INFLUENCE OF SALT STRESS CONDITIONS ON CALCITE FORMATION BY CALCIFYING BACTERIA ISOLATED FROM SALINE ENVIRONMENT

Submitted in partial fulfilment of the requirements for the award of the Degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

SUBMITTED BY:

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled **"Influence of salt stress conditions on calcite formation by calcifying bacteria isolated from saline environment"** in partial fulfilment of the requirement for the award of the degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar university, Patiala, Punjab, is an authentic record of my own work during the period of six months from Jan 2011 to June 2011, under the supervision of Dr. M.S. Reddy, Professor, Department of Biotechnology and Environmental Science, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other University.

Date: 13th July, 2011
Place: Patiala

Roohi Bansal
Roohi Bansal

CERTIFICATE

This is to certify that the thesis entitled **"Influence of salt stress conditions on calcite formation by calcifying bacteria isolated from saline environment"** submitted by Roohi Bansal in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, 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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Abstract

Surface treatments play an important role in the protection of construction materials from the ingress of water and other deleterious substances. Due to the negative side effects of some of the conventional techniques, microbiologically induced calcium carbonate precipitation (MICCP) has been proposed as novel and environmental friendly strategy for the protection of buildings. Most of deterioration of buildings near sea coast takes place because of change in temperature, moisture, physical factors and chemical factors. The basic principles for MICCP are (1) the microbial urease that hydrolyzes urea to produce ammonia and carbonic acid (2) carbonic anhydrase that catalyzes the conversion of carbonic acid to bicarbonate ions which would be available for calcium carbonate precipitation (3) the ammonia released in surroundings subsequently increases pH, leading to accumulation of insoluble calcium carbonate. The technique can be used to improve the compressive strength and durability of cracked concrete of buildings near sea coast. The effect of different salt concentrations on calcite formation by calcifying halobacteria was studied and efficient calcification was observed in saline environments up to salt concentration 5% but above that calcium carbonate precipitation decreased. Scanning electron microscopy (SEM) analysis evidenced the direct involvement of microorganisms in $CaCO_3$ precipitation.

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Chapter 1

Introduction

Concrete in marine environments

Concrete is the main building material used worldwide because of its strength, durability, versatility and economy. For several reasons, effects of marine environments on building structures deserve special attention. First, coastal and offshore sea structures are exposed to the simultaneous action of a number of physical and chemical deterioration processes, which provide an excellent opportunity to understand the complexity of concrete durability problems in practice. Second, oceans make up 80 percent of the surface of the earth; therefore, a large number of structures are exposed to seawater either directly or indirectly.

Most seawater is fairly uniform in chemical composition, which is characterized by the presence of about 3.6 percent soluble salts by weight. The ionic concentrations of Na^+ and Cl^- are the highest, typically 11,000 and 20,000 mg/liter, respectively. However, from the standpoint of aggressive action to cement hydration products, sufficient amounts of Mg^{2+} and SO_4^{2-} are present, typically 1400 and 2700 mg/liter, respectively. The pH of seawater varies between 7.5 and 8.4, the average value in equilibrium with the atmospheric CO_2 being 8.2. (www.theconcrete.org/concrete/concrete-in-seawater/843/).

Concrete exposed to marine environment may deteriorate as a result of combined effects of chemical action of seawater constituents on cement hydration products, alkali-aggregate expansion, crystallization pressure of salts within concrete, frost action in cold climates, corrosion of embedded steel in reinforced or pre stressed members, and physical erosion due to wave action and floating objects. Attack on concrete due to any one of these causes tends to increase the permeability; not only would this make the material progressively more susceptible to further action by the same destructive agent but also to other types of attack.

Durability Problems in concrete:

Environmental factors:

The inferior durability characteristics of concrete may be caused by the environment that the concrete is exposed to or by internal causes within the concrete. Environmental conditions like temperature, moisture, physical factors and chemical factors can affect the durability of concrete leading to steel corrosion, delamination, cracking, carbonation, sulphate attack, chemical attack, scaling, spalling, abrasion and cavitation (www.theconstructor.org/concrete/concrete-durability-problems/852/).

Temperature problem

Temperature variations will cause changes in the concrete volume. When temperature rises, the concrete slightly expands, and when temperature falls, the concrete contracts. If the concrete was unrestrained, these normal volume changes in concrete would have little consequences. Since concrete is usually restrained by foundations, subgrades, reinforcement, or connecting members, volume changes in concrete can produce significant stresses in the concrete. Tensile stresses can cause the concrete to crack.

Moisture problem

Changes in the moisture content in concrete will result in either concrete expansion or contraction. When concrete gains moisture, the concrete will slightly expand or swell. When concrete loses moisture, the concrete will contract or shrink. Concrete will slightly expand if the concrete is kept continuously wet, however, concrete drying causes the concrete to shrink, causing the concrete surface to develop tensile stresses and possible cracks.
The three main problems with moisture and concrete are carbonation, moisture cycle and contaminants.

Carbonation

In addition to shrinkage upon drying, concrete undergoes shrinkage due to carbonation. Carbon dioxide (CO_2) present in the atmosphere reacts in the presence of moisture with the hydrated cement minerals (i.e. the agent usually being the carbonic acid). The alkaline

conditions of hydrated cement paste are neutralized by carbonation. This neutralization, by dropping the pH from over 12 to about 9, affects the protection of reinforcing steel from corrosion. Thus, if the entire concrete cover to steel were carbonated, corrosion of steel would occur in the presence of moisture and oxygen.

Moisture Cycles

Stresses caused by changes in moisture content of the concrete may be additive to stresses caused by temperature changes. Tensile stresses usually increase the tendency for cracking, scaling, spalling, and delamination. Concrete that is subjected to moisture cycles may accumulate salts in the capillaries near the evaporating surface. This accumulation of salt may contribute to the chemical attack and/or salt scaling. Moisture movements in concrete may result in the concentrations of chlorides and sulphates in the concrete. Concentrations of chlorides in the concrete will cause the reinforcing steel to corrode and will also cause the concrete to crack and disintegrate.

Contaminants

Contaminants in the water that is absorbed into the concrete may cause staining, steel corrosion, or sulphate attack. Contaminants include: chloride and sulphate salts, carbonates, etc. Alternate cycles of wetting and drying allow the concentration of salts to increase and thereby increase the severity of their attack.

Physical Factors

Under many circumstances, concrete surfaces are subjected to wear. Concrete wear may be caused by the sliding, scraping or impact of objects that fall onto the concrete. In hydraulic structures, the action of the abrasive materials carried by flowing water generally leads to erosion of the concrete. Another cause of damage to concrete in flowing water is cavitation. Abrasion damage to concrete may be caused by the sliding or scraping of equipment across the concrete and by subjecting the concrete to abrasive materials (such as sand) that are carried by wind or water.

Chemical Factors

Chemical Corrosion

Solid salts do not attack concrete, but when they are in the form of a solution, they can directly react with the hardened cement paste. Some soils contain alkali, magnesium and calcium sulphates. When these sulphates come into contact with groundwater, they form a sulphate solution. Seawater may also contain a significant sulphate content. Formation of the hardened cement paste can occur when the sulphates react with the $Ca(OH)_2$ and the calcium aluminate hydrates. This reaction is called sulphate attack. In damp conditions, SO_2 CO_2, SO_3 and other acid forms that are present in the atmosphere may attack concrete and hence degrading it.

METHODS TO IMPROVE CONCRETE DURABILITY

Physical methods: Adding just an extra inch of concrete cover could double the life of a structure. Another way to prevent chloride intrusion is to reduce the permeability of concrete. Since chlorides are usually introduced in solution, reduction of water permeability will reduce chloride ion permeability. Concrete mixtures with a low water/cement ratio will produce concrete with lower permeability. Reducing the water-cement ratio seems like a simple method for reducing permeability, but in order to produce workable concrete, water-reducing admixtures are necessary.

Addition of pozzolans may also decrease the permeability of concrete. Silica fume, fly ash and ground granulated blast furnace slag in concrete will significantly reduce the water permeability of concrete. These pozzolans react with the calcium hydroxide produced by the reaction of water and cement to produce additional C-S-H, which occupies more of the pore spaces hence reducing the permeability of concrete.

Chemical methods:

i) Epoxy Coated Rebar- Epoxy Coated Rebar is widely available and accepted by ACI 318-95. The layer of epoxy prevents water and oxygen from coming in contact with the steel.

ii) Application of penetrating sealer silane or siloxane- Silanes penetrate the surface more readily and therefore, expected to last longer. These materials polymerize within the concrete

and also combine with siliceous portions of the cement and aggregates, becoming chemically bound adjuncts. Because they are inside the concrete, ultra violet light, a major disintegrator of organic molecules cannot reach them.

Electrochemical method- Cathodic protection systems are recommended for structures with severe exposure to chlorides. Cathodic protection electrically connects rebar in existing structures to a metal anode (usually zinc or magnesium metal), while applying a voltage to the system. Ryu and Otsuki (2002) studied the crack closure of reinforced concrete by electrochemical technique. A cracked specimen is immersed in Zinc sulphate solution and applies with a constant current for 8 weeks. The development of crack closure due to the precipitation of electrodeposits zinc oxide is then evaluated. The results indicate that electrodeposits formed on the concrete surface are able to close the concrete cracks and to decrease the concrete permeability.

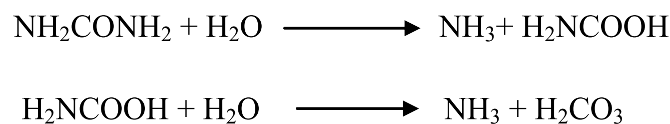
Microbilogically induced calcium carbonate precipitation- Degradation of building materials due to processes like weathering, earthquakes, human activities etc has drawn attention of scientists on the methods for slowing down or eliminating the losses of highly valuable natural and man made structures. Surface treatments with water repellants like epoxy injections, with pore blockers and various synthetic agents like silanes or siloxanes are available in the market today but with number of disadvantages like degradation with time, need for constant maintenance, environmental pollution etc. The emission of green houses like carbon dioxide during manufacturing processes of building materials is contributing a lot to global warming. Its time to put emphasis on reducing the emission of these gases into the atmosphere and save energy by minimizing usage of conventional building materials, methods, techniques and working on some other substitutes. There is a need for some novel, self healing and eco friendly, energy efficient technology that can protect and remediate such materials in a continuous way. And the answer to this question is microbiologically induced calcium carbonate precipitation (MICCP). The bacterially induced carbonate precipitation has received attention as an eco-friendly method of protecting and remediating building materials.

In the environment, natural cementation of geological formation occurs constantly over the geological time due to physical, chemical and biological reactions. Mineral precipitation is induced as of microbial metabolic activities and can persist as a part of environment. Most of

deposits are identifiers as calcium carbonate, phosphorites or sulfide with a few organic compounds. These microbiologically induced substances are pollution free, natural ubiquitous in all environments.

Cracking of concrete is a common phenomenon. Without immediate and proper treatments, cracks in concrete structures tend to expand further and eventually require costly repair. Use of bacteria in concrete remediation is environmentally innocuous, compared to synthetic polymers used for concrete repairs. Numerous conservation treatments have been applied for the protection and consolidation of carbonate stones in monuments. Most of them, however either release dangerous gases during carrying or show very little efficacy. Bacterially induced calcium carbonate mineralization has been proposed as a novel and friendly strategy for conservation of deteriorated ornamental stone.

MICCP in saline environment- MICCP in saline environment can be induced by the halophiles. 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 1.1). Calcite is readily precipitated under these conditions, in the presence of calcium.

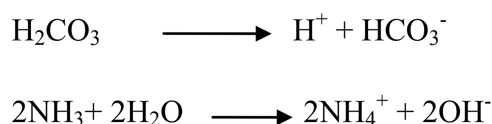


$$NH_2CONH_2 + H_2O \longrightarrow NH_3 + H_2NCOOH$$

$$H_2NCOOH + H_2O \longrightarrow NH_3 + H_2CO_3$$

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

Carbonic anahydrase, EC 4.2.1.1 (Tashian, 1989) is another enzyme which catalyzes the reversible conversion of carbonic acid to bicarbonate ions which would be available for $CaCO_3$ precipitation.



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

$$2NH_3 + 2H_2O \longrightarrow 2NH_4^+ + 2OH^-$$

Released carbonic acid and the two molecules of ammonia are in equillibrium with their deprotonated and protonated forms. Net effect is an increase in pH.

The process of microbiologically induced mineral precipitation can be employed as a method of selective cementation of highly permeable building structures. Our main objective is to develop a methodology for calcite deposition using microbes for repair and restoration of building materials adjoining sea water.

Objectives

1. Isolation and identification of efficient halobacteria isolated from sea water for the production of microbial concrete
2. Physiological and molecular characterization of bacteria
3. Influence of salt stress conditions on enzymatic activity and calcite formation by these bacteria
4. Effect of biological calcification in sand columns.

Chapter 2

Review of Literature

2.1 Microbial calcite

Current concern about the degradation of concrete and the economic impact of the maintenance and repair of concrete structures has drawn the attention to processes of concrete deterioration, and to the methods to slow down or even to eliminate concrete degradation (Hewelett *et al.,*1990) . The service environment, together with the permeation properties of concrete determine the risk of damage and the speed at which it can develop. Many of the physical and chemical deterioration mechanisms of concrete are related to aggressive substances present in aqueous solution. An important measure to protect concrete against damage is then diminishing the uptake of water (Basheer *et al.,*2001) . Surface treatments play an important role in limiting the infiltration of water – and consequently of detrimental components – into concrete. Nowadays a broad array of organic and inorganic products is available on the market for the protection of concrete surfaces, such as a variety of coatings, water repellents and pore blockers. These conventional means of protection show, however, beside their favourable influences also a number of disadvantageous aspects such as: (i) different thermal expansion coefficient of the treated layers; (ii) degradation over time and (iii) the need for constant maintenance. Furthermore the use of certain solvents contributes to environmental pollution (Camaiti *et al.,*1988; Perez *et al.,* 1995 and Le Metayer *et al.,* 1996). To partially offset these disadvantages, more ecologically friendly methods have been suggested.

Within this framework, bacterial induced carbonate mineralization has been proposed as a novel and environmentally friendly strategy for the protection and remediation of stone and mortar (Adolphe *et al.,* 1990). Biomineralization is 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.,* 1996; 1998; Tiano *et al.,* 1999; Castanier *et al.,* 2000; 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).

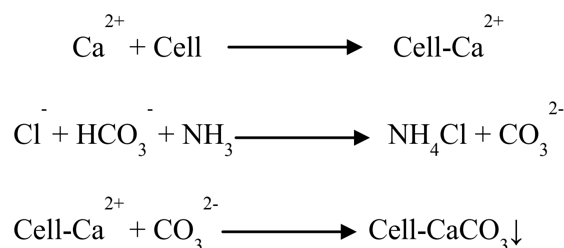
Calcium carbonate precipitation is a general phenomenon in the bacterial world under appropriate conditions (Boquet *et al.,* 1973). Indeed, some bacteria and fungi can induce precipitation of calcium carbonate extracellularly through a number of processes that include photosynthesis, ammonification, denitrification, sulphate reduction and anaerobic sulphide oxidation (Castanier *et al.,* 1999). 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.

Humans have the ability to precipitate minerals in the form of bones and teeth continuously. This ability is not only confined to human beings; even bacteria like *Bacillus Pasteruii*, a common soil bacterium, can continuously precipitate calcite (Stocks-Fischer *et al.,* 1999). This phenomenon is called microbiologically induced calcite precipitation. Under favorable conditions *Bacillus pasteruii* when used in concrete can continuously precipitate a new highly impermeable calcite layer over the surface of the already existing concrete layer. Calcite has a coarse crystalline structure that readily adheres to surfaces in the form of scales. In addition to the ability to continuously grow upon itself it is highly insoluble in water. Due

to its inherent ability to precipitate calcite continuously bacterial concrete can be called as a "Smart Bio Material". Cracks in concrete significantly influence the durability characteristics of the structure (Ramakrishnan *et al.,* 2002). The bacterial remediation technique can be used for repairing structures of historical importance to preserve the aesthetics value, as conventional technique, such as epoxy injection cannot be used to remediate cracks in those structures (Ramachandran *et al.,* 2001).

Biocalcification is a process in which calcite is formed in the soils or civil structures due to action of microbes, especially urease-producing organisms (Ramachandran *et al.,* 2001). This phenomenon, known as microbiologically induced calcite precipitation (MICP), is dependent on the urease enzyme activity, and a large number of soil microorganisms are found to contribute to the process. Undesirable effects of biofilm formation resulting in biodeterioration in civil works have gained attention and have been extensively studied (Brock, 1970; Rose, 1981, Sanchez- Silva *et al.,* 2008; Videla, 1996), whereas Sarda *et al.,* 2009 reports the useful role of a urease-producing microorganism to induce biocalcification in bricks. An endospore-forming soil microorganism, *Bacillus pasteurii*, has been used as the urease producer. A few studies on calcite precipitation for strengthening cement concrete (Bang *et al.,* 2001), plugging of sand (Sirko *et al.,* 2000), remediation of cracks in granite (Gollapudi *et al.,* 1995) and ornamental stone (Dick *et al.,* 2006) have been reported.

In natural environments, chemical $CaCO_3$ precipitation ($Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3\downarrow$) is accompanied by biological processes, both of which often occur simultaneously or sequentially. This microbiologically induced calcium carbonate precipitation (MICCP) comprises of a series of complex biochemical reactions (Stocks-Fischer *et al.,* 1999). As part of metabolism, *B. pasteurii* produces urease, which catalyzes urea to produce CO_2 and ammonia, resulting in an increase of pH in the surroundings where ions Ca^{2+} and CO_3^{2-} precipitate as $CaCO_3$. Possible biochemical reactions in medium to precipitate $CaCO_3$ at the cell surface that provides a nucleation site can be summarized as follows:



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

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

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

Formation of a new additional layer by calcifying bacteria is on the surface of the already existing concrete layer. This new additional calcite layer formed by bacteria is highly insoluble and increases the impermeability of the specimen. Thus it resists the penetration of harmful solutions into the concrete (alkali, sulfate etc.) thereby decreasing the deleterious effects they may cause (Ramakrishanan *et al.,* 2005).

Ghosh *et al.*, (2005) studied the effects of addition of the anaerobic microorganism, *Shewanella* species on the compressive strength of cement-sand mortar. They found that the strength of mortar cubes increased at all levels of anaerobic microbe addition. They reported 25% increase in 28 day compressive strength of cement mortar with the addition of about 10^5 cells/ml of mixing water.

Precipitation of calcium carbonate crystals occurs by heterogeneous nucleation on bacterial cell walls once supersaturation is achieved (De Muynck *et al.,* 2007). Microbial carbonate precipitation (biodeposition) decreases the permeation properties of mortar and concrete. Biologically deposited calcite is less soluble than inorganically precipitated calcite (Morse, 1983). Recently, De Muynck *et al.,* (2010) has shown the protective effect of $CaCO_3$ precipitation by *Bacillus sphaericus* on limestone.

The high alkaline pH of the concrete is a major hindering factor to the growth of a moderate alkalophile, *Bacillus pasteurii,* the common soil bacterium, grows well at pH 9.0 and also has the ability to produce the endospore, a dormant form of the cell, to endure extreme environments. However, due to high pH of the concrete, the growth and urease activity may not be optimal to produce more calcite (Achal *et al.,* 2009). Moreover calcite precipitation is a complex phenomenon, and is a function of the cell concentration, ionic strength and pH of the medium (Ramachandran *et al.,* 2001). Aono *et al.,* (1999) suggested that certain structural components of the cell walls of some alkalophiles, such as teichuronopeptide, may contribute to pH homeostasis at alkaline pH and aid bacteria to survive in alkaline environments.

The microbiological concrete remediation requires further consideration before it can be used with confidence, mainly due to the fact that the pH of concrete remains extremely high even after it is completely cured, i.e., above 12.5. Several reinforcement materials have been considered for providing not only a protection from adverse environmental conditions in concrete but also a higher bonding strength between the crack and the concrete. An immobilization technique for remediation of cracks in concrete, where microbial cells are encapsulated in polymers, has been adopted to enclose $CaCO_3$ precipitation in the gap and to

enhance the strength for selective cementation. The immobilization technique offers several advantages for concrete remediation, in which encapsulated cells retain high metabolic activities and are protected from adverse environmental conditions (O'Reilly and Crawford, 1989). Polyurethanes (PU) have been widely used as a vehicle for immobilization of enzymes and whole cells because of its mechanically strong and biochemically inert characteristics (Fukushima *et al.,* 1978; Wang and Ruchenstein, 1993).

2.1.1. MICCP studies in saline environment

Since the beginning of the century it has been known that bacteria are involved in the formation of carbonates. Carbonate precipitation has been studied both *invitro* and in natural habitats and it has been suggested that this precipitation could be related to the formation of marine calcareous skeletons, carbonate sediments and deposits of carbonates in soils (Doetsch and Cook, 1973; Krumbein, 1979).

Carbonate precipitation is influenced by external factors, among which the concentration of salts within the medium may be the most important. Moderately halophilic bacteria can grow in a wide range of osmotic concentrations which makes them very useful for studying the effect of salt concentration on their capacity to cause mineral precipitation. Some moderately halophilic bacteria have been reported to be capable of forming calcium carbonate precipitates (Ferrer *et al.,* 1988; Rivadeneyra *et al.,* 1991, 1993, 1994).
Cacchio *et al.,* (2003, 2004) also observed that lower the temperature of incubation, the longer it took for the different bacterial species to precipitate carbonates. However this delay in the time to start carbonate precipitation had no effect on the final total crystal production since all the isolates precipitated similar amounts of carbonates at 10°C.

Bacteria can serve as a nucleus of carbonate precipitation upon adsorbing Ca^{2+} and Mg^{2+} cations onto the cell surface (Greenfiled 1963; Morita 1980; Beveridge and Fyfe 1985; Ferris *et al.,* 1991). Each bacterium has a different type of cell surface resulting in a different cell surface charge (Van der Mei *et al.,* 2000). Furthermore, the cell surface charges are strongly influenced by pH, ionic strength and ionic make-up of the water (Ahimou *et al.,* 2002; Lytle *et al.,* 2002). The bacterial metabolism tends to modify these parameters leading to modifications of the cell surface during the culture growth phases. On the other hand, the influence of the physicochemical parameters of the medium on the cell surface varies among

different bacterial species (Pelletier *et al.,* 1968). Liquid media and high concentrations of salts seem to favour the formation of monohydrocalcite (Rivadeneyra *et al.,*1985).

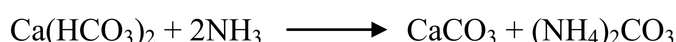
Knorre and Krumbein (2000) found that bacteria from freshwater, marine and hypersaline environments precipitated carbonates when cultivated in artificial laboratory media, the cyanobacteria being most active in this biomineralization process although heterotrophic bacteria were also involved.

According to Rosen (1987) the bacteria pump Ca^{2+} towards the exterior of the cell and Mg^{2+} towards the interior. On the negatively charged surfaces (as in the case of cellular envelopes of bacteria) Ca^{2+} is adsorbed with greater intensity than Mg^{2+} since it has a greater ionic selectivity (Wolt 1994; Maier *et al.,* 2000). This may explain since even even in media with high Mg^{2+}/ Ca^{2+} molar ratios, the minerals precipitated in the greatest quantities and by the highest number of bacteria are minerals rich in Ca^{2+} (Rivadeneyra *et al.,* 2006). *Deleya halophila* is a moderately halophilic microorganism isolated from haplic solochack soils from a saltern near the city of Alicanate (Spain) on the Mediterranean sea coast (Quesada *et al.,* 1984). Its ability to precipitate carbonates, as well as its growth characteristics and salt requirements, make this microorganism a useful aid in the study of carbonate precipitation. *Deleya halophila* mineralizes mainly calcium than magnesium, despite of the fact that the culture medium contained more of the later (Rivadeneyra *et al.,* 1996).

The greater difficulties of biomineralization with increasing salinity may be due to an excess of salts and higher concentrations of Mg^{2+} having a negative influence on CO_3^{2-} precipitation by bacteria (Billy 1980; Ferrer *et al.,* 1988, Rivadeneyra *et al.,* 1985, 1991; Raz *et al.,* 2000). In inorganic precipitation of carbonates, calcite is inhibited and aragonite increased with a higher concentration of Mg^{2+} (Cailleau *et al.,* 1977; Kitano and Hook 1962). Gonzailez (1989) indicated that in natural fluid media with Mg^{2+} to Ca^{2+} molar ratio >1 the precipitation is generally of aragonite (Rivadeneyra *et al.,* 2006).

Moderately halophilic isolates assigned to the genera *Flavobacterium* and *Acinetobacter* make calcite, and *Acinetobacter* also produces aragonite. High temperatures and low ionic strengths favor crystal formation. *Flavobacterium* makes magnesium calcite with 0.04 to 0.32 mol% magnesium; *Acinetobacter* produces magnesium calcite with up to 14% aragonite at the highest salinities (Ferrer *et al.,* 1988). Similarly, 63 strains of *Salinivibrio* isolated from an inland saltern in Spain were found to be involved in crystal formation (Rivadeneyra *et al.,* 1994).

The involvement of marine bacteria in calcium carbonate precipitation was first given prominence by Drew who stated that marine denitrifying forms were capable of bringing about deposition of calcium carbonate by raising alkalinity of sea water, providing sufficient potassium nitrate was present in the medium and these bacteria were responsible for much of the deposition known to occur in the Grand Bahama banks and neighbouring areas. These claims were disputed by Lipman who found that potassium nitrate was not a necessary requisite and that added calcium salts had to be supplied for the process to be completed. Greater significance was given to the activities of ammoniating forms which, by means of their ammonia production could cause the following to occur



$$Ca(HCO_3)_2 + 2NH_3 \longrightarrow CaCO_3 + (NH_4)_2CO_3$$

The argotite crystals produced were the result of metabolism of organisms present in tropical waters together with supersaturation of the water with calcium carbonate (Greenfield, 2006).

2.2 Urease

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 higher purity than is required for biocementation. The ability to produce urease is widespread amongst microbial populations and the enzyme has been well studied from a clinical perspective as it can indicate increased virulence properties in pathogenic bacteria (Collins and D'Orazio, 1993; Lee and Calhoun, 1997; Mobley *et al.,* 1995; Provorov and Vorobyov, 2000) and as a general nitrogen volatilisation phenomenon in agricultural soils (Nielsen *et al.,* 1998; Pettit *et al.,* 1976; Sadeghi *et al.,* 1988; Sloan and Anderson, 1995; Zantua and Bremner, 1977). The urease capability of organisms outside of these two areas is not well investigated.

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.

For 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*.The moderately alkaliphilic organism *Sporosarcina pasteurii* (formerly known as *Bacillus pasteurii* (Yoon *et al.,* 2001) is a commonly found in the soil, sewage and urinal incrustations (Sneath, 1986). *S. pasteurii* has a unique mechanism for the formation of ATP

which involves the coupling of ATP generation with urea hydrolysis. The generation of ATP is controlled by proton motive force (Δp) which is the sum of the transmembrane pH gradient (ΔpH) and the charge gradient or membrane potential ($\Delta\psi$):

$$\Delta p = \Delta pH + \Delta\psi$$

Neutrophilic organisms generate ATP from the chemiosmotic proton gradient generated by pumping protons out of the cell from the electron transport chain. This proton concentration gradient (high outside/low inside) drives protons back into the cell through ATP-synthase, resulting in ATP generation (Prescott *et al.,* 1993). The environment for alkaliphilic growth is high pH (low proton concentration) outside and lower pH (high proton concentration) inside the cell, resulting in a reversed pH with the tendency for protons to move from inside to outside the cell – opposite of the normal direction for ATP generation. To combat this alkaliphiles have developed two alternative means to increase proton motive force and drive protons into the cell for ATP generation:

1. Alkalinization of the cytoplasm, resulting in a reduced ΔpH – this is only tolerated within a small pH range.
2. Efflux of a cation other than H^+, resulting in an increase in the $\Delta\psi$

If the charge separation across the membrane ($\Delta\psi$) is large enough, proton motive force becomes sufficient to drive the influx of protons into the cell, against the concentration gradient. In the case of *S. pasteurii*, the type of ions that are effluxed to increase $\Delta\psi$ depends on the growth history of the culture. For cells grown at low urea concentrations (15 mM), the ions that can be transported out of the cell to increase $\Delta\psi$, have been shown to be K^+, Na^+ or NH_4^+. Cells grown on high urea concentrations (300 mM) however, cannot use K^+ or Na^+ but only NH_4^+ to drive ATP generation (Jahns, 1996). Because this organism has a high level of urease activity, urea is only present at the beginning of batch cultivation and will be fully hydrolysed to NH_4^+ and CO_3^{2-} within the first few hours. After urea is depleted from the medium, NH_4^+ is influxed into the cell via passive diffusion through the membrane. Interestingly, the pH optimum for *S. pasteurii* growth (9.25) is also the half dissociation constant (pK_a) of the NH_3/NH_4^+ equilibrium where NH_3 and NH_4^+ exist in equal proportions. It is fair to assume that the growth optimum for the organism is also the ATP-generating optimum, suggesting that NH_4^+ and NH_3 are required in equal proportions outside of the cell

for maximum ATP generation, but as yet this is not clearly understood in the proposed mechanism.

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 (Dunn and Grütter, 2001; Ha *et al.,* 2001; Marshall *et al.,* 1990). *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:

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

B. pasteurii and the recombinant *E. coli* were tested for the effects of acetohydroxamic acid (AHA) that functions as a competitive inhibitor of urease on microbial calcite precipitation (Bang *et al.,* 2001).

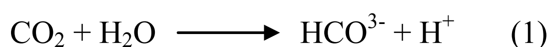
2.3 Carbonic anhydrase

Carbonic anhydrases are very well-known enzymes that are ubiquitous in nature. They can be found in animals and plants and even in the human erythrocyte. They exist in different forms, with different structures and molecular weights, and their activities vary from one to another.(Chegwidden *et al.,* 2000). They are among the fastest enzymes known (Mirjafari *et al.,* 2007).

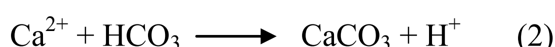
The response of calcifying marine organisms, especially from coral reefs – arguably among the most biologically diverse and ecologically important ecosystems on the planet –

could have a potential mitigating role in buffering atmospheric CO_2 (Rahman *et al.,*2010). Increasing atmospheric carbon dioxide concentrations reduces ocean pH and carbonate ion concentrations, and thus the level of calcium carbonate saturation. Experimental evidence suggests that if these trends continue, key marine organisms, such as corals, will have difficulty maintaining their external calcium carbonate skeletons. Atmospheric CO_2 is expected to reach double the preindustrial levels by the year 2065. It is believed that the increase in CO_2 concentration is responsible for global warming. Hence, it is essential to find ways of reducing CO_2 emissions. Carbonic anhydrase enzyme could be a potential biomolecule for solving this problem. The best way for CO_2 disposal is sequestration based on the chemical fixation of CO_2 in the form of carbonate minerals such as calcite, magnesite, and dolomite. (Rahman *et al.,*2010). Mineralization of CO_2 can be achieved by direct contact of gaseous CO_2 with mineral sources of calcium or magnesium or by dissolving CO_2 in water and then bringing the solution into contact with the minerals. Either way will produce calcium or magnesium carbonate, which are solids and will precipitate (Lackner *et al.,* 1996, 1995).

Carbonic anhydrase (Tashian, 1989, EC 4.2.1.1) is a Zinc-containing enzyme which catalyzes the reversible conversion of CO_2 to HCO_3 (equation 1 below), which would then be available for $CaCO_3$ formation (equation 2) (Rahman M.A. *et al.,*2010).



$$CO_2 + H_2O \longrightarrow HCO^{3-} + H^+ \quad (1)$$



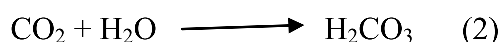
$$Ca^{2+} + HCO_3 \longrightarrow CaCO_3 + H^+ \quad (2)$$

In the indirect method of mineralization of CO_2, calcium carbonate is produced through a reaction between calcium ions and aqueous CO_2. The following reactions take place in this process:

(1) First, gaseous CO_2 dissolves in water to form aqueous CO_2 (reaction 1).

$$CO_{2(g)} \longleftrightarrow O_{2(aq)} \quad (1)$$

(2) Then, aqueous CO_2 reacts with water to form carbonic acid:



$$CO_2 + H_2O \longrightarrow H_2CO_3 \quad (2)$$

(3) In the next step carbonic acid dissociates to bicarbonate and carbonate ions

$$H_2CO_3 \longrightarrow H^+ + HCO_3^- \quad (3)$$

$$HCO_3^- \longrightarrow H^+ + CO_3^{2-} \quad (4)$$

(4) At the end in the presence of Ca^{2+} cations, calcium carbonate forms and precipitates

$$Ca^{2+} + CO_3^{2-} \longrightarrow CaCO_3 \downarrow \quad (5)$$

Among reactions 1-5 reaction 2 is slowest and rate limiting step. It is proposed that a biological catalyst can be used to increase the rate of this reaction (Bond *et al.,* 1999, 2001). The biological catalyst for this reaction is carbonic anhydrase.

In the presence of an anhydrase enzyme, the mechanism of hydration of CO_2 changes completely. The evidence suggests that the catalysis of CO_2 hydration is initiated by the nucleophilic attack on the carbon atom of CO_2, by zinc-bound OH-, to produce bicarbonate, which is then displaced from zinc by a water molecule (Chegwidden *et al.,* 2000). In this study, the mineralization of carbon dioxide is investigated as a method of converting CO_2 to mineral carbonates. The slow rate of hydration of CO_2 has been a limiting factor to make this method widely accepted. Carbonic anhydrase enzyme isolated from soft corals sclerites has been shown to be credible as biological catalysts to overcome this shortcoming. The results showed that this enzyme was a very effective catalyst. It promoted the hydration of CO_2 and, consequently, the precipitation of $CaCO_3$.

Carbonic anhydrase has been reported to be located in the cytoplasmic membrane or periplasmic space (Forkman *et al.,* 1972; Macleod *et al.,* 1981).

Botre and Botre (1989) studied the synergistic effect of urease and carbonic anhydrase in calcite precipitation and reported the increase in the rate of removal of carbon dioxide from the solution facilitated by carbonic anhydrase increases the rate of production of ammonia consequent from urea dissociation.

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 from sea water and stored 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 ml of respective sample in 25 ml nutrient broth (Hi-Media, Mumbai, India) (pH 8.0) containing 2% urea and incubated at 37°C for 120 h under shaking condition (130 rpm). For isolation and enumeration of cultivable bacteria, all the samples were serially diluted plated on sea water agar and the plates were incubated at 37°C. Finally to select urease producing bacteria, the colonies were randomly selected and transferred onto sea water agar containing urea to check the production of urease based on the intensity of pink color.

Sea water media of various salinity concentrations 3.6 %, 5%, 7.5% and 10% were selected to study different parameters. Microbiological urease production, carbonic anhydrase production, change in pH, growth profile and calcite precipitation and growth kinetics parameters were observed in the media containing 2% urea and 25mM $CaCl_2$, The pH of the media was adjusted to 7.5 with 1M Tris HCl (pH 8) prior to autoclaving without urea and $CaCl_2$. Filter-sterilized urea and $CaCl_2$ was added later.

Table 3.1 Composition of sea water (3.6% w/v) media

Component	Quantity(g/l)
NaCl	27.5
$MgCl_2$	5.0
$MgSO_4$	2.0
KCl	0.5
$FeSO_4$	0.001
Peptone	5.0
Yeast extract	1.0
pH adjusted to	7.5

3.1.3. Urease Assay

The urease activity was determined for all the bacterial isolates in all four media (sea water media of 3.6%, 5%, 7.5% and 10%) 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 hours, 48 hours, 72 hours and 96 hours culture was centrifuged at 8000 rpm for 5 minutes. The culture filtrate (250 µl) was added to a mixture containing 1 ml of 0.1M potassium phosphate buffer (pH 8.0) and 2.5 ml of urea (0.1M). The mixture was incubated at 37°C for 5 minutes followed by addition of phenol nitroprusside and alkaline hypochlorite, 1 ml each and incubated at 37°C for 25 minutes. Optical density was measured at 626 nm. One unit of urease is defined as the amount of enzyme hydrolyzing one µmole urea per min. The urease activity was calculated with reference to a caliberation graph plotted from the results obtained by standards.

3.1.4 Carbonic anahydrase assay

The carbonic anhydrase activity was determined for all the bacterial isolates in all four media (sea water media of 3.6%, 5%, 7.5% and 10%) by measuring the amount of p-nitrophenol produced according to spectrophotometric assay of carbonic anahydrase (Armstrong *et al.,* 1966; modified by Smith and Ferry, 1999). 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 hours, 48 hours, 72 hours and 96 hours culture was centrifuged at 8000 rpm for 5 minutes. The culture filtrate (2oo µl) was added to a mixture containing 1.80 ml of 100 mM phosphate buffer, pH 7.0 and 1 ml of 3 mM p-nitrophenyl acetate solution. The increase in A_{348} nm was recorded for 5 minutes. The $\Delta A_{348\ nm}$/ minute was computed using the maximum linear rate for both the test and blank. One unit of carbonic anahydrase activity is defined as the amount of enzyme required to form 1 µmole of p-nitrophenol per minute.

3.1.5 Calcium carbonate estimation

Precipitated calcium carbonate from each inoculated cuture was measured by EDTA titration method (AHPA, 1989) after 5 days. 5ml of the culture of each sample was dissolved with 3N hydrochloric acid. Four ml of Sodium hydroxide (5N) was added to the precipitate so that final pH reaches 12-13. Few drops of hydroxyl napthol blue were added as an indicator and the mixture was finally titrated against 0.05M EDTA. End point was noted from pink to blue which is easily visualized. 1ml of EDTA used for titration is equivalent to 5.004 mg of $CaCO_3$ precipitated.

3.1.6 Microbial sand plugging

To perform microbiological calcite precipitation, the bacterial isolates were grown at 37ºC under shaking condition (130 rpm) in sea water 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 inch; diameter = 3 inch) and bottom side of column was blocked by using Whatman filter paper. A

control reaction was packed in column in which sterile sand was mixed with sea water media only (without cells). All columns were fed continuously with 3.6% concentration sea water media as maximum urease activity was observed in this media. 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 checked for calcite estimation. Precipitated calcite from each layer was measured by EDTA titration method (AHPA, 1989).

3.1.6.1 SEM analysis of microbial sand plug

The morphology and chemical constituents of bacteria and sand consolidated column was analysed with SEM . Samples were completely dried at room temperature, then examined at accelerating voltages ranging from 30 to 35 kV by SEM (Zeiss EVO50) at accelerating voltages ranging from 30 to 35 kV. Samples were gold coated with a sputter coating Emitech K575 prior to examination. Consolidated sand cores of the microbial sand column were cut open and each sand sample was crushed and ground using pestle motor prior to examination.

3.1.7 Extraction of genomic DNA from bacteria

Isolation of genomic DNA generally comprises chemical cell disruption by enzymic digestion and detergent lysis; extractions with organic solvents, and selective recovery of the DNA.

3.1.7.1 Isolation of genomic DNA

A single colony of bacterial isolate was inoculated into 25 ml of nutrient broth in a 250 ml flask and incubated for 14-18 hours 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 50 µl of freshly prepared 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 phenol:chloroform:isoamyl alcohol (25:24:1) solution, and centrifuged 10 min at 12,000 rpm. To precipitate extracted nucleic acids, equal volume of 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 10 min. Finally, the pellets were resuspended in 40 µl TE buffer/milliQ water and stored at 4°C.

3.1.7.1.2 Electrophoresis of DNA on agarose gels

DNA was loaded on agarose gels (0.7 % w/v) prepared in 0.5X TBE, pH 8.0 using a 6X loading dye . 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.

3.1.7.1.3 Quantification of DNA by Nanodrop spectrophotometer

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.

3.1.7.2 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 rDNA 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 rDNA from isolates was carried out with a GenAmp thermocycler (Applied Biosystem, USA). Reaction mixture for the PCR contained 10X PCR buffer (Fermentas, USA), each dNTPs at a concentration of 2 mM, 50 mM $MgCl_2$, each primer at a concentration of 0.1 µM and 2.5U of Taq DNA polymerase (Fermentas, USA) in a final volume of 25 µl. PCR conditions were as follows: Preheating at 92°C for 5 min, 35 cycles of 92°C for 15 s, 55°C 30 s and 72°C for 30 s and final extension 72°C for 5 min. Successful amplifications were confirmed by agarose gel (0.8% w/v) electrophoresis and ethidium bromide staining.

3.1.7.3 Restriction fragment length polymorphism (RFLP)

The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis. The 16s rDNA PCR product of the four strains was digested by two restriction enzymes *AluI* and *RsaI* and then incubated at 37°C.

Restriction digestion reaction mixture

16s rDNA PCR product	5µl
10X enzyme buffer	1µ
Enzyme	1µl
H_2O	3µl

3.2. Physiological characterization of halophilic bacteria

3.2.1 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.

3.2.1.1 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 than washed briefly in water to remove excess crystal violet. Later it was flooded with Gram's iodine for 10 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.

3.2.1.2 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.

3.2.1.3 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, added a 2-3 drops of sulfanilic acid and α-naphthylamine. 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.

3.2.2.4 Salinity test

Different sodium chloride concentrations (3.6, 5, 7.5 and 10%) were amended in the sea water broth (pH 7.5) 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 hr grown cells.

3.2.2.5 Fermentation of carbon substrate by bacterial isolates

Fermentation media consists of peptone, carbohydrate, sodium chloride, phenol red, distilled water, pH 7.3. Three carbohydrate tests were performed with the isolated bacterial species. After inoculating the bacterial cultures in the fermentation media, tubes were incubated at 37° C for 24- 48 hours and change in color was observed.

3. Crystal morphology

Crystals are collected on whattman filter paper and then stained with methylene blue and observed under microscope to know their shape and morphology.

Chapter 4

Results

4.1 Isolation and identification of efficient halophiles for the production of microbial calcite

4.1.1 Isolation of urease producing bacteria

Urease producing bacteria were isolated from various samples collected from sea water by plating on sea water agar containing 2% urea which produce pinkish colonies which were further inoculated in sea water broth containing 2% urea. Four efficient strains designated as A3-5, A3-6, A1-4 and A3-13 producing urease enzyme were selected for further studies.

The bacterial strains isolated from these environments were able to survive at the alkaline range and found to be alkalophiles as due to the production of NH_3 by the hydrolysis of urea, pH of the media increases.

4.1.2 Influence of salt stress conditions on growth of urease producing halophiles

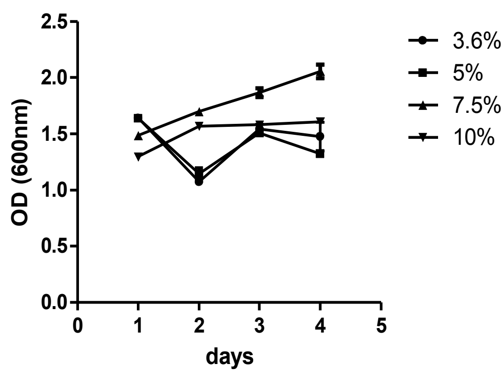
Comparison of survival and growth of halophiles in media of different salt concentrations was done by taking absorbance at 600 nm at all respective four days. Since the strains are halophiles they are able to survive even at high salt concentrations. The growth increased till the fourth day in all conc. of sea water media and as the urease production increases the growth of bacteria decreases. Mineral precipitation is known to reduce the cell surface area available for nutrient uptake, which eventually leads to loss of ATP generating capacity and results in cell death (Southam, 2000). Though there was decline in urease production till fourth day in high salt concentrations, but significant growth of bacteria was observed. In A3-5 strain maximum growth at 1st day was observed in 3.6% salt conc. media but decline in growth was observed till fourth day in 3.6% and 5% sea water media. But in 7.5% and 10% media though the growth was less at 1st day but till fourth day the growth continuously increased (Fig 4.1a). In A3-6 and A3-13 strains maximum growth was observed in 5% salt conc. media (Fig 4.1b and 4.1d). In A1-4 strain maximum growth was observed in 7.5% salt

conc. media bt growth decreased till fourth day (Fig 4.1c). Table 4.1 determines the effect of salt concentrations on the growth of halophiles. Significant differences in growth of halophiles were observed when salt concentration of sea water media was varied. Growth at different time intervals was also significant.

Table 4.1 Growth of halophilic bacteria (O.D. at 600nm) in sea water media containing different salt (NaCl) concentrations (3.6%, 5%, 7.5%, 10%)

Bacterial isolates	1st day	2nd day	3rd day	4th day
		3.6%		
A3-5	1.64 ± 0.05Ab	1.07 ± 0.03Cbc	1.54 ± 0.07Bc	1.48 ± 0.22Bbc
A3-6	1.95 ± 0.04Ba	1.47 ± 0.02Ca	2.04 ± 0.08Aa	2.09 ± 0.01Aa
A1-4	1.38 ± 0.01Bc	0.87 ± 0.03Cc	1.32 ± 0.04Bd	1.41 ± 0.01Ac
A3-13	1.59 ± 0.31Bb	1.17 ± 0.22Cb	1.80 ± 0.09Ab	1.53 ± 0.10Bb
		5.0%		
A3-5	1.64 ± 0.04Ab	1.15 ± 0.09Bb	1.51 ± 0.06Bbc	1.32 ± 0.06Cc
A3-6	1.97 ± 0.02Ba	1.52 ± 0.01Ca	2.11 ± 0.01Aa	2.1 ± 0.01Aa
A1-4	1.55 ± 0.09Abc	1.01 ± 0.08Cc	1.46 ± 0.07Bc	1.39 ± 0.07BCbc
A3-13	1.50 ± 0.05Cc	1.06 ± 0.10Dbc	1.70 ± 0.03Ab	1.62 ± 0.11Bb
		7.5%		
A3-5	1.49 ± 0.01Cb	1.70 ± 0.04Bb	1.87 ± 0.07Bb	2.06 ± 0.11Aa
A3-6	1.40 ± 0.06Cc	1.11 ± 0.02Dd	1.62 ± 0.02Bc	1.71 ± 0.03Ab
A1-4	1.97 ± 0.11Ba	2.00 ± 0.01Aa	2.04 ± 0.05Aa	2.02 ± 0.01Aa
A3-13	1.14 ± 0.08Bd	1.39 ± 0.03Ac	1.37 ± 0.04ABd	1.37 ± 0.10ABc
		10.0%		
A3-5	1.30 ± 0.06Cc	1.57 ± 0.03Bb	1.58 ± 0.01Bb	1.61 ± 0.01Ab
A3-6	1.36 ± 0.04Cbc	1.27 ± 0.09Dbc	1.46 ± 0.02Bc	1.61 ± 0.02Ab
A1-4	1.85 ± 0.03Ba	1.98 ± 0.02Aa	1.96 ± 0.02Aa	1.96 ± 0.07Aa
A3-13	1.43 ± 0.26Ab	1.26 ± 0.07Cc	1.29 ± 0.02Cd	1.37 ± 0.02Bc

Values sharing common lower case letter within the column and upper case letter within the row are not significant at P< 0.05. Values are Mean ± SD (n=3).



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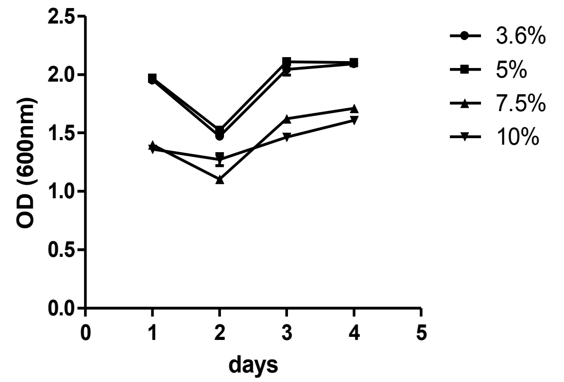

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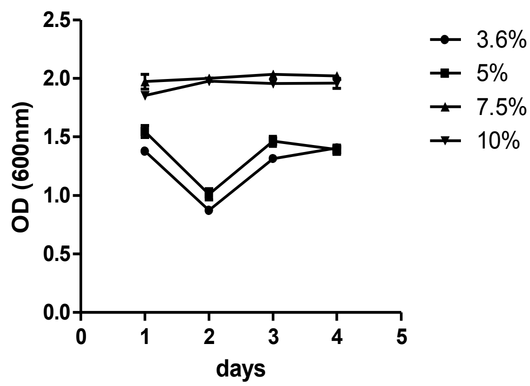

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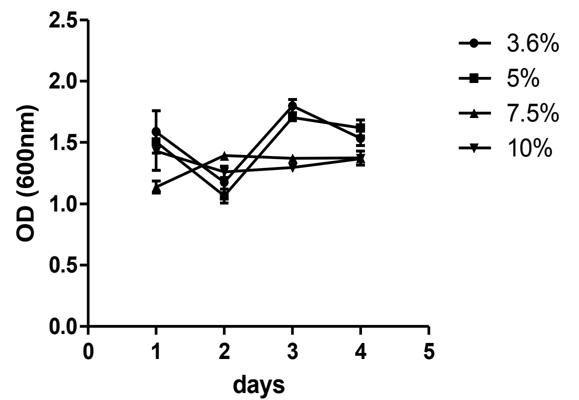


d)

Fig 4.1 Effect of different salt concentrations on the growth of a) A3-5 b) A3-6 c) A1-4 and d) A3-13

Two way ANOVA on the effect of salt concentrations on the growth of bacterial isolates

Source of Variation	Df	Sum-of-squares	Mean square	F
A3-5				
Growth	3	1.034	0.3446	56.15***
Days	3	0.5003	0.1668	27.17***
Growth*Days	9	1.201	0.1334	21.73***
Residual	32	0.1964	0.006138	
A3-6				
Growth	3	2.605	0.8684	521.8***
Days	3	2.040	0.6800	408.6***
Growth*Days	9	0.2235	0.02484	14.93***
Residual	32	0.05325	0.001664	
A1-4				
Growth	3	5.554	1.851	606.5***
Days	3	0.4604	0.1535	50.28***
Growth*Days	9	0.6424	0.07138	23.38***
Residual	32	0.09767	0.003052	
A3-13				
Growth	3	0.3617	0.1206	6.782**
Days	3	0.6762	0.2254	12.68***
Growth*Days	9	0.8389	0.09321	5.244**
Residual	32	0.5688	0.01778	

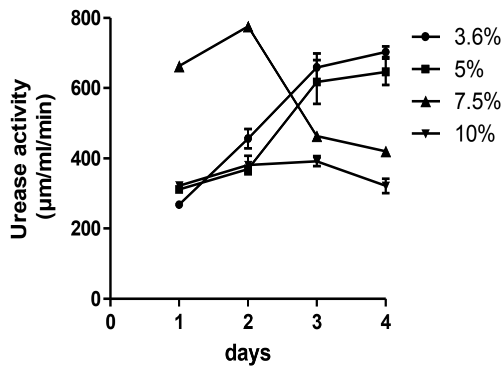
4.1.3 Effect of salt stress conditions on urease activity

Urease activity in sea water media (salt concentrations 3.6%, 5%, 7.5% and 10%) at different time intervals was determined by indophenol assay (Natrajan, 1995). A3-5 and A3-13 strains, showed maximum urease activity till the fourth day in 3.6% conc.sea water media followed by 5% conc.sea water media and in 7.5% and 10% conc.sea water media urease activity declined till fourth day (Fig 4.2a and Fig 4.2d). A3-6 and A1-4 strains showed increase in urease activity till fourth day in sea water media of conc.3.6%, 5% and 7.5% but in 10% conc.sea water media activity declined till fourth day (Fig 4.2c and Fig 4.2c). Urease activity at different time intervals in different salt concentrations was also significantly different, calculated by two way ANOVA test for all the four strains. Table 4.2 determines the urease activity of all strains in different salt concentration sea water media calculated at different time intervals. Significant differences in urease activity of halophiles were observed when salt concentration of sea water media was varied. Urease activity at different time intervals was also significant. Bacteria are known to hydrolyze urea by urease for the purposes of: (1) increasing the ambient pH, (2) utilizing it as a nitrogen source and (3) using it as a source of energy. The subsequent increase in pH in surrounding medium due to the presence of NH_3 ions and the additional release of CO_2 from the enzymatic urea hydrolysis further accelerate the rate of urease induced calcium carbonate precipitation. Thus an active participation of urease is of essence in biochemical calcite precipitation.

Table 4.2 Urease activity (conc in µM/ml/min) of halophilic bacteria in sea water media containing different salt (NaCl) concentrations (3.6%, 5%, 7.5%, 10%).

Bacterial isolates	1st day	2nd day	3rd day	4th day
		3.6%		
A3-5	267.41 ± 9.89Db	456.00 ± 27.12Cbc	659.26 ± 39.09Ba	703.26 ± 15.02Aa
A3-6	208.59 ± 11.51Dbc	514.37 ± 8.15Ca	615.26 ± 6.08Bb	689.78 ± 5.15Ab
A1-4	201.93 ± 6.14Dc	422.52 ± 8.46Cc	539.26 ± 15.27Bd	609.78 ± 22.90Ac
A3-13	454.37 ± 67.51Da	495.26 ± 57.69Cb	593.63 ± 24.51Bc	703.26 ± 14.34Aa
		5.0%		
A3-5	310.96 ± 2.33Dab	369.33 ± 5.26Cd	617.19 ± 6.35Bb	646.37 ± 37.49Aa
A3-6	261.93 ± 9.23Cc	454.96 ± 4.23Bb	572.59 ± 7.44Ac	587.56 ± 4.95Ab
A1-4	316.15 ± 12.75Da	475.11 ± 76.00Ca	624.15 ± 45.69Aa	546.96 ± 19.90Bc
A3-13	286.96 ± 19.87Cb	393.04 ± 11.69Bc	524.59 ± 13.89Ad	537.93 ± 5.57Ad
		7.5%		
A3-5	662.22 ± 2.22Da	775.70 ± 1.03Aa	463.70 ± 1.48Bb	420.15 ± 1.03Cc
A3-6	343.56 ± 4.51Db	395.70 ± 3.46Cb	500.74 ± 10.43Ba	567.41 ± 13.41Aa
A1-4	284.30 ± 2.88Dc	317.04 ± 6.58Cc	354.22 ± 8.48Bc	462.07 ± 13.72Ab
A3-13	344.15 ± 30.94Bb	394.81 ± 3.22Ab	328.89 ± 12.61Cd	221.93 ± 8.22Dd
		10.0%		
A3-5	322.67 ± 3.76Cc	380.89 ± 26.33ABb	391.85 ± 14.50Ab	321.93 ± 20.69Cb
A3-6	348.15 ± 5.44Da	422.52 ± 15.74Ba	460.15 ± 22.41Aa	402.22 ± 3.64Ca
A1-4	327.11 ± 12.38Bb	328.44 ± 37.25Bd	341.63 ± 10.89Ac	289.04 ± 2.88Cd
A3-13	328.59 ± 3.34Bb	342.07 ± 21.55Ac	344.74 ± 14.59Ac	299.41 ± 10.74Cc

Values sharing common lower case letter within the column and upper case letter within the row are not significant at $P < 0.05$. Values are Mean ± SD (n=3).



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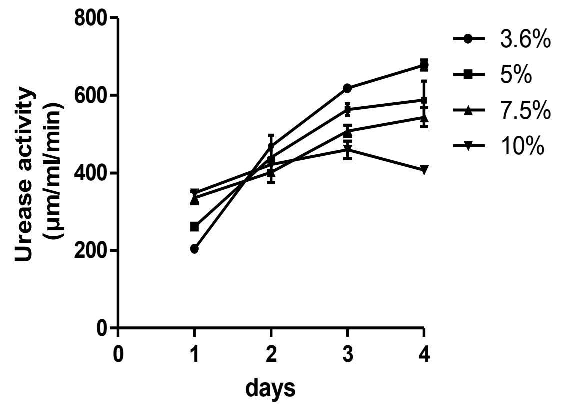

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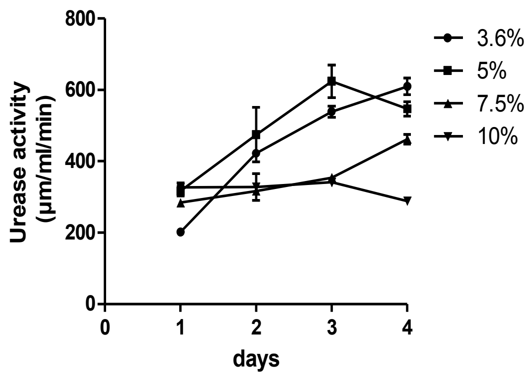

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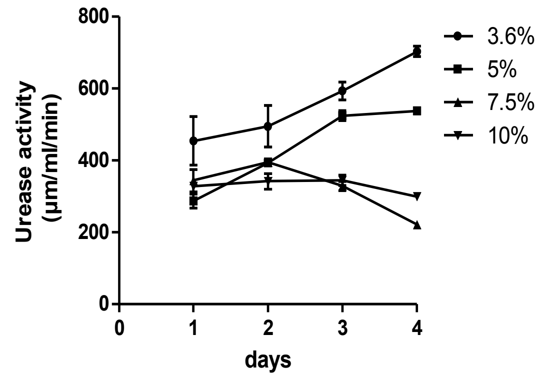


d)

Fig 4.2 Effect of different salt concentrations on the urease activity of a) A3-5 b) A3-6 c) A1-4 and d) A3-13

Two way ANOVA for the stuying the effect of salt concentrations on the urease activity of bacterial isolates

Source of Variation	Df	Sum-of-squares	Mean square	F
A3-5				
Urease	3	330200	110100	63.33***
Days	3	152700	50890	29.28***
Urease*Days	9	736400	81820	47.08***
Residual	32	55610	1738	
A3-6				
Urease	3	42910	14300	11.00***
Days	3	541400	180500	138.8***
Urease*Days	9	161900	17990	13.84***
Residual	32	41590	1300	
A1-4				
Urease	3	219500	73180	34.56***
Days	3	289700	96550	45.59***
Urease*Days	9	210600	23400	11.05***
Residual	32	67770	2118	
A3-13				
Urease	3	454900	151600	70.65***
Days	3	66790	22260	10.37***
Urease*Days	9	222500	24720	11.52***
Residual	32	68670	2146	

4.1.4 Effect on change in pH

Due to release of NH_3 by hydrolysis of urea, pH of the media increases. As the urease activity increases the change in pH occurs linearly. Fig 4.3a, b, c and d depict the increase and decrease in pH with increase and decrease in urease activity respectively for all strains in different media calculated at different time intervals. Change in pH at different time intervals in different salt concentrations was also significantly different, calculated by two way ANOVA test for all the four strains. Table 4.3 determines the change in pH in different salt concentration sea water media calculated at different time intervals. Significant differences in change in pH of media were observed with the change in urease activity. Change in pH at different time intervals was also significant in all strains except in A3-6.

Table 4.3 Change in pH of media with the production of urease enzyme by halophilic bacteria

Bacterial isolates	1st day	2nd day	3rd day	4th day
		3.6%		
A3-5	8.25 ± 0.04Bbc	8.37 ± 0.08Bc	8.52 ± 0.11Abc	8.57 ± 0.12Ab
A3-6	8.29 ± 0.01Cb	8.40 ± 0.03Bb	8.44 ± 0.03Bc	8.56 ± 0.07Ab
A1-4	8.22 ± 0.04BCc	8.35 ± 0.03Bc	8.56 ± 0.06Abc	8.69 ± 0.02Aab
A3-13	8.39 ± 0.21Ca	8.56 ± 0.21Ba	8.70 ± 0.10Aa	8.77 ± 0.12Aa
		5.0%		
A3-5	8.16 ± 0.03Cb	8.26 ± 0.06Bab	8.36 ± 0.08ABab	8.46 ± 0.06Aa
A3-6	8.12 ± 0.02Cc	8.24 ± 0.04Aab	8.28 ± 0.05Ac	8.22 ± 0.02Bab
A1-4	8.13 ± 0.02Cbc	8.29 ± 0.04Aab	8.24 ± 0.11ABc	8.27 ± 0.05Aab
A3-13	8.15 ± 0.01Ca	8.35 ± 0.09Ba	8.50 ± 0.06Aa	8.56 ± 0.08Aa
		7.5%		
A3-5	8.15 ± 0.05Bab	8.15 ± 0.02Bab	8.25 ± 0.03Aa	8.31 ± 0.01Aab
A3-6	8.17 ± 0.04Ba	8.13 ± 0.03Ba	8.21 ± 0.02Aab	8.25 ± 0.04Aab
A1-4	8.16 ± 0.01Cab	8.13 ± 0.03Cab	8.33 ± 0.03Ba	8.57 ± 0.11Aa
A3-13	8.21 ± 0.02Aa	8.14 ± 0.02Aa	8.15 ± 0.02Ab	8.07 ± 0.03ABb
		10.0%		
A3-5	8.15 ± 0.02Bb	8.11 ± 0.02ABab	8.37 ± 0.03Cab	8.19 ± 0.02Aa
A3-6	8.55 ± 0.32Aa	8.12 ± 0.02Bab	8.11 ± 0.07Bc	8.10 ± 0.02Bab
A1-4	8.18 ± 0.03Aab	8.16 ± 0.01Aa	8.31 ± 0.03Aab	8.09 ± 0.02Bab
A3-13	8.15 ± 0.01ABb	8.15 ± 0.04ABa	8.45 ± 0.06Aa	8.12 ± 0.03Ba

Values sharing common lower case letter within the column and upper case letter within the row are not significant at P< 0.05. Values are Mean ± SD (n=3).

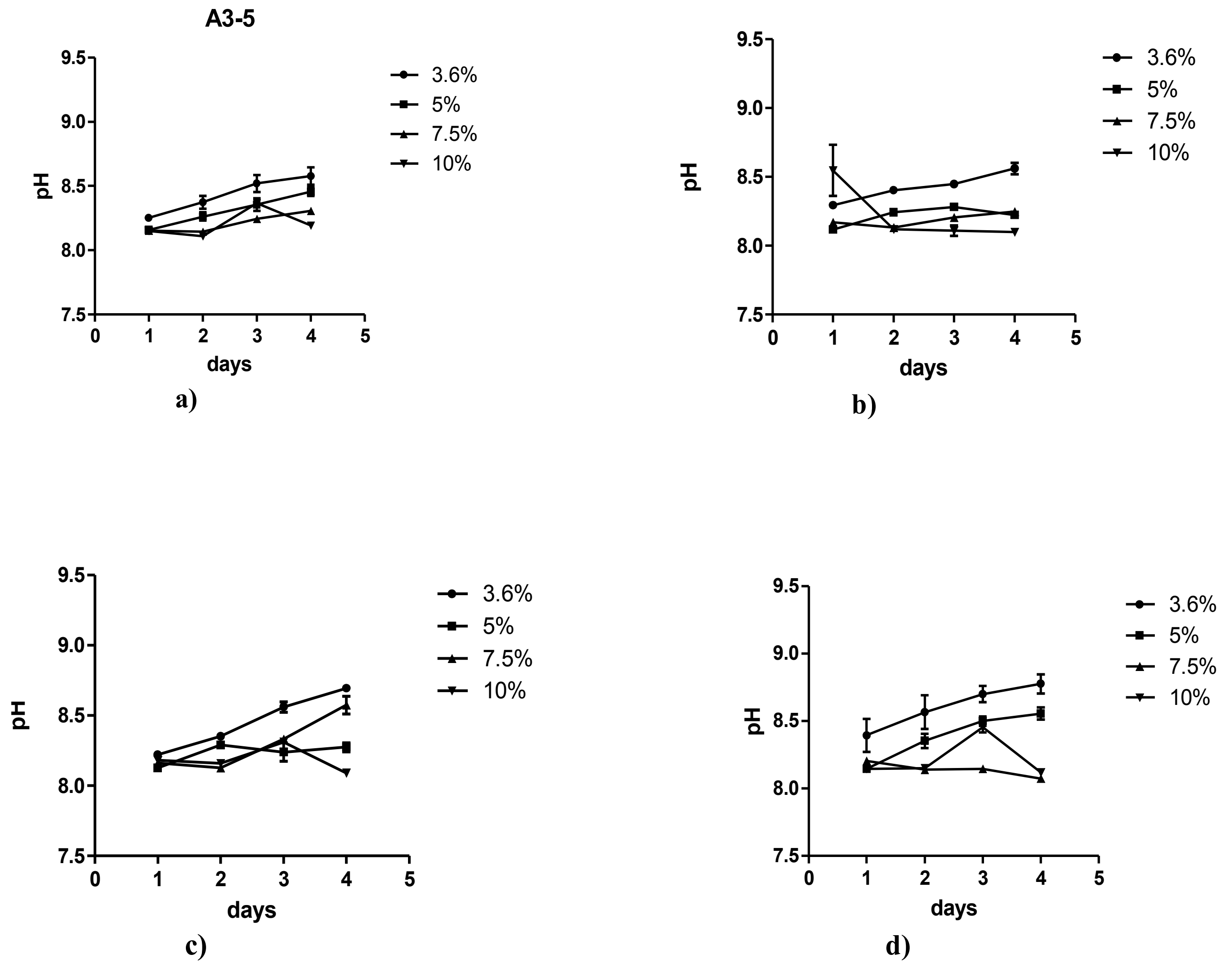


Fig 4.3 Change in pH with the production of urease by a) A3-5 b) A3-6 c) A1-4 and d) A3-13

Two way ANOVA results on the effect on change in pH with production of urease by bacterial isolates

Source of Variation	Df	Sum-of-squares	Mean square	F
		A3-5		
pH	3	0.3996	0.1332	36.94***
Days	3	0.3869	0.1290	35.77***
pH*Days	9	0.1188	0.01320	3.661**
Residual	32	0.1154	0.003606	
		A3-6		
pH	3	0.4324	0.1441	18.25***
Days	3	0.02609	0.008696	1.101
pH*Days	9	0.5799	0.06444	8.159***
Residual	32	0.2527	0.007898	
		A1-4		
pH	3	0.5025	0.1675	64.84***
Days	3	0.4319	0.1440	55.73***
pH*Days	9	0.4685	0.05206	20.15***
Residual	32	0.08267	0.002583	
		A3-13		
pH	3	1.551	0.5168	57.56***
Days	3	0.3462	0.1154	12.85***
pH*Days	9	0.4600	0.05111	5.692***
Residual	32	0.2873	0.008979	

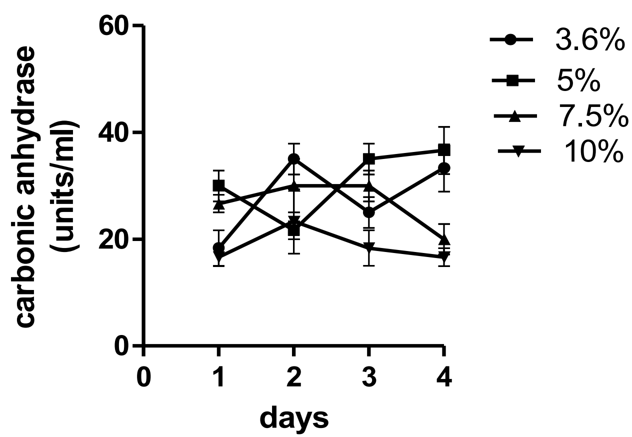
4.1.5 Effect of salt stress conditions on carbonic anhydrase enzyme production by halophiles

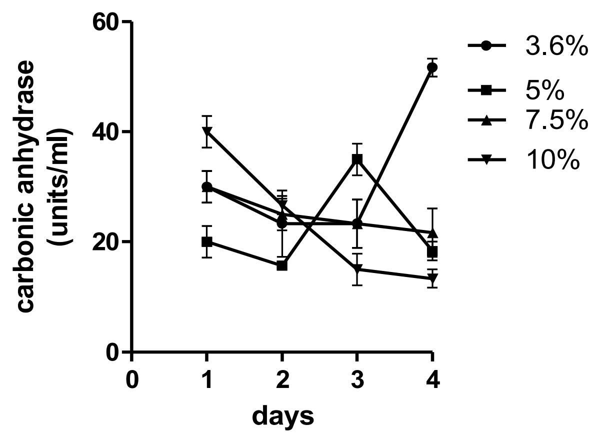
In 3.6% sea water media it was observed that carbonic anhydrase activity was high till fourth day in strains A3-5, A3-6 and A3-13. In 5% sea water media the activity was high till fourth day in strains A3-13 and A1-4 though A1-4 strains showed decline in CA activity at fourth day in 3.6% conc.sea water media. But in 7.5% and 10% sea water media carbonic anhydrase production decreases in all strains till fourth day. The CA curve was not observed linear as the srains showed decrease in CA activity at 2nd and 3rd day and then increase in activity till fourth day. Fig 4.4a ,b, c and d depict the carbonic anhydrase production in different saline environments by halophiles. Table 4.4 shows the comparison of carbonic anhydrase activity by the halophiles under study. Significant differences in carbonic anhydrase activity of strains A3-5, A3-6 were not observed but significant differences in carbonic anhydrase activity of strains A1-4 and A3-13 were observed when salt concentration of sea water media was varied. Carbonic anhydrase activity at different time intervals was not significant in the strains except A3-6.

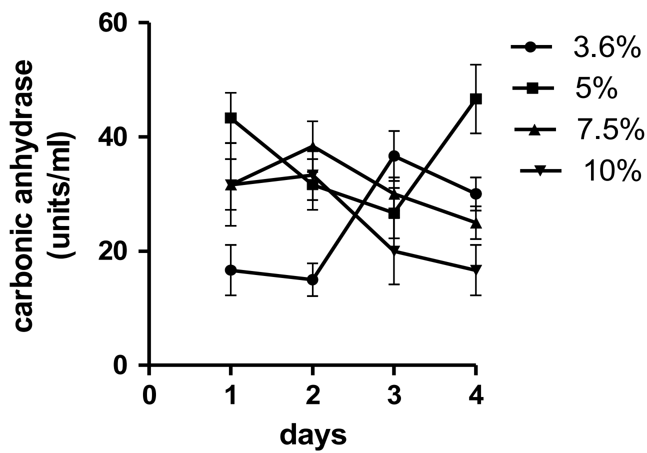
Table 4.4 Carbonic anhydrase enzyme production by halophilic bacteria in sea water media containing different salt (NaCl) concentrations (3.6%, 5%, 7.5% and 10%)

Bacterial isolates	1st day	2nd day	3rd day	4th day
		3.6%		
A3-5	16.67 ± 1.57Dc	40.00 ± 2.00Aa	18.33 ± 2.51Cd	33.33 ± 1.53Bc
A3-6	23.33 ± 3.21Ca	20.00 ± 3.01Dc	30.00 ± 3.6Bb	51.67 ± 0.57Ab
A1-4	16.67 ± 1.52Cc	11.67 ± 1.53Dd	36.67 ± 1.52Aa	26.67 ± 2.08Bd
A3-13	18.33 ± 3.78Db	35.00 ± 4.58Bb	20.00 ± 1.00Cc	53.33 ± 1.53Aa
		5.0%		
A3-5	30.00 ± 1.01Bb	21.67±0.57Dc	35.00 ± 1.03Ab	26.67 ± 4.04Cb
A3-6	20.00 ± 1.06Bd	10.00±1.00Dd	50.00 ± 2.00Aa	18.33 ± 0.58Cc
A1-4	41.67 ± 2.08Ba	26.67±2.51Cb	26.67 ± 1.52Cc	53.33 ± 3.51Aab
A3-13	26.67 ± 3.05Cc	53.33±7.02Aa	33.33 ± 1.53Bbc	55.00 ± 8.71Aa
		7.5%		
A3-5	23.33 ± 1.52Cd	25.00 ± 3.00Bc	30.00 ± 1.00Aa	20.00 ± 1.00Db
A3-6	51.67 ± 5.51Aa	21.67 ± 1.52Cd	23.33 ± 1.52Bb	20.00 ± 2.00Cb
A1-4	38.33 ± 4.72Bb	41.67 ± 2.08Aa	30.00 ± 1.01Ca	28.33 ± 2.08Da
A3-13	25.00 ± 5.29Bc	30.00 ± 1.09Ab	15.00 ± 1.02Cc	6.67 ± 0.57Dc
		10.0%		
A3-5	13.33 ± 1.52Cd	23.33 ± 2.08Ac	16.67 ± 1.53Bb	13.33 ± 1.53Ca
A3-6	43.33 ± 2.51Aa	21.67 ± 2.08Bd	11.67 ± 1.53Cc	10.00 ± 1.00Cb
A1-4	31.67 ± 1.52Bb	33.33 ± 1.53Aa	20 ± 2.00Ca	13.33 ± 2.08Da
A3-13	23.33 ± 3.05Bc	28.33 ± 3.51Ab	16.67 ± 1.52Cb	5.00 ± 1.00Dc

Values sharing common lower case letter within the column and upper case letter within the row are not significant at $P < 0.05$. Values are Mean ± SD (n=3).







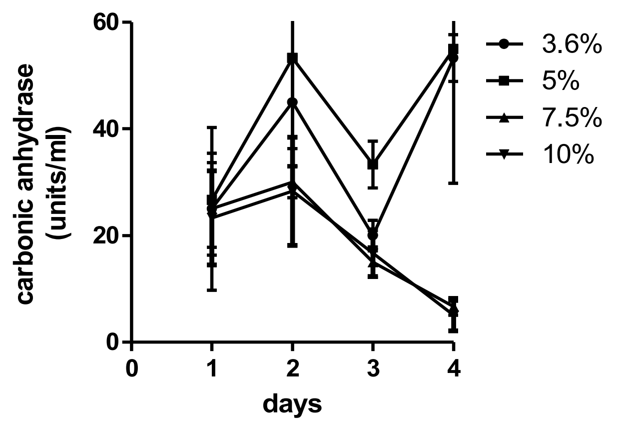


Fig 4.4 Effect of different salt concentrations on the carbonic anhydrase activity of a) A3-5 b) A3-6 c) A1-4 and d) A3-13

Two way ANOVA results on the effect of salt concentrations on the carbonic anhydrase production by bacterial isolates

Source of Variation	Df	Sum-of-squares	Mean square	F
		A3-5		
Carbonic anhydrase CA	3	854.2	284.7	2.862
Days	3	116.7	38.89	0.3909
CA*Days	9	1063	118.1	1.187
Residual	32	3183	99.48	
		A3-6		
Carbonic anhydrase CA	3	679.2	226.4	1.575
Days	3	1671	556.9	3.874*
CA*Days	9	7067	785.2	5.462**
Residual	32	4600	143.8	
		A1-4		
carbonic anhydrase CA	3	1806	602.1	4.718**
Days	3	118.8	39.58	0.3102
CA*Days	9	3690	410.0	3.213**
Residual	32	4083	127.6	
		A3-13		
Carbonic anhydrase CA	3	4597	1532	4.246*
Days	3	1756	585.2	1.621
CA*Days	9	4342	482.5	1.337
Residual	32	11550	360.9	

4.1.5 Calcite precipitation by halophiles in sea water media containing different salt concentrations (3.6%, 5%, 7.5% and 10%)

Calcite precipitation was observed in sea water media (salt concentrations 3.6%, 5%, 7.5% and 10%) amended with 2% urea and 25mM $CaCl_2$. High calcite precipitation was observed in the sea water media with concentration 3.6% and 5%. It is due to the reason that high salt concentration interfere in calcium carbonate precipitation. Fig 4.5 determines the comparison of calcite formation by studied strains in different media. Significant differences in cacium carbonate precipitation by halophiles were observed when salt concentration of sea water media was varied.

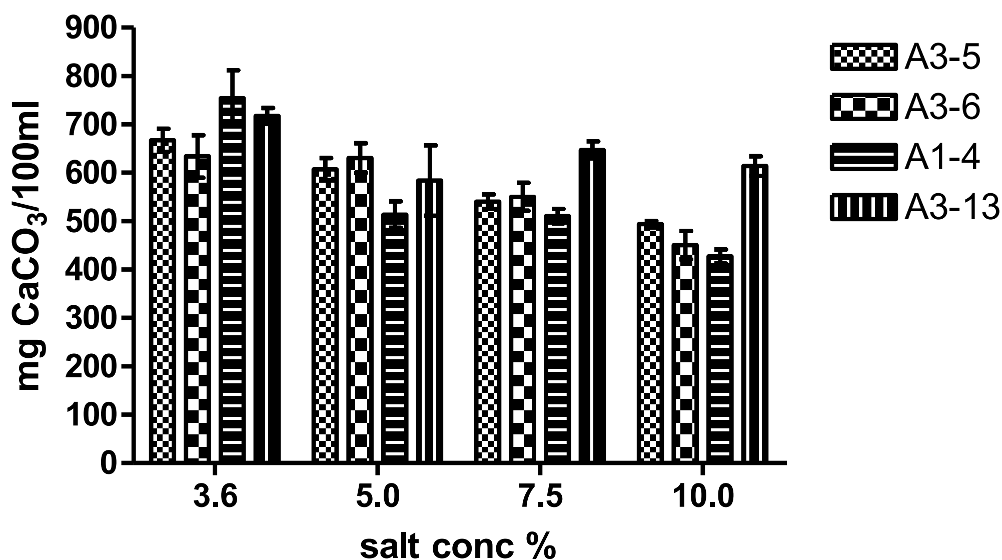


Fig 4.5 Effect of salt concentration on calcite formation by halophiles.

Table 4.5 Effect of salt concentrations on calcite (mg/100ml) formation by halophilic bacteria

Bacterial isolates	3.6%	5.0%	7.5%	10.0%
A3-5	667.02 ± 41.66Ac	607.15 ± 40.44Bb	540.43 ± 26.48Cb	493.72 ± 11.55Db
A3-6	633.84 ± 76.41Ad	630.50 ± 52.95Aa	550.44 ± 50.04Bb	450.36 ± 50.04Cc
A1-4	753.93 ± 100.25Aa	513.74 ± 47.21Bd	510.41 ± 26.48Bc	427.01 ±25.18Cd
A3-13	717.24 ± 28.89Ab	583.82 ± 125.91Dc	647.18 ± 30.57Ba	613.82 ± 35.14Ca

Values sharing common lower case letter within the column and upper case letter within the row are not significant at P< 0.05. Values are Mean ± SD (n=3).

Two way ANOVA results showing the effect of salt concentrations on calcite formation by bacterial isolates

Source of Variation	Df	Sum-of-squares	Mean square	F
calcite precipitation	3	55510	18500	5.880**
conc %	3	240900	80300	25.52***
calcite precipitation*conc%	9	86540	9615	3.056**
Residual	32	100700	3147	

4.1.6 $CaCO_3$ precipitation by halophiles A3-6 and A1-4 in different sand columns with 3.6% salt concentration media

Maximum calcite precipitation was observed in the surface layer of sand by both strains. calcite precipitation in sand columns by both strains was done in replicate. Calcite content in bottom layer and middle layer was significantly less as compared to the bottom layer. Significant differences were not observed in calcite precipitation by A3-6 and A1-4 but significant differences in calcite formation in different layers was observed.

Fig. 4.6 Assembly of microbial sand column

Fig. 4.7 Microbial treated sand column

Table 4.6 Comparison of calcite (mg/100ml) formation in different layers of soil by halophiles A3-6 and A1-4

Layers	A3-6	A1-4
Upper	725.58 ± 199.21Aa	725.58 ± 225.98Aa
Middle	175 ± 30.15Bb	200.16 ± 70.76Ab
Lower	155.12 ± 7.08Ac	150.12 ± 70.67Ac

Values sharing common lower case letter within the column and upper case letter within the row are not significant at P< 0.05. Values are Mean ± SD (n=3).

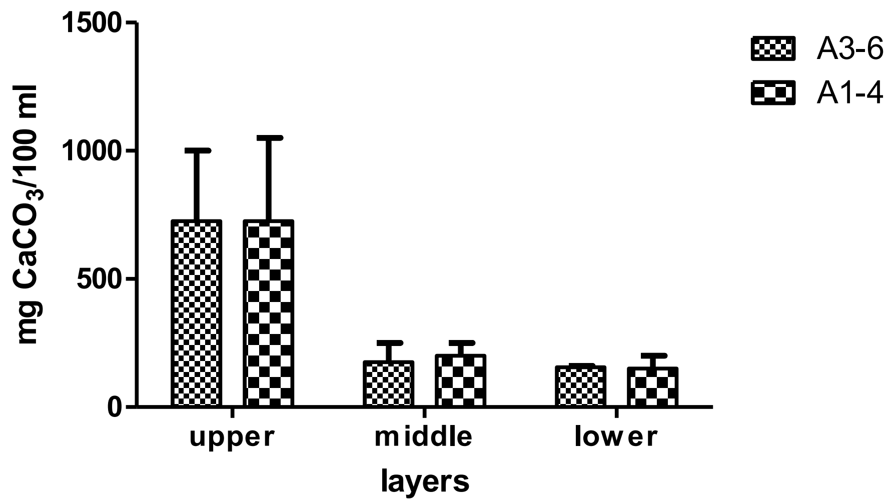
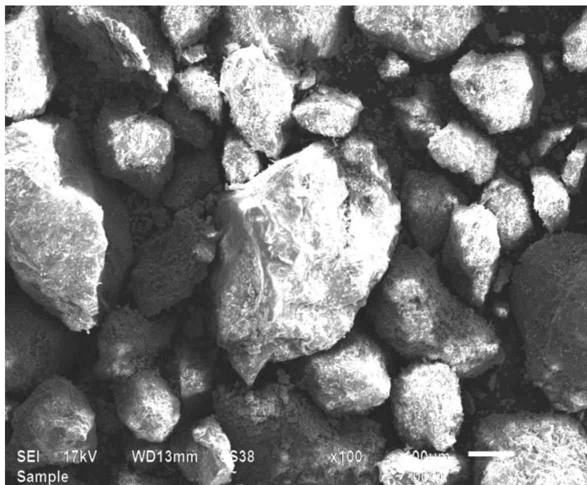


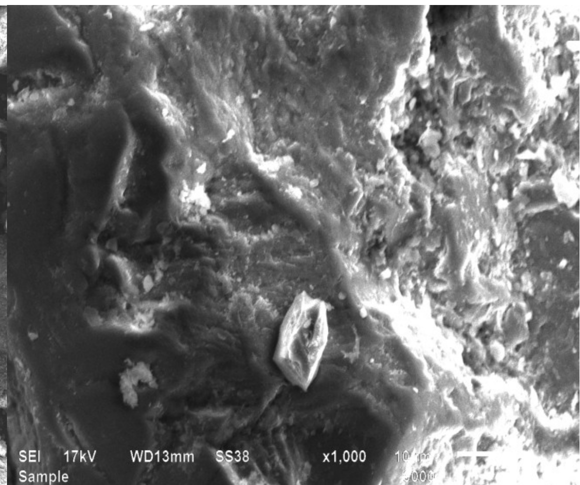
Fig 4.8 Calcite content in different layers of sand columns

Two way ANOVA results showing the calcite content in different layers of sand columns by bacterial isolates

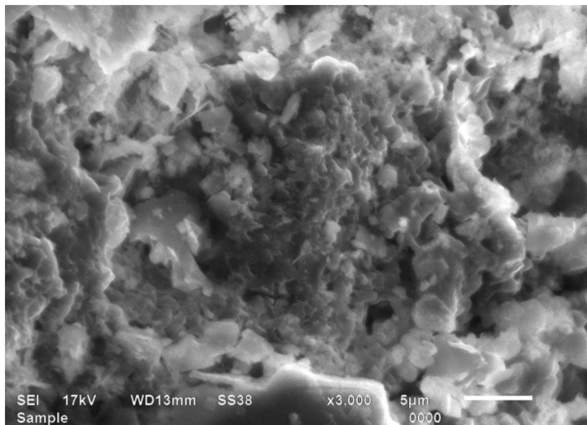
Source of Variation	Df	Sum-of-squares	Mean square	F
Calcite content	1	133.5	133.5	0.002084
Layers	2	825200	412600	6.440*
Calcite content*layers	2	517.5	258.7	0.004039
Residual	6	384400	64070	

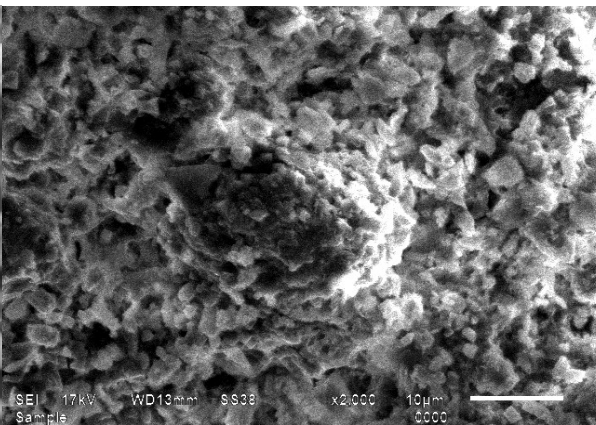
4.1.6.1 SEM analysis of microbial sand plugging



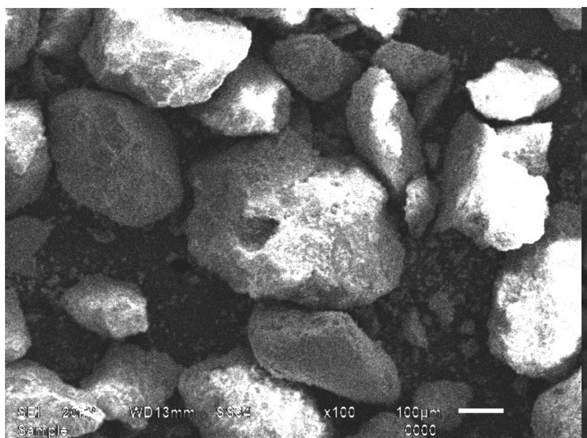


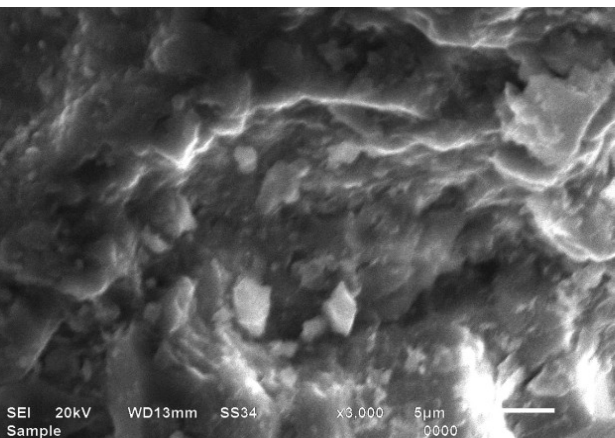
(i) (ii)



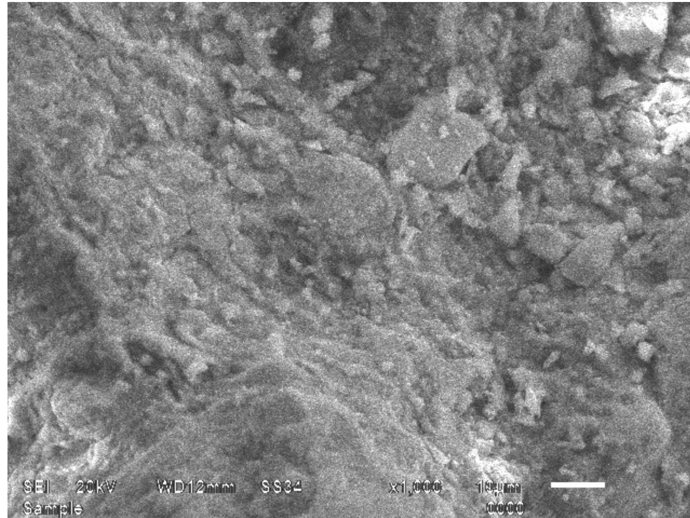


(iii) (iv)





(v) (vi)



(vii)

Fig 4.9 (i)-(vi) represents sand containing bacterial culture and (vii) represents control sand without bacterial culture

4.2 Physiological characterization

4.2.1 Physiological characterization of bacteria

All the bacterial were found to be rod shaped, Gram negative and formed pinkish colonies on sea water agar media containing urea. All isolates were oxidase positive and only A1-4 showed positive nitrate reduction test other strains showed negative test.

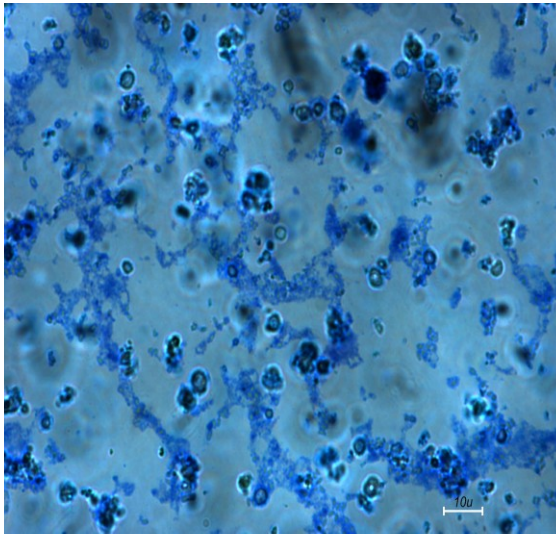
4.2.2 Carbohydrate fermentation test

The ability to ferment various carbohydrates by these bacteria was determined. Majority of the isolates were able to ferment different carbon substrates (Table 4.6). A3-5 and A3-6 were able to ferment two carbon sources, A3-13 and A1-4 fermented three carbon sources.

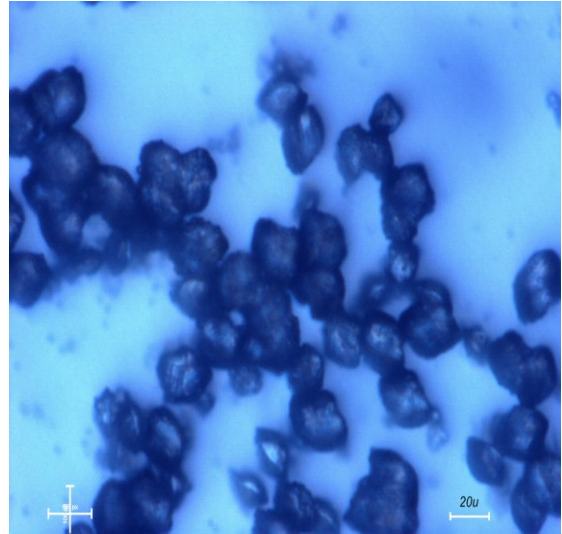
Table 4.6 Biochemical characterization of the bacterial isolates from different sources

Bacterial Isolates	Gram Staining	Shape	Oxidase	Nitrate Reduction	Fermentation of glucose	Fermentation of mannitol	Fermentation of sucrose
A3-5	-	Rod	+	-	+	-	+
A3-6	-	Rod	+	-	+	+	+
A1-4	-	Rod	+	+	+	+	+
A3-13	-	Rod	+	-	+	+	-

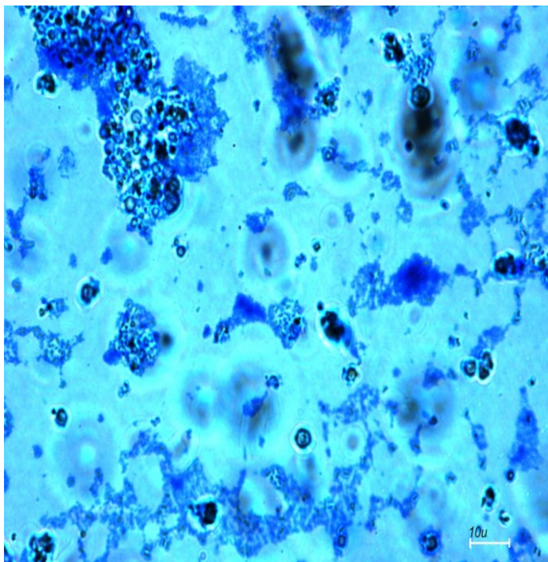
4.2 Microscopic studies of crystal morphology



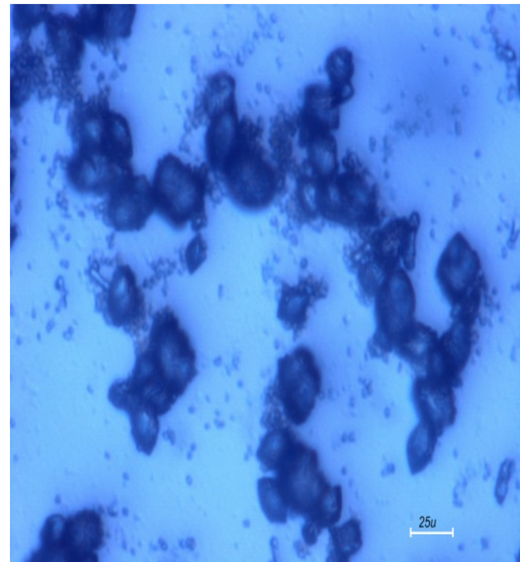
(i)



(ii)



(iii)



(iv)

Fig 4.10 (i)-(iv) represents crystal staining with methylene blue

4.4 Molecular characterization of bacteria

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). The different bacterial isolates selected were identified on the basis of molecular properties.

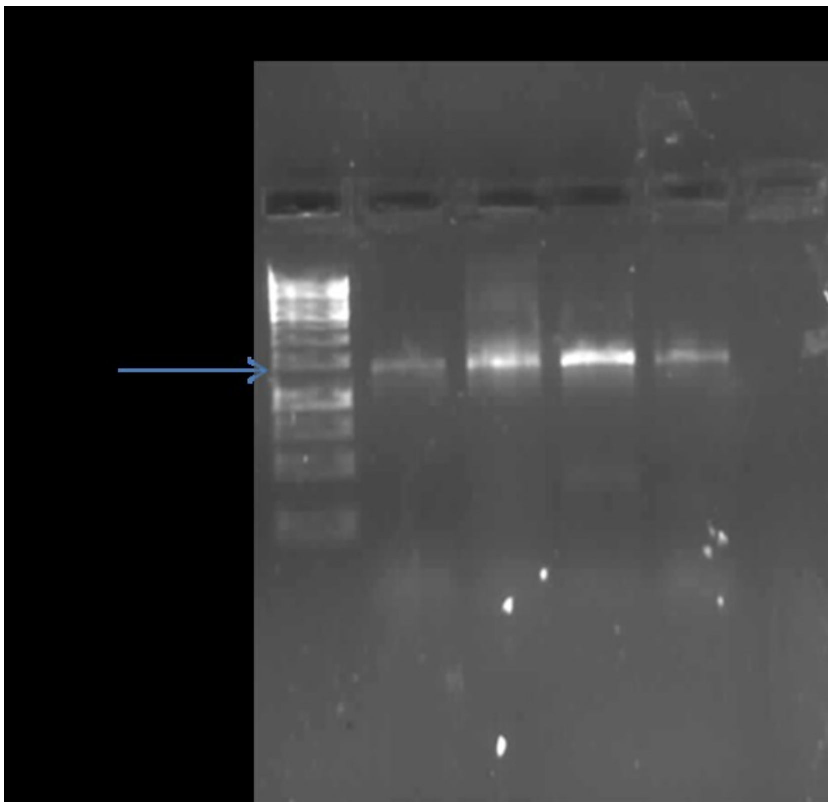


Fig 4.11 16S amplification in lane 1, 2, 3 and 4. Lane 1 contains 16S rDNA product of A3-5, lane 2 of A3-6, lane 3 of A1-4 and lane 4 contains A3-13

Restriction digestion pattern: The 16S amplified products of the 4 strains were restricted digested with two enzymes *AluI* and *RsaI* and following restriction pattern was observed showing that A3-6 and A1-4 strains are of different species and strains A3-5 and A3-13 are of same species due to their same restriction pattern.

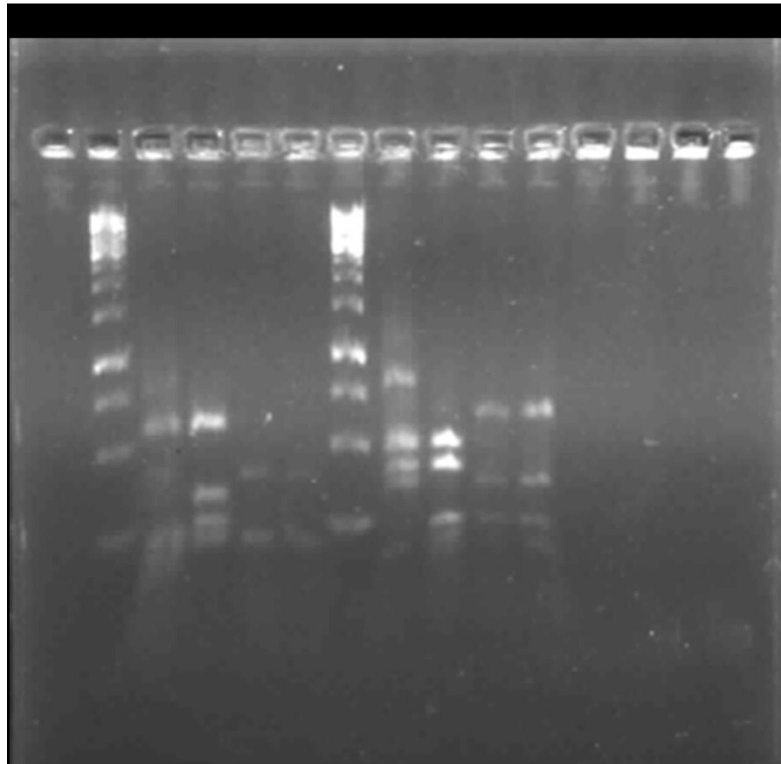


Fig 4.12 Restriction digestion pattern of A3-6, A1-4, A3-5, A3-13 by *AluI* in lanes 1,2,3,4 and restriction digestion by *RsaI* in lanes 5,6,7,8

Chapter 4

Discussion

Urease and carbonic anhydrase enzymes play a key role in microbiologically induced calcium carbonate precipitation. The optimum pH for the growth of halophiles is 7.5. Thus all the experiments to determine urease activity, carbonic anhydrase, growth, change in pH and to study calcium carbonate precipitation were done at pH 7.5.

By comparing the growth of halophiles in sea water media of varying saline concentrations, it was observed that halophiles were able to survive at high salt concentrations. More growth till the fourth day was observed in media having high salt concentration but in media with low salt concentrations the growth declined till fourth day. A plausible reason for such an observation could be attributed to the mineral precipitation known to reduce the cell surface area available for nutrient uptake leading to loss of ATP generation capacity and results in cell death (Southam, 2000). Since the maximum urease activity till the fourth day was observed in low salt concentrations, so the growth decined till fourth day. But in case of high salt concentrations, growth increased till fourth day.

Maximum urease activity was observed in sea water media of low salt concentrations containing 2% urea and 25mM $CaCl_2$ after 4 days incubation. Moreover an increase in urease activity during stationary phase and decline phase of cell growth also indicates that urease was active in degrading urea even after the cells stop dividing. Maximal urease activity after 4 days incubation by A3-5and A3-13 strains was observed in sea water media of 3.6% salt concentration. A3-6 and A1-4 strains showed increase in urease activity till fourth day in sea water media of salt concentration 3.6%, 5% and 7.5% but in 10% concentration sea water media decline in urease activity till fourth day was observed by all four strains. With the increase in urease activity, pH of the media increases due to the release of NH_3 by hydrolysis of urea.

Carbonic anhydrase enzyme catalyzes the reversible conversion of carbon dioxide to bicarbonate ions, which would then be available for calcium carbonate precipitation. In our studies it was observed that carbonic anhydrase production was high till fourth day in sea water media of salt concentrations 3.6% and 5% but in 7.5% and 10% salt concentration enzyme activity decreased till the fourth day. The results are similar to results of Botre and Botre (1989) who studied the synergistic effect of urease and carbonic anhydrase in calcite

precipitation and reported increase in the rate of removal of carbon dioxide facilitated by carbonic anhydrase increases the rate of production of ammonia consequent from urea dissociation.

Due to high urease and carbonic anhydrase enzymatic activities in low salt concentration, maximum calcification was also observed in low salt concentrations. As the salt concentration increases, enzymatic activity and biocalcification decreases. The greater difficulties of biomineralization with increasing salinity may be due to excess of salts having negative influence on calcium carbonate precipitation by bacteria (Billy, 1980; Ferrer *et al.,*1988, Rivadeneyra *et al.,* 1985, 1991, Raz *et al.,* 2000).

Sea water media of 3.6% salt concentration with 2% urea and 25mM $CaCl_2$ was found to be optimum for urease production hence this media was used for studying MICCP in sand columns. Maximum calcite precipitation was observed in the surface layer of sand columns. This may be due to highly aerobic nature of the bacterium. Calcite content in middle and lower layer was found to be less. The results of sand plugging showed that all columns were found to be tightly packed except the control sand column. To determine the presence of microbial calcite precipitation, sand consolidated samples were examined under SEM. The presence of crystalline calcite associated with bacteria indicates that bacteria served as nucleation sites during the mineralization process (Stocks- Fischer *et al.,* 1999).

Crystal morphology was done by staining with methylene blue and crystals were found to be of spherical, triangular and rhomboidal shapes.

Physiological and molecular characterization of bacteria was done. The halophiles were found to be gram negative, rod shaped, oxidase positive, all the other three strains were nitrate reductase negative (except A1-4) , able to ferment glucose, mannitol (except A3-5) and sucrose (except A3-13).

RFLP of all the strains was done with *AluI* and *RsaI* enzymes and was concluded that A3-6 and A1-4 strains are of different species and A3-5 and A3-13 strains belong to same species. Further characterization of the isolates will be done by 16S rDNA sequence analysis.

Conclusion

Disadvantageous impacts of conventional surface treatments necessitate however the development of biodeposition technology. According to the results it can be concluded that urease and carbonic anhydrase enzymes play a key role in microbiologically induced calcium carbonate precipitation. Halophilic bacteria were found to show urease, carbonic anhydrase activity and calcite precipitation, which could be used in the remediation of cracks in buildings near the sea coast. Salt concentration has a profound effect on calcium carbonate precipitation. As salt concentration increases, enzyme activity and biocalcification decreases. Bacterial isolates were found effective in showing microbiologically induced calcite precipitation on the surface of sand samples. SEM analysis evidenced the direct involvement of microorganisms in $CaCO_3$ precipitation. The calcium carbonate crystals were found to be of spherical, triangular and rhomboidal shapes after staining with methylene blue.

Results suggest that bacteria not only act as agents of geochemical change that induce passive biomineralization, but their cell surfaces act directly as highly effective nucleation templates for authigenic precipitation to occur.

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