

BIOMINERALIZATION EFFICIENCY OF HALOPHILIC BACTERIA AND THEIR ROLE IN BUILDING STRUCTURES

A

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by

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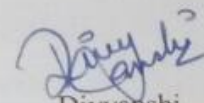
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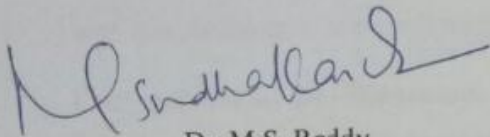
I hereby declare that work presented in dissertation entitled "**Biom mineralization efficiency of halophilic bacteria and their role in building structures**" in partial fulfilment of the requirement for the degree of Masters in Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab, is an authentic record of my own work during the period of six months from January 2019 to June 2019, under the supervision of **Dr. M.S. Reddy**, Professor, Department of Biotechnology, Thapar Institute of Engineering and Technology. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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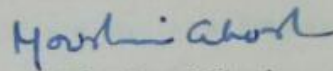

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CERTIFICATE

This is to certify that the thesis entitled "**Biom mineralization efficiency of halophilic bacteria and their role in building structures**" submitted by Divyanshi in partial fulfilment of the requirement for the degree of Masters in Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala, Punjab, is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.



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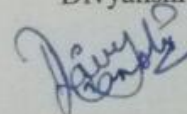
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ABSTRACT

Halophilic microbes have always been a hotspot for researchers due to their ability to survive the extreme conditions, that most of other organisms are unable to endure. Also, during a past few decades, various microbes have been studied for their ability to produce biominerals. Present study focuses on isolation of a novel strain from hypersaline habitat, i.e., Sambhar Lake, Jaipur, Rajasthan. The growth of isolated strain (SL-1) at varying alkalinity stress was monitored along with its proficiency to heal concrete structures in terms of mechanical strength, water and ionic ingress, crystal morphology and amount of CaCO_3 precipitated. It was observed that SL-1 grew best in growth medium with 3.5% NaCl concentration (pH 10) and its inculcation into concrete cubes resulted in an increase of 16% of strength along with significant reduction in water absorption. Present study suggests that CaCO_3 precipitation by halophilic strain can nullify the deterring effect of ionic permeation through salts in saline environment in concrete structures.

CONTENTS

CANDIDATE'S DECLARATION	i
CERTIFICATE	ii
ACKNOWLEDGEMENTS	iii
ABSTRACT	iv
LIST OF TABLES	vii
LIST OF FIGURES	vii
LIST OF ABBREVIATIONS	ix
CHAPTER 1 INTRODUCTION	1
OBJECTIVES	3
CHAPTER 2 LITERATURE REVIEW	4
2.1 Biomineralization	4
2.2 MICCP studies in saline environment	8
2.2.1 Survival strategies of halophilic microflora	10
2.3 Urease enzyme	11
2.4 Polymorphism of carbonate crystals	13
2.5 Optimum conditions for biomineralization	14
2.6 Significance of biomineralization in construction materials	14
CHAPTER 3 MATERIALS AND METHODOLOGY	17
3.1 Sample collection	17
3.2 Bacterial isolation and cultivation	17
3.3 Qualitative analysis of bacterial strain	17
3.4 Quantitative analysis of bacterial strain	18
3.4.1 Urease Activity	18
3.4.2 Calcium Carbonate estimation	18
3.4.3 Microbial sand plugging	19
3.5 Extraction of genomic DNA from bacteria	20
3.5.1 Isolation of genomic DNA	20
3.5.2 Electrophoresis of DNA on agarose gels	21
3.5.3 Quantification of DNA by Nanodrop spectrophotometer	22
3.5.4 Amplification of 16S rDNA and Purification of PCR products	22

3.6 Physiological characterization of halophilic bacteria	22
3.6.1 Growth Kinetics	22
3.6.2 Morphological and biochemical studies of bacterial isolates	23
3.7 Bacterial application on concrete structures	25
3.7.1 Materials	25
3.7.2 Sample Preparation	25
3.7.3 Test Procedures	26
CHAPTER 4 RESULTS AND DISCUSSIONS	28
4.1 Bacterial isolation and cultivation	28
4.1.1 Sample Collection	28
4.1.2 Isolation of bacterial strain	28
4.2 Qualitative analysis of bacterial strain	29
4.3 Quantitative analysis of bacterial strain	30
4.3.1 Urease Activity	30
4.3.2 Calcium Carbonate Estimation	31
4.3.3 Microbial Sand Plugging	32
4.4 Extraction of genomic DNA from bacteria	34
4.4.1 16S rDNA Sequencing and Phylogenetic Analysis	35
4.5 Physiological and biochemical characterization of halophilic bacteria	37
4.5.1 Growth Kinetics	37
4.5.2 Grams' Staining	38
4.5.3 Oxidase Test	38
4.5.4 Catalase Test	39
4.5.5 Nitrate Reduction Test	39
4.6 Bacterial application on concrete cubes	40
4.6.1 Compressive Strength	40
4.6.2 Permeation Properties	42
CONCLUSION	44
REFERENCES	45

LIST OF TABLES

Table 3.1 Curing regimes.....	26
Table 4.1 Colony count with mean and standard deviation.....	28
Table 4.2 Quantification of DNA	35

LIST OF FIGURES

Figure 2.1 Microbial mineralization by bacterial cell	5
Figure 2.2 Bacteria as Nucleation site for CaCO ₃ precipitation	7
Figure 2.3 Pre-stained cells in fluid inclusions	10
Figure 2.4 Miniaturization of Halobacterium salinarum	11
Figure 2.5 Polymorphs of Calcium Carbonate by MICCP	14
Figure 3.1 Setup for Sand Consolidation	20
Figure 3.2 Setup for sorptivity test	27
Figure 4.1 Colony forming units on varying dilutions	29
Figure 4.2 Urease activity shown by ureolytic strain	30
Figure 4.3 Time course of urease activity of SL-1.	30
Figure 4.4 Precipitation in NBU-CaCl ₂	31
Figure 4.5 EDTA titration	32
Figure 4.6 Microstructure analysis (SEM – EDX)	32
Figure 4.7 Sand column consolidation	33
Figure 4.8 Microstructure analysis (SEM – EDX)	34
Figure 4.9 Microstructure analysis (SEM – EDX)	34

Figure 4.10 Gel electrophoresis	35
Figure 4.11 Phylogenetic tree of isolated species with related taxa	36
Figure 4.12 Growth curve for SL-1 at varying salt stresses	37
Figure 4.13 Growth curve for SL-1 at varying salt stresses	38
Figure 4.14 Oxidase Test	399
Figure 4.15 Catalase test	399
Figure 4.16 Nitrate Reductase test	40
Figure 4.17 Microstructure analysis (SEM – EDX)	40
Figure 4.18 Compressive Strength	41
Figure 4.19 Cubes showing carbonate precipitation on surface	41
Figure 4.20 Rate of water absorption by control and bacterial treated specimen.	422

LIST OF ABBREVIATIONS

%:	Percent
&:	and
µg:	microgram
ATP:	Adenosine Tri Phosphate
bp:	base pairs
CFU's:	Colony Forming Units
Conc.:	Concentration
DNA:	Deoxyribonucleic Acid
dNTP's:	Deoxyribonucleotide Triphosphate
Fig.	Figure
g:	Gram
MICCP:	Microbially Induced Calcium Carbonate Precipitation
NA:	Nutrient Agar
NB:	Nutrient Broth
NBU:	Nutrient Broth + Urea
NBU-CaCl ₂ :	Nutrient Broth + Urea + Calcium Chloride
O.D.:	Optical Density
Spp.:	Species
UAB:	Urea Agar Base

CHAPTER 1 INTRODUCTION

Considering the fact, how building structures have been the supporting pillars in the development of society's economy, concrete has become paramount for expedited evolution of socio-economic parameters. A question arises, what is concrete? Commonly used concrete, for building sturdy structures, contains Portland cement along with fine sand, coarse aggregates, binder mixed with water, which sets with time according to the shape of mold it is put into. Although it is one of the maintenance free and strongest building material being used worldwide, there are a few factors, including geological factors such as thermal stress and salinity stress, physical factors such as shrinkage, freeze-thaw reactions, low tensile strength, and high structural stress, chemical factors like carbonation, sulfate attack, chloride ion invasion, and corrosion of metal reinforcements, and poor quality of material and labor which deteriorate the infrastructure of the concrete building. From last few years, the image of being a strong construction material has been tainted as the problem of spalling, leakage, cracking, scaling, delamination, erosion, dusting, disintegration, etc., of concrete tag along, among which cracks in building structures are ineluctable (Koch et al., 2015)

Concrete is most vulnerable to cracks and surface deterioration in saline areas. Ionic ingress (mainly chloride ion) from salts lessens the strength and sturdiness of the reinforced concrete structure and eventually weakens it (Glass & Buenfeld, 2000; Torres-Luque et al., 2014) by corroding the metal reinforcements. Therefore, the damaging of reinforcing metal by corrosion in concrete because of chloride transport in saline environs has received amassed interest during past few years because of its wide spread occurrence and high maintenance. Microbially Induced Calcium Carbonate Precipitation (MICCP) involves intricate biochemical reactions mediated by two important microbial enzymes, carbonic anhydrase and urease. These enzymes utilize urea as substrate, and calcium source for mineralization. Use of synthetic epoxides, grouting, drilling and plugging, routing and sealing, etc., has been prevalent for the remediation of cracks. But many of these techniques require constant maintenance and are expensive. Moreover, such treatments in long run pose destructive effects on the environment, and the aesthetic value of the concrete structure depreciates. Keeping in check, the above-mentioned drawbacks, researchers have

elucidated bio-concrete as the ultimate solution, which works on the principle of MICCP through metabolic pathways. The process involved in the self-healing of concrete due to microbial action is also termed as biomineralization.

Biom mineralization is a technique that involves the deposition of minerals, chiefly carbonate products, formed by the reaction of metabolic products by microorganisms with surrounding materials. Calcium carbonate compounds are precipitated in abundance by this process it is one of the most suitable fillers for cracks and air spaces formed in concrete due to its affinity with concrete, its stability, and obtainability of calcium in cement. CaCO_3 biomineralizes to form different anhydrous polymorphs namely aragonite, calcite, vaterite, monohydrocalcite, amorphous calcium carbonates, depending on the composition of the bacterial wall, temperature, pH, etc. (Dhami et al., 2013a)

Remediation of concrete by MICCP is quite beneficial over the conventional treatments as it is eco-friendly, maintenance free and energy efficient technology. Reportedly, biomineralization is of two types i.e., biologically induced mineralization (BIM), and biologically controlled mineralization (BCM) (Achal & Mukherjee, 2015). In BIM, minerals are deposited in environment as metabolic byproducts while minerals are accumulated intracellularly in BCM. Various organisms ranging from the Monera to Chordata are capable of biomineralization. But most of the existing biominerals are precipitated by prokaryotes according to previous studies (Simkiss & Wilbur, 2012). In 1913, *Drew* was first to discover CaCO_3 precipitating bacteria, when he isolated a denitrifying strain which was capable of producing calcite crystals by utilizing a liquid medium. As the research in this area gained popularity, many other bacterial strains such as *Bacillus* spp., *Lysinibacillus* spp., and *Sporosarcina* spp., (Lee et al., 2017) have been reported as calcifying species. These microbes follow different pathways viz. ureolysis, denitrification, photosynthesis, methane oxidation, ammonification, and sulfate reduction (Castanier et al., 1999; Braissant et al., 2007; Reeburgh, 2007; Van Paassen et al., 2010; Zhu & Dittrich, 2016)

In saline environment having high pH, halophilic and halotolerant bacteria are known to survive best. Also for biomineralization, survival of bacteria inside concrete matrix (high

pH) is a prerequisite (Lee & Park, 2018). As we know that halophilic strains are capable of surviving harsh conditions due to various survival strategies to avoid water loss such as, formation of spheres or vesicles inside the fluid inclusions (Stan-Lotter & Fendrihan, 2015), miniaturization of cells by increasing surface to volume ratio eventually. Hence, this study will be focused on isolation of a calcifying strain from hypersaline environment capable of producing urease enzyme, its optimization at varying salt concentration and pH and its capability to mineralize Calcium Carbonate.

OBJECTIVES

1. Isolation and characterization of halophilic calcifying bacteria from alkaline environmental sources.
2. Evaluation of biomineralization efficiency under altered salt stress conditions.
3. Application of efficient calcifying bacteria on building structures.

CHAPTER 2 LITERATURE REVIEW

2.1 Biomineralization

Current concern about deteriorating concrete structures has drawn the interest to process degradation and restoration of construction materials. The conditions provided during construction along with concretes' permeation properties determine the damage risk and its speed of development. Most of the physicochemical degradation mechanisms are associated to corrosive components present in aqueous solution. Reducing water uptake by the concrete structure is an important measure to lessen concrete damage (Basheer et al., 2001). Surface treatments play prime role in restraining the infiltration of water and destructive elements into concrete. These days a wide range of organic as well as inorganic products are available for surface protection, like, water repellents, pore blockers, etc. Such treatments have been extensively used for restoration and remediation of civil structures because of its uncompromised benefits. Beside their advantages, the conventional methods of protection, however, are lacking in many aspects like: (i) treated layers exhibit varying thermal expansion coefficients; (ii) deterring with time; (iii) maintenance at regular intervals. Moreover, certain solvents contribute to environmental pollution (Pe`rez et al., 1995; Camaiti et al., 1988). To overcome afore mentioned disadvantages, more eco - friendly methods have been proposed.

Keeping in check the given framework, microbially induced carbonate precipitation has been suggested as a novel, eco-friendly technology for protection and remediation of concrete. Biomineralization is a biological process induced and mediated by microorganism for synthesis and precipitation of minerals. The prokaryotic fixation of carbon dioxide with availability of a mineral source, usually calcium, catalyzed by microbial enzymes is termed as Microbially Induced Calcium Carbonate Precipitation (MICCP). Both terms, biomineralization, and MICCP, can be used interchangeably. Further, this process is classified into two categories, BCM and BIM. Biologically Controlled Mineralization is an intracellular process in which precipitation product gets accrued within or on the cytosol or vesicles of microbial cells. While in Biologically Induced Mineralization, microbes produce one or more metabolic products that react with

complexes present in the environment producing mineral particle deposits as their metabolic byproduct (Fig 2.1.) Biominerals formed are unintended and uncontrolled metabolic activity of microbes that often helps to selectively cure concrete by producing organic and inorganic compounds that are relatively insoluble. The mineralized compound used as cementing material sometimes is called "biocement" (Achal & Mukherjee, 2015)

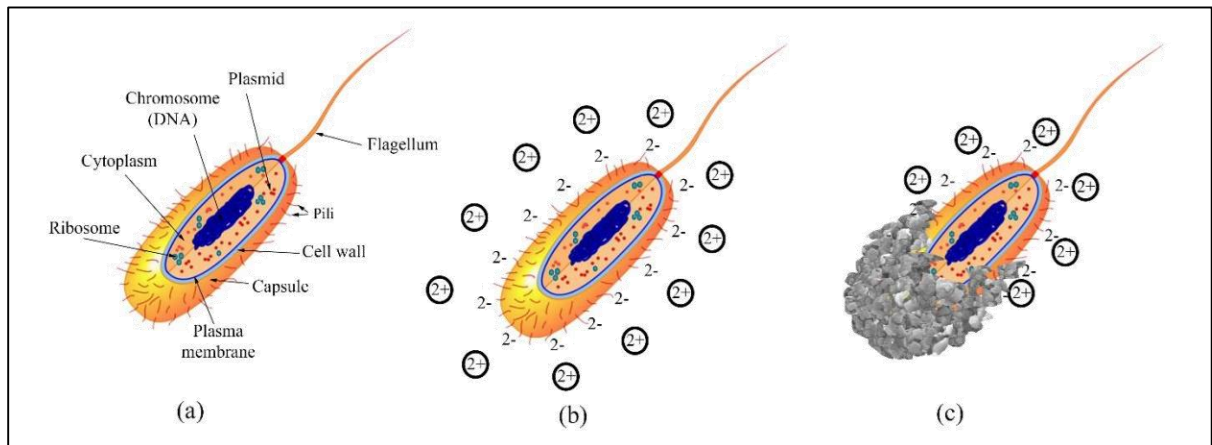
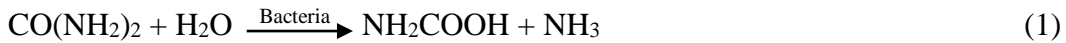


Figure 2.1 (a) Bacterial cell; (b) Negatively charged cell wall and presence of positive charged ions; (c) Microbial mineralization by binding of ions to cell wall. Adapted from: (Seifan et al., 2016)

Concrete structures are more susceptible to degradation in saline due to ionic permeation mainly chloride, which leads to chloride induced corrosion of the metal reinforced concrete structures. When the concentration of chloride reaches a threshold value, it initiates chloride-induced corrosion. It damages the protective layer by formed by deposition of corrosion products (because of high alkalinity of concrete). After corrossions' onset, premature deterioration is triggered by several mechanisms: loss of reinforcement unit, loss of metal-concrete link, cracks and delamination of concrete. After steel corrosion begins, the reinforced concretes' physical and mechanical parameters decay at rate depending on the environmental factors. This debilitation process brings on larger repair cost as well as maintenance tariff, which in turn takes a severe toll on the durability and life-cycle performance (Torres-Luque et al., 2014). MICCP has been known to reduce chloride ingress in concrete. The permeability class for control specimen and specimen treated with bacterial cells (*Sporosarcina pasteurii*) was "moderate" and "low" respectively, regardless of the media used (Achal et al., 2011).

Research in area of this ecofriendly solution gained popularity, and led to discovery of many other bacterial strains such as *Bacillus* spp., *Lysinibacillus* spp., and *Sporosarcina* spp., (Kim et al., 2016) as calcifying species. These microbes follow different pathways viz. ureolysis, denitrification, photosynthesis, methane oxidation, ammonification, and sulfate reduction (Castanier et al., 2000; Fujita et al., 2000; Rodriguez-Navarro et al., 2003; Dupraz et al., 2004; Reeburgh, 2007; Zhu & Dittrich, 2016).

Due to its minimalism, urea hydrolysis by the urease enzyme in presence of rich calcium source, is frequently studied system for applied MICCP. Urease decomposes urea to CO₂ and ammonia, which increases pH as well as carbonate concentration in bacterial surroundings. One mol of urea hydrolyses to ammonia and carbonate (Eq. 1), which extemporaneously breaks down to form 1 mol of ammonia and carbonic acid (Eq. 3) as shown below:



Further the reaction proceeds as follows:



A diverse variety of microbial species contribute in mineralization of carbonates in various environmental biospheres like soil, oceans, saline lakes, geological formations, etc. Some of such bacteria are *Micrococcus* sp., *Bacillus subtilis*, *Bacillus pasteurii*, *Deleya halophila*, *Halomonas eurihalina*, and *Myxococcus xanthus* (Rivadeneira et al., 1996; Tiano et al., 1999; Rodriguez-Navarro et al., 2003). This technology has been explored on concrete cubes by many researchers to study its scope in restoration of various construction

materials (Achal et al., 2011), sterile sand plugging and limestone repair (Stocks-Fischer et al., 1999; Bachmeier et al., 2002; Dick et al., 2006; Dhami et al., 2013b) reduction in water sorptivity and chloride ion permeation in concrete (Achal et al., 2011)

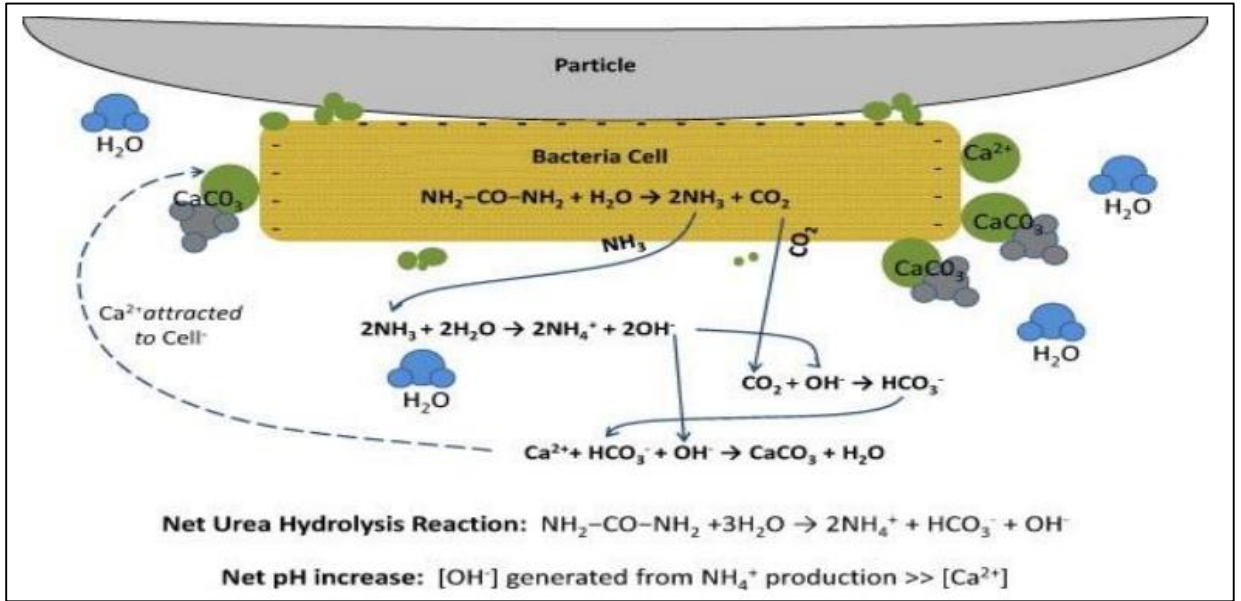


Figure 2.2 Bacteria as Nucleation site for CaCO₃ precipitation Source: (Dejong et al., 2010)

Possible biochemical reactions in urea-CaCl₂ medium for CaCO₃ precipitation (Fig 2.2) at the bacterial surface can be described as below (Joshi et al., 2018):



Sarda et al., (2009), reported in the useful role of a ureolytic microorganism for mineralization in bricks using *Bacillus pasteurii*. A few studies on calcite precipitation for improvement of strength of concrete (Bang et al., 2001), consolidation of sand, restoration of Granite (Gollapudi et al., 1995) & ornamental stone (Dick et al., 2006) have been reported.

An additional layer is formed by calcifying bacteria on surface of pre-existing concrete layer. Impermeability of the concrete specimen increases as the new calcite layer is deposited by bacteria, which in turn is highly insoluble. Thus, permeation of harmful substances (alkali, sulfate etc.) into the concrete is hindered, hence exhibiting a decline in the deleterious effects caused by them (Ramakrishnan et al., 2005).

Ghosh et al., (2005) studied the effects of anaerobic microorganism (*Shewanella* spp.), on the concrete cubes' mechanical strength. It was discovered that the strength of concrete cubes exponentially increases at every addition of anaerobic microbe. About 25% increase in compressive strength of concrete cubes was reported during the period of 28 days by adding 10^5 cells/ml.

Supersaturation of calcium carbonate crystal precipitates was attained by heterogeneous nucleation on microbial cells (DeMuyne et al., 2007). Mineralization efficiency (biodeposition) reduces the permeation properties of concrete and concrete. Calcite deposited biologically is less soluble as compared to calcite precipitated inorganically (Morse, 1983). De Muyeck et al., (2010) has shown the shielding effect of CaCO_3 precipitation by *Bacillus sphaericus* on limestone.

The high alkalinity of concrete is a main barrier for the growth of a moderate alkalophile, *Bacillus pasteurii*, which grows best at pH 9. *Bacillus pasteurii* is also capable to undergo dormancy by producing endospores, in order to endure unfavorable extremities. However, because of very high pH of the concrete (12.5), the growth and urease activity may be retarded for sufficient calcite production (Achal et al., 2009). Moreover, calcite precipitation is an intricate phenomenon, which is a cumulative result of the cell concentration, ionic strength and pH of the medium (Ramachandran et al., 2001).

2.2 MICCP studies in saline environment

Many studies have revealed that microbes, mainly bacteria, produce carbonates by MICCP. Precipitation of CaCO_3 was studied *in vitro* as well as natural habitats and it was proposed that this crystal formation could be associated to the development of calcareous coral reefs, and deposition of carbonates in soils (Krumbein, 1979; Doetsch & Cook, 2012).

Many external factors influence carbonate precipitation, among which the salt concentrations in the medium composition is most significant. Moderately halophilic bacteria are highly suitable for reviewing the effect of different salt concentrations on their ability to precipitate minerals as it flourishes over a broad spectrum of osmotic concentrations. A few moderately halophilic bacteria have been testified for their capacity of forming calcium carbonate crystals (Ferrer et al., 1988; Rivadeneyra et al., 1991; Rivadeneyra et al., 1993; Rivadeneyra et al., 1994). *Deleya halophila* is one of the moderately halophilic microorganisms found in the soils of Alicante (Spain) city situated on the Mediterranean coast (Quesada et al., 1984). Its capability to mineralize carbonates, its growth conditions and salt prerequisite, makes this bacterium a useful tool in the field of carbonate precipitation. Despite the abundance of magnesium as compared to calcium, later is mainly mineralized by *Deleya halophila* (Rivadeneyra et al., 1996).

Different type of cell surface of each bacterium results in a different cell surface charge (Mei et al., 2000). Also, pH, ionic strength and ionic composition of the water strongly influence the charges on cell surface (Lytle et al., 2002; Ahimou et al., 2002). These parameters are modified by the bacterial metabolism leading to changes in the cell surface during the culture growth phases. On the other hand, the impact of the biochemical parameters of medium on microbial cell surface varies with the bacterial species. Formation of monohydrocalcite is favored by liquid growth media with high salt concentrations. It was discovered by Knorre and Krumbein (2000) that when bacteria from freshwater, marine waters and hypersaline ecosystems was cultivated in artificial laboratory media carbonate precipitation was observed.

Twenty-two moderately halophilic bacterial strains exhibited mineral precipitation in solid as well as liquid artificial sea water salts media at varying concentrations and different ratios of Mg^{2+} -to- Ca^{2+} . It was observed by Rivadeneyra et al., (2006) that magnitude of bioliths was inversely proportional and the time required for precipitation was inversely proportional to the salt concentrations. The minerals precipitated displayed a variety in structures as the crystals formed were calcite, aragonite, magnesian calcite, dolomite, hydromagnesite, monohydrocalcite and struvite, subject to the bacterial strain, concentration of salts and the physiology of the medium. Microstructure analysis showed

that generation of the bioliths was triggered by formation of aggregates of calcified cells. Also, it was observed that the dominant morphologies were sphere shaped with fibrous radiated interiors. It was experimentally shown that moderately halophilic bacteria actively participate in carbonate precipitation and this process is termed as biomineralization.

2.2.1 Survival strategies of halophilic microflora

Despite the extreme environmental conditions, hypersaline lakes and salterns harbor diverse species, including both prokaryotic and eukaryotic microorganisms, which form complex functioning colonies in these waters. Survival strategies are needed in all salt concentrations, but especially when the salt level exceeds 15% (w/v). The survival strategies for these microorganisms have required biochemical adaptations to their proteins, lipids, and nucleic acids. To compensate for the high osmotic pressure of hypersaline habitats, microorganisms produce compatible solutes. These solutes maintain the balance of internal osmotic pressure with the external osmotic pressure, to keep the cellular unit intact. It may be either inorganic ions or organic molecules. Few microbes contain certain membrane proteins such as rhodopsin which helps in regulation of osmotic balance. These membrane proteins maintain the osmotic equilibrium of a cell by sustaining the balance between proton efflux and influx of the cell. Also, the survival strategies for these microorganisms have required biochemical modifications and enrichment of their proteins, lipids, and nucleic acids (Litchfield, 1998).

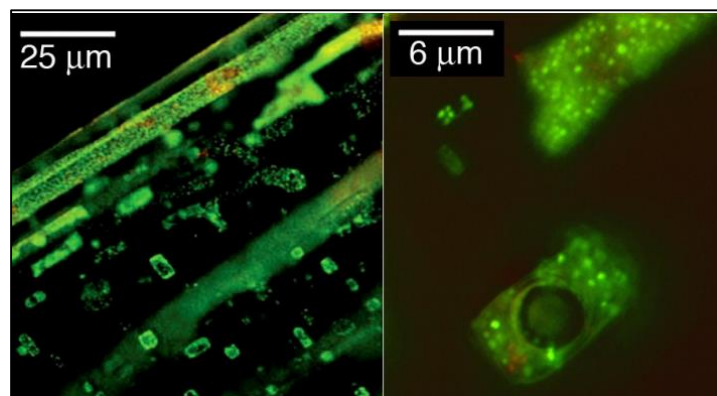


Figure 2.3 Pre-stained cells of *Halobacterium salinarum* NRC-1 in fluid inclusions.
Source: (Stan-Lotter & Fendrihan, 2015)

Halophilic microflora can apparently survive for millions of years under extreme conditions like low water activity, starvation, hypersalinity and radiation. Most of the survival strategies that are adopted by haloarchaea seem specific to this genus such as, synthesis of halomucin, formation of halite fluid inclusions (Fig. 2.3), and a few are similar to that adopted by other prokaryotes. The mechanisms that are involved include miniaturization of cells (Fig. 2.4), production of protective capsules, reduction of ATP and production of dormant stages, like small spherical particles, swarming (Falkinham & Hoffman, 1984). Polyploidy is necessary factor for most of these strategies (Stan-Lotter & Fendrihan, 2015)

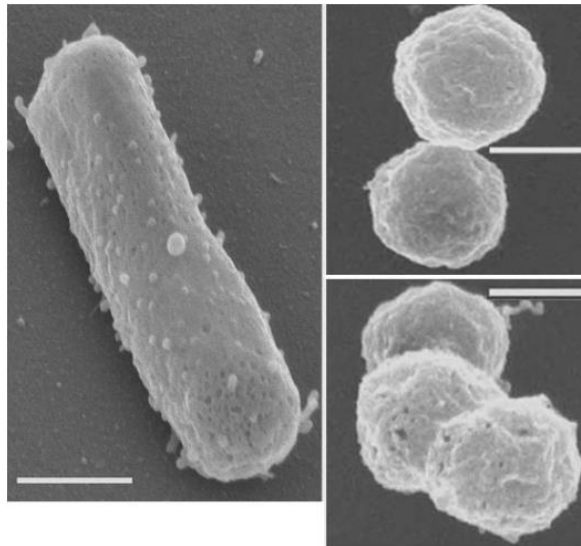


Figure 2.4 Scanning electron micrographs of a rod (left panel) and spheres (right panels) of *Halobacterium salinarum* NRC-1. Photographs taken by Chris Frethem, University of Minnesota

2.3 Urease enzyme

Holm & Sander, (1997) classified urease enzyme under superfamily amidohydrolases. It is distributed abundantly among the living creatures as well as a soil enzyme in soil. It is a nickel containing enzyme with high molecular weight (Krajewska et al., 2012) of about 480 kDa or 545 kDa. Bacterial urease typically contains three discrete subunits, one bulky and two small. The optimum pH and temperature, for proper functioning of enzyme, is 7.4 and 60 °C respectively. Substrates utilized by urease are urea and hydroxyurea. One

mechanism for urea catalysis, to ammonia and carbon dioxide by urease was given by Blakeley and Zerner. The 5-coordinate Nickel (Ni-1) is attacked nucleophilically by carbonyl oxygen of urea molecule. It is displaced by a weakly coordinated water ligand (Dixon et al., 1979). Double bond is formed with the central atom by the lone pair of nitrogen atoms on the urea, and the resultant NH_2^+ interacts with a proximate negatively charged molecule. Blakeley and Zerner suggested this molecule as a Carboxylate ion. A base deprotonates the hydroxide ligand on the six coordinate Nickel. Then, the carbonyl carbon is attacked by the electronegative oxygen. The charge on nitrogen is neutralized by the pair of electrons from double bond between carbon and nitrogen, and an intermediate tetrahedral orientation is assumed by 4-coordinate carbon. Splitting of this byproduct is then aided by a sulfhydryl group of cysteine that is present in the vicinity of the active site. A hydrogen bonds with nitrogen atom breaks its bond with carbon, and releases ammonia. Concomitantly, the bond between 6-coordinate nickel and oxygen is cleaved. This leaves behind a carbamate which then immediately degrades to form another molecule of ammonia and carbonic acid (Zimmer, 2000).

Due to its low demand and availability only in industrial quantities (manufactured by Roche) for diagnostic purposes and application in hi-tech ceramics (Gauckler et al., 1999; Roche, 2001) it is very expensive. Moreover, the purity of enzyme available in market is higher than required for biocementation.

A standard urease producing microbe for biocementation should be capable to withstand elevated concentrations of calcium and urea. Levels of either induced or constitutively produced urease activity for that particular microbe should be large. Also the organism must be environment friendly for meeting the prerequisites for biocementation i.e., the microbe that environment is being exposed to, must not be pathogenic, should be free from genetic alterations, and must not contain any components that may cause mutations or increase pathogenicity of pre-existing wild strains (e.g. antibiotic resistance). Also keeping in mind the high pH of concrete, these organisms must be tolerant to severe alkaline environment. To survive in harsh alkaliphilic conditions with high pH of surroundings, internal pH of the cell should be low which results in reversed pH. This gives rise to movement of protons from inside to outside the cell, which is contrary to the usual path for

ATP generation. In order to overcome this, alkaliphiles have established mechanisms to up surge proton motive force for ATP generation (Mobley & Hausinger, 1989 (Mobley et al., 1995)

For biocementation purposes, an ideal microbial source of urease has the following properties:

- High Urease producing capacity
- Capable to produce urease under ammonium stress
- Robust (highly stable)
- Reliable (consistency in production & availability)
- Involves least upstreaming and down streaming processes prior to biocementation

2.4 Polymorphism of carbonate crystals

CaCO₃ biomineralizes to form different anhydrous polymorphs namely aragonite, calcite, vaterite, ikaite monohydrocalcite, amorphous calcium carbonates, (Fig. 2.5), depending on the composition of the bacterial wall, temperature, pH, amount supersaturation and [Ca²⁺] / [CO₂] ratio (Rodriguez-Navarro et al., 2012; Dhimi et al., 2013a). In microbial systems when the saturation index is above 1, it leads to CaCO₃ precipitation (Arp et al, 2001: Mitchell et al., 2010). As these carbonate polymorphs play important role in various technical applications, the need to characterize these polymorphs is increasing.

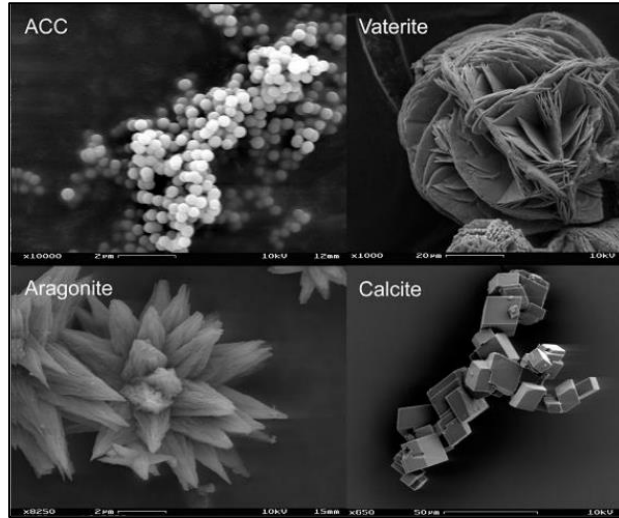


Figure 2.5 Polymorphs of Calcium Carbonate precipitated by MICCP (Source: A. Niedermayr, A. Immenhauser)

2.5 Optimum conditions for biomineralization

Urease enzyme activity is controlled by several factors, including, type of bacterial strain, initial cell concentration of the culture, urea and Ca^{2+} concentration in media, media pH and ionic strength, reaction temperature. These factors have a significant impact on biomineralization. Sharma et al., (2008) reported tremendous increase in enzyme production in the presence of citrate and urea by all the four bacterial species (*Enterobacter gergoviae*, *Enterobacter taylorae*, *Aeromonas hydrophila*, *Aeromonas caviae*).

2.6 Significance of biomineralization in construction materials

During past few decades, research in field of biomineralization has been in the limelight. As the potential of this field is being studied, its applications are increasing, because more knowledge is gained on its properties and applicability. This process is both economically profitable as well as environmentally safe. Some of the applications of biomineralization are listed below:

- elimination of heavy metals and radionucleotides (Warren et al., 2011)

- purging calcium from wastewater and biodegradation of pollutants (Okwadha & Li, 2010; Hammes et al., 2003)
- atmospheric CO₂ sequestration (Barlet-Gouédard, 2009; Wanjari, 2011, Phillips et al. 2013)
- remediation of building materials (Cheng, 2013; Qian et al., 2010)
- modifying the properties of soil (Chu et al., 2012), etc.

Among these applications remediation of building structures has gained a lot of concern, because if deciphered adequately it can be a boon to humanity. Many researchers have investigated the potential of restoration of building materials such as limestone, concrete, sand, bricks, mortar, etc. (Al Qabany, 2011; Chu et al., 2012; Dhami et al., 2013a,b). MICCP is now being used for below listed purposes:

- Biodeposition in soil and sand materials
Soil has always been prone to erosion due to its inadequate mechanical properties. So many researchers such as Whiffin et al., 2007; Ivanov and Chu, 2008 have found a soil strengthening technique by means of MICCP and named it as biogrouting. Also MICCP has been evaluated of par excellence in sand consolidation which reduces the porosity and hence the permeability by deposition of carbonate minerals (Van Paassen et al., 2010).
- Remediation of cracks in concrete
Efficient outcomes have been given by biomineralization in remediating cracks and pores in concrete by slight modifications in the original technique. Few researchers reported positive results on crack remediation by ureolytic strains bacteria encapsulated in polymeric substances (Bang et al., 2001). Achal et al., (2011) discovered potential of strain CT-5 to remediate cracks by carbonate mineralization.

- Restoration of limestone buildings

A study on application of MICCP to microporous and macroporous stones revealed that, bicalcification in macroporous stone was more efficient, where it occurred to a larger magnitude and deeper, than a microporous stone (De Muynck et al., 2011). Zamarreno et al. (2009) revealed that application of biogenic carbonate precipitation on limestone resulted in remarkable decline in the pore size.

- Protection of concrete structures from corrosive chemicals

Calcite precipitation was induced by *Bacillus* strain CT-5 in mortar specimen, resulting in sulfate resistant mortars, which eventually improved the compressive strength of the bacterial treated specimen as compared to the control specimen (Joshi et al., 2019).

Bacteria based coating proved to create a protective coating on concrete specimen. This protective layer of calcite and aragonite mineral prevented infiltration of harmful chemicals into the specimen, hence contributing to the durability of concrete structures (Huynh et al., 2018).

CHAPTER 3 MATERIALS AND METHODOLOGY

3.1 Sample collection

The samples were collected aseptically from the extreme saline environment of sambhar lake saltern located near Jaipur, Rajasthan, India and was stored at 4°C till use.

3.2 Bacterial isolation and cultivation

1. In order to cultivate bacterial flora, the samples needed to be enriched first by inoculating 1% of the sample in Nutrient Broth (NB) supplemented with 2% urea, with an initial pH 8. This broth was incubated at 37°C for 24 hours, 48 hours and 72 hours under shaking conditions (130 rpm).
2. Then the enriched culture was serially diluted and the dilutions were plated on Nutrient Agar (NA) supplemented with 2% urea using spread plate technique.
3. When a required colony count (30-300 CFU's) is attained on NA plates, these plates were selected for further study.
4. Further to acquire pure cultures, the colonies were harvested from NA plates from the previous step and were streaked on fresh NA plates.

3.3 Qualitative analysis of bacterial strain

1. For the selection of strains the colonies obtained on NA plates were transferred to Urea Agar Base (UAB) plates (Hi-Media, Mumbai, India).
2. The UAB plates were then incubated at 37°C and observed for 24 hours to check the presence of urease producing strain with respect to pink coloration obtained on the UAB plates.
3. Once the strain producing urease was identified, it was further subcultured as well as glycerol stock was maintained to preserve the cultures.

3.4 Quantitative analysis of bacterial strain

3.4.1 Urease Activity

- The bacterial strain is then subjected to the phenol-hypochlorite assay (Natarajan, 1995) in order to determine urease activity by measuring the rate of formation of ammonia and carbon dioxide upon the breakdown of urea.
- Standard curve of Ammonium Chloride ranging from 50-1000 μM was used for comparative analysis.
- 1% of overnight grown culture (O. D. = 0.5) was re-inoculated in NB under shaking conditions at 37°C.
- 1 ml culture was sequestered from broth under sterile conditions into sterile microfuge tubes after every 24 hours till urease activity of the culture starts to fall.
- The culture in microfuge tubes was then centrifuged for 5 min at 8000 rpm.
- 250 μl of supernatant was mixed in 1 ml of potassium phosphate buffer (0.1 M, pH 8). Then, 2.5 ml of 0.1 M urea. This step was performed in triplicates.
- After incubating the mixture at 37°C for 5 minutes, 1 ml of phenol nitroprusside and 1 ml of alkaline hypochlorite was added to it and was subjected to incubation at 37°C for 25 minutes.
- Finally, the optical density of the incubated mixture was measured using a UV spectrophotometer (Shimadzu, UV-1800) at 626 nm.
- Urease activity was calculated by calibrating the obtained value with the existing ammonium chloride standard curve. One unit of urease hydrolyzes 1 mole of urea per minute.

3.4.2 Calcium Carbonate estimation

- Calcium carbonate precipitated from inoculated culture was measured by EDTA titration method (AHPA, 1989) after every 24 hours for four days.
- 5ml of the culture was dissolved in HCl acid (3 N). To the mix obtained 4 ml of 5 N NaOH solution was added so that final pH reaches 12-13.

- A pinch of Erichrome Black T was used as indicator. After addition of indicator, solution was titrated against 0.05M EDTA.

End point was observed by change in coloration of mixture from pink to violet. 1ml of EDTA used for titration is equivalent to 5.004 mg of CaCO₃ precipitated.

Also, a small amount of the culture used for calcium carbonate estimation was filtered through a cellulose (pore size = 0.45µm) filter and was then dried using different concentrations of Ethanol. The dried powder was then subjected to SEM-EDX analysis.

3.4.3 Microbial sand plugging

- In order to study calcite precipitation, microbiological sand plugging was carried out. The bacterial isolates were grown at 37°C under shaking condition (130 rpm) in NB. Absorbance at 600 nm was maintained at 1.0.
- 25% of culture (OD₆₀₀ 1.0) was admixed with 100 g of autoclaved river sand. A slurry of sand, consisting of bacterial culture was packed into a plastic column (height = 15 cms; diameter = 3 cms) and Whatman filter paper was used to block the bottom of the column (Fig. 3.1)
- A control column was packed with sterile sand was mixed with sterile NB.
- All columns were supplied continuously with NB every 12 hours. While the column packed with bacterial cell slurry was fed with cultured cells every 2 days.
- The whole procedure was conducted for a time span of 10 days in order to study flow rate kinetics and pH change.
- The column of plugged sand was split into three portions (upper, middle and lower layers). The calcite precipitation was checked for each layer.
- Precipitated calcite from all three layers was measured by EDTA titration method (AHPA, 1989).



Figure 3.1 Setup for Bacterial Sand Consolidation

3.4.3.1 Microstructure analysis of microbial sand column

The morphology and chemical components of the consolidated column of sand and bacteria were analyzed using SEM. Before analysis, consolidated cores of the sand column were split open and each layer was finely crushed and ground using a pestle and mortar. Samples must be completely dry to obtain hassle free and desired results. Each layer of both control and sample with bacterial culture is then individually examined at accelerating voltages for microanalysis of the sand column by SEM. Before subjecting to SEM, each sample is gold-coated using a sputter coating Emitech K575.

3.5 Extraction of genomic DNA from bacteria

Genomic DNA isolation usually involves chemical disruption of cells by enzymic digestion and detergent lysis; organic solvents extractions, and selective recovery of the DNA.

3.5.1 Isolation of genomic DNA

- In a 250 ml conical flask, solitary bacterial colony was inoculated in 25 ml of NB and shaken (130 rpm) for 14-18 hours at 37°C.
- 2 ml of liquid cultures were centrifuged and harvested at 8000 rpm (1 min).

- The pellets obtained were resuspended in 800 µl saline-EDTA, and about 50 µl of freshly prepared lysozyme was added.
- Throughout incubation (37°C for 30 min), eppendorf centrifuge tube containing the cell suspension was gently inverted several times to mix the components thoroughly and shear the cell wall to expose the genomic constituents.
- The components of centrifuge tube were incubated again at 65°C for 15 mins, after adding 10% SDS (200 µl).
- Organic solvents were used to remove cell debris and protein content leaving behind required genome:
 - First, a solution of phenol, chloroform and isoamyl alcohol prepared in ratio 25:24:1, respectively, is added to the cell suspension and centrifuged at 12000 rpm for 10 min.
 - Then, again to the aqueous supernatant, equal volume of isopropanol was added for precipitation of extracted nucleic material. DNase free RNase solution was added and incubated at 37°C for 30 min.
- Extract with equal volume of chloroform and isoamyl alcohol (24:1)
- To precipitate extracted nucleic acid from upper aqueous layer, 2 volume of ethanol (95%) was added. It was centrifuged to obtain the DNA pellet after incubation at -20°C for 1 hour.
- The sequestered DNA pellet was given a washing with 70% EtOH (750µl) and centrifuged for another 10 mins.
- Lastly, DNA pellet was resuspended in 40 µl TE buffer and preserved at 4°C.

3.5.2 Electrophoresis of DNA on agarose gels

- Standard agarose gel (0.8 % w/v) was prepared with ethidium bromide (EtBr) to stain the gel. Once poured into the electrophoresis tray, comb was inserted.
- As the gel solidifies, the comb was pulled off and the gel was kept in electrophoretic chamber containing 0.5X TBE, pH 8.0.
- DNA was loaded into the wells using a 6X loading dye.
- DNA was then electrophoresed for 45 min and observed on a U.V. transilluminator.

3.5.3 Quantification of DNA by Nanodrop spectrophotometer

- The concentration of isolated DNA present in suspension was estimated by spectrophotometric measurement at A_{260} .
- Purity of the DNA was estimated by calculating ratio of the A_{260}/A_{280} and the A_{230}/A_{260} . Preferably, the A_{260}/A_{280} ratio should be in range of 1.8 - 2.0 while the A_{230}/A_{260} ratio should be 0.3 - 0.9. RNA contamination is indicated when the ratios (A_{260}/A_{280}) exceed 2.0 and ratios (A_{260}/A_{280}) less than 1.8 denotes protein or phenol contamination.

3.5.4 Amplification of 16S rDNA and Purification of PCR products

The polymerase chain reaction (PCR) is a quick and extremely sensitive technique for enzymatic production of multiple copies of particular target sequences in the genomic DNA providing an exponential rise in target DNA copies.

Forward Primer 5-AGAGTTTGATCCTGGCTCAG-3 and reverse primer 5'-ACGGGCGGTGTGTTC-3' were used for amplifying 16S rDNA (Weisberg et al, 1991). Reaction mix for the PCR comprised of 10X PCR buffer, each dNTP's at concentration of 2 mM, 50 mM $MgCl_2$, each primer at concentration of 0.1 μM and 2.5U of Taq DNA polymerase in a final volume of 25 μl . Following PCR conditions were provided: Preheated at 92°C for 5 min followed by 35 cycles of 95°C for 1 min, 50°C for 40 s, 72°C for 2 min and final extension of 72°C for 7 min. Successful amplifications were affirmed by agarose gel electrophoresis.

3.6 Physiological characterization of halophilic bacteria

3.6.1 Growth Kinetics

- Different sodium chloride concentrations (3.6, 5, 7.5 and 10%) were amended in NB (pH 7.5) to determine the survival of the bacterial isolate in saline conditions. Growth of bacteria was recorded by measuring the absorbance at 600 nm of 24 hours grown cells.

- Similarly, growth of bacterial isolate grown at different pH (6, 7, 8, 9, 10, 11, 12) was recorded by measuring the absorbance at 600 nm of 24 hours grown cells.

3.6.2 Morphological and biochemical studies of bacterial isolates

For characterization of the isolated bacterial strains conventional biochemical and physiological tests were carried out as defined in Bergey's Manual of Systematic Bacteriology.

3.6.2.1 Gram staining

- Smear from bacterial cells in their log phase of growth was spread heat fixed on a glass slide.
- It was then flooded with primary stain, crystal violet for 60 secs and washed with a gentle stream of distilled water to eliminate excess dye.
- Then it was flooded with mordant i.e., Gram's iodine for 30 secs and washed with water.
- Smear was decolorized using 70% ethanol to remove the unbound stain and washed with distilled water immediately.
- Safranin was used as counterstain for 30 secs and washed with water to remove extra stain.
- Finally, slides were observed under microscope at various magnifications.

3.6.2.2 Oxidase test

Oxidase test was first used for identification of gonococci. It is a test based on the ability of a certain microbe to yield indophenol by oxidation of dimethyl-p-phenylenediamine and α -naphthol (Gordon & McLeod, 1928). Smear of bacterial growth was made on ready to use filter-paper impregnated with 1% (w/v) aqueous tetramethyl-p-phenylenediamine dihydrochloride solution. Oxidase-positive organisms produced a purple coloration on the paper within 10 sec. (Kovacs, 1956)

Growth was picked off the medium with a platinum loop and rubbed on filter-paper impregnated with 1 % (w/v) aqueous tetramethyl-p-phenylene diamine dihydrochloride solution. Appearance of purple coloration immediately or within 10-30 seconds mark a positive reaction. Delayed reactions are ignored. (Steel, 1961).

3.6.2.3. Catalase Test

This test is based on the principle of conversion of hydrogen peroxide into water and oxygen (Whittenbury, 1964). Catalase is mostly produced by either aerobes or facultative anaerobes. Thus, catalase test is used to detect the presence of catalase enzyme in bacteria. Procedure followed to conduct this test is given below:

- A small colony was placed on a clean, grease-free slide.
- Then, 2-3 drops of 3% H₂O₂ were added on the colony.

Test is said to be positive if bubbles are formed, which indicates the release of oxygen from hydrogen peroxide.

3.6.2.4. Nitrate reduction test

Nitrate broth is used to determine the capacity of an organism to reduce nitrate (NO₃) to nitrite (NO₂) by enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen.

Culture was streaked on nitrate agar plates and incubated for 24 hours. After that colony obtained on the plate was tested for nitrate reduction properties of the bacteria by using nitrate reduction discs (Hi Media, India). Disc was placed on the colony and few drops of the reagent were poured on the disc. If the disc turns red after the addition of the nitrate reagents, it was considered a positive result for nitrate reduction.

3.7 Bacterial application on concrete structures

3.7.1 Materials

- Ordinary Portland Cement (OPC) 43 Grade was used in casting of concrete samples in this research (IS:8112-2013, 2013).
- Sterile natural sand from local river confirming to Zone II was used for fraction comprising fine aggregates (IS:383-1970, 1970). Specific gravity for fine aggregates used was 2.70 and water absorption for the same was 1.8%
- Crushed gravel with particle size within the range of 10-20 mm was used for coarse aggregate fraction. The specific gravity and water absorption of 20 mm particles was 2.63 and 1.38% and for 10 mm particles, it was 2.65 and 1.4%, respectively.
- Both fine particles and coarse aggregates confirming to IS: 383–1970 standard were used in preparation of concrete structures (IS:383-1970, 1970).

3.7.2 Sample Preparation

- Two sets of concrete cubes of dimensions (100 mm x 100 mm x 100 mm) were made.
- Ratio of cement: sand: coarse aggregate used to make concrete specimen was 1:1.82:3.24 (by weight) with water to cement ratio of 0.5.
- For molding concrete cubes of bacterial treated specimen, bacterial isolate was grown in NB supplemented with 2% urea and 25 mM calcium chloride solution (w/v) till the optical density (O.D 600) reached 0.5 and was used instead of water for preparing the concrete mix. The bacterial culture to cement ratio was also maintained equal to water to cement ratio.
- Cement and aggregates were blended rigorously for few minutes to form the concrete mixture prior to addition of water or NB containing bacterial culture.
- On addition of water and culture separately, it was blended properly to attain a homogenized concrete mix.
- Fresh mix was poured into the iron molds, while the mixture is still pliable (100 mm x 100 mm x 100 mm).

- Two sets of concrete cylinders of 200 mm x 100 mm dimension were prepared similarly, by using same materials and adhering to same standards as in cubes production.
- After casting, all the specimens were allowed to set in iron molds and set aside in a casting room at room temperature ($27 \pm 2^{\circ}\text{C}$) for 24 hours.
- Thence, the concrete structures were demolded and cured for over 28 days till the testing age. Different regimes for curing of concrete specimen were followed as given in the table below:

Table 3.1 Curing regimes

Specimen	Curing Regimes
Control	Cured in water for 28 days
Bacterial Treated	Submerged in bacterial culture grown in NBU-CaCl ₂ for 28 days

3.7.3 Test Procedures

3.7.3.1 Compressive Strength

- Concrete cubes were subjected to compressive strength measurements.
- Effect of incorporating bacterial cells, grown in NBU-CaCl₂ medium during casting, on the mechanical strength of concrete cubes was investigated after curing all specimen for 28 days following BIS 516: 1959 guidelines.
- Automatic compression testing machine, COMPTTEST 3000, was used for this purpose.
- The average of compressive strength of three concrete cubes was taken as the compressive strength of the cubes.

3.7.3.2 Permeation Properties

- Permeation properties of all the casted specimen were gauged after 28 nights of curing. Sorptivity was calculated with respect to ASTM C1585 (ASTM International, 2004). Underlying principle for this test is the rate of uptake of water by concrete specimen due to capillary action. It correlates the water absorption with the pore network of concrete specimen (Sabir et al., 1998).

- To conduct sorptivity test, the concrete cylinders with diameter 100 mm and 200 mm height were cut to prepare cylinders of diameter 100 mm and height 50 mm. Before the test, circumference and top face of the cylindrical specimen was coated with epoxy resin, leaving the bottom face exposed to water. Initial mass of the samples was noted. The test setup is illustrated in Fig. 3.2.
- The mass change was recorded at the intervals of 60 seconds, 5 min, 10 min, 20 min, 30 min, 60 min and every hour up to 6 h.
- Change in mass of specimen, volume of water absorbed per unit cross sectional area was evaluated for each time interval. A plot between the square root of time and volume of water absorbed was plotted. Slope of the graph depicted the value of sorptivity for that specimen.

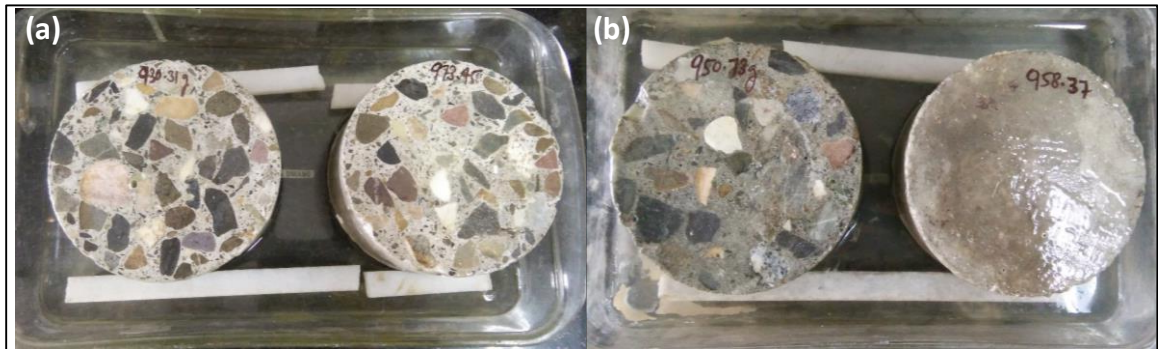


Figure 3.2 Setup for sorptivity test (a) Control Specimen (b) Bacterial Admixed Specimen

CHAPTER 4 RESULTS AND DISCUSSIONS

Microbially induced calcite precipitation on concrete has become an important area of research during the past few decades. This study focused on improvisation of the existing techniques for biomineralization by isolating novel bacterial strains from saline habitats. Sambhar lake saltern was chosen as the sampling site for isolation of a potential strain capable of calcite precipitation.

4.1 Bacterial isolation and cultivation

4.1.1 Sample Collection

Samples were collected randomly from the different areas of the alkaline Sambhar Lake. Temperature at culture collection site was 29/25°C at time of collection. 30% humidity at the location was observed. The pH of the collected sample was 9.58.

4.1.2 Isolation of bacterial strain

Samples were enriched in NBU for at least 72 hours. Bacteria were isolated using spread plate technique (Fig. 4.1). Colony forming units were counted, from plates plated at different dilutions, and recorded (Table 4.1.)

Desired colony count was achieved in dilutions 10^{-3} , 10^{-4} and 10^{-5} as shown in table below:

Table 4.1 Colony count with mean and standard deviation

Sr. No.	Dilutions	CFU's			Mean \pm SD
1	10^{-3}	245	236	237	236 ± 9
2	10^{-4}	145	127	136	136 ± 9
3	10^{-5}	75	64	53	64 ± 11

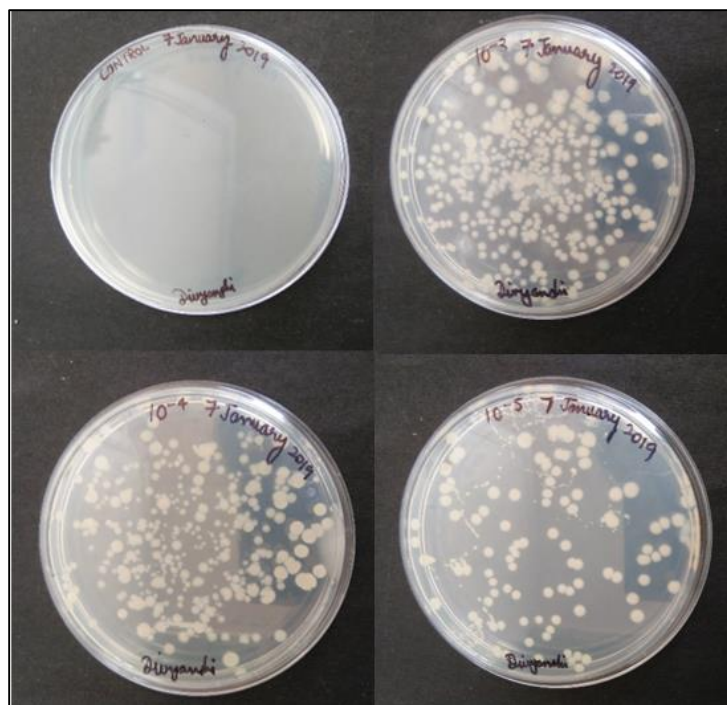


Figure 4.1 Colony forming units on varying dilutions (a) Control (b) 10^{-3} (c) 10^{-4} (d) 10^{-5}

Plates were studied for the morphology of colonies. Colonies with different shape, color and texture were streaked separately on NA plates. Ten different types of colonies were further streaked. Most colonies were elevated & round in shape with entire smooth edges, but few of them were opaque while others were translucent when observed in day light.

4.2 Qualitative analysis of bacterial strain

The isolated bacterial cultures were screened for selection of efficient ureolytic bacteria. Different strains were streaked on UAB plates for selection of urease producing strain with respect to the pink coloration obtained on UAB plates.

Out of ten, only two colonies showed urease activity as the area occupied by the streaked colonies on UAB turned pink from initially yellowish-orange plate. Opaque strain was named SL-1, while the translucent was named SL-2. Intensity of color shown by SL-1 culture was higher as compared to SL-2 (Fig. 4.2). Thus, SL-1 was selected for further research and applications.

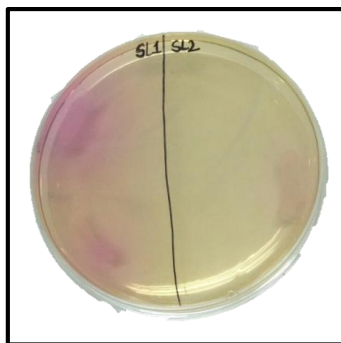


Figure 4.2 urease activity shown by ureolytic strain (left) by giving pink coloration.

4.3 Quantitative analysis of bacterial strain

4.3.1 Urease Activity

Urease activity of SL-1 was assessed every 24 hours over a period of 144 hours and the culture with 3.5% NaCl exhibited maximum urease productivity (909.9 U/mL) after 72 hours followed by the culture without salt (855.5 U/mL). There was a sharp rise in the concentration of urease enzyme between 48 and 72 hours. The urease activity decreased steadily after 72 hours. Urease activity ranging from 100-300 U/ml was observed for cultures with 5% and 7.5% NaCl, which declined progressively (Fig. 4.3)

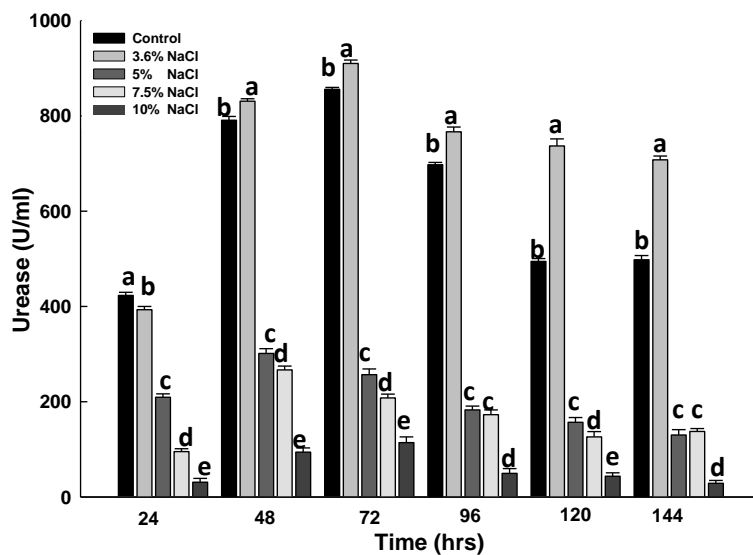


Figure 4.3 Time course of urease activity of SL-1.

The bars sharing a common letter within treatment are not significant at $P < 0.05$. Values bearing different letters in same cluster are significant at $P < 0.05$. The graph was plotted for the mean \pm SD ($n = 3$).

Rangamaran & Shanmugam (2019) recently studied a halophilic strain NIOTVJ5, isolated from deep sea sediments from Barren Island, Andaman Sea. NIOTVJ5 produced a maximum concentration of 554.03 U/mL of urease enzyme after 96 hours. While, in this present investigation, maximum urease concentration of 909.9 U/mL was obtained after 72 hours at 3.5% NaCl concentration using SL-1 strain. So it can be said that SL-1 strain is an efficient urease producer as it produced more enzyme in less time as compared to the previous study.

4.3.2 Calcium Carbonate Estimation

Bacterial culture was cultivated NBU- CaCl_2 (2% urea and 25 mM CaCl_2) to estimate the capacity of bacterial strain for carbonate precipitation. After inoculating 1% culture in NBU- CaCl_2 media, it was incubated for a period of 72 hours. A significant difference was observed in the growth medium supplemented with urea and CaCl_2 as compared to the lacking ones (Fig. 4.4).

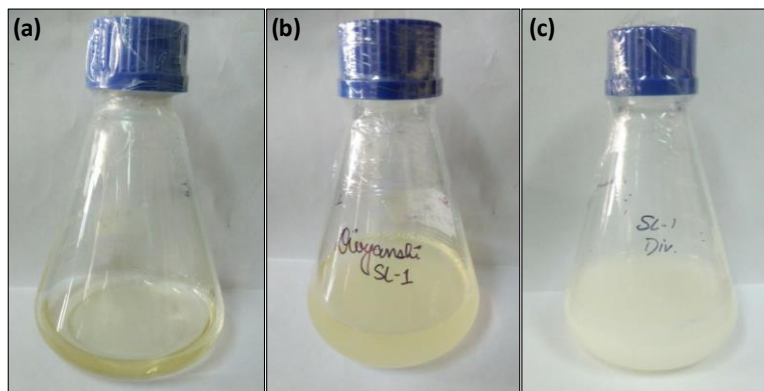


Figure 4.4 (a) Sterile NB, (b) Bacterial culture without precipitation, (c) Bacterial culture with precipitation.

Further when subjected to EDTA titration procedure, end point was observed by change in coloration of mixture from pink to violet (Fig. 4.5). 2.5 mg/ml CaCO_3 was detected in the culture medium after 24 hours, which ascended to 3.9 mg/ml after 96 hours. Microstructure analysis revealed the presence of calcite crystals, which was confirmed by SEM – EDX analysis (Fig. 4.6).

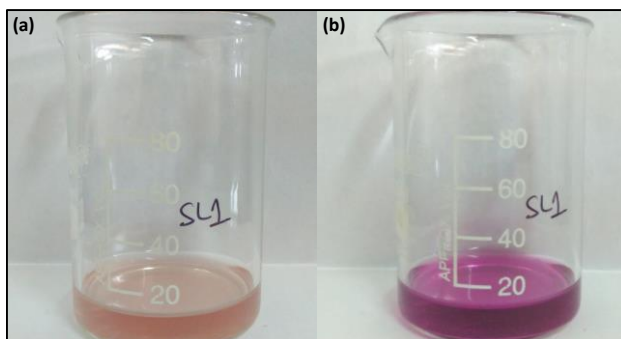


Figure 4.5 EDTA titration of carbonate precipitation in NBU- CaCl_2 (a) before and (b) after

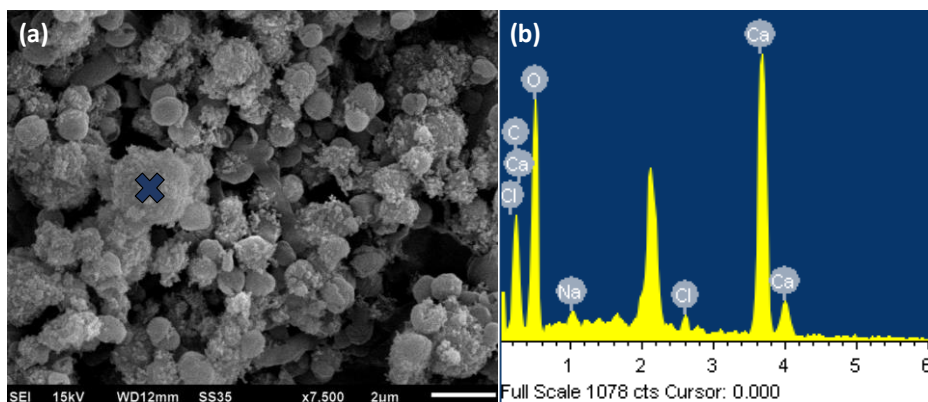


Figure 4.6 (a) SEM image (b) EDX of calcite precipitation by bacterial cells in NBU- CaCl_2 . “X” represents the spot of EDX.

4.3.3 Microbial Sand Plugging

The isolate (SL-1) obtained in this study showed a significant calcite precipitation. The sand column prepared using the strain SL-1 was tightly packed (Fig. 4.7a.) while the

control sand column crumbled and lost its form after the removal of plastic mold. The flow rate of sand columns was measured regularly for 10 days. In the control column, initial flow rate was 4.7 ml/min, and on day 10 it was 3.2 ml/min. A clear change in flow rate was observed over time as cementation and pore clogging progressed in column containing bacterial admixed sand. This column was completely clogged on the 9th day as shown in Fig. 4.7 (b). A fraction of each layer (top, middle and bottom) from control as well as test column was titrated against EDTA. Top layer of bacteria fed column exhibited maximum (38%) and bottom layer had least (12%) carbonate precipitation. While precipitation in control was negligible.

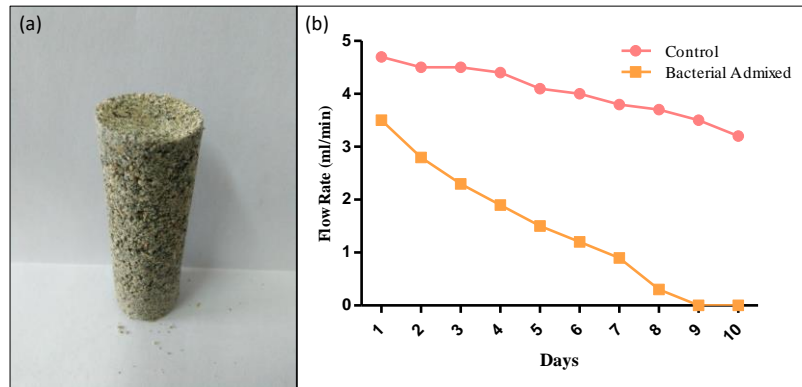


Figure 4.7 (a) Consolidated sand column, (b) Flow rate of media from sand columns with respect to time.

Achal et al., (2011) studied Microbial sand plugging by *Sporosarcina pasteurii* using different growth media, i.e., Corn steep liquor and NB. Calcite precipitation ranging from 7-33% was observed in sand columns treated with *S. pasteurii*. Top layer of the column had maximum precipitation, while the precipitation was low in the bottom layer of the column. Similarly, in the present study, precipitation decreased from top (38%) to the bottom (12%) of the column.

4.3.3.1 SEM analysis of microbial sand column

Calcite precipitation was clearly visible in SEM images of column treated with bacterial culture and peak for carbon was seen in the EDS {Fig. 4.8 (a) & (b)} with a concentration

of 13.97 ± 2.01 % (by weight). On the other hand, no such crystals were seen in control specimen SEM images and peak for carbon was not perceived in EDS results {Fig. 4.9 (a) & (b)}.

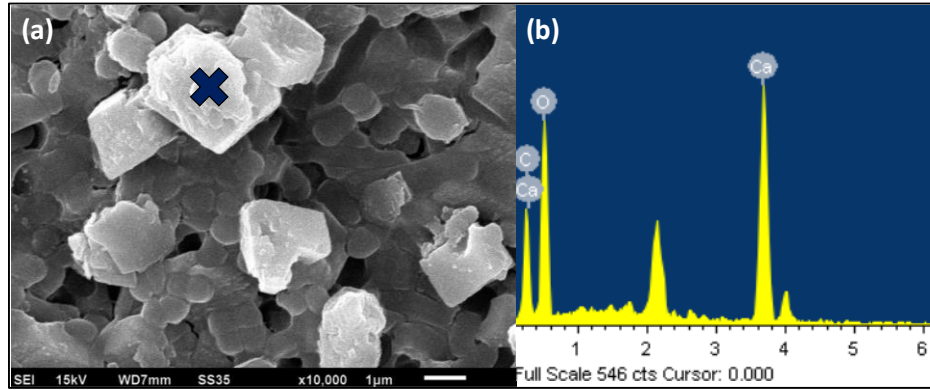


Figure 4.8 (a) SEM image representing calcite crystals associated with bacterial cells. “X” represents the spot of EDX (b).

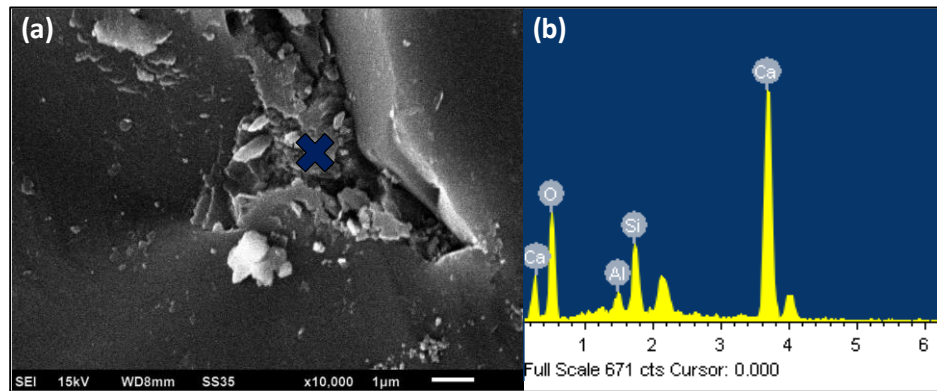


Figure 4.9 (a) SEM image representing control sand column. “X” represents the spot of EDX (b).

4.4 Extraction of genomic DNA from bacteria

Amplification and purification of isolated DNA was done by PCR. Then, crude and purified (amplified) DNA was quantified using Nanodrop spectrophotometer. A_{260}/A_{280} ratio for crude DNA and PCR purified product was 2.02 and 1.88, respectively (Table 4.2).

Table 4.2 Quantification of DNA

Sr No.	DNA	A ₂₆₀ /A ₂₈₀	A ₂₆₀ /A ₂₃₀	Conc. (ng/μl)
1	Crude	2.02	2.18	486.3
2	Purified PCR Product	1.88	1.07	108.1

Gel electrophoresis for PCR product was performed (Fig. 4.10). The size of DNA obtained was 1.5 kb.

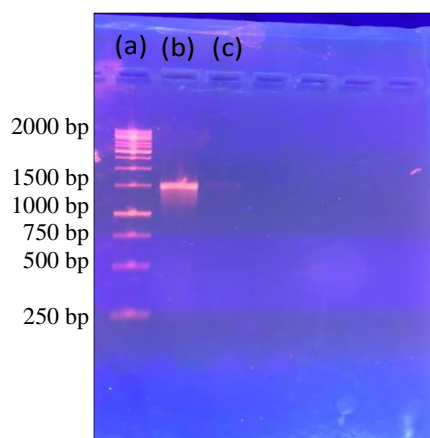


Figure 4.10 Gel electrophoresis with (a)ladder and PCR products (b) undiluted & (c)1/10th dilution.

4.4.1 16S rDNA Sequencing and Phylogenetic Analysis

Purified samples were sent to First Base Asia, Malaysia. The results for sequencing of SL-1 bacterial isolate were:

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TGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCATAAGACTGGGATAACTCCGGGA
AACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAA
GGCGGCTTCGGCTGTCACCTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGT
GAGGTAACGGCTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGAT
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGCTTTCGGGTCGTAAACTCTGTTGTTAGGGAAGAACAAGTGCTAG
TTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGG
CTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGA
AAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACC
```

AGTGGCGAAGGCGACTTTCTGGTCTGTAACCTGACACTGAGGCGCGAAAGCGT
 GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
 GCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCA
 CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGG
 GGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAAC
 CTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCG
 GGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGTCGTGAGATG
 TTGGGTTAAGTCCCGCAACGAGCGCACCCCTTGATCTTAGTTGCCATCATTAAAT
 TGGGCACTCTAAGTGACTGCCGGTGACAACCGGAGAAGGGGGGATGACGTC
 AATCATCTGCCCTTATGACTGGGCTACCACGTGCTACATGGACGGTACAAGN
 GCTGCAGACCGCAGGGGAGCTAATCCAAGAACGTTTCATTTCGATTGAGCTGC

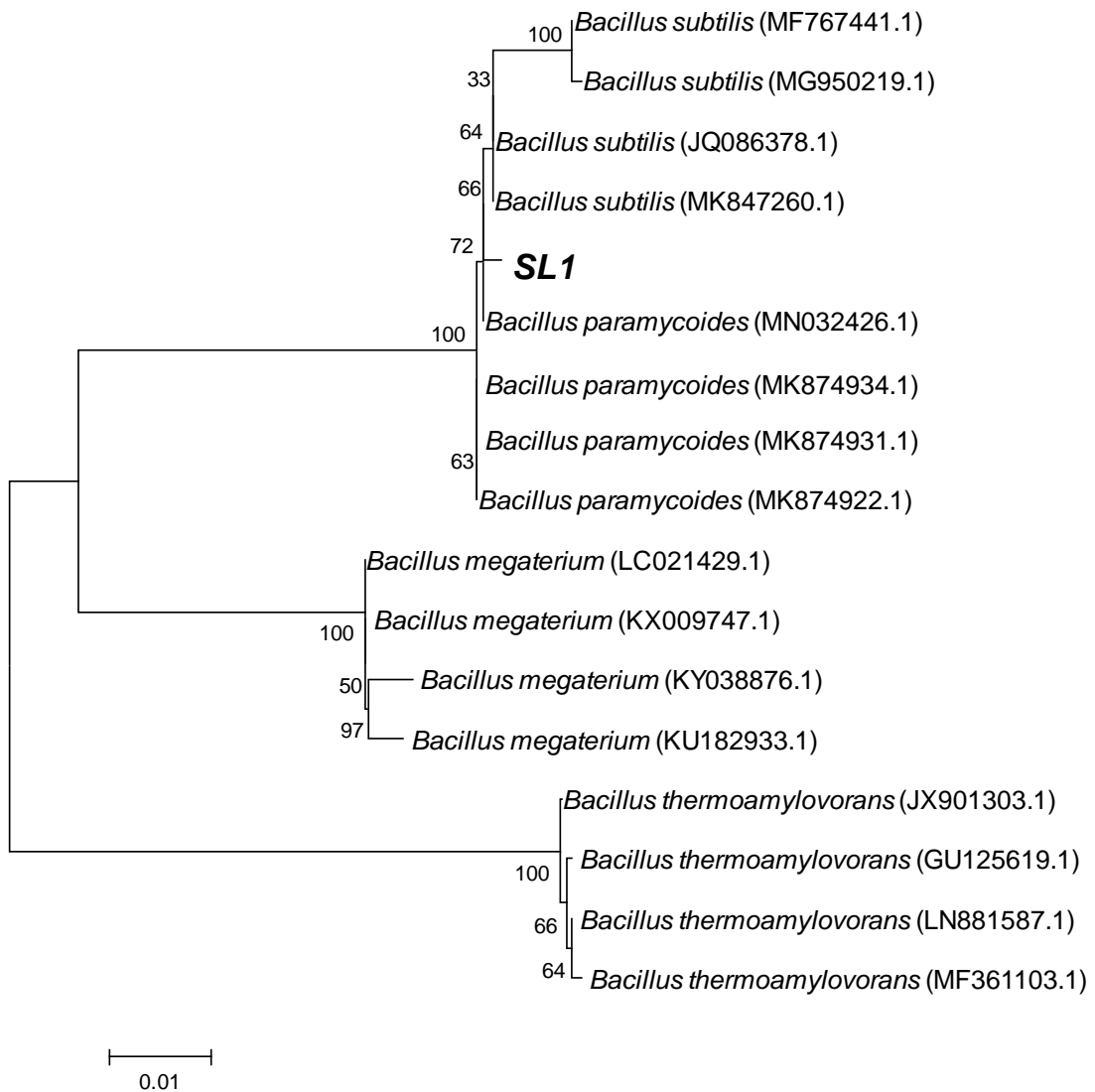


Figure 4.11 Phylogenetic tree of isolated species with related taxa

4.5 Physiological and biochemical characterization of halophilic bacteria

4.5.1 Growth Kinetics

Growth of the isolated strain SL-1 was studied at varying salinity stress. This study revealed that SL-1 grew best at 3.5% NaCl concentration (Fig. 4.12), and pH 10 (Fig 4.11) and the log phase lasted up to 12 hours followed by 12 hours of stationary phase. While a short log phase of 4 hours for cultures grown at lower pH (6 – 8) was observed and growth started to decline after 12 hours. Although log phase for culture grown at 5% salt concentration lasted up to 12 hours but the cell density was low as compared to the culture grown at 3.5% NaCl concentration. It was also inferred that this strain was unable to survive at high salinity stress as there was negligible growth at 7.5% and higher NaCl concentration as well as pH 11 and above.

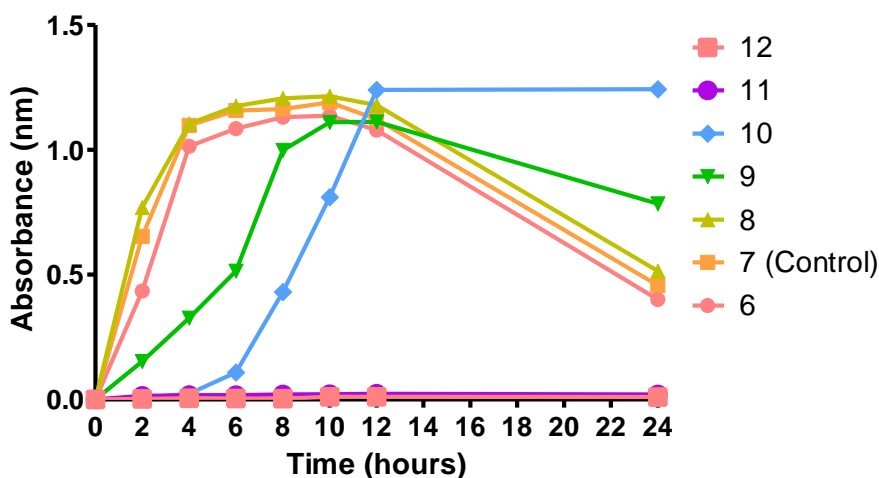


Figure 4.12 Growth curve for SL-1 at varying pH

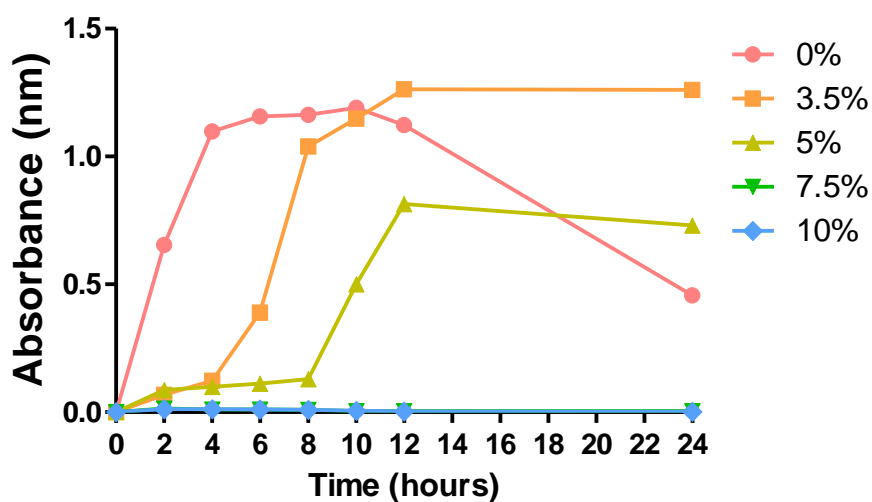


Figure 4.13 Growth curve for SL-1 at varying salt stresses.

4.5.2 Grams' Staining

Gram stain is usually performed to differentiate bacterial cells on the basis of composition of their cell wall. Bacteria are said to be gram positive when it retains crystal violet stain, i.e., its cell wall is abundant in peptidoglycans. On the other hand, if the bacteria release the primary stain and appears pink or red by uptake of the secondary stain on treating with decolorizer, it is Gram negative strain. On performing Grams' Test on SL1 strain, it was elucidated to be a Gram positive bacteria, as violet rods were observed under the microscope.

4.5.3 Oxidase Test

Oxidase test is based on the ability of microorganisms to form indophenol by oxidation of dimethyl-p-phenylenediamine and α -naphthol. Oxidase discs (Hi-Media, India) were used for this test. Positive oxidase test is indicated by appearance of purple color on the disc. As we can see below, there was no change in color of discs. Hence, the bacterial strain is oxidase negative (Fig. 4.13)



Figure 4.14 Oxidase Test for SL-1 strain

4.5.4 Catalase Test

Catalase test classifies the microbes into two categories, the ones which can reduce hydrogen peroxide to water and releases oxygen, while others cannot. The ones which show bubbles (release of oxygen) are catalase positive while which do not show any kind of effervescence are catalase negative. SL-1 strain showed no effervescence on treating with hydrogen peroxide, so it is catalase negative (Fig. 4.14)



Figure 4.15 Catalase Test for SL-1 strain

4.5.5 Nitrate Reduction Test

Nitrate reduction test is used to check the ability of bacteria to reduce nitrate to nitrite with nitrate reductase enzyme as a catalyst. Positive nitrate reduction test is assured by change in nitrate reduction disc to blood-red color (Fig. 4.15).



Figure 4.16 Nitrate Reductase Test for SL-1 strain

4.6 Bacterial application on concrete cubes

4.6.1 Compressive Strength

Mechanical properties of the concrete cubes were determined by subjecting them to automatic compressing machine (COMPTEST 3000). This test is used to check the endurance of bacterial treated concrete cubes with respect to control. When the specimens in this study were subjected to compression, an increase of 16% in compressive strength of bacterial treated specimen was observed when compared to control specimen (Fig. 4.17). $17.56 \pm 1.59 \%$ (n = 4) carbon was detected by microstructure analysis (Fig. 4.16).

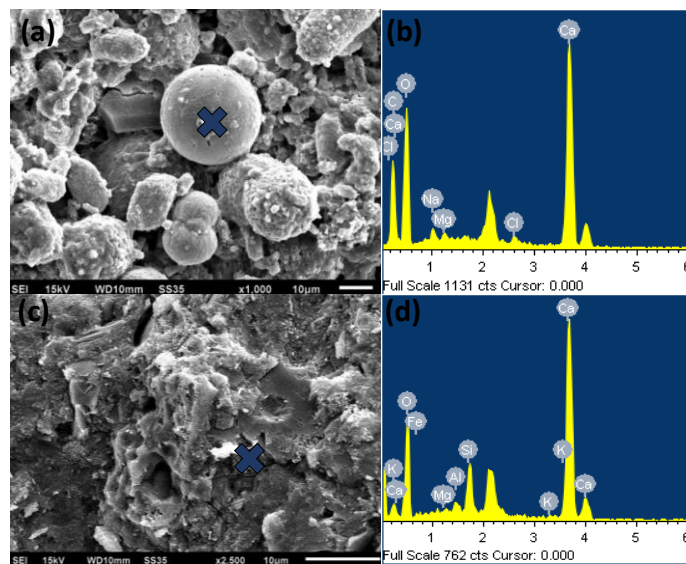


Figure 4.17 SEM images of (a) bacterial treated specimen and (c) control along with EDX (b) bacterial treated specimen and (d) control. “X” represents the spot of EDX.

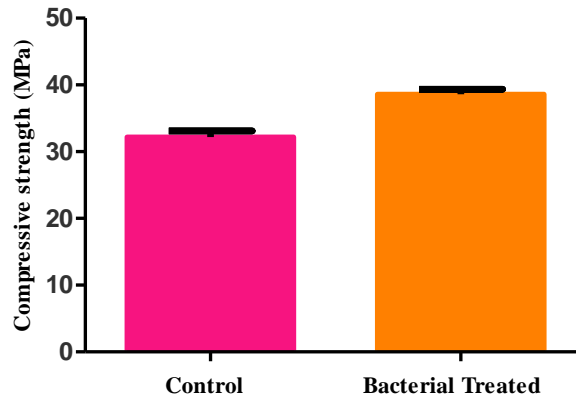


Figure 4.18 Compressive Strength of control (●) and bacterial treated specimen (●) after curing of 28 days.

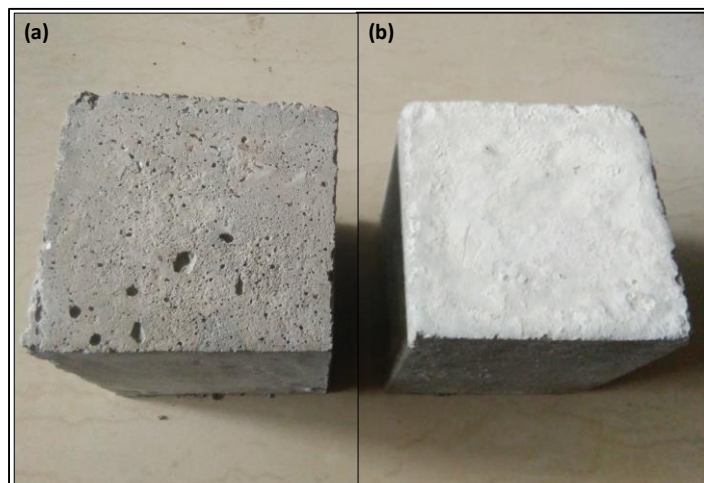


Figure 4.19 Cubes showing carbonate precipitation on surface of (b) bacterial treated specimen while there is no formation of any layer in (a) control specimen

After 28 days of curing, the concrete cube made with admixed bacterial culture and cured in bacterial culture as well showed formation of a white flaky layer on its surface (Fig. 4.18b). Microanalysis of the surface revealed this layer to be a mixture of calcite and aragonite crystals (Fig. 4.16a)

4.6.2 Permeation Properties

Deposition of carbonate crystals reduces the pore size as well as the pore number of the concrete structure, making it impermeable to water. Less the penetration of water, lower will be the penetration of other deterring chemicals, hence increasing the durability of the structure. Fig. 4.19 represents rate of water absorption by cylindrical specimen.

The graph (Fig. 4.19) depicts the rate of water absorption into concrete specimen. Treated specimen showed low water penetration while high water penetration was observed in control specimen. During initial absorption lower 'k' value (0.002) was observed in bacterial treated specimen as compared to the control specimen ('k' value = 0.005), which shows reduction of water penetration into treated specimen.

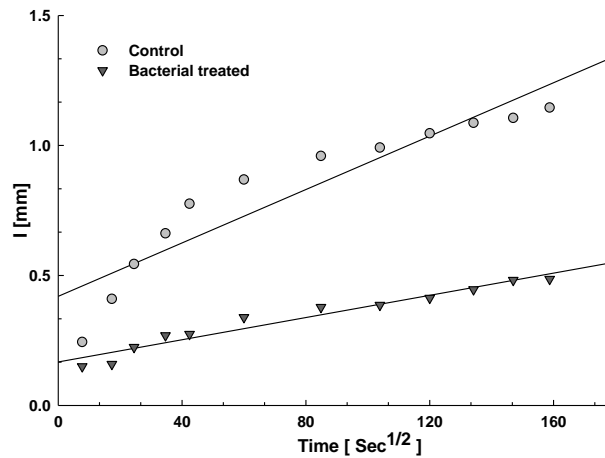


Figure 4.20 Rate of water absorption by control and bacterial treated specimen.

Tripathi et al., (2019) studied the mechanical properties of *Bacillus* sp. CT-5 in concrete by five different ways of bacterial treatment. Five regimes of bacterial treatment were;

- (i) Control specimen cured in water,
- (ii) Control specimen cured in bacterial culture
- (iii) Specimen casted with bacterial culture cured in water,
- (iv) Specimen casted with bacterial culture cured in bacterial culture, and
- (v) Specimen casted with bacterial culture and cured by spraying bacterial culture.

In present study a control cured in water and specimen casted with bacterial culture cured in bacterial culture were subjected to investigation of mechanical and permeation properties.

Control cured in water had compressive strength about 32 MPa after 28 days, while specimen casted with bacterial culture and cured in bacterial culture hasd compressive strength of about 44.5 MPa (Tripathi et al., 2019). While in present study, compressive strength of control and bacterial treated specimen was 33.2 and 38.6, respectively.

CONCLUSION

Microbially induced biominerals have gained a lot of attention due to its wide applicability in present and unexplored future potential. Many researchers have already deciphered MICCP as a revolutionary technique for rehabilitation building materials, still this field is being explored for bringing its applications to large scale. This study focuses on studying novel strain isolated from Sambhar Lake, Jaipur, Rajasthan, for its biomineralization properties.

- Isolated strain was named SL-1 and characterized successfully. From biochemical tests it was revealed that this strain is gram positive, catalase negative, oxidase negative and nitrate reductase positive.
- For a microorganism to be an ideal mineralizer possessing a high urease activity is a prerequisite. The isolated strain SL-1 exhibited significant urease activity. Rapid increase of the enzyme concentration was observed after 48 hours of growth. When the culture was grown under salt stress, maximum activity was shown at 3.6% NaCl concentration, while it was negligible at 10% NaCl concentration.
- Precipitation of calcium carbonate on bacterial cell surface was revealed by the microstructure analysis of the filtered culture pellet from broth supplemented with urea and CaCl_2 . Calcite precipitation was observed in sand column as the flow from the bacterial treated column completely stopped after certain period of time, which represents blocking of pores in the column by CaCO_3 .
- After affirmation of biomineralizing properties of this strain, it was tested on concrete specimen. A significant increase was achieved in mechanical and permeation properties of the treated specimen.

For successful application of this particular strain in bio-healing of concrete structures on a large scale this strain needs to be studied more for it to be released in environment safely. As it is a multidisciplinary research, extensive investigation in this field needs to be done by collaborating with experts from concerned fields.

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