactgcg tgaagctgga atcgctagta atcgcggatc agcatgccgc ggtgaatacg
     1381 ttcccggccc ttgtacacac cgcccgtcac accacgagag tttgtaacac ccgaagtcgg
     1441 tgaggtaacc ttttaggagc cagccgccga aggtgggaca gatgattggg gtgaagtcgt
     1501 aacaaggtaa cc
```

Bacillus subtilis strain AS-4 16S ribosomal RNA gene, partial sequence

```
LOCUS       GU301921                1503 bp    DNA     linear   BCT 24-JAN-2010
DEFINITION  Bacillus subtilis strain AS-4 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   GU301921
VERSION     GU301921.1  GI:284018995
KEYWORDS    .
SOURCE      Bacillus subtilis
  ORGANISM  Bacillus subtilis
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1503)
  AUTHORS   Achal,V., Reddy,M.S. and Mukherjee,A.
  TITLE     Isolation and characterization of urease producing and calcifying
            bacteria from alkaline soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1503)
  AUTHORS   Achal,V., Reddy,M.S. and Mukherjee,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2009) Department of Biotechnology, Thapar
            University, Bhadson Road, Patiala, Punjab 147001, India
FEATURES             Location/Qualifiers
     source          1..1503
                     /organism="Bacillus subtilis"
                     /mol_type="genomic DNA"
                     /strain="AS-4"
                     /isolation_source="alkaline soil"
                     /db_xref="taxon:1423"
     rRNA            <1..>1503
                     /product="16S ribosomal RNA"
ORIGIN
        1 tggctcagga cgaacgctgg cggcgtgcct aatacatgca agtcgagcgg acagatggga
       61 gcttgctccc tgatgttagc ggcggacggg tgagtaacac gtgggtaacc tgcctgtaag
      121 actgggataa ctccgggaaa ccggggctaa taccggatgg ttgtttgaac cgcatggttc
      181 aaacataaaa ggtggcttcg gctaccactt acagatggac ccgcggcgca ttagctagtt
      241 ggtgaggtaa cggctcacca aggcaacgat gcgtagccga cctgagaggg tgatcggcca
      301 cactgggact gagacacggc ccagactcct acgggaggca gcagtaggga atcttccgca
      361 atggacgaaa gtctgacgga gcaacgccgc gtgagtgatg aaggttttcg gatcgtaaag
      421 ctctgttgtt agggaagaac aagtaccgtt cgaatagggc ggtaccttga cggtacctaa
      481 ccagaaagcc acggctaact acgtgccagc agccgcggta atacgtaggt ggcaagcgtt
      541 gtccggaatt attgggcgtg aagggctcgc aggcggtttc ttaagtctga tgtgaaagcc
      601 cccggctcaa ccggagaggg tcattggaaa ctggggaact tgagtgcaga agaggagagt
      661 ggaattccac gtgtagcggt gaaatgcgta gagatgtgga ggaacaccag tggcgaaggc
      721 gactctctgg tctgtaactg acgctgagga gcgaaagcgt ggggagcgaa caggattaga
      781 taccctggta gtccacgccg taaacgatga gtgctaagtg ttaggggttt tccgcccctt
      841 agtgctgcag ctaacgcatt aagcactccg cctggggagt acggtcgcaa gactgaaact
      901 caaaggaatt gacgggggcc cgcacaagcg gtggagcatg tggtttaatt cgaagcaacg
      961 cgaagaacct taccaggtct tgacatcctc tgacaatcct agagatagga cgtccccttc
     1021 gggggcagag tgacaggtgg tgcatggttg tcgtcagctc gtgtcgtgag atgttgggtt
     1081 aagtcccgca acgagcgcaa cccttgatct tagttgccag cattcagttg ggcactctaa
     1141 ggtgactgcc ggtgacaaac cggaggaagg tggggatgac gtcaaatcat catgcccctt
     1201 atgacctggg ctacacacgt gctacaatgg acagaacaaa gggcagcgaa accgcgaggt
     1261 taagccaatc ccacaaatct gttctcagtt cggatcgcag tctgcaactc gactgcgtga
     1321 agctggaatc gctagtaatc gcggatcagc atgccgcggt gaatacgttc ccgggccttg
     1381 tacacaccgc ccgtcacacc acgagagttt gtaacacccg aagccggtga ggtaaccttt
     1441 taggagccag ccgccgaagg tgggacagat gattggggtg aagtcgtaac aaggtaacca
     1501 atc
```