Potential of extracellular polymeric substances and biofilm produced by bacterial isolates in calcium carbonate mineralization

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in

Microbiology

Under the Guidance of

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled **"Potential of extracellular polymeric substances and biofilm producing bacterial isolates in calcium carbonate mineralization."** in partial fulfilment of the requirement for the award of Master of Science, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period of six months from January 2014 to July 2014, under the supervision of Dr. M Sudhakara Reddy, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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This is to certify that above statement made by the candidate is correct and true to the best of our knowledge.

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CERTIFICATE

This is to certify that the dissertation entitled "**Potential of Extracellular Polymeric Substances and Biofilm producing Bacterial Isolates in Calcium Carbonate Mineralization.**" Submitted by Amrita Bains in partial fulfilment of the requirement for the award of Master of Science, Department of Biotechnology, Thapar University, Patiala, is an authentic record of student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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ABSTRACT

Microbially induced calcium carbonate precipitation (MICCP) is a biomineralization process. It has emerged as a novel and eco-friendly technology for protecting consolidating decayed constaruction materials. Various factors including presence of nucleation site on bacterial surface affects the microbially induced calcite precipitation. Bacterial surface formed of extracellular polymeric substances (EPS) plays a very important role in capturing calcium carbonate and resulting in precipitation. In the present study, best ureolytic alkalophilic bacterial isolate (*Bacillus megaterium*) was screened and grown in best nutritive media (Specific media) for the maximum production of extracellular polymeric substances. *B. megaterium* showed highest production of EPS in presence of urea and calcium chloride. EPS production came out to be 4881 µg/100ml. Specific media was optimized by one factor at a time approach (OFAT) by taking variable carbon, nitrogen, calcium source and pH profile for maximum EPS production and enhancing calcium carbonate precipitation. EPS production by *Bacillus megaterium* was maximum in media with glucose as carbon source (3651 µg/100ml); peptone as organic (6661 µg/100ml) and ammonium sulphate (4365 µg/100ml) as inorganic nitrogen source, calcium chloride as calcium source (4192 µg/100ml) at pH 7.0 with value of 3762 µg/100ml. EPS and calcium carbonate crystals produced by *B. megaterium* were characterized by staining, scanning electron microscopy (SEM), X-ray diffraction (XRD) and fourier transform infra red (FTIR) spectrum analysis. SEM analysis of EPS revealed smooth and rough surface in nutrient broth and specific media whereas SEM analysis of crystals depicted the shape and size of calcite crystals in smooth, circular, triangular forms in nutrient broth and rhombohedral in specific media. XRD and FTIR analysis of EPS and calcium carbonate crystals depicted the presence of functional groups on their surface. In case of, XRD of EPS of *B. megaterium* in nutrient broth and specific media the highest peak of carbon and calcium ions were depicted at 45 and 56° 2theta positions whereas in case of XRD of calcite crystals of *B. megaterium* in nutrient broth and specific media the highest peak was observed at 30° 2theta positions. FTIR analysis of EPS of *B. megaterium* in nutrient broth and specific media was showing highest absorption peaks at λ=1400 cm^{-1} and λ=1000 cm^{-1}, whereas in case of FTIR analysis of calcite crystals of *B. megaterium* in nutrient broth and specific media the highest absorption peak was observed at λ=800 cm^{-1} and λ=1000 cm^{-1}. It was concluded that extracellular polymeric substances (EPS) produced by alkalophilic ureolytic *Bacillus megaterium* has the potential for efficient calcium carbonate precipitation and hence can be serve as new remediation tool in MICCP.

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ABBREVIATIONS

Abbreviations	Word (s)
µg	micro gram
µl	micro liter
µM	micro moles
mg	milli gram
ml	milli liter
g	gram
°C	degree celcius
rpm	rotation per minute
min	minute
hrs	hours
w/v	weight by volume
v/v	volume by volume
OD	optical density
EPS	Extracellular polymeric substance
CPS	Capsular polysaccharides
PMSF	Phenyl methyl Sulphonyl fluoride
SEM	Scanning electron microscopy
XRD	X-ray diffraction
FTIR	Fourier transform infra red
OFAT	One factor at a time
MICCP	Microbially induced calcium carbonate precipitation

CHAPTER 1

INTRODUCTION

1.1. General introduction

Over so many years, priorly prokaryotes and then eukaryotes developed the ability to form minerals. Microorganisms have some properties of interest in various biotechnological applications. They hold promise for diverse and multidisciplinary fields like biotechnology, geotechnology, paleobiology to civil engineering. Bacteria are the primary agents for the geochemical changes due to their high surface area to volume ratio, abundant distribution, evolutionary adaptiveness and incredible diverse enzyme and nutritional possibilities (Warren & Hack, 2001). Geomicrobiology recognizes that microorganisms are important active and passive promoters of redox reactions that can influence "biomineralization" (Ehrlich, 2002).

Biomineralization is the process which leads to the formation of biominerals by the action of microorganisms. Biominerals can be present in the form of beautiful corals, ant hills, caves, shells of molluscs, teeth, bones and rocks.

Fig.1.1. Biomineralization of calcium carbonate in natural environment (a) Corals, (b) Ant hills, (c) Limestone Caves, (d) Molluscs (www.wikipedia.org).

Biomineralization is a universal process occurring in both prokaryotes and eukayotes. The term biomineralization was first coined by Japanese researcher in 1940s. The biominerals formed by biomineralization could be silicates in algae, and diatoms, carbonates in invertebrates and calcium, phosphate, and carbonates in vertebrates (Dhami et al., 2013). Bacteria is capable of forming crystals either intracellularly or extracellularly (Konishi et al., 2006; Langley et al., 1999).

Minerals formed via biomineralization includes Fe, Mn, & other metal oxides like ferrihydrite ($5Fe_2O_3.9H_2O$), hematite (α-Fe_2O_3), metal sulfates, phosphates, carbonates etc. (Dhami et al, 2013). Carbonates are the most common minerals formed amongst all. Carbonate minerals are considered as most reactive minerals as they are involved in the process of dissolution and precipitation. Calcium carbonate (calcite) is the most widespread minerals on the earth. It is naturally found in sedimentary rocks, limestones, marbles, calcareous stones in oceans and terrestrial environment (Klein & Hurlbut, 1999). It has been shown that in eukaryotic organisms, mitochondria (or chloroplast) are the cellular organelles which play an important role in the production of carbonate shells. As these organelles are considered as primitive endosymbiotic bacteria which supports the bacterial contribution to carbonate precipitation (Castanier et al., 2000).

Naturally seen structures like limestone caves, corals, ant hills etc all are the result of biomineralization of calcium carbonate. These structures are not only highly durable but also posses the property of self-healing. These structures remain intact even at extreme weather conditions as they are unaffected by natural environmental effects. This occurs through the deposition of calcium carbonate crystals by the activity of microorganisms. These deposits acts as binders between the loose substrate particles and reduce the pores inside the particles. This idea lay down the base for the strength and durability of any building material that can be enhanced by microbial deposits.

In the present era, the degradation of building materials is the major topic of concern. The durability and structural safety is important while designing building or concrete structures. Broad ranges of products are available in the market for protection of concrete surfaces like organic coatings consisting of volatile organic compounds (Basher et al., 1997; Ibrahim et al., 1997; Basher et al., 2006). Surface treatment of concrete with water repellents like epoxy, pore blockers and synthetic agents like silanes are also available but have various

disadvantages i.e. degradation with time, constant maintenance & environment pollution (De muynck et al., 2006).

The drawbacks of conventional treatments have invited the usage of novel, eco-friendly, self-healing and energy efficient technology where microorganisms are used for remediation of building materials and enhancement in the durability characteristics. This technology of using microbes for calcium carbonate deposition or microbial concrete, called as microbially induced calcium carbonate precipitation (MICCP), may bring new approaches in the construction industry.

Microorganisms and microbially mediated mineralization process is active in every environment on earth. Under natural conditions, the carbonate precipitation occur very slowly so in order to produce large amount of carbonates in short span of time there is a need to look for the microbes which have the ability to create conditions for carbonate precipitation in short period of time. Different bacterial species have the ability to precipitate carbonates in alkaline environment rich in Ca^{2+} ions. Alkaline bacteria are diverse in nature but *Bacillus* sp. have the ability for calcite precipitation in alkaline environment. Various mechanisms have been proposed in which precipitation could be induced by bacteria in natural habitats (Ehrlich, 1996; Rivadeneyra et al., 2004). The role of bacteria and bacterial activities in carbonate crystallization falls under 3 categories:

1.1.1. As a first hypothesis, mineralization is a byproduct of microbial metabolism which involves either autotrophic or heterotrophic pathways (Rivadeneyra et al., 1994; Douglas & Beveridge, 1998; Castanier et al., 1999; Lian et al., 2006). In the passive processes, enzymatic reactions like urea hydrolysis or dissimilatory nitrate and sulphate reduction, causes pH increase thus shifting the bicarbonate-carbonate equilibrium towards the production of more CO_3^{2-} ions and ultimately the $CaCO_3$ precipitation, if free Ca^{2+} ions are present. Alternatively, $CaCO_3$ precipitation can be controlled by intracellular calcium metabolism rather than changing CO_3^{2-} concentration (Anderson et al., 1992; McConnaughey & Whelan, 1997; Hammes & Verstraete, 2002).

1.1.2. As a second hypothesis, carbonate nucleation takes place on the cell wall, either through the cell membrane (i.e. active process given by Castenier et al., 2000) or due to some specific negatively charged functional groups that absorb divalent cations, Ca^{2+}, (Schultze-Lam et al., 1996; Rivadeneyra et al., 1998). These Ca^{2+}-cell wall

interaction modifies the cell wall thus allowing interactions between differently charged bacteria. As a result of these interactions there is a change in the overall ionic charge, bacteria aggregrate to magnify the size of biomineral and in turn bacteria become the nucleus of the biomineral (Ferrer et al., 1988; Rivadeneyra et al., 1996, 1998; Zamarreno et al., 2009).

1.1.3. The third hypothesis involves the extracellular polymeric substances [i.e. exopolysaccharides (EPS) and capsular polysaccharides (CPS)]. These substances produced by bacteria have some implications in various processes such as the formation of structure and architecture of biofilm matrices and calcium carbonate precipitation (Ercole et al., 2007; Decho, 2009).

However, it has been noticed that bacterial surface also plays an important role in calcium precipitation (Fortin et al., 1997). Prokaryotic (Bacteria & Archae) and eukaryotic (Algae & Fungi) microorganisms have the property of producing extracellular polymeric substances (EPS). EPS is a complex material composed of various biopolymers. EPS is a hydrated gel matrix in which microbial cells can establish synergistic consortia. EPS matrix serve to determine properties like cohesion, adhesion, morphology, structure, biological functions, mechanical strength, stability, diffusion etc. EPS represents the 'house of biofilm cells'. EPS comprises of proteins, polysaccharides, nucleic acids. Microorganisms are embedded in the matrix of EPS. These aggregated forms of microorganisms are called as biofilm. Biofilms containing mixed populations of microorganisms are ubiquitously distributed in natural soil, aquatic environment, on plant and animal tissues, as well as in technical systems like pipelines, porous materials, reservoirs, separating materials etc. EPS creates a sessile environment for the carbonate precipitation in which precipitation takes place on the external surface of bacterial cell by successive stratification.

Carbonatogenesis has various implications for-

1. Atmospheric CO_2 fixation through carbonate sediment formation and lithification (Krumbein, 1979; Monger et al., 1991; Folk, 1993).
2. Solid-phase capture of inorganic contaminants (Warren et al, 2001).
3. Pathological formation of mineral concretions such as gallstones and kidney stones in humans (Keefe, 1976).

4. Possibility of understanding extraterrestrial processes such as martian carbonate production by bacteria (Mckay et al., 1996; Thomas-Keprta et al., 1998).

The production of high amounts of carbonates by bacterial cells is dependent on high urease and EPS production. Their production is highly influenced by media components like carbon source, nitrogen source, inoculum concentration, calcium source, pH and other physical parameters. The conventional approach for optimizing analytical methods in the laboratory is the one-factor-at-a-time approach (OFAT), where each experimental factor or parameter is optimized separately and independently of other factors. In contrast, factorial designs involve simultaneous optimization of all factors at once. Factorial designs offer a simple, efficient, and statistically valid method for optimizing analytical methods.

Though bacterial extracellular polymeric substances has been shown to play a major role in calcium carbonate precipitation by many researchers. But not much work has been done on contribution of these extracellular molecules in bacterially induced calcium carbonate precipitation. So it is imperative to study and characterize these extracellular polymeric substances (EPS) and investigate its role in calcium carbonate precipitation.

1.2. Objectives

- Screening of ureolytic and calcifying bacteria for production of extracellular polymeric substances (EPS)
- Optimization of media components for EPS and calcium carbonate production
- Characterization of extracellular polymeric substances and calcium carbonate crystals.

CHAPTER 2

REVIEW OF LITERATURE

2.1. BIOMINERALIZATION

Biominerals are present everywhere. Biominerals refers to the minerals produced by microorganisms and also to the composite mineralized products comprised of both minerals and organic components. The study of biomineral formation is perhaps fascinating and is called as biomineralization. In early 1980's this discipline was called as 'calcification' reflecting the predominance of biologically formed calcium containing minerals. As the time progress more and more biogenic minerals were discovered that contained other cations, this field came to known as 'biomineralization'. The first book on biomineralization was published in 1924 in german by W.J.Schmidt (Schmidt, 1924).

Biomineralization is an important and ubiquitous process resulting in biomineral formation by a range of taxa i.e. from Bacteria and Archae to Eukarya (Lowen stam and Weiner, 1989; Mann, 2001; Gower, 2008; Maldrum and Colfen, 2008; Dittrich and Sibler, 2010). . Biomineralization is defined as the biologically induced process in which an organism creates a local micro-environment with conditions that allow optimal extracellular chemical precipitation of mineral phases (Hamilton, 2003).

The process of biomineralization can be either intracellular or extracellular. The synthesis of minerals by prokaryotes is broadly classified into two classes:-

a. Biologically induced mineralization (BIM)
b. Biologically controlled mineralization (BCM) (Lowenstam, 1981; Lowenstam & Weiner, 1989).

Microorganisms play an active role in creation of biominerals. Biologically controlled mineralization (BCM) occurs as a result of direct synthesis of minerals at a specific location within or on the cell and only under certain conditions. BCM occurs intracellularly, lipids, polysaccharides, proteins etc. makes a suitable matrix for cations to condense and minerals to grow in a constrained space. Examples of controlled biomineralization are magnetite

formation in magnetotactic bacteria (Bazylinski *et al*., 2004) and silica deposition in unicellular algae coccolithophores and diatoms, respectively (Barabesi *et al*., 2007).

Whereas in case of biologically induced mineralization (BIM), minerals generally nucleate and grow extracellularly as a result of organismal metabolic activities and subsequent chemical reactions involving metabolic byproducts. BIM appears to be more important and depends on environmental conditions such as pH, temperature etc. This process tends to produce amorphous and poorly crystalline minerals. Bacterial surfaces such as cell walls or polymeric materials (exopolymers) exuded by bacteria including slimes, sheaths, or biofilms, and even dormant spores can act as important sites for adsorption of ions, mineral nucleation and growth (Beveridge, 1989; Konhauser, 1998; Banfield and Zhang, 2001; Bäuerlein, 2003). Boquet *et al.* (1973) reported that almost all bacteria are capable of $CaCO_3$ precipitation while Knorre and Krumbein (2000) concluded Microbially induced calcium carbonate precipitation (MICCP) as a by product of microbial metabolic processes.

Calcite ($CaCO_3$) precipitation is a well-known example of extracellular bacterial biomineralization. Certain species of marine bacteria have been shown to precipitate minerals in water supplemented with artificial marine salt media and differing ratios of Mg^{2+} and Ca^{2+} concentrations (Rivadeneyra et al., 2006). Currently Researchers and engineers have great interest in the development of mimic materials based on biomineralization (Aizenberg et al., 2003).

2.2. MICROBIALLY INDUCED CALCIUM CARBONATE PRECIPITATION (MICCP)

Microbially induced calcium carbonate precipitation is a wide subject that comes under the category of science called biomineralization. It is a process by which living organism forms inorganic minerals as a part of their basic metabolic activities. MICCP is highly desirable because calcium carbonate precipitation occurs as a result of microbial activities (Achal et al., 2011a).

Carbonate minerals are represented by the chemical formula $CaCO_3$ (Deer et al, 1960). Ca is the cation of choice for most of the organisms. About 50% of biominerals are comprised of Ca^{2+} ions. The $CaCO_3$ precipitation occurs mainly by abiotic as well as biotic pathways. In abiotic pathway, precipitation occurs in supersaturated solutions through evaporation,

temperature increase and pressure decrease. (Castenier et al., 1999). Biotic precipitation can be either bioticaly controlled or bioticaly induced (Frankel & Bazylinski, 2003).

For calcification and polymerization to take place ambient conditions are required as can be seen from sustainability of ant hills and coral reefs. These structures are highly durable and possess the property of self-healing. This occurs through the application of microorganisms which deposits carbonates. These deposits are commonly called as $CaCO_3$ crystals (calcite crystals, microbial concrete) act as binders between loose substrate particles and reduce the pores inside the substrate particles (Dhami, 2013). The technology of using microbes for $CaCO_3$ deposition or microbial concrete is called as MICCP (microbially induced calcium carbonate precipitation). MICCP has been developed as a potential technique to remediate & restore various building materials & enhancement in durability characteristics. This technology is novel, eco-friendly, have self-healing property, energy efficient. This technology may bring new approaches in construction industries (Dhami et al., 2012a).

MICCP involves various research disciplines such as geology, crystal chemistry, aquatic chemistry, microbial ecology & microbial technology (Mckenity & Sellwood, 1999). MICCP done by microorganisms has been observed in wide range of natural environment. It has been widely studied in saline aquatic environment, fresh water environment, saline soils (Basker et al., 2005; Novistky, 1981; Rivadeneyra et al., 2004).MICCP has various applications in:

i. Remediation (fixation) of metal contaminated soil and ground water (Warren et al. 2001).
ii. Consolidation and restoration of various building materials (Dhami et al. 2012a), atmospheric CO_2 sequestration (Mitchell & Ferris, 2006).

Number of applications involving MICCP have been attempted in various fields as in the removal of heavy metals (Warren et al., 2001; Wu et al., 2011; Achal et al., 2011, 2012) and radionuclides (Fujita et al., 2004), removal of calcium from wastewater (Hammes et al., 2003), biodegradation of pollutants (Simon et al., 2004; Chaturvedi et al., 2006), remediation (fixation) of metal-contaminated soil and groundwater (Warren et al., 2001; Mitchell and Ferris, 2006), polychlorinated biphenyl removal (Okwadha and Li, 2011) and atmospheric CO_2 sequestration (Dupraz et al., 2009; Ramanan et al., 2009; Shaffer, 2010; Mitchell et al., 2010; Sharma and Bhattacharya, 2010; Yadav et al., 2011).

Recently, bacterial precipitation of fluorescent calcium carbonate with potential applications as filler in rubber and plastics, in stationery ink, and as fluorescent marker, has also been reported (Yoshida et al., 2010).

MICCP is a research subject widely studied in past decades (Banfield & Hammes, 1997). Biologically induced precipitation of $CaCO_3$ is called as carbonatogenesis. This technology is highly desirable because calcite precipitation is induced as a result of microbial activities. It is pollution free and natural process. Carbonate precipitation by MICCP is achieved by different pathways including (Castenier, 2000):-

2.2.1. Autotrophic pathway-

Three different kind of bacteria are involved in autotrophic pathway. all of these bacteria obtain carbon as a source of energy either from gaseous or dissolved CO_2 (atmospheric eukaryotic & prokaryotic respiration, fermentation). The bacteria involved are:

i. **Non- methylotrophic methanogenesis**- it is carried out by methanogenic archaebacteria. Bacteria use CO_2 & CH_4 in strict anaerobic conditions to give CH_4 (Marty, 1983). These bacteria live deep in sediments. CO_2 and H_2 they use are the products of activity of fermentative bacteria.

ii. **Methylotrophic methanogenesis**- methylotrophic methanogenic bacteria uses organic matter rather than CO_2 & H_2 to release CH_4 products.

iii. **Anoxygenic photosynthesis-** it is carried out by either sulphurous or non-sulphurous purple & green photosynthetic bacteria. They do not produce O_2 & live in anaerobic conditions. They uses IR light as a source of energy (735-755nm for green bacteria & 800-1000nm for purple bacteria). Sulphur bacteria use H_2S as an electron donor. These bacteria live in anaerobic conditions. They may be found in gypsum deposits (Pfenning & Truper, 1989).

iv. **Oxygenic photosynthesis**- it is performed by cyanobacteria. It uses visible light as source of energy (680-685 nm) and produces oxygen (Hammes and Verstraete, 2002).

All the above processes use CO_2 as carbon source in the production of organic matter. This pathway induces CO_2 depletion either of the medium or of the immediate environment of the bacteria. When Ca^{2+} ions are present such depletion favours $CaCO_3$ precipitation.

2.2.2. Heterotrophic pathway- This pathway involves two types of precipitation:

i. **Active precipitation**-

Active carbonatogenesis is an independent process (Castanier, 1987; Castanier et al. 1997). Carbonate ions are produced by ionic exchange through the cell membrane by the activation of Ca/Mg ionic pumps or channels coupled with CO_3^{2-} ion production. The CO_3^{2-} precipitation environment comprises of high extracellular calcium ion concentration and low extracellular proton concentration. Figure2.1 schematises the probable events under such conditions. The combination of high extracellular alkaline pH and Ca^{2+} ions creates a stressfull condition for bacteria. The passive Ca influx due to complementary Ca^{2+}/ H^+ electrochemical gradient leads to high intracellular Ca^{2+} concentration and excessive proton expulsion.

At cellular levels, this could be detrimental due to the : disruption of intracellular Ca regulated signal processes, alkalisation of intracellular pH, depletion of H^+ pool (Norris et al., 1996). Survival of bacteria under such conditions require active export of intracellular Ca via ATP-dependent Ca pumps which would compensate the H^+ loss by reducing the intracellular Ca^{2+} ions. This event resulted in the localised increase in pH, due to H^+ uptake, in the same region Ca^{2+} ions also increases, this would form an ideal localised precipitation microenvironment (McConnaughey & Whelan, 1997).

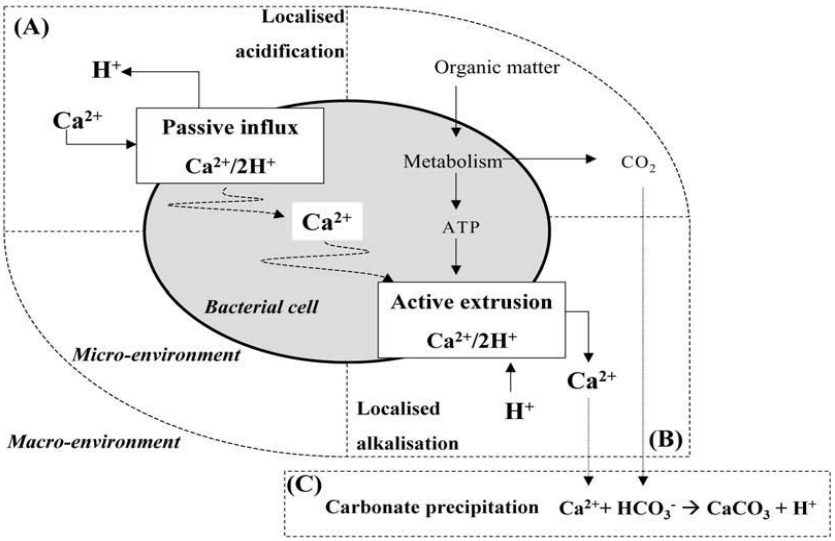


Fig. 2.1. Schematic presentation of suggested bacterial calcium metabolism and subsequent $CaCO_3$ precipitation under high-pH and high-Ca^{2+} extracellular conditions

ii. **Passive precipitation-**

Passive carbonatogenesis operates by producing CO_3^{2-} & HCO_3^- ions including various chemical modifications that would lead to $CaCO_3$ precipitation in the medium. Two metabolic cycles can be operated:-

A. **Sulphur cycle-** it is specifically a dissimilatory sulphate reduction, which is carried out by sulphate reducing bacteria (SRB). Precipitation via this pathway has been described to occur in seawater, geological formations (Peckman et al.1999) during the biological treatment of acid mine drainage (Kaufman et al. 1996). The reaction often starts with the dissolution of gypsum ($CaSO_4.2H_2O/CaSO_4$), which is a pure physicochemical process (Equation 1). Under these circumstances, organic matter can be consumed by SRB, and sulphide (H_2S) and metabolic CO_2 is released (Equation 2) (Peckman et al. 1999; Wright 1999).

$$CaSO_4.\ 2H_2O \rightarrow Ca^{2+} + SO_4^{2-} + 2H_2O \qquad (1)$$

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \qquad (2)$$

Castanier et al. (1999) further showed that removal of the produced hydrogen sulphide (H_2S) and the resultant pH increase is a prerequisite for carbonate precipitation to occur. In natural environments, sulphide can combine with iron to produce pyrite (Wright 1999), or it can be converted by anoxygenic sulphide phototrophic bacteria to elemental sulphur (Castanier et al. 1999). In several of the described examples for this pathway, dolomite and aragonite, instead of calcite, seems to be the most predominant forms of calcium carbonate to precipitate (Warthmann et al. 2000; Peckman et al. 1999; Wright, 1999).

B. **Nitrogen cycle -** this passive bacterial precipitation follows three different pathways:

- Ammonification of amino acids (aerobic process, in the presence of organic matter & Ca).
- Dissimilatory reduction of nitrates (anaerobic/microaerophilic process, in the presence of organic matter & Ca^{2+} & NO_3^-).
- Degradation of urea & uric acid (aerobic process, in the presence of organic matter & Ca^{2+} & urea/uric acid).

This pathway is more often associated with precipitation in soils and geological sediments (Fujita et al. 2001), as well as precipitates linked to the urinary tract (Kramer et al. 2000) (Equation 3). These three pathways induce the production of metabolic carbonate ions (CO_3^{2}) bicarbonate ions (HCO_{3-}) & ammonium ions (NH_4^+) which induces the increase in pH (Equation 4). When H^+ concentration decreases, the CO_3^{2-}—HCO_3^- equillibrium shifts towards the production of more CO_3^{2-} ions. In the presence of calcium ions, the $CaCO_3$ precipitation occurs. If divalent Ca^{2+} ions are lacking in the medium, the CO_3^{2-} and HCO_3^- ions accumulate resulting in pH increase as a result bacterial activity may favour the zeolite formation (Castanier et al., 1993). Urea or uric acid hydrolysis via the urease enzyme is the simplest model and has often been used in technological applications of MICCP (Stocks-Fischer et al., 1999). The reaction takes place according to the following equations (Wright, 1999):

$$CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2NH_3 \qquad (3)$$

$$2NH_3 + CO_2 + H_2O \rightarrow 2NH_4^+ + CO_3^{2-} \qquad (4)$$

2.3. MECHANISM OF MICCP

Bacteria from various natural habitats have been reported to be able to precipitate calcium carbonate both in natural and in laboratory conditions (Krumbein, 1979; Rodriguez et al., 2003). Different types of bacteria, as well as abiotic factors (salinity and composition of the medium) contribute in a variety of ways for calcium carbonate precipitation to occur in a wide range of different environments (Knorre & Krumbein, 2000; Rivadeneyra et al., 2004).

$CaCO_3$ precipitation requires sufficient calcium and carbonate ions so that the ion activity product (IAP) exceeds the solubility constant (K_{so}) (Eqs. (5) and (6)). From the comparison of the IAP with the K_{so} the saturation state (Ω) of the system can be defined; if $\Omega > 1$ the system is oversaturated and precipitation is likely (Morse, 2003):

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

$$\Omega = \alpha(Ca^{2+})\alpha(CO_3^{2-})/ K_{so} \text{ with } K_{so,calcite,25^\circ} = 4.8x10^{-9} \qquad (6)$$

The concentration of carbonate ions is related to the concentration of dissolved inorganic carbon (DIC) and the pH of a given aquatic system. In addition, the concentration of DIC depends on several environmental parameters such as temperature and the partial pressure of carbon dioxide (for systems exposed to the atmosphere). The equilibrium reactions and

constants governing the dissolution of CO_2 in aqueous media (25°C and 1 atm) are given in Eqs. (7)–(8) (Stumm & Morgan, 1981):

$$CO_2\ (g) = CO_2\ (aq)\ (pK_H = 1.468) \quad (7)$$

$$CO_2\ (aq.) + H_2O = H_2CO_3^*\ (pK = 2.84) \quad (8)$$

$$H_2CO_3^* = H^+ + HCO_3^-\ (pK1 = 6.352) \quad (9)$$

$$HCO_3^- = CO_3^{2-} + H^+\ (pK2 = 10.329) \quad (10)$$

$$H_2CO_3^* = CO_2\ (aq.) + H_2CO_3 \quad (11)$$

Microorganisms can influence precipitation by altering the precipitation parameters, either separately or in various combinations with one another (Hammes & Verstraete, 2002).

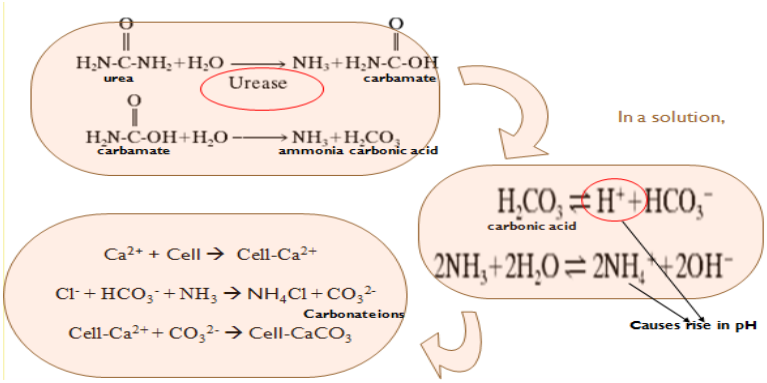


Fig. 2.2. Mechanisms of MICCP in media (www.wikipedia.org)

2.4. FACTORS AFFECTING MICCP

Calcium carbonate precipitation is a straight forward chemical process governed by various key factors:

2.4.1. Calcium concentration- calcium concentration plays major role in carbonate precipitation. More than half of the biominerals discovered yet has been found to be composed of calcium carbonates. Due to its cationic nature binds easily to the negatively charged carbonate ions and results in the formation of calcium carbonates. This activity is enhanced by microbes at alkaline pH in the process of biomineralization (Hammes & Verstraete, 2002).

2.4.2. Concentration of Dissolved Inorganic Carbon (DIC)- DIC($C_{T)}$ is the sum of inorganic carbon species in the solution. It includes carbon dioxide, carbonic acid, bicarbonate anion and carbonate ion (Eqn. 12). All these carbon species plays an important role in enhancing the calcite precipitation. The concentration of carbonate ions is related to the concentration of DIC and DIC in turn depends on several environmental parameters such as temperature and the partial pressure of carbon dioxide (Stumm & Morgan, 1981).

$$C_T = [CO_2] + [HCO_3^-] + [CO_3^{2-}] \quad (12)$$

Each of these species are related by the following pH-driven chemical equillibria:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-} \quad (13)$$

2.4.3. pH- the solubility of $CaCO_3$ depends strongly on pH. Calcium carbonate precipitation occurs at high pH (10). It gives rise to alkaline conditions thus favouring the growth of alkalophiles and more carbonate precipitation. Ammonium and hydroxide ions increase the pH and results in carbonate precipitation. (Eqn. 14)

$$NH_3 + H_2O \longrightarrow NH_4^+ + OH^- \quad (14)$$

The lower the pH, more soluble is the $CaCO_3$. At lower pH, bicarbonate form (HCO_3^-) predominates and at higher pH, more & more of the carbonate form (CO_3^{2-}) exists. As the pH changes, so does the amount of CO_3^{2-} ions in the solution. At higher pH, the solubility of $CaCO_3$ lowers (Whiffin, 2004).

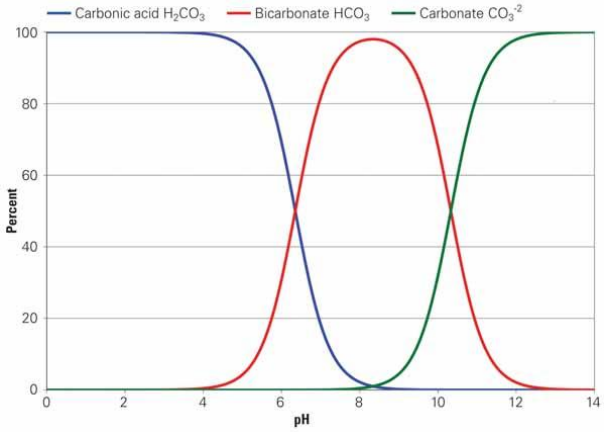


Fig.2.3.Chemical speciation of H_2CO_3, HCO_3^- and CO_3^{2-} at 25°C (Atkins, 2002)

2.4.4. Availability of nucleation sites- Bacterial surfaces play an important role in calcium precipitation (Fortin et al., 1997). Due to the presence of several negatively charged groups,

at a neutral pH, positively charged metal ions could be bound on bacterial surfaces, favouring heterogenous nucleation (Douglas,1998 ; Bauerlein, 2003). Commonly, carbonate precipitates develop on the external surface of bacterial cells by successive stratification (Pentecost & Bauld, 1988; Castanier et al., 1999) and bacteria can be embedded in growing carbonate crystals (Rivadeneyra e al., 1998; Castanier et al., 1999).

Possible biochemical reactions in urea-$CaCl_2$ medium to precipitate $CaCO_3$ at the cell surface can be summarized as follows:

$$Ca^{2+} + Cell \longrightarrow Cell\text{-}Ca^{2+} \quad (15)$$

$$Cl^- + HCO_3^- + NH_3 \longrightarrow NH_4Cl + CO_3^{2-} \quad (16)$$

$$Cell\text{-}Ca^{2+} + CO_3^{2-} \longrightarrow Cell\text{-}CaCO_3 \quad (17)$$

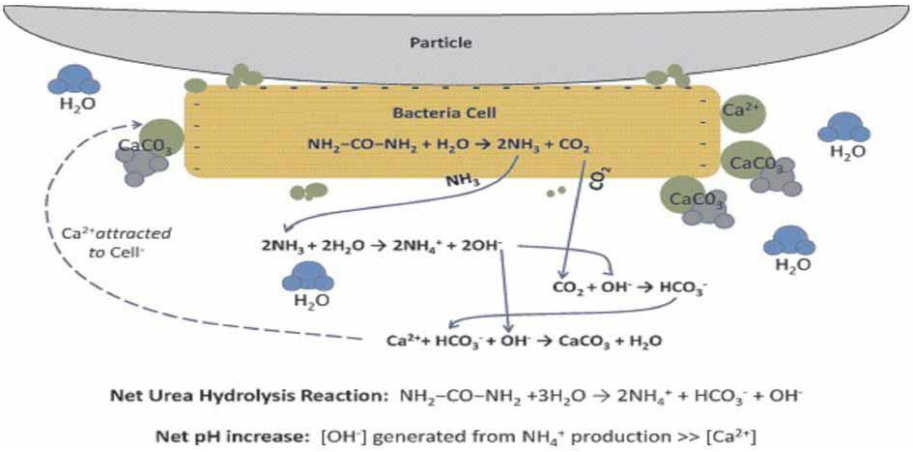


Fig.2.4. Bacteria serving as nucleation site for $CaCO_3$ precipitation (Source: De Jong et al., 2010)

2.4.5. Ca^{2+}/ CO_3^{2-} ratio- In the natural environments, carbonate precipitation theoretically occurs by increasing the concentration of calcium and/or carbonate in solution or by decreasing the solubility of calcium and/or carbonate. Bacterial cells have been shown to be excellent nucleation sites for growing of minerals as seen in the formation of rocks (Ferris *et al*., 1986; 1987). If excess calcium and carbonate is present, then it is possible for precipitation of calcium carbonate to begin, and it can continue until saturation is reached. In other words, if either (or both) calcium or carbonate ions are very high, then precipitation of calcium carbonate can be reduced.

2.4.6. Saturation Index- In order for precipitation to take place, supersaturation of the precipitating species must exist (Randolph and Larson, 1988). As the solubility product (*Ksp*) of calcite is extremely low (3.3 x 10-9 mol L-1 at 25°C), the supersaturation can be achieved straightforwardly (Sawada, 1998). The precipitation can be achieved by simply mixing together moderate concentrations of soluble Ca^{2+} and CO_3^{2-} ions. The difference between the

saturation concentration and supersaturation concentration forms one of the prime factors which controls the rate of precipitation (Bodek et al., 1988). In general, the higher the supersaturation concentration, the faster the rate and the smaller the crystals. The supersaturating product concentration and thus the degree of disequilibrium remains relatively low (compared to chemical example), which allows larger crystals to form over an extended period with higher cementation strength (Whiffin, 2004).

2.4.7. Enzymes-

2.4.7.1. Urease enzyme- Urease (UA; EC 3.5.1.5) is a nickel-containing metalloenzyme which is found in a wide range of microorganisms and plants, some of which produce this enzyme in large quantities (Mobley et al., 1989). Urease from Jack bean (*Canavalia ensiformis*) was the first enzyme to be crystallized and the first enzyme found to contain nickel (Sumner, 1926). A compound urea is widespread in the environment, a diverse section of the biota has evolved with the ability to hydrolyse urea, through the action of urease. The occurene of urease is found commonly in 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). The process of urea hydrolysis can be easily controlled, has the potential to produce high amounts of carbonates within a short period of time and is also energy efficient process (De Muynck et al. 2012; DeJong et al., 2010). During this process, enzymatic hydrolysis of urea by bacterial enzyme urease causes an increase in pH that shifts the bicarbonate–carbonate equilibrium towards the production of more CO_3^{2-} and ultimately leads to the precipitation of $CaCO_3$ (Castanier et al. 2000) (Eqns. 18-19).

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Bacteria}} NH_2COOH + NH_3 \quad (18)$$

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3 \quad (19)$$

These products equilibrate in water to form bicarbonate, 1mol of ammonium and hydroxide ions which give rise to pH increase (Eqns. 20-22).

$$H_2CO_3 \longrightarrow 2H^+ + 2CO_3^{2-} \quad (20)$$

$$NH_3 + H_2O \longrightarrow NH_4^+ + OH^- \quad (21)$$

$$Ca^{2+} + CO_3^{2-} \longrightarrow CaCO_3 \ (K_{sp} = 3.8\times10^{-9}) \quad (22)$$

K_{sp} is the solubility product in Equation 22.

2.4.7.2. Carbonic Anhydrase- Carbonic Anhydrase (EC4.2.1.1) is a zinc-containing metalloenzyme that is widespread in animals, plants and micro organisms (Karlsson et al., 1998; Smith and Ferry, 2000; Sharma et al., 2009). This enzyme facilitates the interconversion of CO_2 and H_2O to CO_3^{2-} and H^+ (Eqn. 24). It plays a role in $CaCO_3$ precipitation in the presence of free Ca^{2+} ions (Smith and Ferry 2000). Reaction catalyzed by CA:

$$CO_2 + H_2O \xleftrightarrow{CA} H_2CO_3 \quad (23)$$

$$HCO_3^- + H^+ \longrightarrow H_2CO_3 \longrightarrow CO_2 + H_2O \quad (24)$$

The enzyme employs two steps mechanisms:-

- There is a nucleophilic attack of a zinc-bound OH^- ions on CO_2.
- The active site is regenerated by the ionization of the zinc-bound H_2O molecule & the removal of a proton (H^+) from the active site. The active site can exist in two forms.
- At high pH- active in hydration of CO_2.
- At low pH- active in dehydration of bicarbonates (HCO_3^-)

2.4.8. Bacterial species- Some authors believe this precipitation to be an unwanted and accidental byproduct of the metabolism (Knorre and Krumbein , 2000) while others think that it is a specific process with ecological benefits for the precipitating organisms (Ehrlich,1996; McConnaughey and Whelan,1997). These bacteria include *Bacillus pasteurii*, *Bacillus megaterium*, *Bacillus cereus*, *Bacillus foraminis*, *Bacillus sphaericus*, *Bacillus psychrophiles*, *Bacillus globisporus*, *Pseudomonas* sp., *Variovorax* sp., *Leuconostoc mesenteroides*, *Micrococcus* sp., *Bacillus subtilis*, *Deleya halophila*, *Halomonas eurihalina* and *Myxococcus xanthus* (Mobley and Hausinger,1989 ; Rivadeneyra et al., 1991,1996, 1998; Ferris and Stehmeier,1992; Stocks-Fischer et al.,1999; Tiano et al.,1999; Castanier et al.,2000; Fujita etal., 2000; Rodriguez-Navarro et al., 2003).

2.4.9. Alkalinity- Calcium carbonate's solubility also depends strongly on the water's alkalinity. The higher the alkalinity (at a fixed pH), the more carbonate is present. In fact, the amount of carbonate present is directly proportional to the alkalinity. Lower calcium carbonate solubility at higher alkalinity implies that precipitation of calcium carbonate can be more extensive. In other words, as the alkalinity rises, the amount of calcium that can be kept in solution without precipitation decreases. Likewise, as the alkalinity is reduced, the amount

of calcium that can be kept in solution without precipitation is increased (Hammes and Verstraete, 2002).

2.4.10. Solubility of $CaCO_3$ - Calcium carbonate precipitates also dissolve naturally or under biological influence. The precipitation and dissolution processes are equilibrium reactions governed by solubility constants (Eqns. 25-27). Solubility constant for $CaCO_3$ is temperature dependant and defined as:

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3 \quad (25)$$

$$K_{so} = [Ca^{2+}][CO_3^{2-}] = 4.83 \times 10^{-9} \text{ (calcite, 25°C)} \quad (26)$$

$$IAP = (Ca^{2+})(CO_3^{2-}) \quad (27)$$

$$\Omega = IAP/K_{so}$$

IAP > K : over-saturated system (precipitation is likely)
IAP = K : equilibrium
IAP < K: under-saturated system (dissolution is likely)

2.5. EXTRACELLULAR POLYMERIC SUBSTANCES (EPS)

The contribution of both prokaryotic and eukaryotic microbial cells in mineral precipitation has widely been studied by researchers (Braissant et al., 2002; Castanier et al., 2000). Bacteria in common with all living organisms of other taxa can synthesize diverse structural, functional and valuable biopolymers. These biopolymers are referred to as extracellular polymeric substances. These diverse biopolymers are produced with varied chemical properties via utilization of simple to complex substrate (Anderson et al., 1990). With respect to cellular location, biopolymers could be either intracellular or extracellular. The intracellular biopolymers are few, however, there is a vast range of extracellular biopolymers and the extracellular biopolymers are composed of organic substances, including mostly polysaccharides and proteins, nucleic acids, lipids and uronic acids (Nichols & Nichols, 2005).

They can be present in two forms including exopolysaccharides (EPS) and capsular polysaccharides (CPS). EPS is also reffered to as “loosely bound extracellular polymeric substances” (slime type, free or released or in the form of free dissolved matter) whereas CPS is also refered to as “cell bound extracellular polymeric substances” (cell attached or capsular) (Nielson & Jahn, 1999; Bhaskar & Bhosle, 2005). These are located on the outermost surface of wide range of bacterial cells. In contrast to free or loosely bound EPS,

the CPS remain connected to the cell surface by means of a covalent attachment to phospholipid or lipid A molecules present on the gram-positive bacterial cell surface (Deng et al., 2000; Whitfield & Valvano, 1993). To differentiate between CPS and free EPS using the extent of attachment it has been seen that capsular polysaccharide may themselves be released into the growth medium (or become free) due to the low stability of phosphodiester linkage between the polysaccharides & the phospholipid membrane (Roberts, 1996; Troy et al., 1971).

Based on the monomeric composition EPS are divided into two groups- *homopolysaccharides* and *heteropolysaccharides* (Ruas-Madiedo,2002). Homopolysaccharides contain only one type monomers, these monomers are clustered into four groups- α-d-glucans, fructans & polygalactans (Stewart-Tull,1980) whereas heteropolysaccharides are composed of repeating units of d-glucose, D- galactose, L-rhamnose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) or glucuronic acid (GlcA) (Ruas-Madiedo et al., 2002).

EPS exist in a wide variety of unique and complex chemical structures. Hence, they are believed to provide self protection against anti microbial substances (Kumon et al., 1994). It has been shown that a potential controlling factor in EPS biosynthesis is the availability of sugar nucleotides which are necessary for the construction of polymers (Boels et al., 2001). EPS plays a crucial role in the bacterial colonization of surfaces by facilitating cell adhesions to biotic (i.e. epithelial & endothelial cells) and abiotic surfaces (i.e. mineral surfaces) and to each other (Beveridge & Graham, 1991; Bruno & Yves, 2002; Decho, 1990; Flemming, 1995). Adhesion of the cells to solid surfaces involves a combination of electrostatic interactions, hydrogen bond and London dispersion forces. This may result in the formation of 'Biofilm' & EPS- mediated interspecies co-aggregation within biofilms that can boost their individual potential for colonization of various ecological niches (Costerton et al., 1987; Mayer et al., 1999).

A Biofilm is defined as an aggregation of bacteria, algae, fungi, protozoa enclosed in an EPS matrix. Over 99% of microorganisms live within these biopolymers (Flemming, 2001b ; Sutherland, 2001). Biofilm has been dubbed as "city of microbes" & extracellular biopolymers as "house of the biofilm cells" (Schembri, M.A., 2004; Daegelen, P, 2009). Aside from polysaccharides, biofilm consists of proteins, nucleic acids, lipids. The composition & quantity of the EPS will vary depending on the type of microorganism, age of

biofilm & the different environmental conditions like different level of oxygen and nitrogen, extent of dessication, temperature, pH & availability of nutrients under which the biofilm exists (Mayer, C.; Moritz, R.; Flemming, H.C., 1999).

The growth of a biofilm is the result of a complex process as shown in figure 2.5, that involves the transport of organic & inorganic molecules and microbial cells to the surface, adsorption to the surface & finally an irreversible attachment aided by the production of EPS (Beech, 2004).

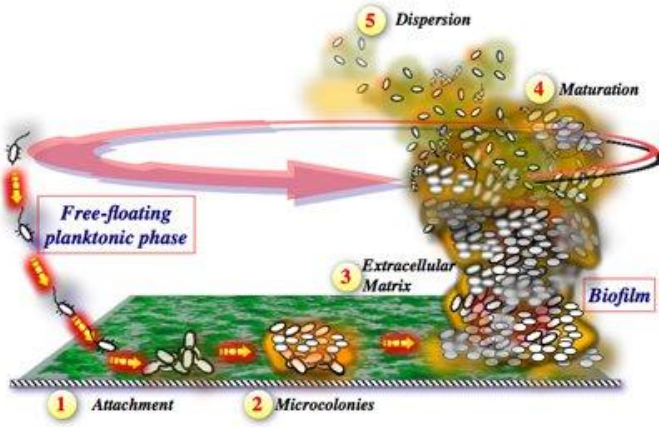


Fig. 2.5. Biofilm formation (Source Montana State University Center for Biofilm Engineering)

- ✓ *Attachment-* adhesion of few cells to solid substrate
- ✓ *Colonization*- proliferation of cells & imtercellular communication
- ✓ *EPS and BIOFILM formation*- growth & polysaccharide formation
- ✓ *Maturation*- more growth & polysaccharide formation
- ✓ *Dispersion*- detachment & release of cells to planktonic form

The most studied regulatory mechanism that has been found to control the production of EPS, biofilm formation and differentiation is quorum sensing (QS) regulation (Donlan, 2002; Ruiz et al., 2008; Waters et al., 2005; Von Bodman et al., 1998; Rivas et al., 2005; Davies et al., 1998). QS allows bacteria to maintain cell-cell communication. In gram-positive bacteria the communication is carried out with modified oligopeptide generating the signals and membrane-bound sensors histidine-kinase acting as receptors. Signalling is mediated by many phosphorylation steps which controls the activity of a response regulation (Waters et al., 2005; Miller et al., 2001).

Researchers have made attempts to investigate the role of bacterial biofilms and extrapolymeric substances in biomineralization (calcium carbonate precipitation) (Ercole et al., 2007; Decho, 2009; Dittrich & Sibler, 2010). Merz-Preiss and Riding (1999) showed that a biofilm formed on the surface of the stones acts as nucleation site for extracellular calcium carbonate precipitation. The carbonate crystal deposition is promoted by extracellular polymeric materials and presence of calcium ions increases the production of biofilms. It suggested that calcium ions modulate the protein composition of extracellular fraction which further impacts bioprecipitation. The shape of the crystals precipitated by CPS fraction from *Nocardia calcarea* was similar to the crystals precipitated by living cells (Ercole et al.,2012) . It has been observed through various studies that mutants which are unable to synthesize EPS are also unable to form biofilms (Sutherland, 2001).

EPS has been reported to play an important role in precipitation, either through trapping and concentration or through the action of specific proteins (Tsuneda et al., 2003; Rodriguez–Navarro et al., 2012; Hammes et al., 2003). The matrix of extracellular polymeric secretions has been described to influence the calcium carbonate precipitation in a positive way (Kawaguchi and Decho, 2002). The gram-positive bacterial cell surface has lipopolysaccharides (LPS) and peptidoglycan (PDG) layers which have net negative charge, so due to this anionic nature of the bacterial extracellular biopolymers it can trap calcium ions at a neutral pH, thus, positively charged divalent cations could be bound on bacterial surfaces, favouring heterogenous nucleation (Douglas, 1998; Bauerlein, 2003). The trapped divalent cations (Ca^{2+}) serves as a growth modifiers, thus controlling the crystallization and influencing the polymorphic development of $CaCO_3$ during mineralization, as observed in cyanobacteria (Kawaguchi and Decho, 2002; Braissant et al., 2003; Dupraz et al., 2009). The divalent cations (Ca^{2+},Mg^{2+}) ensures the stability of peptidoglycan (PDG), lipopolysaccharides (LPS) and S-layers. During mineral precipitation, microorganisms may act as crystal nucleating agents (Ferris & Stehmeier, 1992) and due to high surface to volume ratio, bacterial surface act as an ideal crystal nucleation sites (Warren & Haack, 2001). Bacterial cell surface is composed of different types of functional groups which enhance their capability to interact with metals; these functional groups are in turn affected by cell wall composition, presence of S layers, sheaths, capsules or extracellular polymeric substances (EPS).

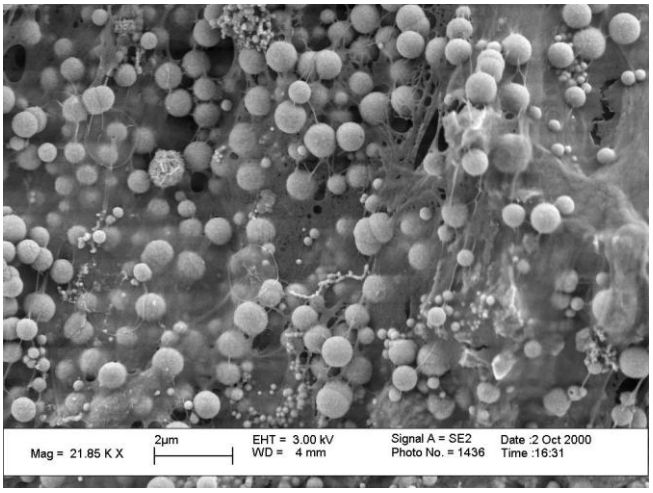


Fig. 2.6. Surface of a biofilm with hydrated matrix of polysaccharide and protein formed by aggregates of bacteria (http://dujs.dartmouth.edu/fall-2009/biofilms-bacteria's-elixer-of-survival).

In aquatic environments, the anionic nature of bacterial cell is result of pKa values of carboxyl and phosphoryl groups (Warren & Haack, 2001; Ferris & Beveridge, 1985). The binding of divalent cations to the cell wall forms the beginning of crystallization (Southam, 2000) and the formation of $CaCO_3$ crystal moieties on the cell surface (Nilsonn and Sternbeck, 1999). Bacterial cell surface not only contribute as a nucleation site for crystal formation but also influence cellular processes surrounding environment of the bacteria (Schultze-Lam *et al*., 1996). Bacteria are surrounded by thin watery layer, which physically creates micro-environment, this forms an interface between microbial cell and outside environment, in which different concentration of chemical species (Ca^{2+}, DIC, H^{+}) can prevail (Warren and Haack, 2001; Schultze-Lam *et al*., 1996) (Fig.2.7). Microorganisms have the potential to concentrate cations (Wright, 1999; McConnaughey and Whelan,1997), while EPS is known to slow down or limit the diffusion of ions (HCO_3^-, CO_3^{2-}, Ca^{2+}), thus resulting in the increased localized concentrations (Kawaguchi and Decho 2002). Commonly, carbonate precipitates develop on the external surface of bacterial cells by successive stratification (Pentecost & Bauld, 1988; Castanier *et al*., 1999) and bacteria can be embedded in growing carbonate crystals (Rivadeneyra *et al.*, 1998; Castanier *et al*., 1999).

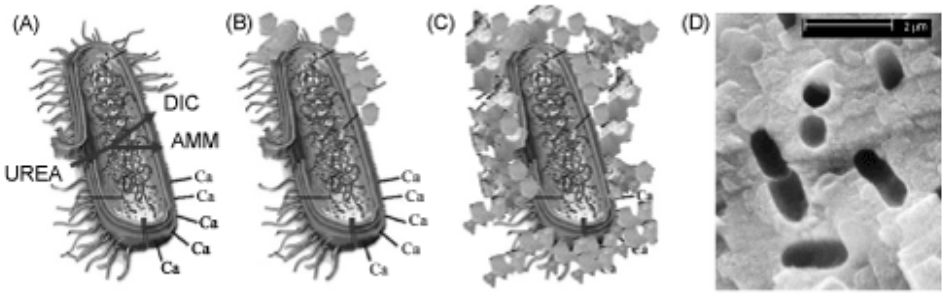


Fig. 2.7.Simplified representation of the events occurring during the microbially induced carbonate precipitation Calcium ions in the solution are attracted to the bacterial cell wall due to the negative charge of the latter. Upon addition of urea to the bacteria, dissolved inorganic carbon (DIC) and ammonium (AMM) are released in the microenvironment of the bacteria (A). In the presence of calcium ions, this can result in a local supersaturation and hence heterogeneous precipitation of calcium carbonate on the bacterial cell wall (B). After a while, the whole cell becomes encapsulated (C), limiting nutrient transfer, resulting in cell death. Image (D) shows the imprints of bacterial cells involved in carbonate precipitation (Source: Hammes & Verstraete 2002).

2.6. POLYMORPHISM OF CARBONATE CRYSTALS

Microorganisms are abundant in nature and pave a way for the massive production of biominerals. They can design and shape the minerals to a desirable conformation and orientation. In the past decades, organisms from many different phyla evolved the ability to form many different minerals which are known till date (Knoll, 2003).

All of the organic and inorganic minerals synthesised by microorganisms is by the process of biomineralization. Amongst them carbonates are the most obvious ones (Dhami et al., 2013). Calcium is the cation of choice for most organisms. The calcium-bearing minerals comprise about 50% of known biominerals (Lowenstam & Weiner, 1989) because calcium fulfills many fundamental functions in cellular metabolism (Lowenstam and Margulis 1980; Simkiss and Wilbur 1989; Berridge et al. 1998). This dominance of calcium-bearing minerals led to the usage of the term “calcification”.

The calcium carbonate minerals are the most abundant biogenic minerals, both in terms of the quantities produced and their widespread distribution among many different taxa (Lowenstam and Weiner, 1989). Most bacteria are capable of inducing precipitation in a given environment (Boquet et al., 1973). Bacterially induced precipitation of calcium carbonate has attracted much attention from both basic and applied points of view (Rodriguez-Navarro et al., 2003). Biologically induced calcium carbonate precipitation results in the production of

different phases (polymorphs) of calcium carbonate mineral (Rodriguez-Navvaro et al, 2012; Rusznyak et al, 2012; Dhami et al, 2013a). Out of the different polymorphs of calcium carbonate, some are in crystalline and some are in amorphous phases. Three of the polymorphs are pure anhydrous forms—calcite, aragonite and vaterite (Fig. 2.8) while two are hydrated crystalline forms—monohydrocalcite ($CaCO_3.H_2O$) and ikaite ($CaCO_3.6H_2O$) and various amorphous calcium carbonate forms (ACC) with difference in short range order and degree of hydration (Lippmann, 1973; Rieger *et al.*, 2007; Gower, 2008; Gebauer *et al.*, 2010). The transient forms of amorphous calcium carbonate do not contain water (Addadi et al. 2003).

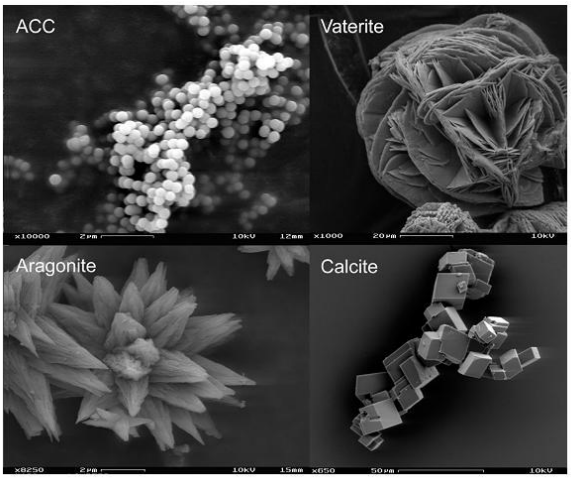


Fig.2.8. Polymorphs of CaCO3 (http://www.ruhr-uni hum.de/sediment/forschung.html)

The most common bacterial calcium carbonate polymorphs are vaterite and calcite (Ben Chekroun et al., 2004; Rodriguez Navarro et al., 2007; González-Muñoz et al., 2011), mineralization of monohydrocalcite (Krumbein, 1975) and aragonite (Krumbein, 1974, Sánchez-Navas et al., 2009) have also been reported. The evidence that bacterial calcium carbonate mineralization also involves the formation of ACC precursor phases (Hammes et al., 2003; Benzerara et al., 2006; Chen et al., 2009).

The bacterially induced carbonate precipitation is a genetically controlled process, hence, it brings some evolutionary advantage to organisms (Barabesi et al., 2007). For instance, it helps in fixating excess/toxic metal ions (Obst et al., 2009) or help bacteria to survive under unfavourable conditions (Yoshida et al., 2010). Hammes et al. (2003) suggested that certain proteins present in the biological EPS may cause the formation of different & polymorph $CaCO_3$ bioliths.

Exopolysaccharides (EPS) and amino acids isolated from *Bacillus firmus* and *Bacillus sphaericus*, plays an essential role in calcium carbonate morphology and mineralogy (Rodriguez-Navarro et al., 2003; Ercole et al., 2007). Kawaguchi and Decho, (2002) has suggested that specific proteins present in extracellular polymeric substances (EPS) produced by different bacteria controls aragonite or calcite polymorph selection. Many researchers reported the species specific precipitation of carbonate biominerals by various bacterial isolates (Hammes et al., 2003; Benzerara et al., 2006; Chen et al., 2009).

During bacterial calcium carbonate mineralization, it has become challenging to understand the mechanism(s) by which biological system determines which polymorph will precipitate. Studies suggested that phase, amount and morphology of calcium carbonate minerals depend on supersaturation, temperature, pH and $[Ca^{2+}]$ / $[CO_3^{2-}]$ ratio. The saturation index, $SI = \log \Omega = \log IAP/K_s$; where Ω is the saturation state of the system, IAP is the ion activity product and K_s is the thermodynamic solubility product of the relevant phase. Calcium carbonate precipitation in microbial systems occurs when the saturation index (with respect to calcite) is above 1 (Arp et al., 2001; Mitchell and Ferris, 2006). It follows that bacterial presence and activity are a prerequisite for the precipitation of calcium carbonate. The bacterial metabolic activity produces the necessary increase in supersaturation as to induce the heterogeneous crystallization of calcium carbonate.

Calcium carbonate polymorph selection can have important technical implications (Meldrum and Cölfen, 2008). For instance, the bacterial conservation of building materials requires the formation of a coherent, durable, calcium carbonate cement i.e., calcite which is achieved if metastable vaterite (more soluble than calcite) is formed during a bacterial conservation treatment (Rodriguez-Navarro et al., 2003). Calcite has the industrial applications for the preservation, remediation, and restoration of buildings, stone statues and historic monuments because of its high purity and good coherency for better restoration.

2.7. APPLICATIONS OF BIOMINERALS

Bacteria are capable of forming inorganic crystals either intracellularly (Konishi et al., 2006; Langley et al., 1999) or extracellularly (Kashefi et al., 2001). Few microorganisms produce glycocalyx and a variety of organic polymers outside the cell wall (Lappin-Scott et al., 1988; MacLeod et al., 1988), while others accumulate inorganic compounds such as phosphates, carbonates, silicates, iron, and manganese oxides in cytoplasm (Beveridge et al., 1983; Ghiorse, 1984; Knoll, 1985; Ruiz et al., 1988; Rivadeneya et al., 1991).

The use of bacterially induced carbonate biominerals is becoming increasingly popular day by day. These biominerals has various biotechnological applications.

2.7.1. Removal of heavy metals and radionuclides

The disposal of heavy metals and radionuclides has been posing a greatest threat to human lives and environment. Several measures like traditional methods (using pump & treatment) renders ineffective in remediation of ground water from radionuclides (Warren et al., 2001; Fujita et al., 2004, 2008, 2010; Wu et al., 2011). In this, competitive co-precipitation reaction takes place in which divalent cations (metal contaminants) incorporate into calcite lattice, thus, forming low-strontium carbonate minerals having low solubility (Fujita et al., 2000, 2004; Smith et al., 2004) (Eqn. 28).

$$Sr^{2+} + OH^- + HCO_3^- \longrightarrow SrCO_3\,(s) + H_2O \qquad (28)$$

It has been reported that enriching the native ureolytic organisms in situ for remediation of ground waters can capture about 95% of total strontium in solid phase (Warren et al., 2001, Fujita et al., 2008). Due to the strong adsorption of metal contaminants on soil particles has led to the use of many conventional methodologies but these methods are quite expensive so MICCP has come up as a novel solution to this problem. Recently, the bioremediation of arsenic and copper has been successfully reported by the action of ureolytic organisms, *Sporosarcina ginsengisoli* CR5 and *Kocuria* flava CR1 (Achal et al., 2011b, 2012).

2.7.2. Removal of calcium ions and polychlorinated biphenyls (PCBs)

The presence of calcium ions in high concentrations in waste waters causes severe scaling of pipeline due to the formation of carbonates, phosphates. MICCP plays a great role in removal of calcium ions from industrial waste waters (Hammes et al., 2003). Biomineralization has also been applied in the removal of recalcitrant contaminants like PCBs. Okwadha and Li (2010) reported the use of *Sporosarcina pasteurii* for the treatment of PCB-coated cement cylinders.

2.7.3. Carbon dioxide sequestration

The exponentially growing CO_2 concentration in the environment has become one of the most important societal challenges. The growing CO_2 is the cause of global warming and hence it is most likely linked to human activities (IPCC, 2007). To overcome such a problem researchers have used various methods to capture/sequester the CO_2 like conversion of CO_2

to carbonates offers a safe, stable and environmental friendly process for long term (Herzog and drake, 1996; Reichle et al., 1999; Shaeffer, 2010; Sharma and Bhattacharya, 2010). Scientists proposed biomimetic CO_2 sequestration to fix CO_2 in the form of carbonate biominerals such as calcite, dolomite, aragonite and magnesite by using biological catalyst like carbonic anhydrase (CA) to minimize the CO_2 concentration in a local environment (Smith & Ferry, 2000). Bovine CA has been used to enhance the co2 hydration & carbonate precipitation (Liu e al., 2005).Many researchers have recently studied the successfull sequestration of CO_2 by bacterial CA from different bacterial isolates (Ramanan et al., 2009; Wanjari et al., 2011; Yadav et al., 2011). MICCP serves as a safe and novel solution to capture/store CO_2 in the form of carbonates or bicarbonates (Mitchell et al., 2010). Ureolytic microorganisms are potentially able to induce CO_2 transformations into carbonates (Menez et al., 2007). Kamennaya et al. (2012) showed that cyanobacteria utilize solar energy through photosynthesis to convert CO_2 to recalcitrant $CaCO_3$ biominerals.

2.7.4. Filler for rubber, plastics and ink-

Yoshida et al. (2010) has recently reported the use of moderately thermophilic bacterium *Geobacillus thermoglucosidasius* isolated from thermophilically composted organic waste to produce material that can be used as filler in rubber and plastics, fluorescent particles in stationary ink and fluorescent marker. The calcite crystals formed by *G. thermoglucosidasius* have the property of fluorescence (emission wavelength is from 350-600nm) are used as filler in rubber, plastics, fluorescent ink and fluorescent marker.

2.7.5. Biominerals in construction materials-

The potential of MICCP technology in restoration of cement mortar cubes, sand consolidation and limestone monument repair, reduction of water and chloride ion permeability in concrete, filling of pores and cracks in concrete, enhanced strength of bricks via urea hydrolysis pathway has been investigated by many researchers (Ramakrishnan, 2007; Sarda et al., 2009; Al Qabany, 2011; Chu et al., 2011; Dhami et al., 2012a; Cheng et al., 2013 and references within).

2.7.5.1. Biodeposition in soil and sand materials-

Mechanical properties of soil are often insufficient so stabilization of soil is highly desirable because of increasing infrastructure. Recently techniques aiming at changing the soil properties i.e. in situ soil strengthening technique (biogrouting), involves MICCP (Van Meurs

et al., 2006; Whiffin et al., 2007). The potential use of MICCP has been increased by ureolytic bacteria by providing urea and calcium sources in various sand plugs (Stocks-Fischer et al., 1999; Dick et al., 2006; Achal et al., 2009b). Improvement in sand and soil strength i.e. reduced porosity (50%) and permeability (90%) by bacterial carbonation has been successfully reported (Kantzas et al., 1992; Dejong et al., 2006; Chiffin et al., 2007). Harkes et al. (2010) and Chu et al. (2012) reported the evaluated potential of MICCP in soil for ground reinforcement and improvement in shear strength of soil.

2.7.5.2. Biomineralization in cementitious materials-

Concrete is strong and cheap construction material found worldwide. Concrete is very liable to cracking, hence, it is less durable. Its durability depends upon the characteristics of its pore size (Khan, 2003). Degradation of concrete depends upon many factors like permeability, compressive strength & porosity of pores. Deterioration of concrete involves the movement of aggressive substances and gases or liquids from surrounding environment into the concrete (Claisse et al., 1997).Several studies have revealed the application of MICCP for improving the compressive strength of concrete (Bang et al., 2001; Ramachandran, 2001; Ghosh et al., 2005; De Muynck et al., 2008a,b; Jonkers et al., 2010; Park et al., 2010; Achal et al., 2011a,c). The application of different ureolytic bacteria has improved the properties of concrete to a large extent. Ramakrishnan et al. (1998) found increase in resistance of concrete toward alkali, freeze thaw attack, drying shrinkage and reduction in permeability upon application of bacterial cells. De Muynck et al. (2008b) enhanced the permeability characteristics of mortar by *B. sphaericus*. Achal et al. (2009a) treated the mortar cubes with *Sporosarcina pasteurii* and observed 17% improvement in compressive strength.

2.7.5.3. Remediation of cracks in concrete-

Microbial concrete has proved its efficacy in remediation of cracks in various structural formulations (Gollapudi et al., 1995; Stocks-Fischer et al., 1999). In microbiologically enhanced crack remediation (MCER) *Bacillus pasteurii* has been used to induce calcite precipitation (Bang and Ramakrishnan, 2001). Ramachandran et al. (2001) has reported MECR in concrete. Bang et al. (2001) reported positive potential of MECR by polyurethanes immobilized bacterial cells in which ureolytic bacterial cells are encapsulated in polyurethanes. *B.sphaericus* has been shown for its positive potential in biomineralized calcite in crack remediation of concrete (De Belie and De muynck, 2008). Qian et al. (2010) reported that compressive strength of biomineralized specimens could be restored to 84%.

Recently Achal et al. (2013) reported the positive potential of bacterial calcite in crack remediation by *Bacillus* sp.CT-5.

2.7.5.4. Restoration of limestone buildings-

The durability of calcareous stone buildings decreases with time due to progressive increase in porosity and subsequent decrease in mechanical characteristics of concrete (Amoroso, 1983). Various researchers studied the positive effects of MICCP in reducing the permeability and increasing durability of stone (Le Metayer-Levrel et al., 1999; Rodriguez-Navarro et al., 2003; Dick et al., 2006).Le Metayer-Levrel et al. (1999) reported that bacterially induced calcites reduced the water sorptivity of treated stones. Adolphe et al. (1990) were among the first to consider the use of MICCP for protection of ornamental stones. Some scientists reported the use of *Myxococcus xanthus* in precipitation of carbonates, phosphates and silicates in a wide range of solid and liquid media (González-Munoz et al., 1993, 1996; Ben Omar et al., 1995, 1998; Ben Chekroun et al., 2004; Rodriguez-Navarro et al., 2007). Dick et al. (2006) reported half of the reduction in water absorption after the application of *B. sphaericus* to limestone cubes. Zamarreno et al. (2009) investigated the application of calcite crystals precipitated by freshwater bacteria on limestone and found significant reduction in pore sizes of the substrate in treated specimens.

2.8. One Factor At A Time (OFAT)

For the commercial production of various biotechnological products there is need to optimize the conditions required for their maximal production. There are various factors which effect the production and that have to be optimized. In case of maximum production of calcium carbonate, the factors effecting its production need to be optimized through several conventional methods out of which one is OFAT i.e. one factor at a time. In this methodology, only one factor (effecting the carbonate precipitation) is considered at a time and others are kept constant. This approach is simplest to implement, and primarily helps in selection of significant parameters affecting the enzyme yield. However, this method is not only time restrictive, but also ignores the combined interaction(s) among various physical and nutritional parameters (Singh et al., 2011). OFAT method was used to investigate the effect of media constituents, such as carbon and nitrogen source (Seetha Ram Kotra, 2012).

Though lot of studies have proved the successful application of OFAT in production of various enzymes, fermentation products and biomolecules by optimizing various parameters and interactions involved in the final product but till now, not much has been done on the

standardization of conditions for maximum carbonate production by OFAT. So, there is need to investigate and optimize the major factors involved in production of enzymes involved in carbonate precipitation so as to enhance the production of these biominerals.

CHAPTER 3
MATERIALS & METHODS

3.1. Organism and culture conditions

Alkalophilic bacterial cultures isolated earlier from various alkaline soil source (Achal et al., 2011) were characterised.

3.2. Culture revival

Different bacterial isolates (C7, S5, CT5,S13, S18, S3, AI-4, AS4, C13, AN2, S20, S32, S15, SN3, A35, LS2, LS4, *Bacillus pulvis*, *Bacillus megaterium*, *Bacillus cereus*) were revived on nutrient agar (NA: 0.5% peptone, 0.3% beef extract/yeast extract,1.5% agar, 0.5% NaCl, distilled water, pH-7) and incubated overnight at 37°C and 120 rpm shaking conditions.

3.3. Screening of bacterial isolates

The qualitative screening of bacterial isolates was done in order to confirm the production of urease. The grown bacterial colonies from the NA plates were sub cultured on the urea agar base (UAB) media plates (HiMedia, India) and urea broth (UB) (HiMedia, India) for the screening of ureolytic bacteria. The urea agar base plates and urea broth was supplemented with 2% urea (out of 40% urea stock). The cultures were inoculated in UAB plates or Urea broth + 2% urea and incubated overnight at 37°C and 120 rpm shaking conditions for the growth of urease producing isolates. To select the efficient urease producers, the formation of pink colour was checked on UAB plates and Urea broth.

After confirming urease production qualitatively on plates and broth, the selected 7 bacterial isolates were inoculated (0.5%) into nutrient broth supplemented with 2% urea + 10μM $ZnSO_4$ + 25mM $CaCl_2$ and incubated at 37°C & 120 rpm for 5 days. Samples were taken from the culture flasks after interval of 24 hrs and checked for growth curve, pH profile, enzyme assays (urease activity, carbonic anhydrase activity, protease activity), EPS/biofilm production and calcium carbonate precipitation.

3.4. Optimization of different media

After checking different characteristics, out of 7 ureolytic isolates, one isolate with best urease, EPS and calcite production selected was *B. megaterium*. To optimize its best activity, it was grown in different media i.e. nutrient broth (NB), basal media (BM), B4 media (B4M), specific media (M).

Three different sets of each media were prepared. First set is comprised of all above listed media supplemented with 2% urea and 25mM $CaCl_2$. Second set of media is comprised of all corresponding media without 2% urea and 25mM $CaCl_2$ and third set have all media with 2% urea, 25mM $CaCl_2$ and 10 µM $ZnSO_4$. The pH of different medias (NB-7.4, BM-5.2, B4M-6.5, M-7.2) was adjusted with 1N HCl & 1N NaOH prior to autoclaving without urea and $CaCl_2$. Filter sterilized urea and $CaCl_2$ were added to media later. The final pH of each media was adjusted to 8.0. Media were inoculated with *B. megaterium* and incubated at 37°C & 120 rpm for 5 days. Samples were taken from the culture flasks after interval of 24 hrs and checked for growth curve, pH profile, enzyme assays (urease activity, carbonic anhydrase activity, protease activity), eps/biofilm production and calcium carbonate precipitation. The media in which highest urease, EPS & calcium carbonate was produced have been selected as best optimized media for the better growth of the isolate.

3.5. Enzyme assays

3.5.1. Urease assay

The urease activity was determined for all bacterial isolates in all corresponding media by measuring the amount of ammonia released from urea according to phenol-hypochlorite assay method (Natarajan, 1995) at different time intervals. 1% of overnight grown culture of OD_{600} =0.8 was inoculated in corresponding autoclaved media supplemented with 2% urea (pH 8·0) and incubated under shaking conditions (120 rpm) at 37°C for 5 days (120 hrs). 1 ml culture broths from each flask were taken at an interval of every 24 hrs, centrifuged (8000 rpm for 5 mins) and the supernatant was collected. The culture supernatant (250 µl) was added to a mixture containing 1 ml of 0.1 M potassium phosphate buffer (pH 8.0) and 2.5 ml of urea (0.1 M). The mixture was incubated at 37°C for 5 minutes followed by addition of 1ml each phenol nitroprusside and alkaline hypochlorite and the assay mixture was then incubated at 37°C for 25 minutes. Optical density was measured at 626 nm. Ammonium

chloride (50-1000 µM) was used as the standard. One unit of urease is defined as the amount of enzyme hydrolyzing one µmole urea per min.

3.5.2. Carbonic anhydrase assay

The carbonic anhydrase activity of all bacterial isolates was investigated in all media for quantitative production. The bacterial cells (1%, OD_{600} =0.8) of all the isolates were inoculated into corresponding media supplemented with 10µM $ZnSO_4$ and incubated at 37°C under shaking condition (120 rpm) for 5 days (120 hrs). The CA production was then estimated by measuring the amount of p-nitrophenol produced according to spectrophotometric assay of Smith and Ferry (1999). After an interval of every 24 hours, 1ml culture was centrifuged at 8000 rpm for 5 minutes (4°C) and the supernatant comprising extracellular CA was collected. The culture supernatant (200 µl) was added to a mixture containing 1·8 ml of 100 mM phosphate buffer (pH 7·0) and 1 ml of 3 mM p-nitrophenyl acetate solution. OD was measured at 348nm. p- nitrophenol (100µM-1mM)was used as standard. One unit of carbonic anahydrase activity is defined as the amount of enzyme required to form 1 µmole of p-nitrophenol per minute.

3.5.3. Protease assay

Alkaline protease activity was determined according to the method of Fukumoto et al. (1971). The bacterial cells (1%, OD_{600} =0.8) of all the isolates were inoculated into corresponding media and incubated at 37°C under shaking condition (120 rpm) for 5 days (120 hrs). 1 ml culture was centrifuged at 8000 rpm for 5 minutes (4°C) after an interval of every 24hrs and the supernatant was collected. 1ml of the supernatant was added to 5 ml of 0.6% casein and incubated at 37°C for 10 minutes. The reaction was stopped by adding 5 ml of a TCA mixture containing 36 ml of 50% (w/v) TCA solution, 220 ml of 1 M sodium acetate solution, 330 ml of 1M acetic acid solution in a total volume of 1000 ml. The unreacted casein was precipitated, and the resulting precipitate was filtered off using Whatman filter paper No. 1. The optical density of the filterate was measured at 275 nm against a blanck. A standard graph was generated using standard tyrosine solutions of 5-50 µg/ml. One unit of protease activity was defined as the amount of enzyme, which liberated 1 µg tyrosine per min. at 37°C.

3.6. Biofilm production

Biofilm production from all the isolates was established aseptically in each corresponding media according to method developed by Conway et al., (2002) and Ercole et al., (2012). The bacterial cells (1%, OD_{600} =0.8) of all the isolates were inoculated into corresponding media. 200 µl of each culture was aliquoted per well on 96-well polystyrene microtiter plate (Falcon; Becton; Dickinson and Company, Franklin Lakes, NJ, USA). Microtiter plates with fitted lids were incubated at 37°C for 120 hrs under 120 rpm shaking conditions. After an interval of every 24 hrs, plates were washed with saline solution (NaCl, 9g/l) to remove planktonic bacteria; the remaining biofilm associated cells were stained for 20 min with 125 µl 1% (w/v) crystal violet. After thorough washing with saline solution, 200 µl of 98% (v/v) ethanol was added to the well at room temperature for 15 min to release the dye in the solution. The extent of biofilm, related to crystal violet bound to biofilm-associated cells, was determined by measuring the absorbance of the resulting solution at 590 nm.

3.7. Extraction of extracellular polymeric substances (EPS)

The EPS produced by different bacterial isolates was estimated as per the method outlined by Rodrigues and Bhosle (1991). The bacterial cells (1%, OD_{600} =0.8) of all the isolates were inoculated into corresponding media and incubated at 37°C under shaking condition (120 rpm) for 5 days (120 hrs). To 1 ml of cell-free supernatant, 2 ml of distilled alcohol (90%) was added and incubated for 12 h at -20ºC. The EPS was obtained by centrifugation at 8000 rpm for 15 min. The precipitate was air-dried and then dissolved in 2 ml of distilled water. The EPS in this solution was estimated by phenol sulfuric acid method (Dubois et al. 1956) using glucose (1 mg/ml) as standard.

The PSA method involved adding 200 µl of 5% (w/w) phenol solution (Fisher Scientific), followed by addition of 2 ml of highly concentrated sulfuric acid 98% (Fisher Scientific) to 200 µl of resuspended EPS solution. The phenol, acid, and EPS suspension was incubated at room temperature for 10 min, followed by incubation in a water bath for 20 min at 25–30°C. Total carbohydrate content was measured at optical density of 490 nm.

3.8. Optimization of extracellular polymeric substances (EPS)

Different EPS concentrations have been optimized for the maximal carbonate precipitation. Specific media was selected for eps characterization with nutrient broth taken as standard. Bacterial cells (1%, OD_{600} =0.8) was inoculated and incubated overnight at 37°C &120 rpm.

Overnight grown culture was taken and different concentrations of bacterial culture (20, 40, 60, 80, 100 mg/100 ml) were made with corresponding media. Incubated the cultures at 37°C for 24 hrs. After 24 hrs, bacterial culture was taken and centrifuged at 8000 rpm for 15 min at 4°C. EPS in 300 ml supernatant was precipitated by the addition of two volumes of chilled absolute ethanol. The mixture was incubated for 12 h at -20ºC. The EPS was obtained by centrifugation at 8000 rpm for 15 min. The pellet was air-dried and then dissolved in 100 ml of distilled water. A constant volume of filter-sterilized 25mM $CaCl_2$ was added to the precipitate. Then soluble Ca^{2+} content in the solution was quantified according the EDTA titration method (APHA et al., 1989). Culture solution was dissolved with 3N hydrochloric acid (HCl). Four ml of 5N Sodium hydroxide (NaOH) was added. 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.

3.9. Characterization of extracellular polymeric substances (EPS)

EPS was characterized for the estimation of total polysaccharides and total proteins. Polysaccharides were extracted as described by Del Gallo and Haegi (1990). Bacterial cells (1%, OD_{600} =0.8) was inoculated in 500 ml flasks and incubated for 14 days at 37°C and 120rpm. Following growth for 14 days, bacterial cultures were centrifuged at 30 × g for 3 min to remove mineral precipitates. The culture obtained was centrifuged at 2300 × g at 4°C for 20 min. Supernatant and pellet were processed as follows:

Supernatant, containing EPS, protein, glycoprotein and residual microbial cells, was added to phosphate-buffered saline (appendix), (one fifth of initial volume) and protease inhibitors (Ercole et al., 1999) and incubated for 4 days at 4°C. After 4 days, mixture was centrifuged at 9800 × g at 4°C for 20 min. The pellet containing microbial cells was discarded and the EPS fraction was isolated from the supernatant by incubation overnight in 2 volumes of ethanol at 4°C and then centrifuging the solution at 15,300 × g at 4°C for 30 min. The resulting pellet, containing ethanol-insoluble EPS, was resuspended in 1/10 of initial volume of PBS supplemented with protease inhibitor. This solution was treated with thioglicolic acid 0.1M (Himedia, India) at pH 8 at room temperature for 30 min to cleave disulphide bridges present in mucous secretions. The solution was lyophilized and weighed (Manca et al., 1996).

Pellet, from the first centrifugation (CPS, proteins and glycoproteins), was resuspended in 1/10 of initial volume of HEPES-Triton-X 1% (Himedia, India) and kept for 3 days at 4°C. Thereafter, cells were removed by centrifugation (2300 × g, 20 min, 4°C) and the CPS

containing supernatant CPS was supplemented with protease inhibitors (Ercole et al., 1999). The solution was lyophilized and weighed. The total carbohydrate content in the sample was determined by PSA method and total protein content was estimated by Lowry method (Lowry et al., 1951) by using bovine serum albumin as a standard. BSA (Bovine serum albumin) standard solutions were prepared in the range of concentrations 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mg/ ml. From these solutions, 0.2 ml protein solutions were pipette out into different test tubes. Simultaneously bacterial samples were also taken. 2 ml of alkaline copper sulphate reagent [prepared by (a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water) (b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Prepared analytical reagents by mixing 2 ml of (b) with 100 ml of (a)] was then added and the solutions were mixed well. The final volume in each of the test tubes was 5 ml. These solutions were incubated at room temperature for 10 min. This was followed by addition of 0.2 ml of Folin Ciocalteau reagent solution (1N) to each tube and incubation for 30 min. The absorbance was measured at 660 nm. The absorbance was plotted against protein concentration to get a standard calibration curve. The absorbance of sample was checked and concentration of the sample was determined from the standard curve.

3.10. Estimation of bacterially induced calcium carbonate precipitation

For estimation of bacterially induced $CaCO_3$ precipitation via ureolytic pathway, bacterial cells of each strain were inoculated with inoculum of 1% into 250 ml flasks containing 100 ml corresponding media supplemented with 2% urea and 25 mM $CaCl_2$ (pH 8·0) and incubated at 37 °C with shaking conditions of 120 rpm for the interval of 120hrs. After every 24 hr, the amount of insoluble Ca^{2+} in the pellets and soluble Ca^{2+} in the supernatant by centrifuging the broth and quantifying the Ca^{2+} content according to the EDTA titration method (APHA et al., 1989). Culture pellet of each sample was dissolved with 3N HCl and 4 ml of 5N NaOH was added to the precipitate and final volume was made upto 50 ml with distilled water. 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. The amount of $CaCO_3$ formed was calculated by the volume of edta used × 0.005 × 1000/ ml of sample used.

3.11. Characterization of calcium carbonate crystal polymorphs

In order to assess the applicability of different calcium carbonate polymorphs for different biotechnological applications, it is pre requisite to gather information on the various polymorphs produced by different calcifying bacteria. Best ureolytic, EPS producing and $CaCO_3$ precipitating bacterial strain was grown nutrient broth (OD_{600} = 0.8) and then 1% of the bacterial isolate was inoculated into 1000 ml flasks containing 500 ml best optimized media and nutrient broth as standard media (pH 8·0) and incubated at 37 °C with shaking (120 rpm) for14 days. After14 days, the carbonate crystals precipitated at the bottom of each flask were filtered on Whatman No.1 filter paper (90 mm in diameter) and collected after washing with sterile distilled water. The crystals were air dried at 37°C for 24 hrs. The filters were weighed before and after collection of crystals to estimate the amounts of carbonate crystals precipitated by the bacterial isolate. This was followed by analysis of the crystals for morphological and chemical constituents.

3.11.1. Gram staining

Bacterial smear prepared from actively growing cells was spread on a glass slide and fixed by heating. Crystal violet was flooded for 10 sec. It was followed by brief washing in water to remove excess crystal violet. Gram iodine was flooded for ten seconds and washed with water. Further, it was decolourised with ethyl alcohol until the moving dye front has passed the lower edge of the section and washed immediately in tap water. Safranin was used to counter stain for 15 sec and washed with water to remove the excessive stain. The slides were visualized under microscope at different magnifications.

3.11.2. Capsule Staining

The capsule stain is a differential stain, which selectively stains external capsules surrounding bacterial cells. Capsules are highly ordered polymers of sugars and proteins that surround some bacterial cells, and can be easily dislodged by heat or water. A loopful of culture and 10% (w/v) Nigrosan Stain (S025) each, was placed on the clean glass slide. Mixed well and heat fixed the culture and stain. Secondary stain, Methylene Blue (S021) was applied for 30 seconds. Rinse the silde with water and air dried before observing under microscope. At the end of the staining procedure, the capsule appears as a faint blue or white halo around a purple cell observed under microscope.

3.11.3. Staining of crystals

The crystals were mounted on glass slides and stained with Loeffler's methylene blue (methylene blue, 3 g; potassium hydroxide, 10 mg; 95% ethanol; 300 ml per liter of dH_2O) for 1 min, rinsed with dH_2O, dried by blotting, and observed with white light by the use of 100 X magnification and immersion oil. The bacteria inside and around the crystals were observed.

To investigate the polysaccharides associated with carbonate crystals, the crystals were stained with alcian blue – periodic acid - Schiff stain (PAS). Firstly, 100% ethanol was applied on the crystals for 5 min followed by rinsing with dH2O for 5 min, application of alcian blue for 5 min followed by 1% Schiff's reagent for 5 min. The final rinsing was done with dH_2O for 5 min.

3.12. Scanning electron microscopy (SEM)

Morphology and size of crystals deposited from living bacterial cells and EPS & CPS fractions were studied by scanning electron microscopy (SEM, Jeol, JSM 6510 LV) equipped with EDX. Elemental analysis was done with energy dispersive X-ray (EDX) analyzer (Jeol, JSM 6510 LV).

3.13. X-ray diffraction analysis (XRD)

XRD spectra were obtained using an X' Pert PRO diffractometer with a Cu anode (40kV and 30mA) and scanning from 5°- 80°2θ. The components of the sample were identified by comparing them with standards established by the International Center for Diffraction Data.

3.14. Fourier transform infra red spectra (FTIR)

The FTIR spectrum of all the crystals was recorded on Chary Eclipse 630 (Agilent Technologies) apparatus by the diffuse reflectance accessory technique. The spectra of the crystals were scanned in the range of 400 - 4000 cm^{-1}.

3.15. Optimization of conditions for EPS and carbonate precipitation by OFAT

In order to optimize the production of $CaCO_3$ for maximal precipitation, there is need to standardize the media for maximum EPS production. For this, it is crucial to optimize the medium constituents. Specific media (1 g Glucose, 5 g Peptone, 1 g Yeast Extract, 1 g K_2HPO_4 , 2 g $MgSO_4$.$7H_2O$, 25mM $CaCl_2$, 0.1 g NaCl, 2 g $(NH_4)_2$ SO_4 , 3 g Agar per liter)

was optimized for EPS production in *Bacillus megaterium* using one factor at a time. Afterwards, in order to find the most significant media parameters for EPS production by *B. megaterium*. *B. megaterium* was inoculated in media with variable ranges of media components and were investigated for EPS production after incubation at 37°C and 120 rpm for 144 hrs.

3.15.1. Optimization of production medium by OFAT method

Growth conditions and chemical components were varied one at a time to check their impact on EPS production. Various parameters such carbon source, nitrogen (organic and inorganic) source, calcium source and variable pH were optimized. *B. megaterium* was inoculated in specific media with varying carbon, nitrogen and inoculums concentrations. Various carbon sources tested were: glucose, fructose, maltose, lactose, sucrose, starch and mannitol. Different organic nitrogen sources tested were beef extract, peptone, and inorganic nitrogen sources were ammonium sulphate, ammonium acetate, ammonium carbonate, urea and ammonium chloride. Different calcium sources tested were calcium chloride, calcium acetate, calcium phosphate, calcium nitrate and calcium hydroxide. Variable pH profile tested were 7, 8, 9, 10. The initial inoculum added was 1% to the different sets of specific media and incubated at 37°C and 120 rpm for 5 days (144 hrs).

3.16. Statistical analysis of data generated

Results were expressed as mean value and standard deviation. Generated data was analysed by analysis of variance (ANOVA) for each response and the mean were compared by Tukey's test at probability value of less than 5%. Analysis of data was done by using software Graph pad prism 5.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Screening of bacterial isolates

Soil harbours various microbes which play great role in biomineralization processes and precipitate inorganic solids of great technical importance. The most ubiquitous organisms in the soil are bacteria present in high concentrations. Alkaline soils are the prime home of alkalophilic bacteria and are areas of potential interest because of being carbonate dominated habitats (Satyanarayana et al., 2005). Alkaliphilic ureolytic bacteria represent the most favoured pathway for precipitation of carbonates.

The bacterial urease simply hydrolyzes the urea to form bicarbonate, ammonium and hydroxide ions. These products gives rise to pH increase, which in turn results in the formation of carbonate ions, which in the presence of soluble calcium ions, precipitate as $CaCO_3$ either on the soil or on capsule, comprising of extracellular polymeric materials. Because of the carbonate precipitating ability of these alkalophilic bacteria, alkaline soils of various sites were investigated for isolation of calcifying bacteria.

In the present study, twenty ureolytic alkalophiles isolated from alkaline soils (pH >8.0) were studied for their ability to produce urease. These cultures were screened for the production of urease by plate assay method on urease selective media i.e. Urea Agar Base plates and urea broth. This media contains phenol red and the urease producing bacteria utilize the urea present in the media degrading phenol red to give pink colour in alkaline environment (Andrews et al., 1995). Formation of intense pink colour confirmed the presence of Urease in the isolates. Out of twenty isolates screened initially, seven were found to be positive (Figure 4.1a,b).

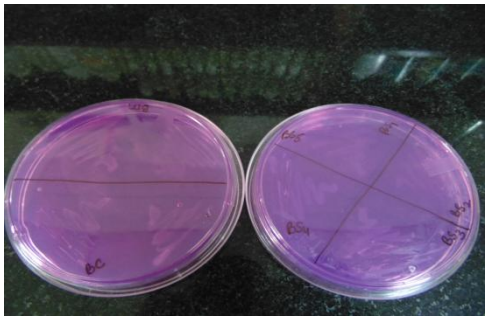
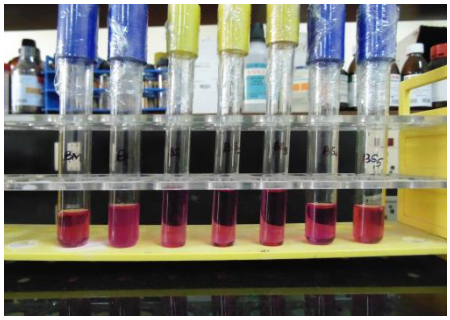
a)  b) 

Fig. 4.1. Urease positive different Bacillus sp. isolates in a) urea agar plates, b) urea broth

4.1.1. Growth curve and pH profile of different bacteria isolates

The growth curves and pH profile of different isolates were also studied over an interval of 24 hrs in nutrient broth. It was noticed that the isolates showed increasing growth and pH till 48 hours while after that it was found to be static. Bacterial growth and pH profile studies reveals a sigmoidal curve (S-shaped). Bacteria shows maximum growth till exponential phase (log phase) as after stationary phase, due to deprivation of nutrients, the bacterial cells start with the death phase. In our study the exponential phase was found upto 48 hours (Figure 4.2. and 4.3)

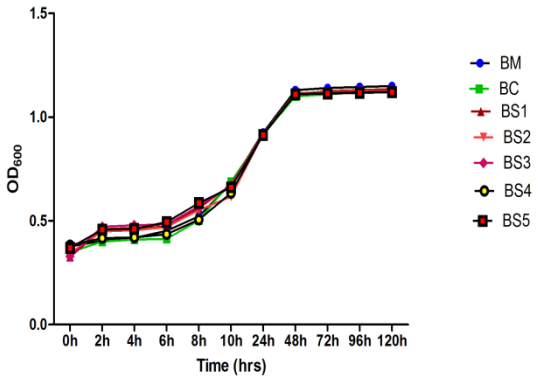


Fig. 4.2.Growth Curve of different *Bacillus* isolates

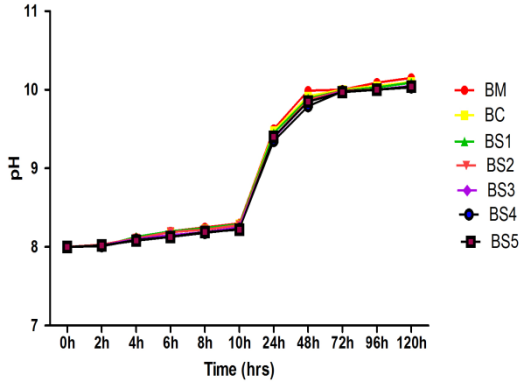


Fig. 4.3. pH profile of different *Bacillus* isolates

4.1.2. Quantitative analysis of bacterial isolates

The quantitative analysis of seven ureolytic bacterial isolates was performed. Nutrient broth was inoculated with different bacterial isolates and was quantified for the production of highest amount of different enzymes (urease, carbonic anhydrase, protease), extracellular polymeric substances (EPS), biofilm production and calcium carbonate precipitation.

4.1.2.1. Enzyme Assays

4.1.2.1.1. Urease assay

Urease production by *Bacillus megaterium* (BM), one of the alkalophilic ureolytic isolate, increased significantly with time and maximum production was recorded on 3rd day (185 U/ml),while after that; there was sharp decrease in the activity of the enzyme. Bacteria hydrolyze urea by means of urease to increase the ambient pH and either allows urea to be used as nitrogen source or as a source of energy (Mobley and Hausinger 1989; Burne and Chen 2001) (Table 4.1. and Figure 4.4.).

Table 4.1.Comparison of urease activity (U/ml) of different *Bacillus* isolates in nutrient broth

Isolates	**Urease Activity (U/ml)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM	132 ± 3b	144 ± 7b	186 ± 8.7b	154 ± 4.7b	145 ± 8.2b
BC	124 ± 4cd	135 ± 5cd	138 ± 9.4cd	131 ± 5.3cd	128 ± 2.1cd
BS1	149 ± 4a	164 ± 1.3a	172 ± 6.6a	138 ± 5.2a	106 ± 8.4a
BS2	122 ±4.2cd	133 ± 5.3cd	155 ± 8.7cd	121 ± 5.1cd	110 ± 5.2cd
BS3	132 ± 0.7bc	141 ± 5.2bc	154 ± 3.4bc	140 ± 2.1bc	136 ± 3.0bc
BS4	117 ± 1d	130 ± 2.6d	128 ± 3.0d	124 ± 6.3d	120 ± 1.6d
BS5	100 ± 6.1d	132 ± 2.3d	167 ± 6d	139 ± 2.9d	129 ± 4.6d
Control	83 ± 1.1e	88 ± 2.1e	94 ± 3.8e	88 ± 1.5e	81 ± 0.6e

Values bearing different letters in the same column differ significantly ($P<0.05$). All values are mean ± SD (n = 3).

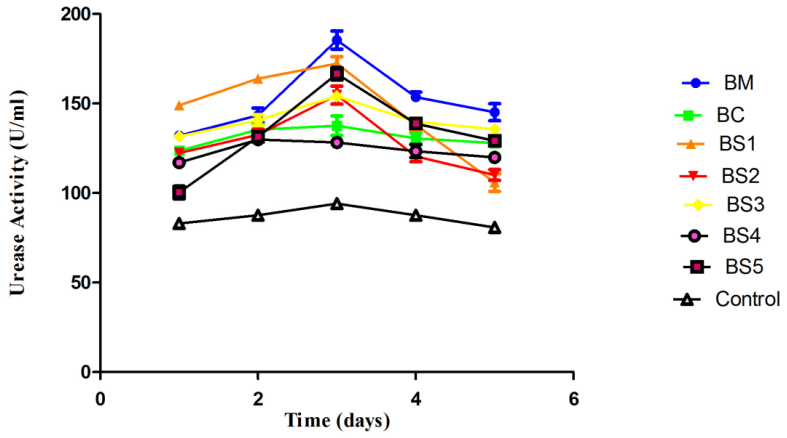


Fig.4.4. Comparison of urease activity (U/ml) of different *Bacillus* isolates in nutrient broth.

4.1.2.1.2. Carbonic Anhydrase Assay

In case of Carbonic Anhydrase (CA), similar trend in the enzyme activity was observed with maximum activity after 48h of incubation and then decrease was noticed which shows its correlation with urease. Maximum Carbonic anhydrase was noticed in *B. megaterium* (BM) (861U/ml) on 3rd day (Table 4.2. and Figure 4.5.).

Table 4.2. Comparison of Carbonic anhydrase activity (U/ml) of different *Bacillus* isolates in nutrient broth

Isolates	**Carbonic Anhydrase activity (U/ml)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM	576 ± 4.7a	671 ± 24a	861 ± 42.3a	458 ± 32.7a	333 ± 27.6a
BC	532 ± 32.5b	612 ± 3.6b	747 ± 27.5b	459 ± 16.5b	441 ± 15b
BS1	548 ± 4a	636 ± 19.5a	728 ± 17a	427 ± 23a	383 ±12.8a
BS2	592 ± 29.5ab	661 ± 12.5ab	725 ±19.8ab	482 ± 8.1ab	364 ± 24.4ab
BS3	641 ± 7.5ab	716 ± 18.5ab	822 ±18.5ab	454 ± 11.1ab	448 ± 16.2ab
BS4	589 ± 18.2a	709 ± 31.3a	839 ± 34.6a	463 ± 21.3a	339 ± 20.4a
BS5	574 ± 24.6a	696 ± 29.2a	739 ± 26.9a	483 ± 16.1a	342 ± 18.3a
Control	277 ± 21.7c	341 ± 4c	475 ± 20.4c	376 ± 19c	270 ± 20.5c

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3).

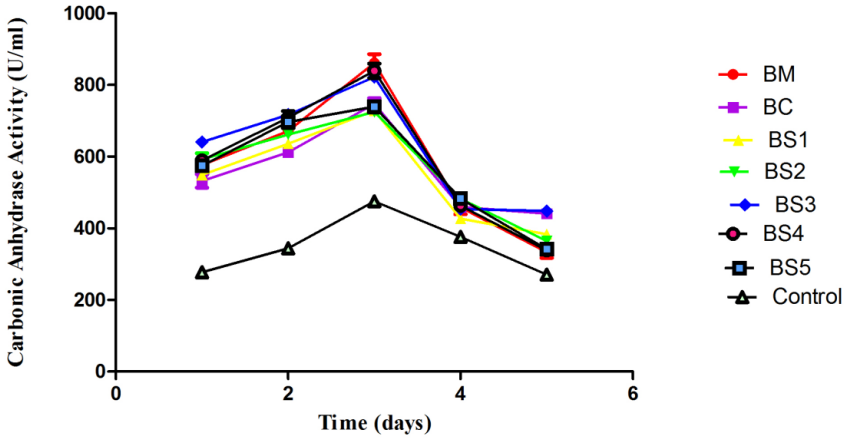


Fig. 4.5. Comparison of carbonic anhydrase activity (U/ml) of different *Bacillus* isolates in nutrient broth.

4.1.2.1.3. Protease assay

Extracellular protease activity of different bacterial isolates was also determined in the culture supernatant by method of Fukomoto *et al.* (1971) at different time intervals. It was noticed that the protease activity increases abruptly after 4 - 5 days in all the isolates. The production of protease was higher in *B. megaterium* (BM) (57 U/ml) as compared to other isolates at the end of 5th day (Table 4.3. and Figure 4.6.)

Table 4.3. Comparison of protease activity (U/ml) of different *Bacillus* isolates in nutrient broth

Isolates	**Protease activity (U/ml)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM	28 ± 0.9a	37 ± 2.0a	43 ± 0.2a	57 ± 1.1a	58 ± 1.1a
BC	37 ± 0.7a	38 ± 1.3a	43 ± 1.2a	54 ± 1.3a	56 ± 1.4a
BS1	33 ± 0.2a	34 ± 1.6a	42 ± 0.6a	49 ± 0.7a	50 ± 0.3a
BS2	34 ± 0.4a	38 ± 1.9a	39 ± 0.4a	50 ± 0.7a	52 ± 1a
BS3	33 ± 0.8a	36 ± 1.3a	38 ± 0.3a	39 ± 0.2a	43 ± 0.4a
BS4	29 ± 0.4a	38 ± 1.2a	39 ± 0.8a	41 ± 0.6a	41 ± 1.1a
BS5	36 ± 0.4a	39 ± 1.5a	41 ± 0.6a	43 ± 0.4a	45 ± 1.6a
Control	25 ± 0.9b	27 ± 1.2b	29 ± 0.7b	31 ± 0.4b	32 ± 0.4b

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3).

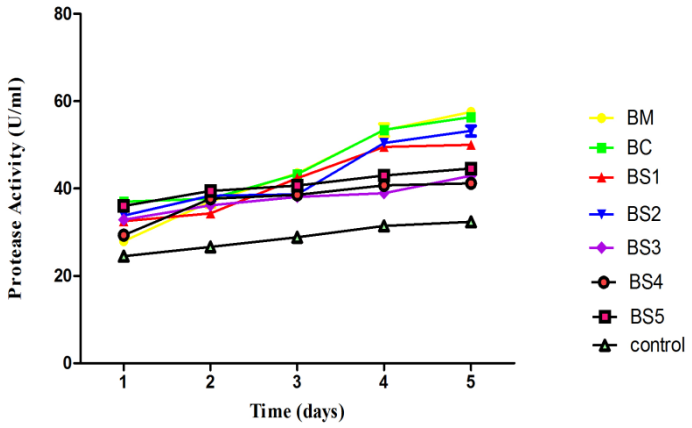


Fig. 4.6. Protease activity (U/ml) of different *Bacillus* isolates in nutrient broth.

4.1.3. Extracellular Polymeric Substances (EPS)

Both Extracellular Polymeric Substances (EPS) and Biofilm have been reported to play important role in the microbial $CaCO_3$ precipitation (Ercole *et al.*, 2012). In the present study, all the bacterial isolates produced significant amount of EPS and biofilm. Noticeable differences were observed in the production of EPS and Biofilm by different bacterial isolates. *Bacillus megaterium* (BM) produces highest amount of EPS (1173µg/100ml) on 3rd day after that significant decrease was observed in its production as seen in (Table 4.4.and Figure 4.7.)

Table 4.4. Comparison of EPS production (µg/100ml) by different *Bacillus* isolates in nutrient broth

Isolates	**EPS production (µg/100ml)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM	807 ± 9.7a	852 ± 10.9a	1173 ± 77.2a	741 ± 24.2a	449 ± 17.2a
BC	494 ± 5.5d	678 ± 27.4d	931 ± 52.5d	702 ± 17.6d	569 ± 13.1d
BS1	450 ± 4e	859 ± 42.1e	941 ± 8.6e	852 ± 25.4e	477 ± 6e
BS2	583 ± 18.1c	752 ± 30.4c	937 ± 5.1c	856 ± 15.2c	645 ± 8.1c
BS3	481 ± 6.4d	926 ± 19.7d	1090 ± 25.6d	806 ± 15d	527 ± 6.1d
BS4	644 ± 11.8b	598 ± 10.4b	937 ± 40.1b	877 ± 12.8b	459 ± 15.0b
BS5	469 ± 30.8de	803 ± 13de	1050 ± 45.1de	870 ± 16.8de	519 ± 8de
Control	104 ± 3.5f	245 ± 8.9f	343 ± 12.9f	259 ± 11.1f	121 ± 5.3f

Values bearing different letters in the same column differ significantly ($P<0.05$). All values are mean ± SD (n = 3)

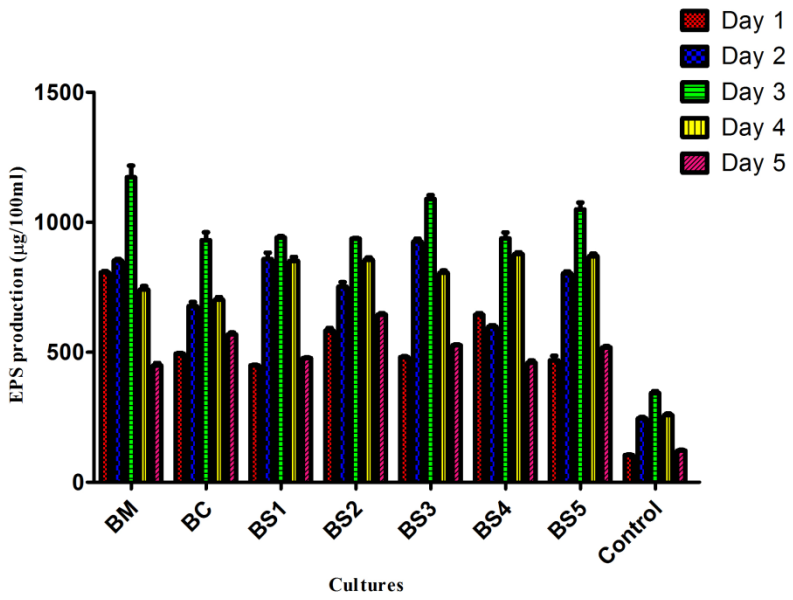


Fig. 4.7. EPS production (µg/100ml) by different *Bacillus* isolates in nutrient broth.

4.1.4. Biofilm

Biofilm produced by bacterial isolates were estimated by method of Ercole, 2012. Biofilm estimation was done in 96-well microtiter plates. 200µl culture was inoculated in the wells of plate and covered by lids and incubated at 37°C and 120 rpm for 3 days. Biofilm production seen after 72 hrs in each culture came to be highest in *Bacillus megaterium* (BM). It shows highest production at absorbance 0.05 at 590 nm (Table 4.5. and Figure 4.8.).

Table 4.5. Comparison of biofilm production (OD_{590}) by different *Bacillus* isolates in nutrient broth

Cultures	Biofilm production
BM	0.05 ± 0.0a
BC	0.04 ± 0.0b
BS1	0.04 ± 0.0b
BS2	0.04 ± 0.0b
BS3	0.04 ±0.0b
BS4	0.04 ± 0.0b
BS5	0.04 ± 0.0b
Control	0.02 ± 0.0c

Values bearing different letters in the same column differ significantly ($P<0.05$). All values are mean ± SD (n = 3)

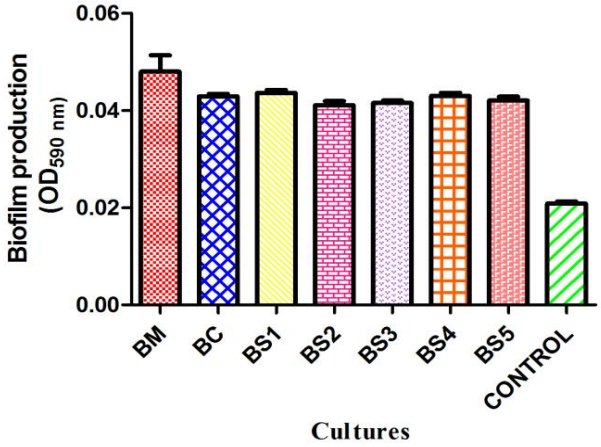


Fig. 4.8. Biofilm production (OD_{590}) by different *Bacillus* isolates in nutrient broth

4.1.5. Calcium Carbonate ($CaCO_3$) precipitation

Alkalophilic ureolytic bacterial isolates are very effective in $CaCO_3$ precipitation.Ca^{2+} concentration in the ureolytic bacterial culture supernatant reduced with time while the pH of the culture filtrate increased leading to precipitation of $CaCO_3$.$CaCO_3$ precipitation is dependent on the concentration of Ca^{2+} and CO_3^{2-} in solution. An increase in CO_3^{2-} concentration occurs under alkaline conditions abundant of calcium (Ca^{2+}) and carbonate (CO_3^{2-}) ions (Qian et al. 2010). In initial days, maximum soluble Ca^{2+} ions was noticed in *B. megaterium* (BM) as compared to other *Bacillus* isolates BC, BS1, BS2, BS3, BS4, BS5 (Table 4.6. and Figure 4.9.)

Table 4.6. Amount of soluble Ca^{2+} (mM) present in the nutrient broth

Isolates	**Soluble calcium ions (mM)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM	0.08 ± 0.0a	0.05 ± 0.0a	0.03 ± 0.0a	0.02 ± 0.0a	0.01 ± 0.0a
BC	0.06 ± 0.0ab	0.04 ± 0.0ab	0.03 ± 0.0ab	0.01 ± 0.0ab	0.01 ± 0.0ab
BS1	0.06 ± 0.0a	0.05 ± 0.0a	0.02 ± 0.0a	0.02 ± 0.0a	0.01 ± 0.0a
BS2	0.07 ± 0.0ab	0.04 ± 0.0ab	0.03 ± 0.0ab	0.01 ± 0.0ab	0.01 ± 0.0ab
BS3	0.08 ± 0.0a	0.05 ± 0.0a	0.01 ± 0.0a	0.02 ± 0.0a	0.01 ± 0.0a
BS4	0.07 ± 0.0a	0.05 ± 0.0a	0.01 ± 0.0a	0.01 ± 0.0a	0.01 ± 0.0a
BS5	0.06 ± 0.0ab	0.04 ± 0.0ab	0.01 ± 0.0ab	0.01 ± 0.0ab	0.01 ± 0.0ab
Control	0.02 ± 0.0b	0.01 ± 0.0b	0.01 ± 0.0b	0.01 ± 0.0b	0.01 ± 0.0b

Values bearing different letters in the same column differ significantly ($P<0.05$). All values are mean ± SD (n = 2)

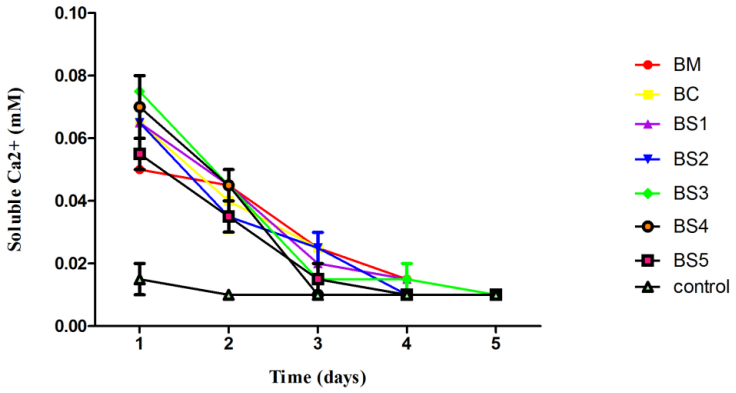


Fig. 4.9. Soluble Ca^{2+} (mM) of different *Bacillus* isolates in nutrient media.

4.1.6. Conclusion

In this study, it has been observed that different ureolytic alkalophilic bacterial isolates associated with calcium carbonate precipitation by enriching the medium with urea and $CaCl_2$. The best ureolytic bacterium, *Bacillus megaterium* (BM), of all the screened have been found to show highest enzyme activities (urease, carbonic anhydrase, protease), EPS/biofilm synthesis and carbonate precipitation.This alkalophile have the property of producing high amount of enzymes, EPS & calcite which is of most valuable importance for biotechnological applications. Further, the results from this study suggest that bacteria are enhancing calcium carbonate precipitation by providing well-suited surface for calcification. This work refers the *B. megaterium* as most active *Bacillus* sp. in calcification.

4.2. Optimization of different media

Culture medium plays an important role in supporting the growth and enzyme activity of bacterial isolates. Culture medium comprises of variety of nutrients as energy sources for bacterial cells and hence it is critical to provide proper and sufficient nutrients for growth and survival of ureolytic bacterial isolates. In the present study, the best found bacterial isolate, *B. megaterium*, was grown in different nutritional media as Nutrient broth (NB), Basal media (BM), B4 media (B4M), Specific media (M). All the media were supplemented with 2 % urea & 25mM $CaCl_2$ and labelled as BMU, NBU, B4M, MU in first set. Second set comprised of all the listed media but without 2% urea & 25mM $CaCl_2$ and labelled as Nutrient broth (NB-), Basal media (BM-), Specific media (M-). Third set involves the above listed media with 2% urea, 25mM $CaCl_2$ & 10µm $ZnSO_4$ and labelled as Nutrient broth (NB), Basal media (BM), B4 media (B4M), Specific media (M). It was recorded that maximum EPS production occurred in specific media with urea and $CaCl_2$ (MU). Specific media proved to be better medium for best ureolytic *Bacillus* species in production of highest EPS. As the production of enzyme, extracellular biopolymer is related to its biomass under conditions of favourable growth. The amount of enzyme expressed, EPS synthesized and carbonate produced by a specific microorganism is directly dependent on the nutrients available in the culture media.

4.2.1. Growth curve & pH profile in different media

The growth of the bacterial isolate was studied upto 144 hours in different media supplemented with 2% urea and 25 mM $CaCl_2$ and without it. The growth pattern and pH profile was highest in specific media. The cell growth was noticed to increase upto 48 hours. Cell concentration was determined from measurement of optical density (OD_{600}) of media. The growth curve showed that all the cells started to grow around 2-3 hours followed by inoculation and log phase was continued till 48 hours. In the present study, the media containing urea-CaCl2 supports maximum microbial growth. NH_4^+ and Cl^- react with OH^- and H^+ and urea serves as energy source and facilitates the growth of bacteria. From the above studies, it was noticed that both urea and calcium play most important role in determining the highest production of EPS by best ureolytic bacterial isolate. The synthesis of EPS was observed from early log phase; though it is quite low in initial timings, but enhances significantly later on. In the present study also, bacterial isolate was seen to produce significant EPS in exponential phase i.e. 48 hours. (Figure 4.10. and 4.11.).

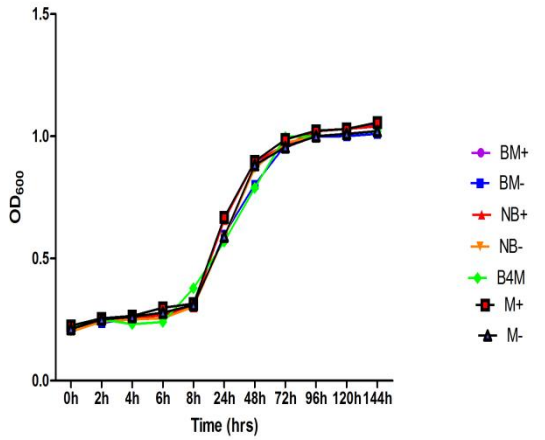


Fig. 4.10. Growth Curve of *B. megaterium* in different media

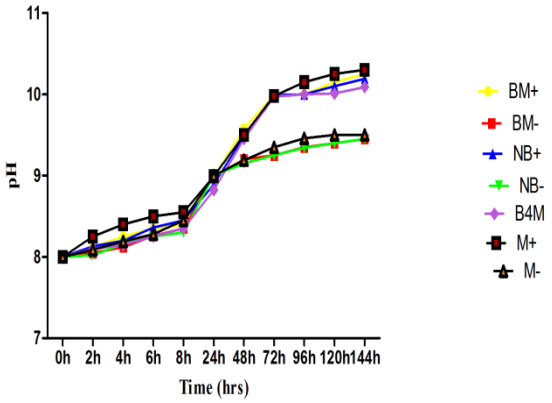


Fig.4.11. pH profile of *B. megaterium* in different media

4.2.2. Evaluation of different media for quantitative analysis

4.2.2.1. Urease production

Different media were used as nutrient source for the maximum production of urease by *B. megaterium*. *B. megaterium* was grown in each media supplemented with 2% urea & 25mM $CaCl_2$ and in media without urea & $CaCl_2$. Maximum urease production was seen with media containing urea and $CaCl_2$ with highest productivities on 3rd day (72hrs). After 72hrs, urease production decreased in all media. Highest enzyme activity was shown by *B. megaterium* on 3rd day (232 U/ml) in specific media (MU) (Table 4.7 and Figure 4.12). Whereas in case of media without urea and $CaCl_2$, urease activity was highest in specific media (157 U/ml) but less as compared to in media supplemented with urea and $CaCl_2$ (Table 4.8. and Figure 4.13).

Table 4.7. Urease activity of *B megaterium* in different media in the presence of urea and $CaCl_2$.

Media	Urease activity (U/ml)				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM U	194.57±0.0b	196.13±8.0b	223.46±2.3b	212.35±3.8b	197.69±0.3b
NBU	136.13±4.3c	181.68±4.0c	198.35±2.0c	167.24±1.0c	147.24±3.0c
B4M	114.13±3.5d	125.46±1.1d	159.46±5.4d	128.46±3.1d	125.46±1.1d
MU	195.47±0.0a	211.46±0.8a	232.02±15.2a	215.46±3.5a	196.57±2.1a
Control	107.91±5.0e	108.46±8.4e	119.80±0.3e	112.80±5.8e	103.35±2.2e

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

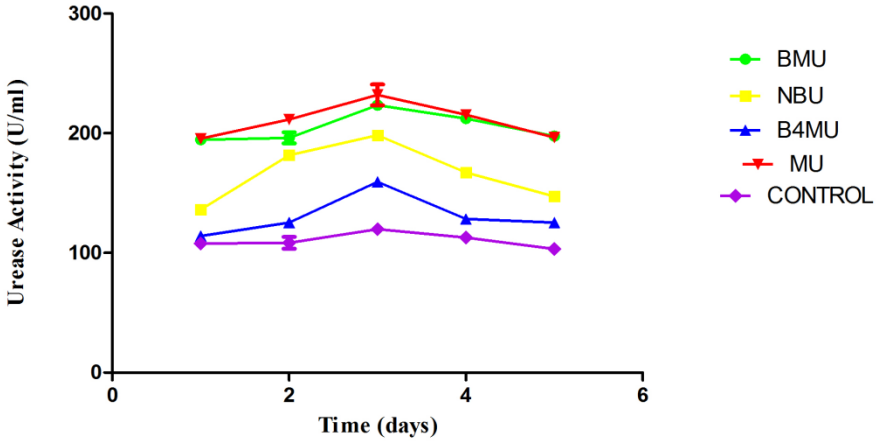


Fig.4.12. Urease Activity (U/ml) of *B. megaterium* in different media supplemented with urea & $CaCl_2$

Table 4.8. Urease activity of *B megaterium* in different media in absence of urea and $CaCl_2$

Media	Urease activity (U/ml)				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM -	107 ± 2b	118 ± 3.9b	137 ± 8.3b	109 ± 4.6b	100 ± 0.7b
NB-	113 ± 1.6b	116 ± 4.4b	135 ± 6.9b	120 ± 5b	115 ± 3.6b
M-	111 ± 3.7a	136 ± 4.5a	157 ± 5.2a	128 ± 5.8a	119 ± 1.3a
Control	92 ± 1.2c	97 ± 1.5c	103 ± 2.4c	100 ± 1.2c	94 ± 2.8c

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

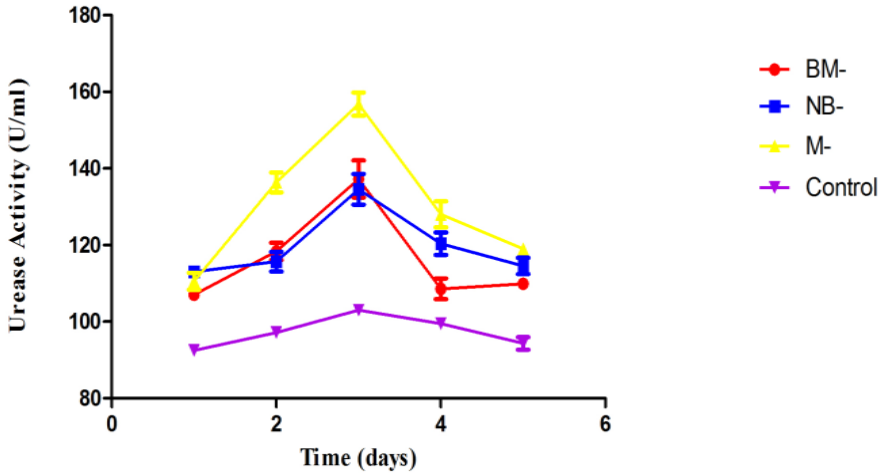


Fig.4.13. Urease Activity (U/ml) of *B. megaterium* in different medias without urea & $CaCl_2$

4.2.2.2. Carbonic Anhydrase production

The maximum production of Carbonic Anhydrase by *B. megaterium* was seen in different media. *B. megaterium* was grown in each media supplemented with 2% urea & 25mM $CaCl_2$ and 10µm $ZnSO_4$. Maximum CA production was observed in specific media (M) with highest productivities on 3rd day (72hrs). After 72hrs CA production decreases in all media. *B.megaterium* shows highest enzyme activity (929 U/ml) on 3rd day after that gradual decrease in enzyme activity (Table 4.9. and Figure 4.14)

Table 4.9. CA activity of *B. megaterium* in different media in presence of urea, $CaCl_2$ & $ZnSO_4$

Media	**CA activity (U/ml)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM	655 ± 20c	821 ± 10.5c	880 ± 32c	853 ± 17.7c	726 ± 13.2c
NB	696 ± 8.14a	808 ± 17.7a	847 ± 12.5a	808 ± 30.7a	698 ± 31.6a
B4M	650 ± 25.9b	715 ± 33.5b	761 ± 3.0b	712 ± 36.6b	622 ± 18.5b
M	774 ± 20.8a	817 ± 17.3a	929 ± 20.1a	774 ± 32.1a	750 ± 20.8a
Control	462 ± 17.1c	566 ± 15.9c	633 ± 12.8c	573 ± 23.3c	438 ± 14.0c

Values bearing different letters in the same column differ significantly ($P<0.05$). All values are mean ± SD (n = 3)

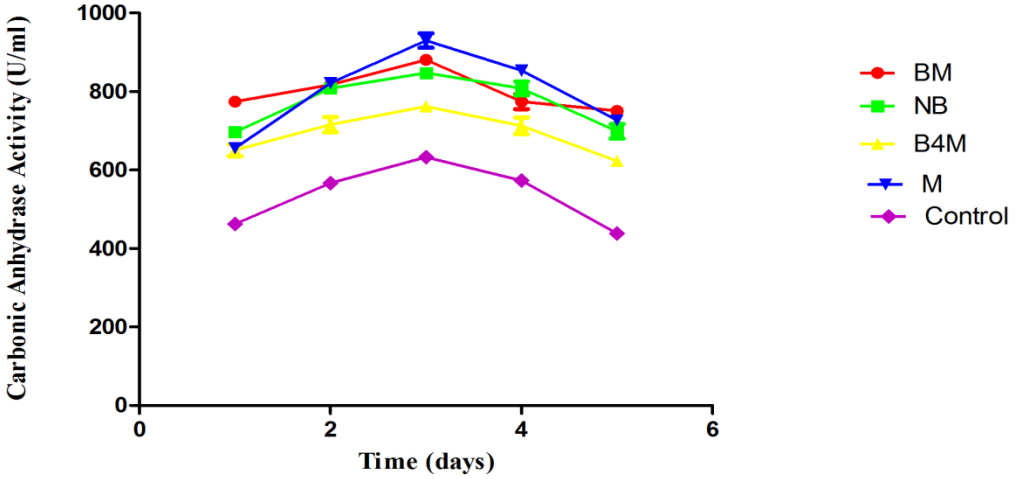


Fig.4.14. Carbonic Anhydrase activity (U/ml) of *B. megaterium* in different media supplemented with urea, $CaCl_2$ and $ZnSO_4$.

4.2.2.3. Protease Activity

Protease activity produced by *B. megaterium* grown in different media was detected in the culture supernatant from 24hrs to120 hrs and a sudden increase in its activity was found at end of 5^{th} day (120hrs). Protease activity of *B. megaterium* was maximum in specific media (MU) supplemented with urea and $CaCl_2$ (57 U/ml) at the end of 5^{th} day (Table 4.10 and Figure 4.15) whereas it decreases in the media without urea and $CaCl_2$ (51 U/ml) (Table 4.11 and figure 4.16).

Table 4.10. Comparison of protease activity of *B megaterium* in different media in the presence of urea and calcium chloride

Media	**Protease activity (U/ml)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BMU	31.26±1.8b	35.75±0.6b	46.46±0.2b	49.61±0.6b	56.06±0.8b
NBU	32.1±0.3b	34.35±1.3b	39.75±0.5b	47.99±1.5b	52.40±1.0b
B4M	25.00±1.8c	31.22±0.5c	38.02±0.8c	44.33±0.2c	49.39±0.8c
MU	37.20±1.0a	41.38±1.1a	46.22±0.6a	51.23±0.8a	56.82±0.9a
Control	11.69±1.0d	13.22±0.8d	14.57±0.6d	16.30±0.7d	19.50±0.4

Values bearing different letters in the same column differ significantly ($P<0.05$). All values are mean ± SD (n =3)

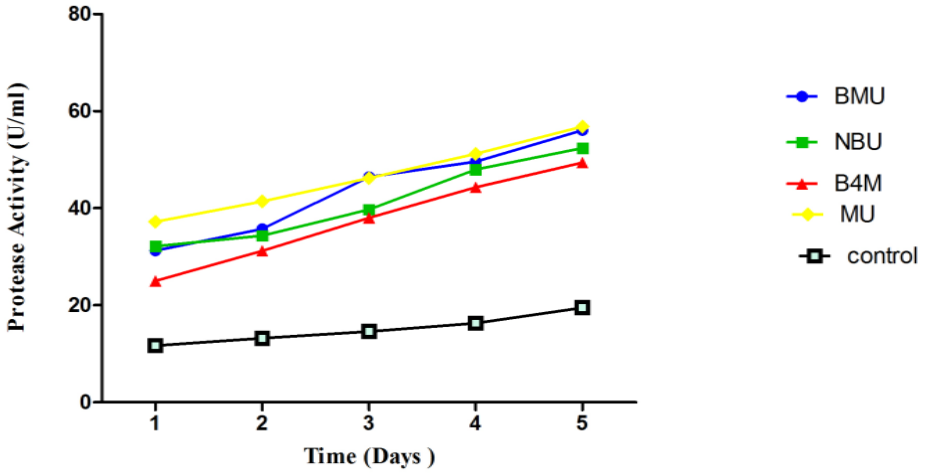


Fig. 4.15. Protease Activity (U/ml) of *B. megaterium* in different media supplemented with urea & $CaCl_2$

Table 4.11. Comparison of protease activity (U/ml) of *B. megaterium* in different media in absence of urea and $CaCl_2$

Media	**Protease activity (U/ml)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM-	22.86±1.0b	29.33±1.2b	36.55±0.6b	39.62±0.4b	48.88±0.7b
NB-	22.25±0.7b	30.08±0.4b	34.95±0.1b	37.39±0.6b	45.13±1.0b
M-	29.74±0.3a	35.93±0.4a	40.15±0.2a	44.53±1.2a	51.62±0.9a
Control	11.49±1.1c	12.69±0.7c	14.50±1.0c	17.49±0.5c	19.95±0.3c

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n =3)

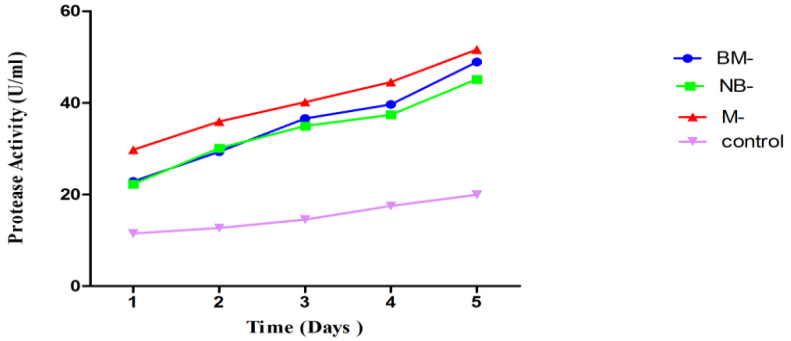


Fig. 4.16. Protease activity (U/ml) of *B. megaterium* in different media without urea and $CaCl_2$

4.2.3. Extracellular polymeric substances (EPS) and biofilm production

The ability of bacteria to adhere on surfaces is associated with the extensive production of extracellular biopolymers (EPS). EPS of bacteria serves as nucleation site for calcium carbonate to precipitate. EPS matrix enables bacteria to capture nutrients present in different nutritive media. Highest EPS and biofilm produced by *B. megaterium* in specific media (M) media (MU, 4881µg/100ml) supplemented with urea and $CaCl_2$; followed by media with urea, $CaCl_2$ and $ZnSO_4$ (M, 4320 µg/100ml) and media without urea and $CaCl_2$ (M-, 4115 µg/100ml) (Table 4.12, 4.13, 4.14 and Figure 4.17, 4.18, 4.19) respectively. Biofilm production by *B. megaterium* grown in specific media (MU) supplemented with urea and $CaCl_2$, shows highest absorbance (0.05) at 590 nm (Table 4.15 and Figure 4.20).

Table 4.12. EPS production (µg/100ml) by *B. megaterium* in different media in presence of urea & $CaCl_2$

Media	**EPS production (µg/100ml)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM U	975 ± 45.9b	1543 ± 14b	1661 ± 33b	1632 ± 24.7b	1468 ± 43.3b
NBU	1097 ± 56.6c	1144 ± 49.8c	1330 ± 55.4c	1095 ± 16.8c	939 ± 35.5c
B4M	669 ± 18.9d	910 ± 4.5d	1122 ± 32.3d	740 ± 59.8d	595 ± 11.4d
MU	3845 ± 17.8a	4398 ± 33.1a	4881 ± 93.5a	3636 ± 47.7a	3277 ± 50.3a
Control	244 ± 10.3e	344 ± 10e	428 ± 45.7e	325 ± 12.6e	384 ± 4.8e

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

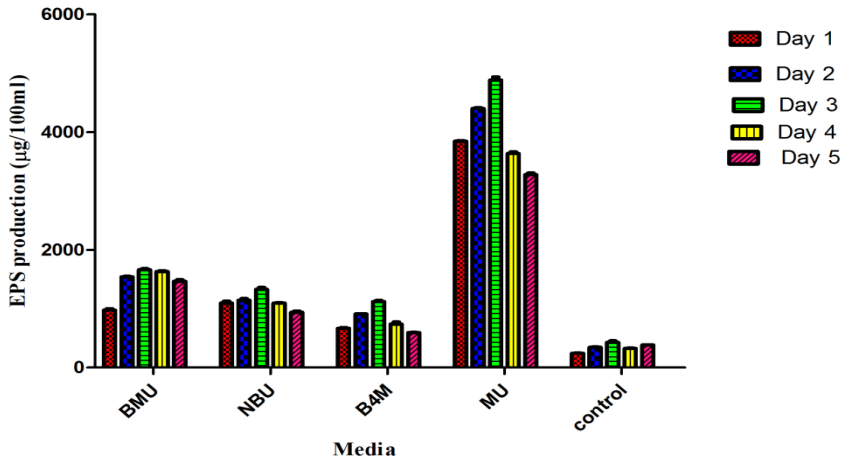


Fig. 4.17. EPS production (µg/100ml) of *B. megaterium* in different media supplemented with urea & $CaCl_2$

Table 4.13.EPS production (µg/100ml) of *B. megaterium* in different medias in absence of urea & $CaCl_2$

Media	EPS production (µg/100ml)				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM-	937 ± 28.1b	1208 ± 34.9b	1469 ± 42.2b	1080 ± 56b	1029 ± 29.5b
NB-	658 ± 35.4c	761 ± 17.9c	759 ± 33.9c	635 ± 12.1c	646 ± 16.8c
M-	3270 ± 71.5a	3505 ± 64.1a	4115 ± 75.1a	3236 ± 100.2a	2935 ± 106.2a
Control	262 ± 39.8d	367 ± 21.9d	447 ± 27.8d	344 ± 14.9d	254 ± 6.8d

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

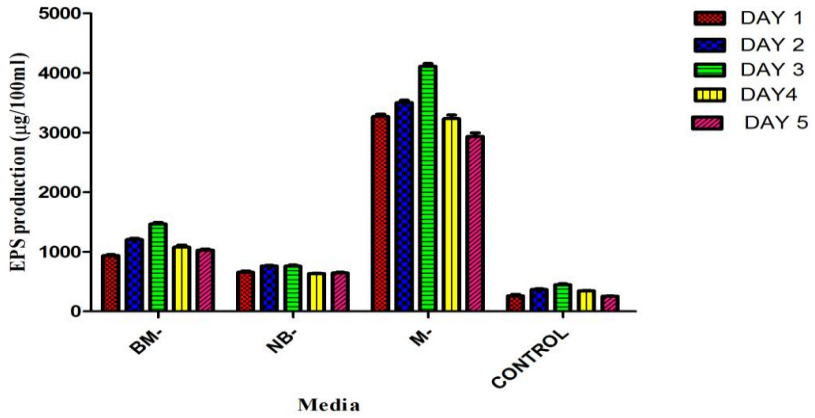


Fig. 4.18. EPS production (µg/100ml) of *B. megaterium* in different media without urea & $CaCl_2$

Table 4.14. EPS production (µg/100ml) of *B. megaterium* in different media in the presence of urea, $CaCl_2$, $ZnSO_4$

Media	EPS production (µg/100ml)				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM	1654 ± 54b	2216 ± 62.7b	2514 ± 35.2b	1523 ± 64.7b	1327 ± 23.3b
NB	1271 ± 77.4c	1450 ± 35.7c	1652 ± 39.3c	1401 ± 70.8c	1226 ± 49.2c
B4M	659 ± 29d	828 ± 9.9d	964 ± 26.6d	699 ± 30.3d	608 ± 23d
M	3400 ± 50.5a	3926 ± 71.6a	4320 ± 74.9a	3855 ± 10.7a	3272 ± 102.7a
Control	313 ± 13e	441 ± 32.7e	469 ± 9.4e	366 ± 20.4e	268 ± 17.7e

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

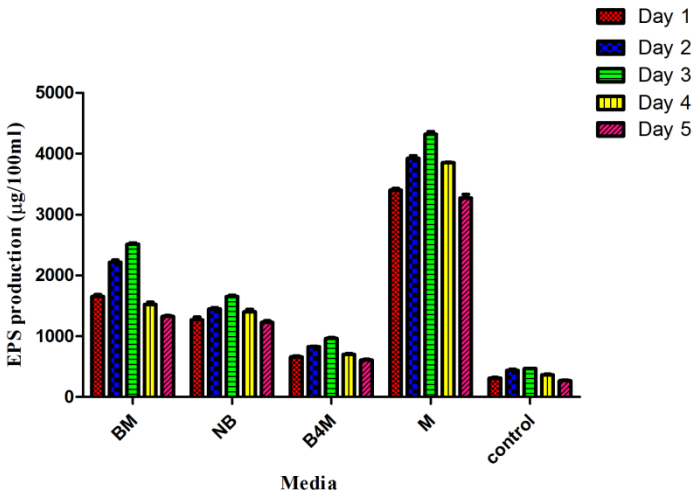


Fig.4.19. EPS production (µg/100ml) of *B. megaterium* in different media supplemented with urea, $CaCl_2$, $ZnSO_4$)

Table 4.15. Biofilm production (OD_{590}) by *B megaterium* in different media in presence of urea, $CaCl_2$

Media	Biofilm production
BM	0.04 ± 0.0a
NB	0.04 ± 0.0a
B4M	0.03 ± 0.0ab
M	0.05 ± 0.0a
Control	0.02 ±0.0b

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 3)

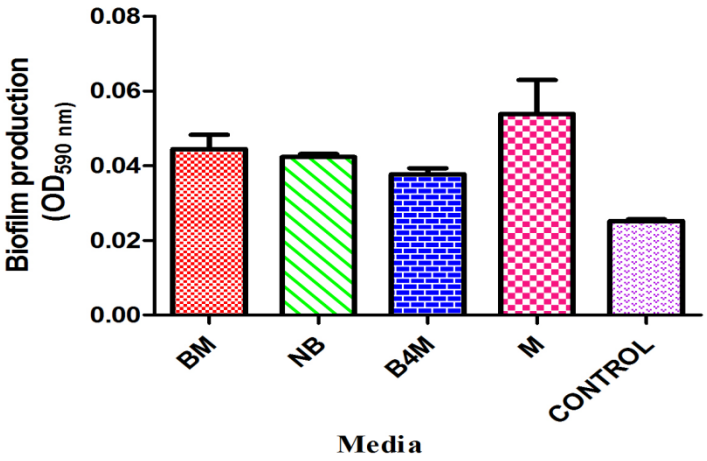


Fig. 4.20. Biofilm production by *B. megaterium* in different media supplemented with urea and $CaCl_2$

4.2.4. Calcite precipitation in different media

In case of carbonate precipitation, it was observed that concentration of soluble calcium was high initialy in early hours after bacterial inoculation. Soluble calcium (Ca^{2+}) decreases with time due to increase in the precipitation of calcium carbonate. Soluble calcium (Ca^{2+}) was highest in media with urea, $CaCl_2$, $ZnSO_4$ (0.3mM) as compared to media without urea and $CaCl_2$ (0.07mM) and media with urea and $CaCl_2$ (0.2 mM). All the ureolytic bacterial isolates exhibited their potential for efficient carbonate precipitation (Tables 4.16, 4.17, 4.18 and Figures 4.21, 4.22, 4.23).

Table 4.16. Soluble calcium (Ca^{2+}) (mM) in different media in presence of urea and $CaCl_2$

Media	**Soluble calcium (mM)**				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM U	0.12 ± 0.0ab	0.08 ± 0.0ab	0.02 ± 0.0ab	0.01± 0.0ab	0.01 ± 0.0ab
NBU	0.1 ± 0.0bc	0.07 ± 0.0bc	0.02 ± 0.0bc	0.01 ± 0.0bc	0.01 ± 0.0bc
B4M	0.08 ± 0.0cd	0.05 ± 0.0cd	0.02 ± 0.0cd	0.01 ± 0.0cd	0.01 ± 0.0cd
MU	0.2 ± 0.0a	0.1 ± 0.0a	0.06 ± 0.0a	0.03 ± 0.0a	0.01 ± 0.0a
Control	0.02 ± 0.0e	0.01 ± 0.0e	0.01± 0.0e	0.01 ± 0.0e	0.01 ± 0.0e

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

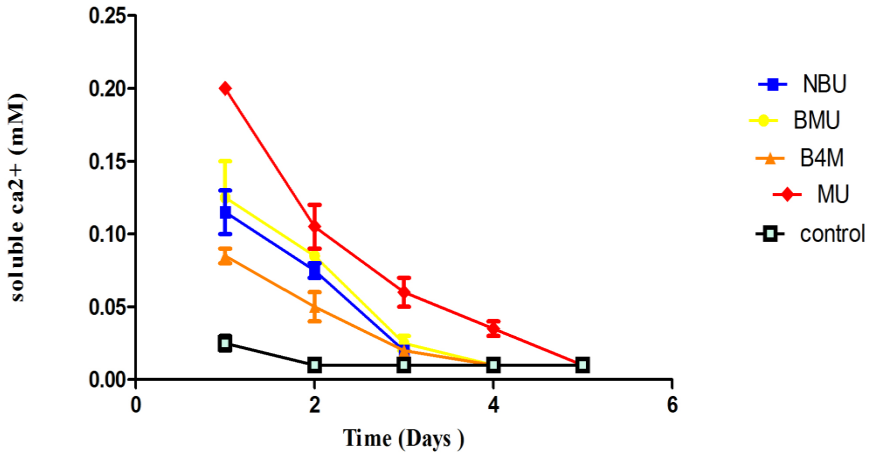


Fig. 4.21. Soluble calcium (Ca^{2+}) in media supplemented with urea and $CaCl_2$.

Table 4.17. Soluble calcium (Ca^{2+}) (mM) in different media in absence of urea and $CaCl_2$

Media	Soluble calcium (mM)				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM-	0.050±0.028a	0.030±0.014a	0.025±0.01a	0.010±0.0a	0.010±0.0a
NB-	0.045±0.007a	0.025±0.007a	0.012±0.00a	0.010±0.0a	0.010±0.0a
M-	0.07±0.014a	0.025±0.007a	0.018±0.00a	0.010±0.0a	0.010±0.0a
Control	0.035±0.007a	0.015±0.007a	0.010±0.00a	0.010±0.0a	0.010±0.0a

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

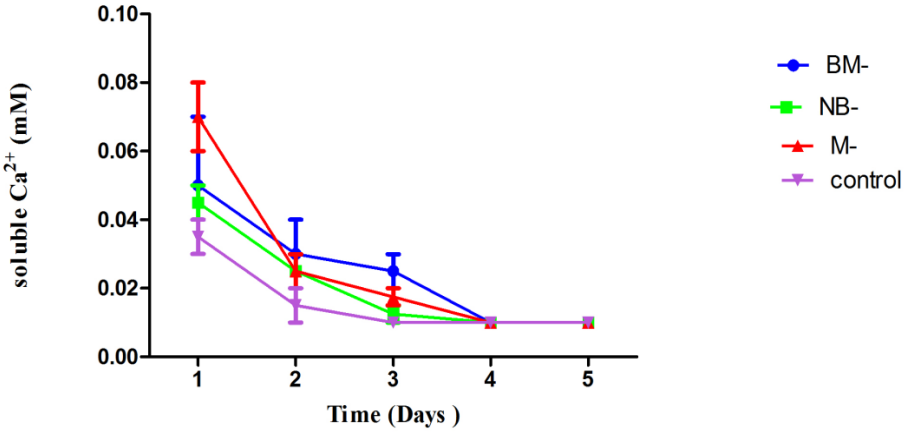


Fig. 4.22. Soluble calcium (Ca^{2+}) in media without urea and $CaCl_2$.

Table 4.18. Soluble calcium (Ca^{2+}) (mM) in different media in presence of Urea, $ZnSO_4$ and $CaCl_2$

Media	Soluble calcium (mM)				
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**
BM	0.3 ± 0.0ab	0.2 ± 0.0ab	0.17 ± 0.0ab	0.13 ± 0.0ab	0.01 ± 0.0ab
NB	0.2 ± 0.0ab	0.17 ± 0.0ab	0.13 ± 0.0ab	0.11± 0.0ab	0.01 ± 0.0ab
B4M	0.17 ± 0.0b	0.15 ± 0.0b	0.12 ± 0.0b	0.10 ± 0.0b	0.01 ± 0.0b
M	0.3 ± 0.0a	0.2 ± 0.0a	0.17 ± 0.0a	0.14 ± 0.0a	0.01 ± 0.0a
Control	0.03 ± 0.0c	0.01± 0.0c	0.01 ± 0.0c	0.01± 0.0c	0.01± 0.0c

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

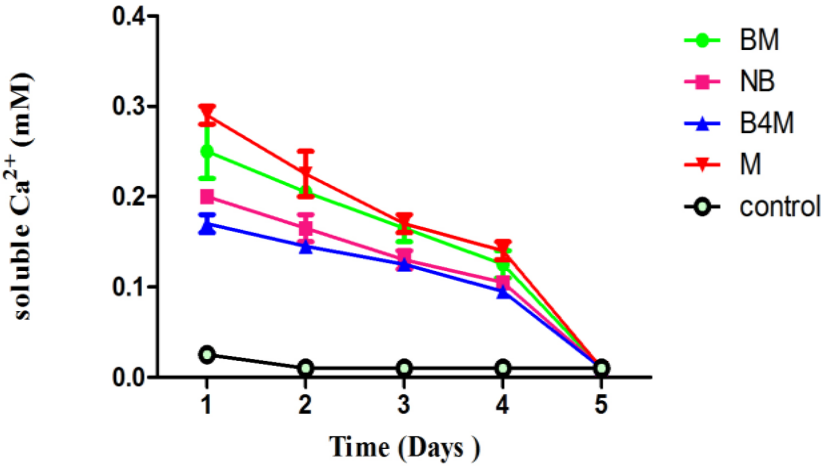


Fig. 4.23. Soluble calcium (Ca^{2+}) (mM) in media supplemented with Urea, $ZnSO_4$ and $CaCl_2$

4.2.5. Conclusion

Based on higher productions of enzymes (urease, carbonic anhydrase, protease), extracellular biopolymers and calcite precipitation by bacterial isolate after utilizing different nutritive medias, it can be concluded that specific media (M) is the best suited media for the better quantitative production. The *B. megaterium* showed maximal production activity in specific media (M) followed by Basal media (BM), Nutrient broth (NB) and B4M. Further, the results from this study suggest that bacteria can proliferate to its maximum or increase its amount in the presence of urea and $CaCl_2$. Urea by releasing NH_4 increases the pH of the media which is better suited for the growth of bacteria. Media without urea & $CaCl_2$ showed less quantitative productions. $ZnSO_4$ in addition to urea & $CaCl_2$ also play a major role in determining the best growth media for bacteria so that it can capture more and more nutrients on its surface by adhesion and cohesion properties and results in higher calcification.

4.3. Optimization of media components by using one factor at a time (OFAT) approach

As it was envisaged that ureolytic pathway is very efficient and economical way for generating large amounts of carbonate precipitates in short periods of time, an attempt was made to enhance the synthesis of extracellular polymeric substances for enhancing the production of carbonates by optimizing the media components through OFAT.

4.3.1. Selection of constituents of growth media

The constituents of the growth media and process variables play a significant role in synthesis of EPS. Among these, the major players are: carbon source, nitrogen source, calcium source, pH, temperature, time of incubation, concentration of urea, $CaCl_2$, size of inoculum, trace salts, supplements etc. As many of these factors have already been investigated in earlier work, in the present study physicochemical parameters that were studied by one factor at a time (OFAT) were carbon source, nitrogen source (organic & inorganic), calcium source & pH. These parameters were varied and optimized for highest production of EPS. The various carbon sources tested were: glucose, fructose, maltose, lactose, sucrose, starch and mannitol while different organic nitrogen sources tested were beef extract, peptone and inorganic nitrogen sources estimated were ammonium sulphate, ammonium acetate, ammonium carbonate, urea and ammonium chloride. Different calcium sources tested were calcium chloride, calcium acetate, calcium phosphate, calcium nitrate and calcium hydroxide whereas different pH conditions were also investigated that were at pH 7, 8, 9, 10.

Glucose (1%) was found to be the best carbon source compared to sucrose, fructose, lactose and starch. In case of organic nitrogen sources, peptone (0.5%) and in case of inorganic nitrogen source, ammonium sulphate (2%) was found to be better source of nitrogen compared to beef extract, ammonium carbonate, ammonium acetate, urea, ammonium chloride while pH 7 was found to be the optimal cell concentration for highest EPS synthesis on 3rd day (Tables 4.19 - 4.23).

4.3.1.1. Effect of different carbon sources

Carbon is an important factor in the formation of EPS. Bacterial cell wall is composed of polysaccharide as major factor. These polysaccharides provide mechanical and tensile strength to bacteria to survive in external harsh environment. Gram positive bacteria (*Bacillus* sp.) is composed of large number of polysaccharide layer which provide it electronegative nature, helping in capturing large amount of cationic calcium ions to bind with carbonate ions

and induce calcite precipitation. Glucose serves as the best carbon source in enhancing the EPS production (3651 µg/ml) by *B. megaterium* (Table 4.19. and Figure 4.24.)

Table 4.19. Effect of different carbon sources on EPS production (µg/100ml) by *B. megaterium*

Carbon source	EPS production (µg/100ml)
Glucose	3651 ± 24.8a
Fructose	3400 ± 109.2bc
Maltose	3280 ± 38.2cd
Lactose	3348 ± 114.8bc
Sucrose	3172 ± 55.8d
Starch	3352 ± 57.7bc
Mannitol	3465 ± 4.5b
Control	2930 ± 68.8e

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

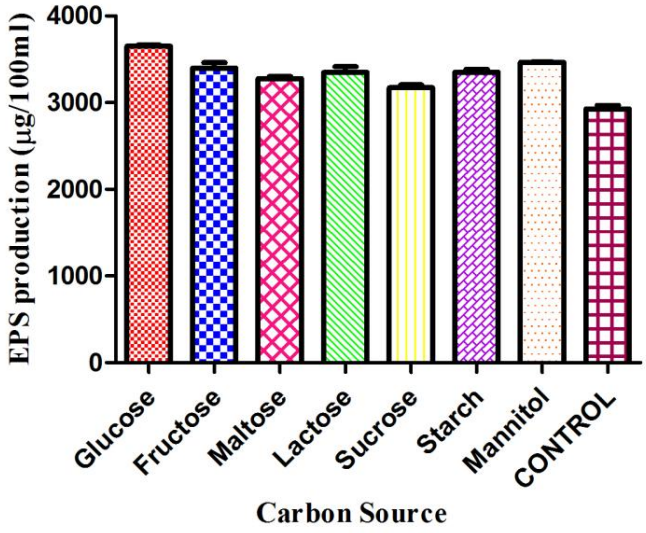


Fig. 4.24. Effect of different carbon sources on EPS production (µg/100ml) by *B. megaterium* in specific media.

4.3.1.2. Effect of different nitrogen source on EPS production

Nitrogen is main factor responsible for calcite precipitation either extracellularly or intracellularly. Nitrogen in organic and inorganic form acts a source of energy for bacteria for synthesis of cell wall, locomotion and to carry out basic metabolisms. Peptone as organic nitrogen source shows highest EPS production 6661 µg/100ml and ammonium sulphate and urea as inorganic nitrogen source shows highest EPS production 4365 µg/100ml and 4121µg/100ml as compared to other nitrogen sources. (Tables 4.20-4.21. and Figure 4.25-4.26)

Table 4.20. Effect of different organic nitrogen source on EPS production (µg/100ml) by *B. megaterium*

Organic nitrogen source	EPS production (µg/100ml)
Peptone	6661 ± 108.9a
Beef	4910 ± 100.0b
Control	3511 ± 69.8c

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

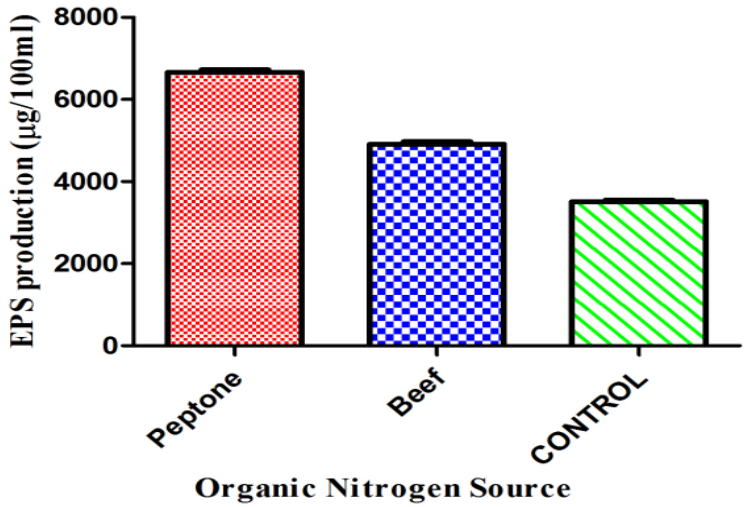


Fig. 4.25. Effect of different organic nitrogen source on EPS production (µg/100ml) by *B. megaterium* in specific media

Table 4.21. Effect of different inorganic nitrogen source on EPS production (µg/100ml) by *B. megaterium*

Inorganic nitrogen source	EPS production (µg/100ml)
Ammonium sulphate	4365 ± 57.5a
Ammonium acetate	3368 ± 15.3c
Ammonium carbonate	3241 ± 121.6c
Urea	4122 ± 124.3b
Ammonium chloride	3227 ± 152.8c
Control	2668 ± 85.5d

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

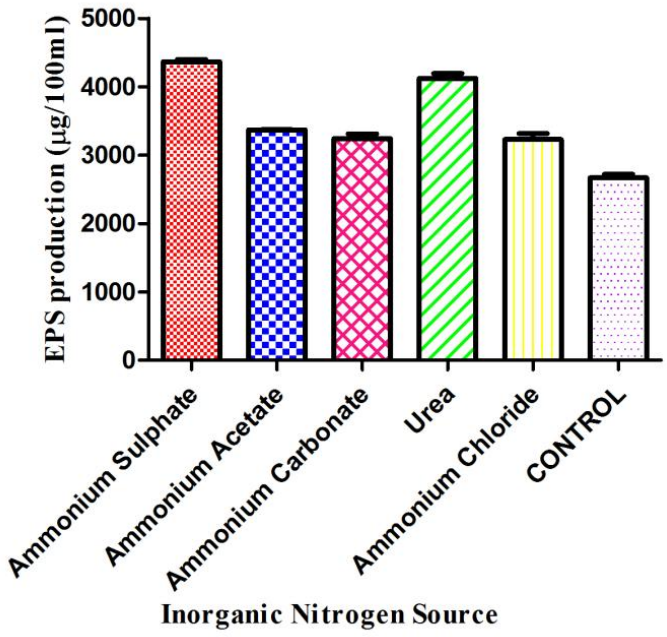


Fig. 4.26. Effect of inorganic nitrogen source on EPS production (µg/100ml) by *B. megaterium* in specific media.

4.3.1.3. Effect of calcium sources on EPS production

Calcium plays a very important role in calcite precipitation. Higher the amount of calcium higher will be the carbonate precipitation. Due to its cationic nature calcium ions bind to

negatively charged EPS layer of bacteria and gets surface for capturing more and more carbonate ions for precipitation to take place as a result bacterial cell become nucleus of microbial calcite crystals. In this study, different calcium sources are used to enhance calcite precipitation on EPS layer. Calcium chloride was studied as best source in production of highest EPS (4194 µg/ml) (Table 4.22 and Figure 4.27)

Table 4.22. Effect of different calcium sources on EPS production (µg/100ml) by *B. megaterium*

Calcium source	EPS production (µg/100ml)
Calcium chloride	4194 ± 49.7a
Calcium acetate	3502 ± 36.0b
Calcium phosphate	3488 ± 17.1b
Calcium nitrate	3411 ± 18.6b
Calcium hydroxide	3257 ± 49.2c
Control	2637 ± 96.69d

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

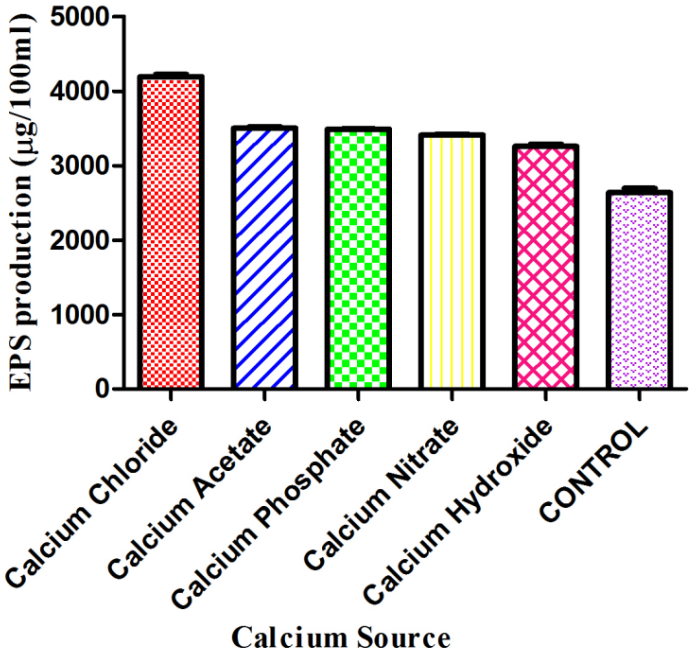


Fig. 4.27. Effect of calcium sources on EPS production (µg/100ml) by *B. megaterium* in specific media

4.3.1.4. Effect of pH on EPS production

pH is the key factor in microbially induced calcite precipitation. Calcite precipitation takes place at high pH (9-10). The release of ammonium ions by the urea hydrolysis increases the pH of the medium resulting in dominance of bicarbonate ions in the media and formation of more and more carbonate crystals on the bacterial surface (EPS matrix). EPS production by *B. megaterium* was observed to be highest (3762µg/100ml) at pH 7, which increases to 9 during the growth phase in the medium (Table 4.23 and figure 4.28).

Table 4.23. Effect of variable pH on EPS production (µg/100ml) by *B. megaterium*

pH	EPS production (µg/100ml)
7	3762 ± 88.5a
8	3332 ± 92.4b
9	3198 ± 48.2b
10	2922 ± 66.7c
Control	2526 ± 64.9d

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

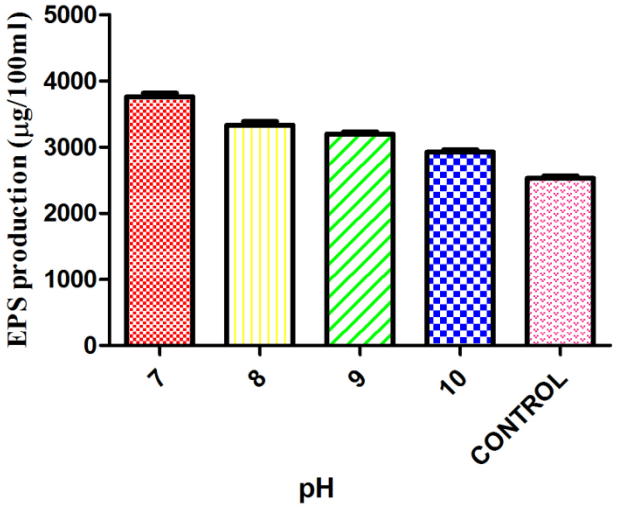


Fig. 4.28. Effect of variable pH on EPS production (µg/100ml) by *B. megaterium* in specific media.

4.3.2. Conclusion

The present work has culminated in production of higher EPS and carbonate precipitates by the application of conventional method OFAT. The physical and chemical factors which affect the production of EPS in *Bacillus megaterium* were varied one at a time. Concentration of glucose, peptone, ammonium sulphate, calcium chloride at pH 7 was found to be most significant factors for the EPS synthesis. Based on the results of OFAT, the optimized media and conditions for maximum EPS production comprised glucose = 2g, peptone = 5g, $(NH_4)_2SO_4$ = 2 g, $CaCl_2$ = 25mM per litre at pH 7.0 and temperature of 37°C for incubation of 5 days which resulted in 1.2 folds increase in the activity and 1.7 folds increase in the production of carbonates in the optimized media.

4.4. Optimization of EPS production by *B. megaterium*

EPS production by *B. megaterium* has been optimised for the better precipitation of calcite as EPS surface enhance the calcite biomineralization by capturing $CaCO_3$ due to its adhesion properties and electronegative nature of EPS. Varying concentrations of EPS was made and after centrifugation supernatant was treated overnight with ethanol. In resulting pellet, constant volume of 25mM $CaCl_2$ was added. The soluble Ca^{2+} was the estimated at known concentration of EPS by EDTA titration method. Maximum soluble Ca^{2+} concentration was found in pellet with minimum weight. Soluble calcium concentration decreases with increase in EPS concentration of *B. megaterium* in both specific media (M) and nutrient broth (NB). Maximum soluble Ca^{2+} was estimated at 20mg/100ml EPS concentration in specific media (15mM) (Table 4.24 and Figure 4.29).

Table 4.24. Relation between EPS produced by *B. megaterium* and soluble Ca^{2+} in media

EPS concentration (mg/100ml)	25mM $CaCl_2$ concentration (ml)	Soluble Calcium (mM)		
		NB	M	Control
20	2.5	14.5 ± 0.7a	15 ± 1.4a	5 ± 1.4a
40	2.5	9 ±1.4b	13 ± 0.7b	3 ± 0.3b
60	2.5	7.4 ± 0.2bc	11 ± 0.7bc	1 ± 0.0bc
80	2.5	6 ± 0.5c	9.5 ± 0.7c	1 ± 0.0c
100	2.5	1.5 ± 0.7d	3 ± 0.8d	1 ± 0.0d

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

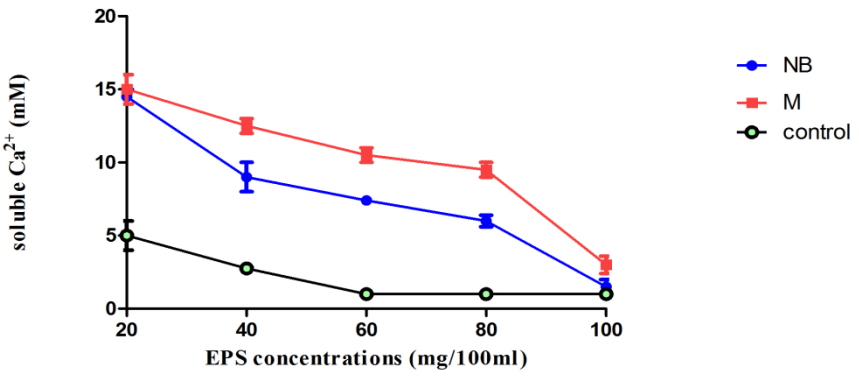


Fig. 4.29. Relation between EPS produced by *B. megaterium* and soluble Ca^{2+} in media

4.5. Characterization of EPS produced by *B. megaterium*

Due to the presence of negatively charged surface bacteria enables to perform calcite precipitation in a form of successive stratification. Different carbohydrate and protein components are responsible for the electronegative nature of EPS resulting in bounding of calcium ions to capture carbonate ions and formation of calcite on EPS layer and protein in the presence of Ca^{2+} highly influence the carbonate precipitation. Proteins present in the EPS layer determines the polymorphism of carbonate crystals. EPS has been characterized for determining the presence of total amount of carbohydrate and protein thus influencing the calcite biomineralization.

4.5.1. Gram's Staining and capsule staining of bacteria

Bacterial cells were stained by gram's staining to identify the morphology. *Bacillus megaterium* which is a rod shaped, gram positive bacteria was observed under microscope as it appeared pinkish in colour. Bacterial surface is surrounded by a capsular layer which protects bacteria under harsh conditions. Capsule of bacteria was stained by nigrosin and methylene blue dye. Capsule of *B. megaterium* appeared bluish in colour (figure 4.30).

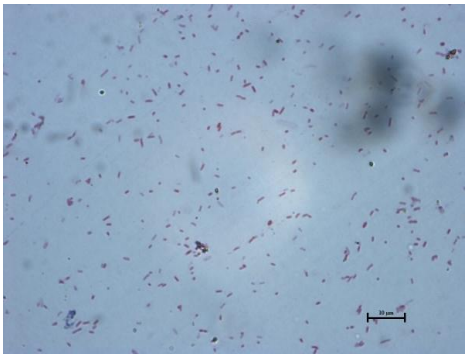
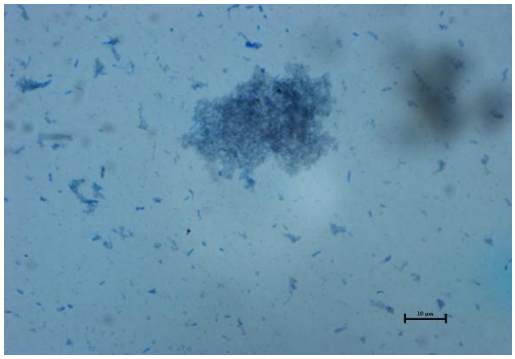
a)  b) 

Fig. 4.30. a) Pink coloured, rod shaped *B. megaterium* cells, b) Blue coloured outer surface (capsule) of *B. megaterium*

4.5.2. Total carbohydrate and protein content in EPS of *B. megaterium*

Total carbohydrate content was determined in the EPS by phenol-sulphuric acid method. *Bacillus megaterium* was grown in two media with nutrient broth as standard media. Carbohydrates present in the extracellular polymeric substances (EPS) layer were highest in specific media (M, 3016 µg/100ml) as compared to nutrient broth. Carbohydrates in capsular polysaccharides (CPS) were less produced than in EPS. Whereas in case, of total protein

content, proteins were found to be highest in CPS (45 mg/ml) as compare to EPS in case of specific media (SM) (Table 4.25 and Figure 4.31).

Table 4.25. Total carbohydrate and protein content in EPS of of *B. megaterium*

Media	Total carbohydrate content(µg/100ml)			Total protein content (mg/ml)		
	EPS	CPS	Control	EPS	CPS	Control
NB	1573 ±16.4c	1385 ± 15d	1080 ± 54e	14.6 ± 0.5d	20.35 ± 1.7c	10.13 ± 0.6e
M	3016 ± 128a	2076 ± 85b		39.3 ± 0.4b	45.33 ± 0.6a	

Values bearing different letters in the same column differ significantly (P<0.05). All values are mean ± SD (n = 2)

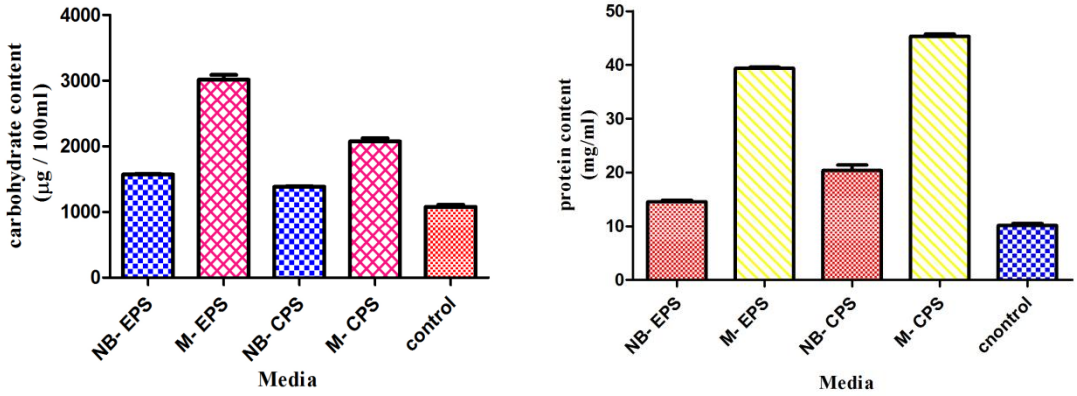
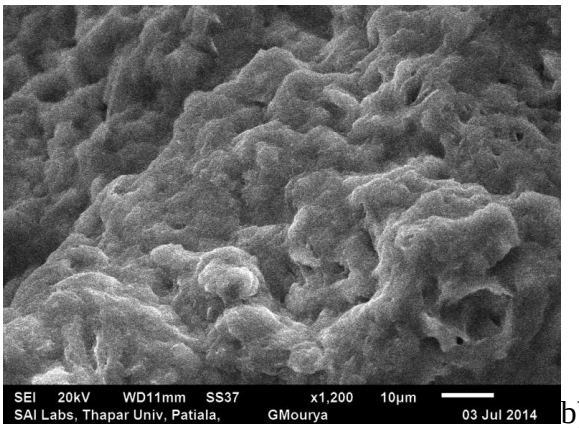
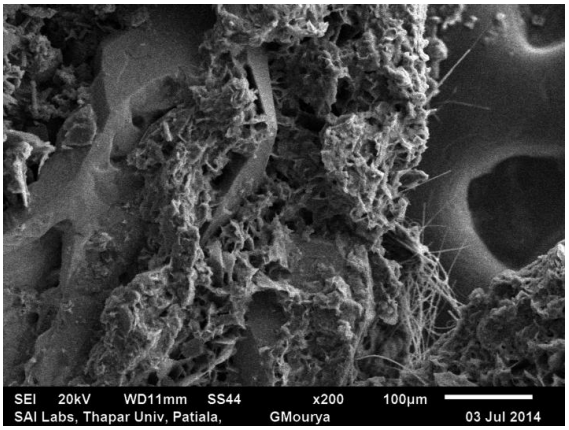


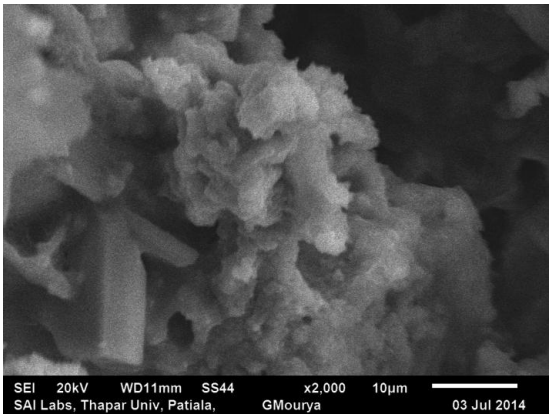
Fig. 4.31. a) Total carbohydrate content , b) Total protein content in EPS and CPS of *B. megaterium* in nutrient broth (NB) and specific media (M)

4.5.3. Scanning electron microscopic (SEM) of EPS & CPS of *B. megaterium*

Scanning electron microscopic images of EPS and CPS of *B. megaterium* were clearly seen. SEM analyses of bacterial exocellular biopolymers depicted the magnified images of EPS and CPS. EPS in nutrient broth was smooth and have mucous matrix whereas EPS in specific media have rough surface with some needle like structures present. SEM analysis of CPS images also depicted the smooth surface whereas CPS in specific media was of rough surface (Figure 4.32).

a) 

b) 

c) 

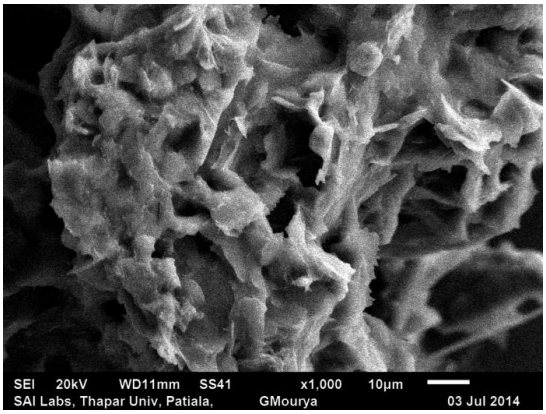
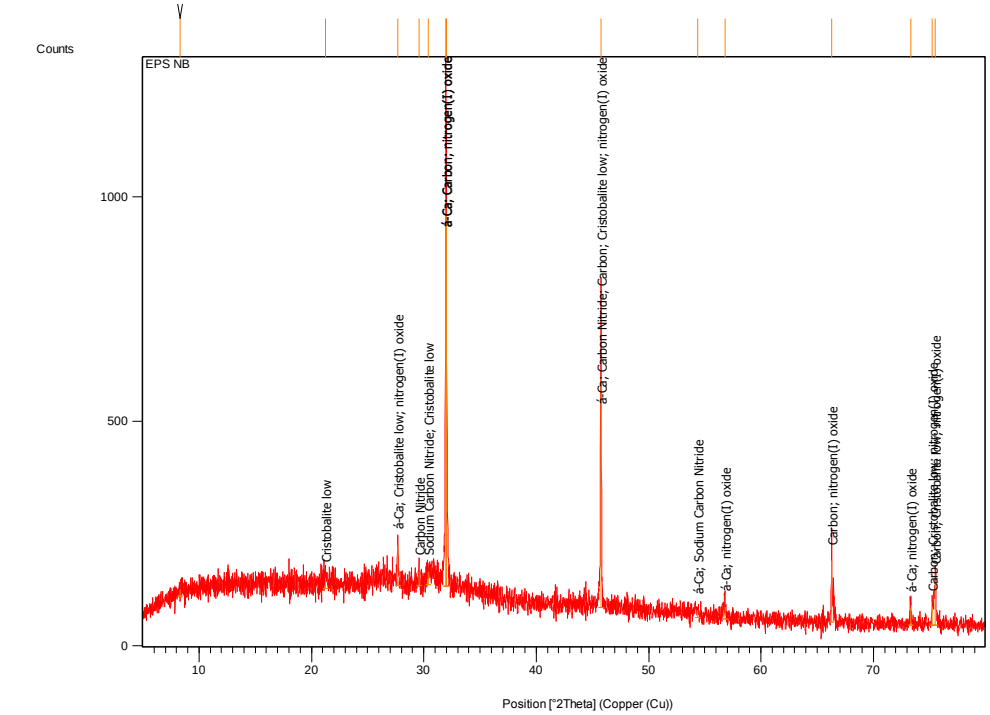
d) 

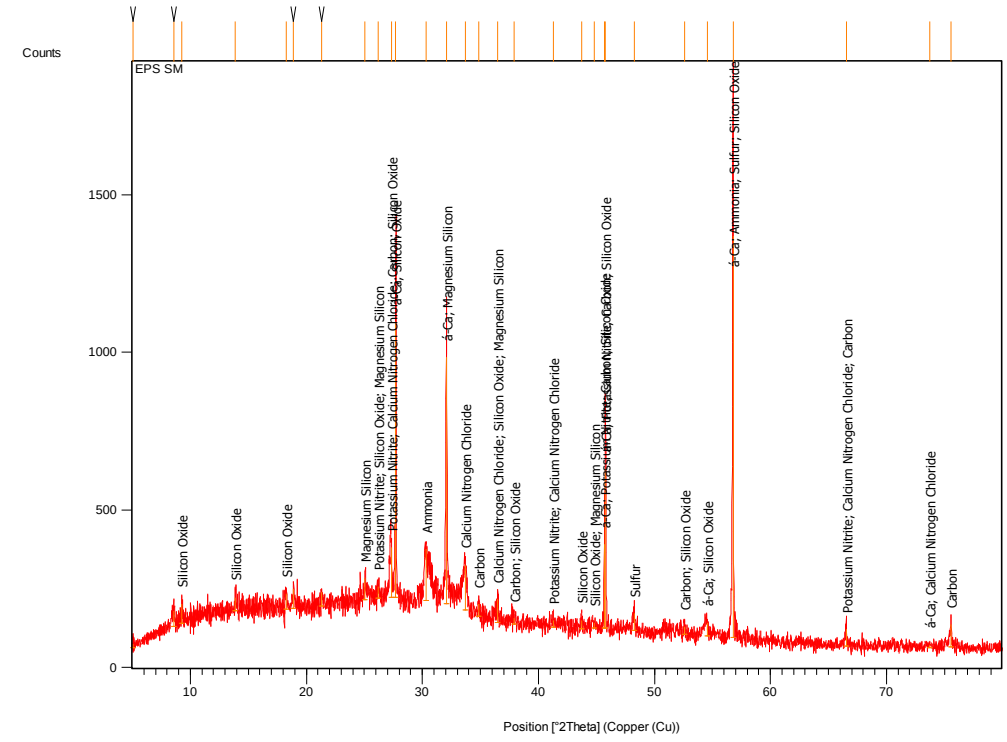
Fig.4.32 1) SEM images of EPS of *B. megaterium* in a) nutrient broth, b) specific media.
2) SEM images of CPS of *B. megaterium* in c) nutrient broth, d) specific media.

4.5.4. X-ray diffraction (XRD) of EPS of *B. megaterium*

XRD analysis showed that maximum amount of calcium ions was present on the EPS layer of of *B. megaterium* due to the binding of calcium with the anionic EPS layer. Calcium ions along with carbon were present in high amount. Ca and carbon with nitrite showed highest peaks at 31,32,45 °2 theta positions in nutrient broth whereas in case of specific media XRD images depicted the presence of highest amount of calcium, carbon, nitrogen, ammonia, sulphur . Ca along with carbon, nitrogen , sulphur, ammonia showed highest peaks at various °2Th. positions. Highest peaks came at 27, 32, 45, 56 °2Th. positions (Fig. 4.33.)



a)

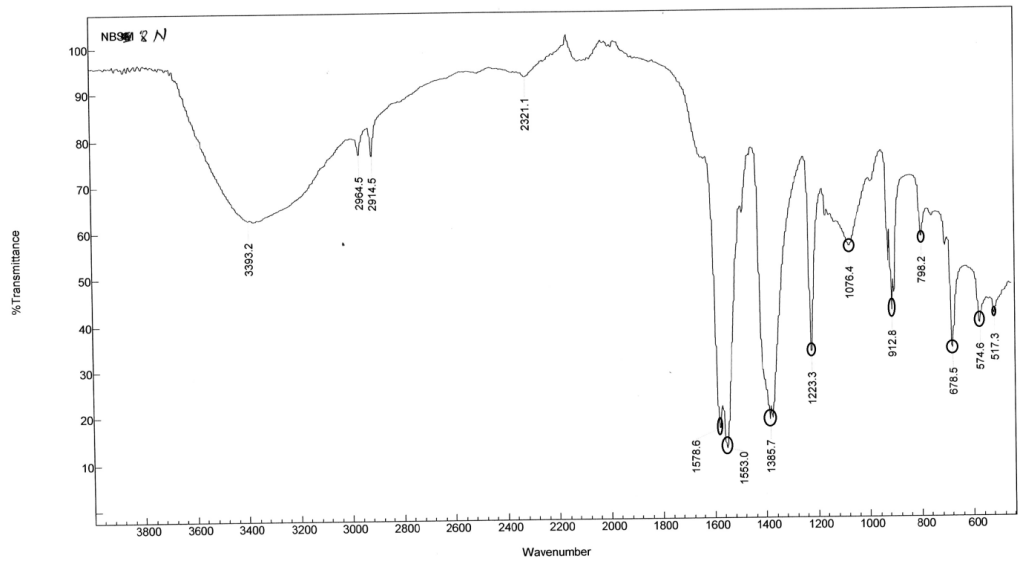


b)

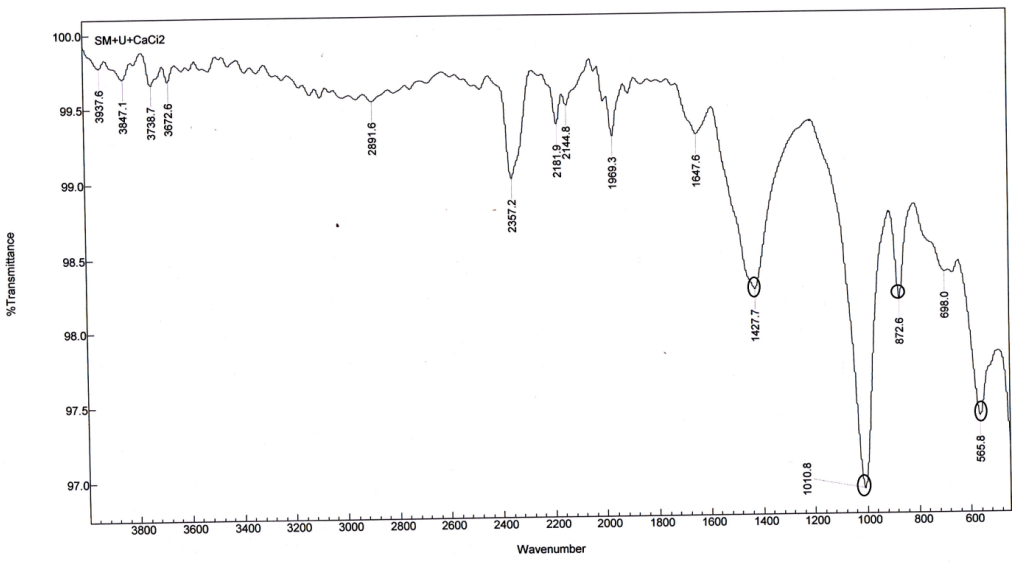
Fig. 4.33. XRD analysis of EPS of *B. megaterium* in a) nutrient broth, b) specific media.

4.5.5. Fourier transform infra red spectroscopy (FTIR) of EPS of *B. megaterium*

FTIR analysis of EPS of *B. megaterium* in different media was determined. FTIR analyses were done for the characterisation of EPS. FTIR analyses depict the presence of functional groups on the surface of EPS. Maximum peaks in FTIR spectra of EPS depicted the presence of carbon around wavelength 1400 cm^{-1} in nutrient broth whereas in case of specific media the FTIR analysis of EPS, revealed the presence of carbon around absorbance peak at wavelength 1000 cm^{-1} (Fig. 4.34).



a)



b)

Fig. 4.34 FTIR analysis of EPS of *B. megaterium* in a) nutrient broth, b) specific media.

4.5.6 Conclusion

In this study, characterization of EPS of *B. megaterium* was performed. Gram staining and capsular staining revealed the morphology of *B. megaterium,* which showed the rod shaped bacterial cells having capsular layer. SEM images of EPS in nutrient broth depicted the smooth , mucous membrane whereas in specific media it appeared rough surface. XRD and FTIR analysis of EPS showed the presence of highest concentration of calcium and carbon on the EPS layer. XRD analysis of EPS formed by *B. megaterium* showed the presence of chemical compounds i.e. Ca, carbon at 45°2theta position in nutrient broth and 56°position in specific media. Fourier transmission infra red (FTIR) spectra of EPS were also determined. The IR spectra of EPS formed by *B. megaterium* isolate showed strong absorption peak at λ = 1000-1400 cm^{-1} in nutrient broth and specific media.

4.6. Characterization of calcium carbonate crystals of *B. megaterium*

4.6.1. Methylene blue and PAS staining of carbonate crystals

The isolated carbonate crystals were stained by Loeffler's methylene blue stain and observed with white light by the use of 100 x magnification. Different crystals displayed different shapes and sizes. The morphology of the crystals precipitated by ureolytic bacterial isolates have been shown. Crystals formed by *B. megaterium* were spherical, rhombohedral as well as few oval. Few bacterial cells were also found to be associated to the crystals.

The polysaccharides associated with all the bacterial isolates were detected using alcian blue periodic acid Schiff stain. Sections of crystals showed that bacteria were embedded inside as well as distributed on the outer surface of the carbonate crystals in all the cases. Acidic (blue) as well as neutral polysaccharides (pink) were clearly seen located in different areas of the carbonate crystals. Rod shaped bacterial cells were also visible in the centre as well as along the outer surface of the carbonate crystals. Methylene blue and alcian blue staining of carbonate crystals has also been reported by Zamarreno *et al.* (2009) and they found different polysaccharides in which bacteria were embedded inside the crystal structures (Figure 4.35).

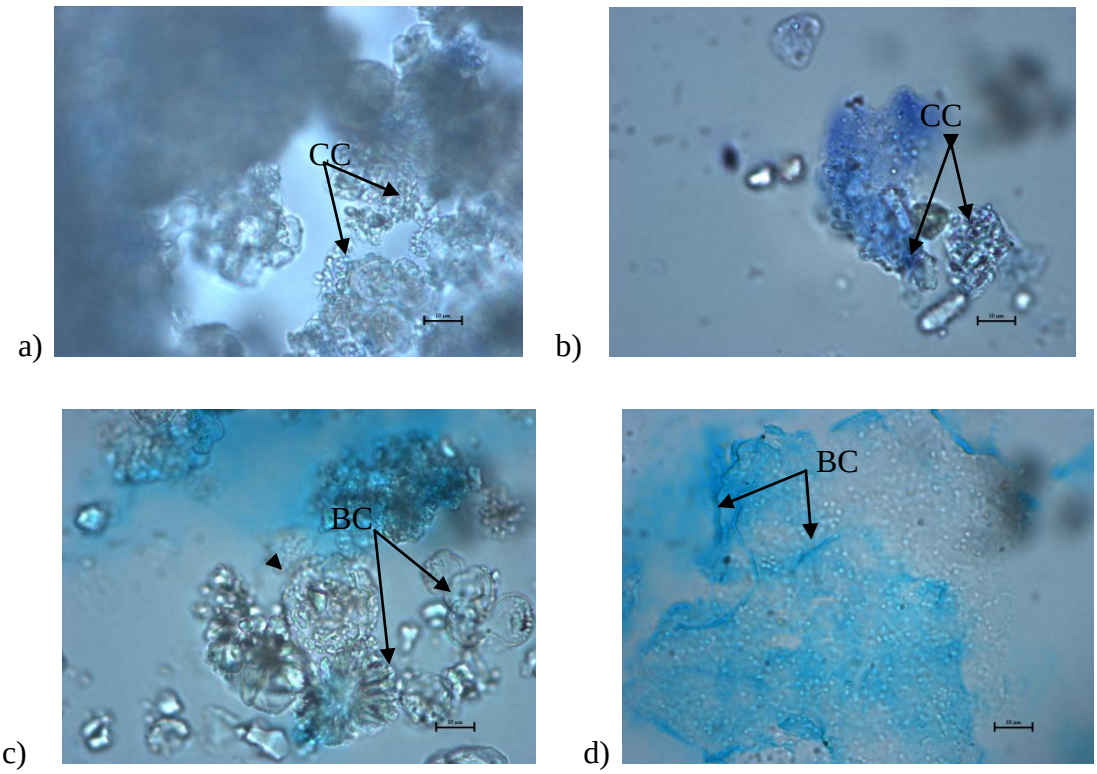
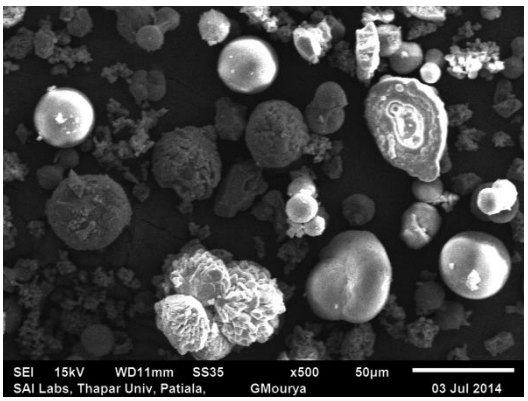
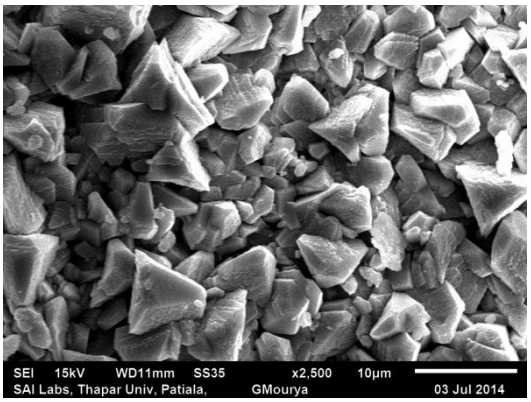


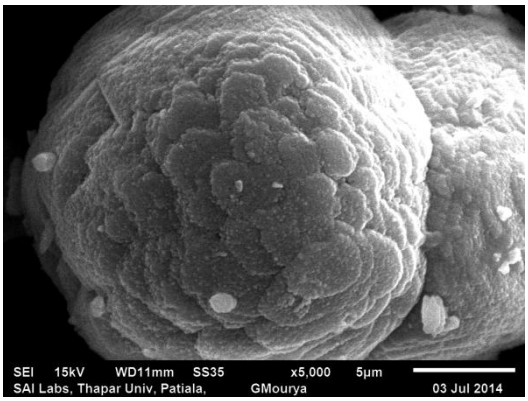
Fig.4.35. Calcite crystals (CC) of *B. megaterium* in a) nutrient broth, b) specific media Capsule (BC) of *B. megaterium* in c) nutrient broth, d) specific media

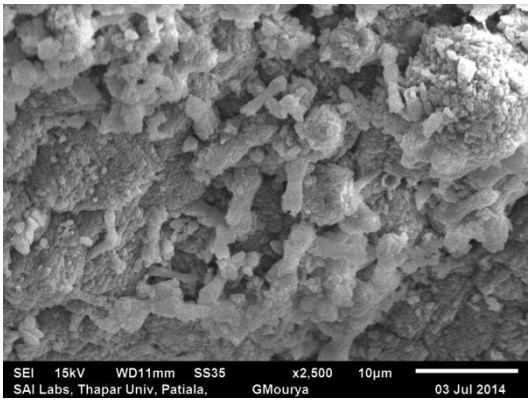
4.6.2. SEM of calcium carbonate crystals of *B. megaterium*

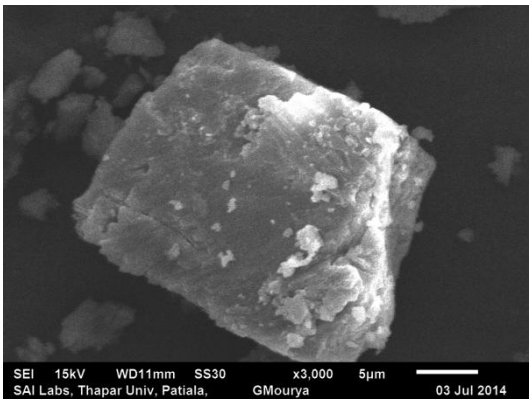
Scanning electron microscopic study depicted detailed analysis of crystals formed by *B. megaterium* and showed obvious differences in size and shape. Distinct carbonate crystals embedded with bacterial cells were clearly seen. Size of crystals varied from 2 - 50 µm. Crystals formed by *B. megaterium* were spherical, oval, rhombohedral as well as triangular in shape with smooth and rough surfaces. Number of crystals with smooth surfaces was relatively more abundant than with rough surfaces. In certain cases, the surface of crystals was very rough which was due to the deposition of new carbonates. Few bacterial cells were also seen on the surface of crystals. SEM images of calcite crystals of *B. megaterium* revealed the presence of smooth and rough surfaced, triangular shaped crystals (Fig.4.36).

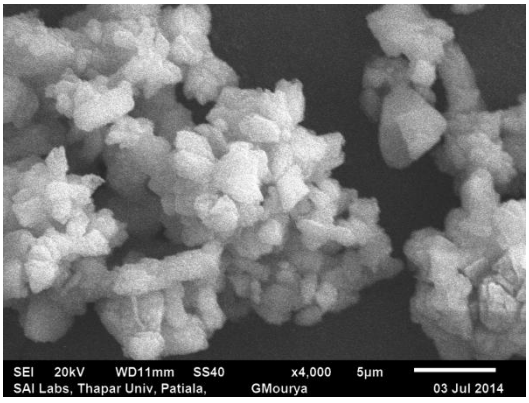
a) 

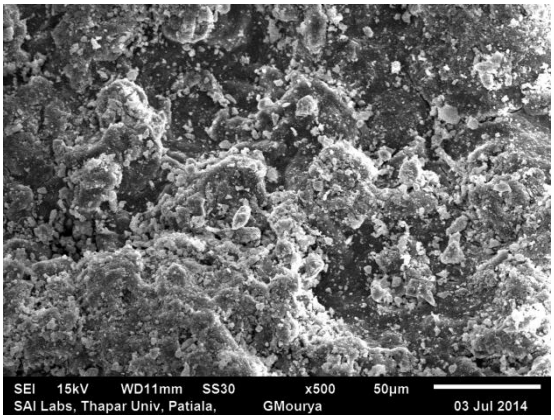
b) 

c) 

d) 

e) 

f) 

g) 

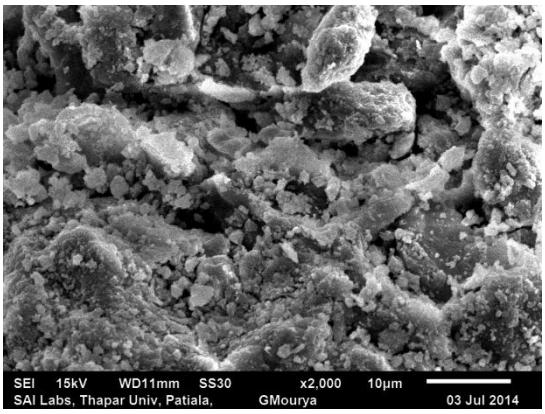
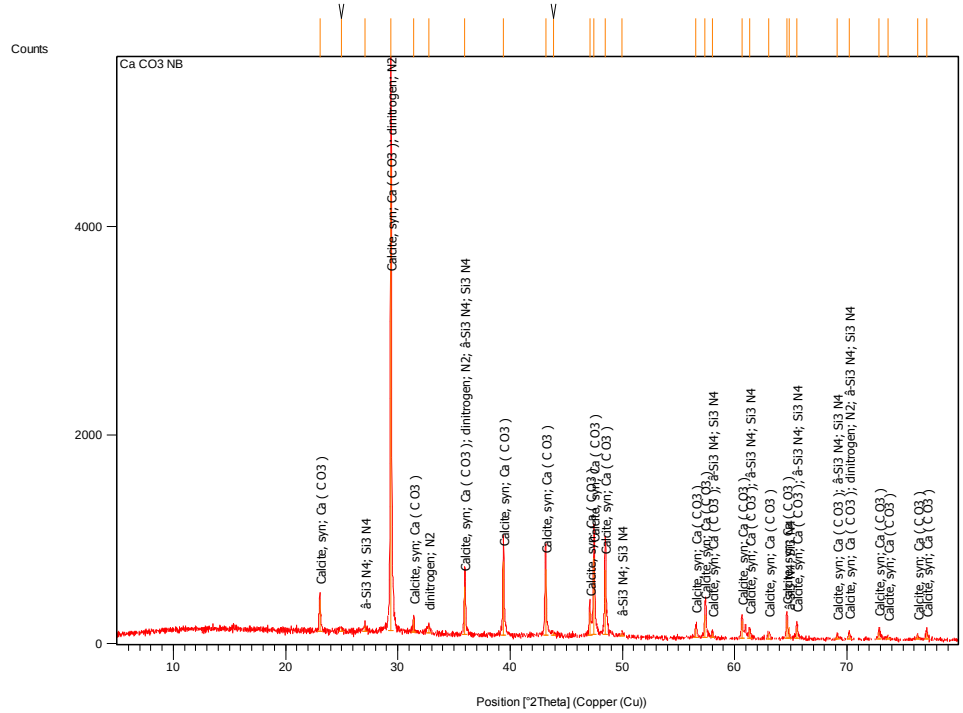
h) 

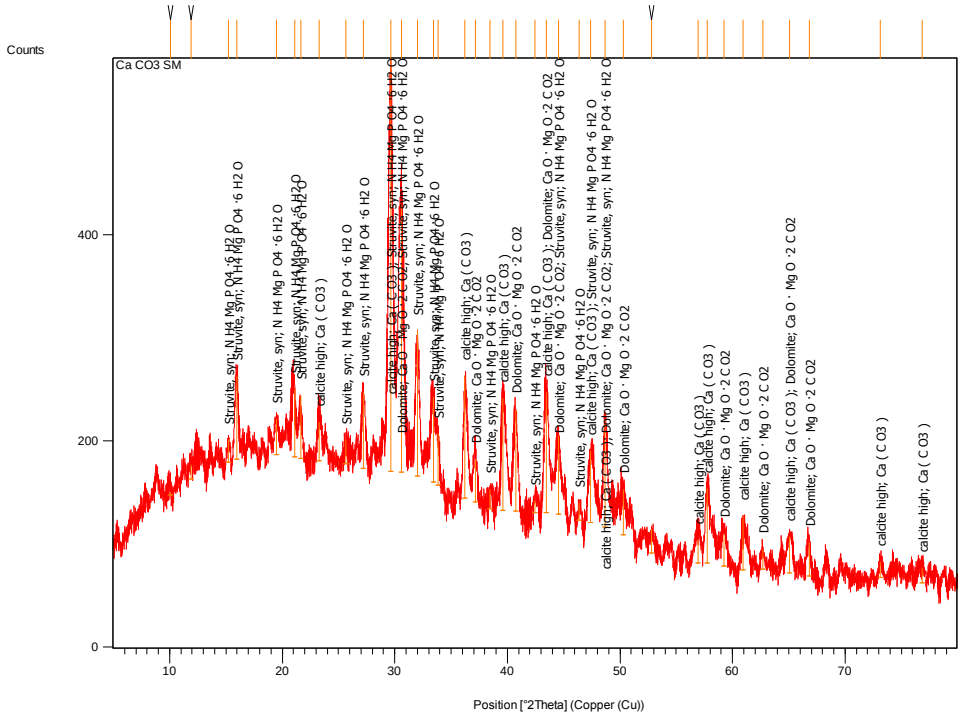
Fig. 4.36. SEM images of calcium carbonate crystals of *B. megaterium* in a-d) nutrient broth, e-h) specific media

4.6.3. XRD of calcium carbonate crystals of *B. megaterium*

X ray diffraction analysis showed that in both cases calcite were formed. XRD analysis of crystals precipitated by *B. megaterium* showed that calcite phase formed the major form of crystals. Maximum amount of calcite crystals were formed rather than any other polymorph of carbonate. Calcite showed highest peak of calcite at 30 °2 theta positions in nutrient broth whereas in case of specific media XRD images depicted the presence of calcite along with dolomite and struvite crystals. The highest peaks of calcite were found at 30 °2Th. positions as depicted by figure



a)

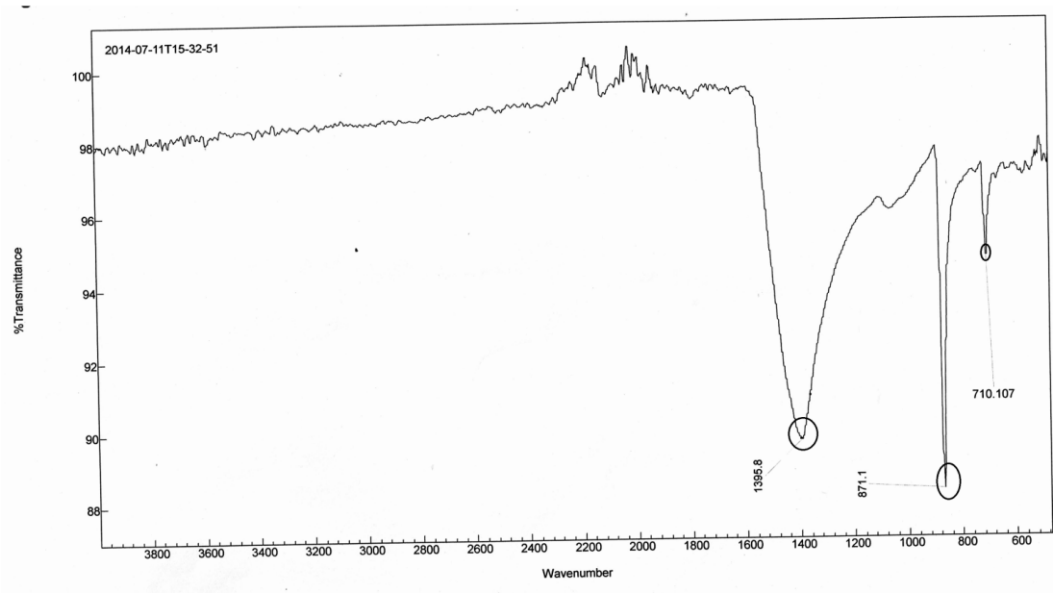


b)

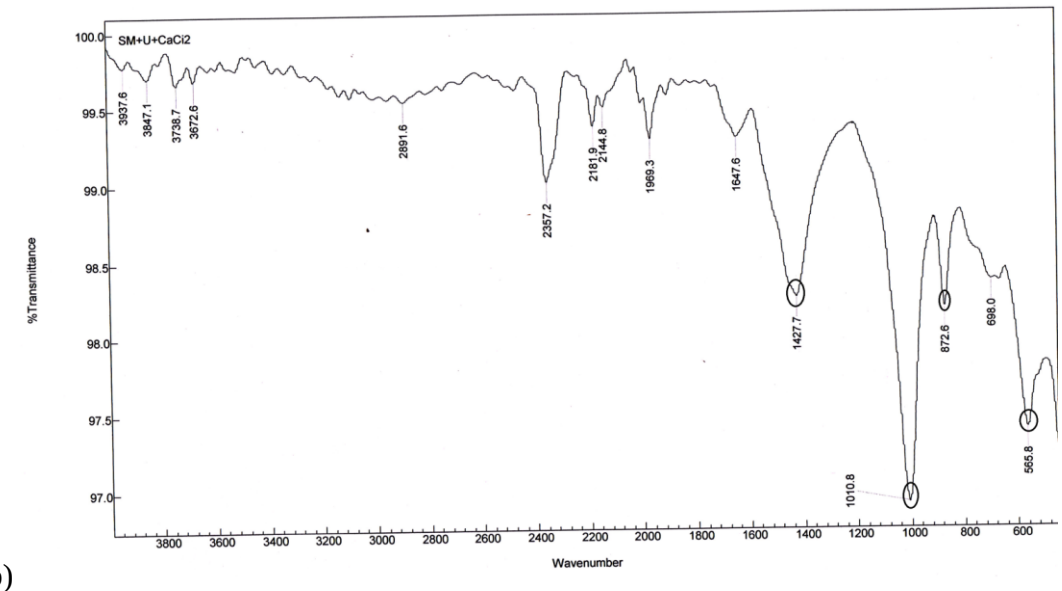
Fig. 4.37. XRD analysis of calcium carbonate crystals of *B. megaterium* in a) nutrient broth, b) specific media.

4.6.4. Fourier transform infra red (FTIR) images of calcite crystals of *B. megaterium*

Fourier transform infra red (FTIR) spectra of all the crystals were also determined. The spectra of crystals formed by *B. megaterium* isolate showed strong absorption peak at λ = 800-1000 cm-1 which corresponds to calcite. This further confirmed XRD results. The morphological differences of different carbonate crystals were further found to be different as per XRD and FTIR analysis. The FTIR analyses of calcite crystals in nutrient broth showed maximum absorption peak at λ= 800 cm^{-1} whereas in specific media maximum absorption peak was at λ= 1000 cm^{-1} (Figure 4.38).



a)



b)

Fig. 4.38. FTIR images of calcite crystals of *B. megaterium* in a) nutrient broth, b) specific media

4.6.5. Conclusion

Crystal staining of calcite crystals revealed the presence of white coloured crystals of *B. megaterium* in nutrient broth whereas they appeared blue coloured in specific media. SEM images of crystals in nutrient broth appeared smooth, circular and triangular with some bacterial cells associated with them whereas in case of crystals in specific media they appeared rough and rhombohedral in shape. X-ray diffraction analysis showed both cases, calcite and other polymorphs were formed. XRD analysis of crystals precipitated by *B. megaterium* showed that calcite phase formed the major form of crystals. X ray analysis of pure bacterial carbonate crystals has been recently determined by Rodriguez - Navarro *et al.* (2012) who investigated the effect of substrate on calcium carbonate polymorph. Fourier transform infra red (FTIR) spectra of all the crystals were also determined. The spectra of crystals formed by *B. megaterium* isolate showed strong absorption peak at λ = 800-1000cm^{-1} in different media which corresponds to calcite. This further confirmed XRD results. The morphological differences of different carbonate crystals were further found to be different as per XRD and FTIR analysis. XRD and FTIR studies showed the presence of calcite as main polymorph in three isolates.

All the samples produced very similar XRD results, while clear morphological differences were evident. This suggests that these differences were a result of variations in crystal growth rates along different planes of the crystal structure.

4.7. Salient findings

- *Bacillus megaterium* was found as an efficient ureolytic *Bacillus* isolate for maximum production of extracellular polymeric substances (1173 µg/100ml).
- Specific media was obtained as a best nutritive media for the better growth of *B. megaterium* and for highest production of EPS (4881 µg/100ml).
- Specific media was optimized for the maximum production of EPS and $CaCO_3$ by using one factor at a time (OFAT) method.
- SEM of EPS and calcite crystals showed smooth surfaces and different morphological forms of crystals
- XRD and FTIR analysis depicted the highest peaks of calcite polymorphs.

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ANNEXURE

A) Nutrient broth

Ingredient	Quantity (g/l)
Peptone	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0

Boiled to dissolve the medium completely, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min, pH 7.4 ± 0.2

B) Nutrient broth – Urea $CaCl_2$ Media (NBUC)

Ingredient	Quantity (g/l)
Peptone	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0
Urea	20
$CaCl_2$	25 mM

The pH of the media was adjusted to 6.5 with 1 N HCl prior to autoclaving without urea and $CaCl_2$. Filter-sterilized urea and $CaCl_2$ was added later. The final pH was adjusted to 8.0 by adding 1N HCl.

C) Basal media

Ingredients	Quantity (g/l)
Glucose	10
Yeast extract	3
Malt extract	3
Peptone	5
$MgSO_4.7H_2O$	1

KH_2PO_4	0.3
Vitamin B1	0.01

pH adjusted to 5.2 with 1N HCl.

D) Basal – Urea $CaCl_2$ Media (BUCM)

Ingredients	**Quantity (g/l)**
Glucose	10
Yeast extract	3
Malt extract	3
Peptone	5
$MgSO_4.7H_2O$	1
KH_2PO_4	0.3
Vitamin B1	0.01
Urea	20
$CaCl_2$	25mM

pH adjusted to 5.2 with 1N HCl before adding urea and $CaCl_2$. Then pH was adjusted to 8.0 with 1N HCl.

E) B4 Media (B4M)

Ingredients	**Quantity (g/l)**
Calcium acetate	2.5
Yeast extract	4.0
Glucose	10

F) B4 – Urea $CaCl_2$ Media (B4MUC)

Ingredients	**Quantity (g/l)**
Calcium acetate	2.5
Yeast extract	4.0
Glucose	10
Urea	20
$CaCl_2$	25mM

G) Specific Media

Ingredients	Quantity (g/l)
Glucose	1
Peptone	5
Yeast extract	1
K_2HPO_4	1
$MgSO_4.7H_2O$	2
$CaCl_2$	25mM
NaCl	0.1
Diammonium Sulphate	2
Agar	3

pH was adjusted to 7.2 with 1N HCl.

H) **Specific Media – Urea $CaCl_2$ (SMUC)**

Ingredients	Quantity (g/l)
Glucose	1
Peptone	5
Yeast extract	1
K_2HPO_4	1
$MgSO_4.7H_2O$	2
$CaCl_2$	25mM
NaCl	0.1
Diammonium Sulphate	2
Agar	3

pH was adjusted to 7.2 with 1N HCl before adding urea and $CaCl_2$. Then pH was adjusted to 8.0 with 1N HCl.

I) Urea agar base

Ingredients	Quantity (g/l)
Peptic digest of animal tissue	1
Dextrose	1
NaCl	5

Monopotassium Phosphate	2
Phenol red	0.012
Agar	15
Urea	40% (add externally)

pH was adjusted to 6.8 with 1N HCl.

J) Phosphate buffer

0.5L of 1M K_2HPO_4 at 174.18g mol^{-1} = 87.09g

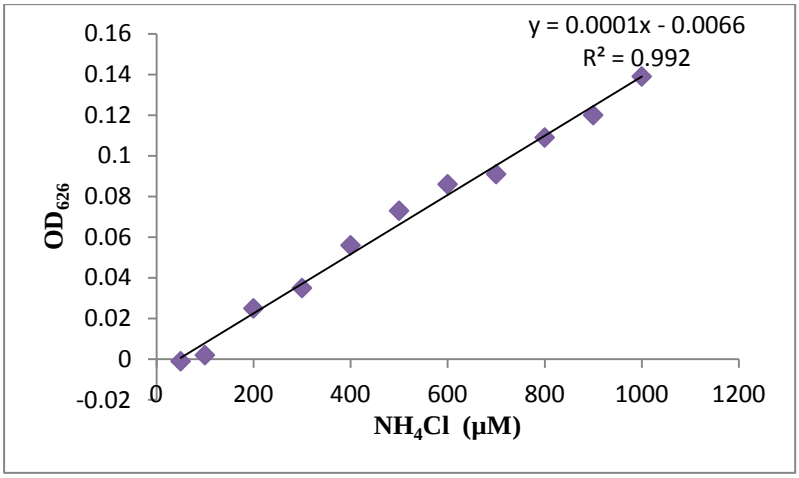
0.5L of 1M KH_2PO_4 at 136.09g mol^{-1} = 68.045g

Preparation of 0.1 M potassium phosphate buffer at 25°C

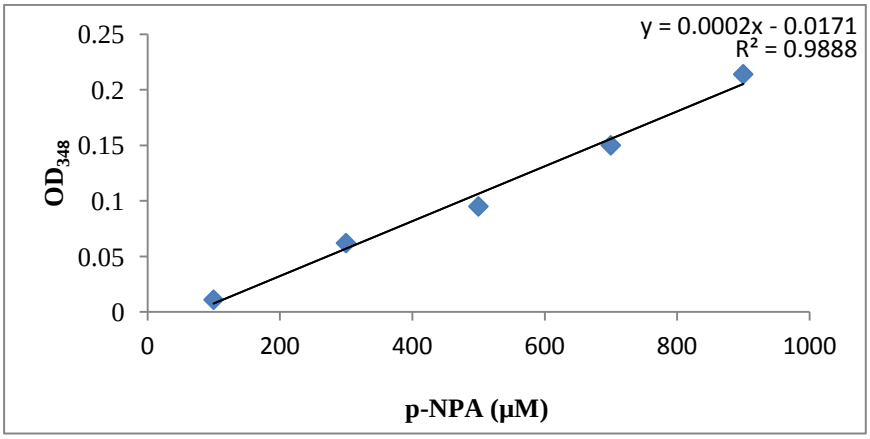
pH	Volume of 1M K_2HPO_4 (ml)	Volume of 1M KH_2PO_4 (ml)
5.8	8.5	91.5
6.0	13.2	86.8
6.4	27.8	72.2
6.8	49.7	50.3
7.0	61.5	38.5
7.4	80.2	19.8
7.8	90.8	9.2
8.0	94.0	6.0

K) STANDARD CURVES

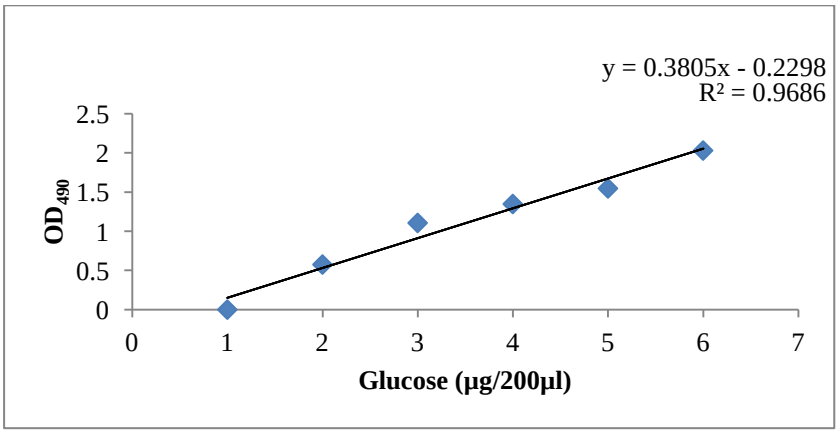
1) Standard curve of ammonium chloride for urease estimation



2) Standard curve of p-NPA (para nitro phenol acetate) for carbonic anhydrase estimation



3) Standard curve of glucose for carbohydrate estimation in EPS



4) Standard curve of Bovine Serum Albumin for protein estimation in EPS