Characterization of Novel Carbonic Anhydrase from Halophilic Bacterial Isolates and it's Role in Microbially Induced Calcium Carbonate Precipitation

A

DISSERTATION

Submitted in the partial fulfillment of the requirements
For the Award of the Degree of

Masters of Science
(Biotechnology)

Under the Guidance of
Dr. M. Sudhakara Reddy
(Professor and Head)
D.B.T.E.S.

By
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(July 2013)

Dedicated To
My Parents and Sister

Candidate's Declaration

I, hereby declare that the work presented in the dissertation entitled "**Characterization of Novel Carbonic Anhydrase from Halophilic Bacterial Isolates and its Role in Microbially Induced Calcium Carbonate Precipitation**" in partial fulfillment of the requirement for the award of the degree of Masters of Science, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of six months from January 2013 to July 2013, under the supervision of Dr. M. Sudhakara Reddy, Professor and Head, , Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Place: Patiala

Date: 17/07/2013

(Othima Sharma)

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

Dr. M. S. Reddy
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Certificate

This is to certify that the dissertation entitled "**Characterization of Novel Carbonic Anhydrase from Halophilic Bacterial Isolates and its Role in Microbially Induced Calcium Carbonate Precipitation**" submitted by **Othima Sharma** in partial fulfillment of the requirement for the award of the degree of Masters of Science, Department of Biotechnology and Environmental Sciences, 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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Date: 17/07/2013

Place: Patiala

(Othima Sharma)

CONTENTS

ABSTRACT

Microbially Induced Calcium Carbonate Precipitation (MICCP) has been widely used technology now a day for remediation of building structures. It has been found that along with Urease, Carbonic anhydrase (CA) is also involved in the carbonate precipitation process. In the present study, CA was isolated and characterised from halophilic bacteria (A3-5 and A1-4) and its role in microbially induced calcium carbonate precipitation was studied. The catalytic activities for the crude and lyophilised CA were estimated by using p-Nitrophenyl Acetate (p-NPA) as the substrate. K_M and V_{max} values for crude enzyme were found to be 0.31 mM and 1.88 µmoles/min and 0.67 mM and 1.49 µmoles/min, respectively for A3-5 and A1-4. The lyophilized enzyme showed an increased K_M value of 0.20 mM and 0.37 mM for A3-5 and A1-4, respectively whereas V_{max} value was found to be slightly lower for A3-5 (1.14 µmoles/min) but increased in case of A1-4 (1.69 µmoles/min) at 25°C. The optimum range of pH temperature for the crude CA was found to be between 7 - 9 and 50°C - 60°C respectively. The crude CA was investigated to enhance $CaCO_3$ precipitation. MICCP was also studied at varying pH and morphologies of the different carbonate polymorphs formed were studied. Best MICCP activity by CA influenced precipitation was obtained at 100 mM $CaCl_2$ + 75mM $NaHCO_3$ + 100 µM $ZnSO_4$ at pH 9. It was concluded that the extracellular CA from halophilic bacteria has the potential for efficient calcium carbonate precipitation and hence can be looked as a new tool in MICCP.

Chapter 1: Introduction

CHAPTER 1

INTRODUCTION

CO_2 has been regulated as a "devil" molecule that must be reduced or prevented by various researchers. But atmospheric concentration of carbon dioxide (CO_2) has been increasing day by day due to human induced (anthropogenic) activities. The Kyoto Protocol signed in 1997 stated that the industrialized nations need to reduce their CO_2 emissions to 95% of their 1990 levels by 2012 (Bolin 1998). Thus, many researchers have focused on the development of methods for the mitigation of CO_2. One of the strategies to control the CO_2 concentration in the atmosphere is to reduce its production and release into the atmosphere. The other strategy is to increase the efficiency of the energy use, from production to its end use (Reichle *et al.,* 1999). However, these two options are not practically possible. Therefore, a third option was looked upon by various researchers which can capture and dispose the produced CO_2 in a safe manner i.e. sequestration of CO_2 (Anjana Sharma *et al.,* 2010, Reichle *et al.,* 1999, Herzog and Drake 1996). A number of CO_2 sequestration options have been proposed which include the placement of CO_2 in deep oceans; in geologic formations such as deep saline aquifers, abandoned oil or gas reservoirs, unmineable coal seams and consumption via advanced chemical and biological processes (Reichle *et al.,* 1999). However, none of these options proved to be ideal.

The alternative for CO_2 disposal in sequestration form paved way for fixing it in the form of carbonate minerals as these are very stable and environmentally benign. But hydration of CO_2 to form carbonic acid is the rate-limiting step in the conversion of CO_2 into carbonate ions (Bond *et al.,* 1999; Sullivan *et al.,* 1993). The most suitable catalyst for this reaction was found to be Carbonic anhydrase (Ho and Sturtevant 1963). Carbonic anhydrase (CA) is one of the fastest enzymes that catalyses CO_2-hydration reaction with typical rates between 10^4 and 10^6 reactions per second (Heck *et al.,* 1994). It catalyzes the hydration of carbon dioxide and the dehydration of bicarbonate as:

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \qquad \text{(eq. 1.1)}$$

Many studies have now reported the involvement of biological Carbonic anhydrase in fixing large quantities of CO_2 into calcium carbonate (Smith and Ferry 2000; Sharma *et al., 2*008; Wanjari *et al., 2*011).

Microbially induced calcium carbonate precipitation (MICCP) is the process by which an organism creates a local micro-environment, with conditions that allow optimal extracellular chemical precipitation of mineral phases (Hamilton 2003). The use of these carbonate biominerals for remediation of building structures has gained wide attention as an eco-friendly and novel method of protecting and remediating building materials. But, till now, most of the work on MICCP has been targeted on Urease enzyme (Stocks Fischer *et al.,* 1999; De Muynck *et al., 2*008; Achal *et al., 2*009). Though recent researchers have reported the participation of CA in carbonate precipitation but not much has been done to study the role of this enzyme.

CA enzyme is ubiquitously distributed in organisms and fundamental to many eukaryotic biological processes like photosynthesis, respiration, CO_2 and ion transport etc. (Smith and Ferry 2000). The role of biological CA in biological calcification of marine invertebrates (mollusks), fish otoliths, corals, hard tissues of vertebrates has been widely studied but not much progress has been done on the role of bacterial CA in biomineralization (Tambutte *et al., 2*007; Beier and Anken 2006). So, it is imperative to study and characterize this enzyme and investigate its role in $CaCO_3$ precipitation.

Objectives

1 Isolation and identification of efficient halobacteria for the production of carbonic anhydrase
2 Characterization of bacterial Carbonic anhydrase
3 Purification of bacterial Carbonic anhydrase
4 Effect of bacterial CA in MICCP
5 Optimization on CA mediated MICCP

Chapter 2:
Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

2.1 The Problem of CO_2 and Global Warming

Along with the industrialization, the atmospheric concentrations of greenhouse gases (GHGs) such as carbon dioxide (CO_2), methane (CH_4), chlorofluorocarbons (CFCs), and nitrous oxides (NOx) are increasing due to human induced (anthropogenic) activities (Hansen *et al.,* 1997). CO_2 is the most abundant greenhouse gas, which is produced mainly by the burning of fossil fuels such as coal, oil, and natural gas. The CO_2 concentration in the atmosphere has increased from 280 ppm during the preindustrial era to about 380 ppm in 2007 with an accumulation rate of about 1.5 ppm per year (Halman and Steinberg 1999). The predictions indicate that the CO_2 emissions will continue to increase in the future as the fossil fuels will continue to be the major energy sources (IPCC 1996).

Table 2.1: Estimated Sequestration cost for CO_2 (Wong and Bioletti 2002)

Sequestration Step	Cost
Separation (Amine process) ($/tonne CO_2)	30-50
Compression ($/tonne CO_2)	8-10
Transportation ($/tonne/100km)	0.7-4
Injection ($/tonne CO_2)	2-8
Total	41-72

The sequestration cost could be reduced if the CO_2 is captured and directly converted into stable products at the production sites without further compression, transportation, and injection processes but hydration of CO_2 (equation 2.1) is reported as the slowest step (Bond *et al.,* 2001, Mirjafari *et al., 2*007). It was found that biological catalyst for this reaction is enzyme Carbonic anhydrase.

$$CO_2 + H_2O \xleftrightarrow{CA} H_2CO_3 \qquad \text{(eq. 2.1)}$$

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., 2*009). This enzyme promotes the interconversion between CO_2 into carbonate form (Khalifaht *et al.*, 1972). Therefore, the on-site capturing process will serve not only for the CO_2 sequestration but also the production of nano-sized $CaCO_3$ particles which could serve solution to many sequestration problems (Wanjari *et al., 20*11). Carbonic anhydrases have since then been studied vastly for $CaCO_3$ precipitation studies. Liu *et al* (2005) reported that use of Bovine CA increased the rate of precipitation of $CaCO_3$ in brines along with Mirjafri *et al* (2007) who also demonstrated that bovine CA could promote the formation of $CaCO_3$. Tambutté *et al* (2007) observed that CA plays important role in the formation of calcified hard tissues in vertebrates, invertebrates and calcification of corals. Recent studies have highlighted that bacterial CAs could also play important roles in $CaCO_3$ precipitations.

2.2 Biomineralization

Biomineralization is defined as a biologically induced precipitation in which an organism creates a local micro-environment, with conditions that allow optimal extracellular chemical precipitation of mineral phases (Hamilton 2003). The synthesis of minerals by prokaryotes is broadly classified into two classes: Biologically controlled mineralization (BCM) and Biologically induced mineralization (BIM) (Lowenstam 1981; Lowenstam & Weiner 1989). BCM happens intracellulary. Minerals that form by biologically induced mineralization processes generally nucleate and grow extracellularly as a result of metabolic activity of the organism and subsequent chemical reactions involving metabolic byproducts. Bacterially induced and mediated mineralization is a research subject widely studied in the past decades (Banfield & Hamers 1997; Douglas & Beveridge 1998; Ehrlich 2002). Bacteria from various natural habitats have frequently been reported to be able to precipitate calcium carbonate both in natural and in laboratory conditions (Krumbein 1979; Rodriguez *et al.*, 2003). Different pathways appear to be involved in calcium carbonate precipitation processes that include photosynthesis, ammonification, denitrification, sulphate reduction and anaerobic sulphide oxidation (Castanier *et al.*, 1999, 2000; Riding, 2000).

Because of its simplicity, the most commonly studied system of applied MICCP is urea hydrolysis via the enzyme urease in a calcium rich environment. Urease catalyzes the hydrolysis of urea to CO_2 and ammonia, resulting in an increase of pH and carbonate concentration in the bacterial environment. During microbial urease activity, 1 mol of urea is hydrolyzed intracellularly to 1 mol of ammonia and 1 mol of carbonate (Eq.2.2), which spontaneously hydrolyzes to form additional 1 mol of ammonia and carbonic acid (Eq.2.3) as follows:

$$CO(NH_2)_2 + H_2O \xrightarrow{bacteria} NH_2COOH + NH_3 \quad \text{(eq 2.2)}$$

$$NH_2COOH + H_2O \longrightarrow NH_3 + H_2CO_3 \quad \text{(eq 2.3)}$$

These products equilibrate in water to form bicarbonate, 1 mol of ammonium and hydroxide ions which give rise to pH increase

$$H_2CO_3 \longrightarrow 2H^+ + 2CO_3^{2-} \quad \text{(eq 2.4)}$$

$$NH_3 + H_2O \longrightarrow NH^{4-} + OH^- \quad \text{(eq 2.5)}$$

$$Ca^{2+} + CO_3^{2-} \longrightarrow CaCO_3 (K_{SP} = 3.8 \times 10^{-9}) \quad \text{(eq 2.6)}$$

This technology has been investigated for its potential in consolidation and restoration of various building materials by many research groups on cement mortar cubes (Achal *et al.,* 2011), sand consolidation and limestone monument repair (Stocks – Fischer *et al.,* 1999; Dhami *et al.,* 2013a; Bachmeier *et al.,* 2002; Dick *et al.,* 2006; Achal *et al.,* 2009b), reduction of water and chloride ion permeability in concrete (Achal *et al.,* 2010), filling of pores and cracks in concrete (Bang *et al.,* 2001; Ramakrishnan 2007; De Muynck *et al.,* 2008a,b) and enhanced strength of bricks (Sarda *et al.,* 2009; Dhami *et al.,* 2012).

Though application of CA in CO_2 sequestration has been successfully proved by many but till now, the effect of bacterial CA on $CaCO_3$ precipitation has not been elucidated in depth (Wanjari rt a., 2011; Ramanan *et al.,* 2008; Yadav *et al.,* 2011). Li *et al* (2011; 2012) reported that bacterial CA can promote $CaCO_3$ as an activator. Botre and Botre (1989) reported that in enzyme catalysed reactions, increase in the rate of removal of CO_2 from solution facilitated by CA increases the rate of production of NH_3 consequent from urea dissociation. Achal and Pan (2011) recently found that along with Urease, CA might also be involved in $CaCO_3$ precipitation, but no detailed studies were conducted to understand the effect of both these enzymes in carbonate precipitation and their

relationship in calcium carbonate mineralization. Li *et al* (2011) hypothesized following reactions involving UA and CA that might be taking place during $CaCO_3$ precipitation:

CO_2 (g) ↔ CO_2 (aq) Dissolution (eq. 2.7)

CO_2 (aq) + H_2O ↔ H_2CO_3 Hydration (eq. 2.8)

H_2CO_3 ↔ $H^+ + HCO_3^-$ Dissociation (eq. 2.9)

HCO_3^- ↔ $H^+ + CO_3^{2-}$ Carbonate Formation (eq. 2.10)

$Ca^{2+} + CO^{2-} \rightarrow CaCO_3\downarrow$ Precipitation (eq. 2.11)

Fortin *et al* (1997) Bacterial surfaces play an important role in calcium precipitation (Fig. 2.1). 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 *et al.,* 1998; Castanier *et al.,* 1999).

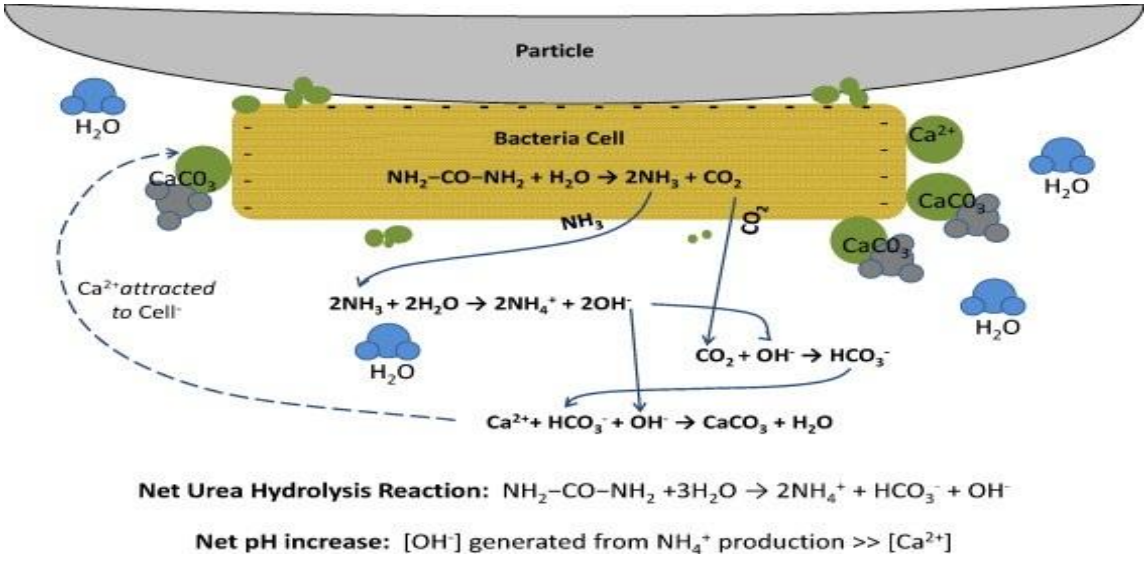


Fig 2.1: Bacteria as Nucleation site for $CaCO_3$ precipitation (Source: De Jong *et al* 2010)

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

$$Ca^{2+} + Cell \longrightarrow Cell - Ca^{2+} \quad \text{(eq 2.12)}$$

$$Cl^- + HCO^{3-} + NH_3 \longrightarrow NH_4Cl + CO_3^{2-} \quad \text{(eq 2.13)}$$

$$Cell - Ca^{2+} + CO_3^{2-} \longrightarrow Cell - CaCO_3 \quad \text{(eq 2.14)}$$

The primary role of bacteria (Fig. 2.2) has been ascribed to their ability to create an alkaline environment through various physiological activities.

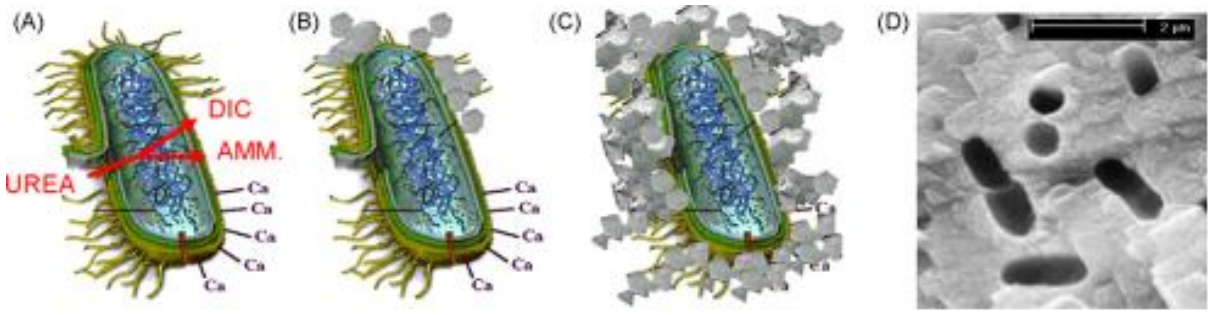


Fig 2.2: Detailed Sketch of MICCP: Simplified representation of the events occurring during the ureolytic induced carbonate precipitation. Calcium ions in the solution are attracted to the bacterial cell wall due to the negative charge of the latter. Upon addition of urea to the bacteria, dissolved inorganic carbon (DIC) and ammonium (AMM) are released in the micro-environment of the bacteria (A). In the presence of calcium ions, this can result in a local super saturation, 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: De Muynck *et. al*., 2008)

2.2.3 MICCP studies in saline environment

Since the beginning of the century it has been known that bacteria are involved in the formation of carbonates. Some moderately halophilic bacteria have also been reported to be capable of forming calcium carbonate precipitates (Ferrer *et al.,* 1988; Rivadeneyra *et al.,* 1991, 1993, 1994). The involvement of marine bacteria in calcium carbonate precipitation was first given prominence by Drew (1914) who stated that marine denitrifying forms were capable of bringing about deposition of calcium carbonate by raising alkalinity of sea water, and these bacteria were responsible for much of the deposition known to occur in the Grand Bahama banks and neighbouring areas.

2.3 Enzyme Studies

2.3.1 Urease

Urease has been the major player in MICCP (Stocks Fischer *et al.,* 1999; De Muynck *et al., 2*008). The ability to produce urease is widespread amongst microbial populations and

the enzyme has been well studied from a clinical perspective as it can indicate increased virulence properties in pathogenic bacteria (Collins and D'Orazio 1993; Lee and Calhoun 1997; Mobley *et al.,* 1995; Provorov and Vorobyov 2000) and as a general nitrogen volatilisation phenomenon in agricultural soils (Pettit *et al.,* 1976; Stump *et al.,* 1984; Sullivan DM and Havlin JL 1988; Zantua and Bremner 1977). The role of Urease in MICCP is well documented by researchers (Stocks-Fischer *et al.,* 1999; Bang *et al., 2*002; Ramakrishnan *et al., 2*007; van Paassen *et al., 2*009). Its hydrolysis has been found to be a pathway for biotechnological applications as it can be controlled easily, allows the production of high concentrations of carbonate precipitates within a short period of time, common occurrence of ureolytic bacteria in soil and aquatic environments and also because urea is fairly inexpensive substrate (De Muynck *et al., 2*010).

Along with Urease, various researchers hypothesised the involvement of bacterial CA in MICCP (Li *et al.,* 2011; Achal and Pan 2012).

2.3.2 Carbonic Anhydrase

Although ubiquitous in highly evolved organisms from the eukarya domain, Carbonic anhydrase enzyme has received scant attention in prokaryotes from the bacteria and archaea domains. In case of bacterial system, Carbonic anhydrase activity was first reported in *Neisseria sicca* by Veitch and Blankenship (1963) and this enzyme was the first carbonic anhydrase purifed from a prokaryote (Silverman and Lindskog 1988). The *N. sicca* enzyme is a monomer with a molecular mass of 28.6 kDa (Adler *et al.,* 1972). There are five independent Carbonic Anhydrase gene families such as α-CA, β-CA, γ-CA, δ-CA, and ε-CA. These families have no significant amino acid sequence similarity and in most cases are thought to be an example of convergent evolution. This enzyme has been purified from few more bacterial species since *N. sicca* (Alber and Ferry 1994; 1997; Braus *et al.,* 1997; Yagawa *et al.,* 1984). Buzolyova and Somov (1999) have reported the presence of CA in *Yersina pseudotuberculosis* and *Listeria monocytogenes,* the pathogens isolated from soil and water. Recently, CA from *Bacillus pumilis, Citrobacter freundii, Bacillus mucilaginous, Bacillus* megaterium, *Methanobacterium thermoautotrophicum, Enterobacter gergoviae, Enterobacter taylorae, Aeromonas hydrophila, Aeromonas caviae, Pseudomonas fragii (*Sharma *et al., 2009) etc* . has also been reported. The mechanism of action of CA on the CO_2 hydration is that hydroxyl ion that is coordinated by zinc of the enzyme attack CO_2 to form bicarbonate at the active

site of the enzyme. After that the bicarbonate of the enzyme displaces with the water molecule (Mirjafar *et al., 2*007). The k_{cat}/K_M value for the Human Carbonic Anhydrase II (HCAII) which is the fastest isozyme is $1.5*10^8 M^{-1}s^{-1}$ for CO_2 (Host 2007). Carbonic anhydrase has many isozymes which have different kinetic parameters with different substrates. Kinetic constants of CA isoenzymes are shown in Table 2.2.

Table 2.2. Kinetic constants of CA isoenzymes with different substrates.

Carbonic Anhydrase	**Substrate**	**Kinetic Constants**		**Reference**
		K_M (mM)	**K_{cat}/K_M ($M^{-1}s^{-1}$)**	
Bovine CA II	m-nitrophenyl acetate	13	240	Thobslund *et al.,* 1967
	p-nitrophenyl acetate	76.9	960	
	o-nitrophenyl acetate	-	100	
	p-nitrophenyl propionate	-	68	
Bovine CA II	p-nitrophenyl propionate	-	61	Drevon *et al., 2*003
Human CA III	CO_2	-	$3*10^5$	Duda *et al., 2*003
Bovine CA I, II	p-nitrophenyl acetate	-	266.6	Pocker *et al.,* 1966
Bovine CA	p-nitrophenyl acetate	-	153.3	Pocker *et al.* 1968
Human CA I	p-nitrophenyl acetate	3.025	753	Innocenti *et al., 2*008
Human CA II		30.53	2607	
Human CA I	p-nitrophenyl phosphate	.935	65.55	
Human CA II		2.195	14.89	
Human CA I	CO_2	4	$5*10^7$	Supuran *et al., 2*008
Human CA II		9.3	$1.5*10^8$	
Human CA		52	$2.5*10^5$	

III				
Bovine CA	CO_2	.65	36.31	Mirjafari *et al., 2*007
Carbonic anhydrase	CO_2	12	$8.3*10^7$	Whitford 2005

2.3.2.1 Esterase activity of Carbonic Anhydrase

CA catalyses the hydrolysis of nitrophenyl esters with different efficiencies depending on the structure of the acyl part of substrate. But, as shown in Table 2.3, most efficient ester substrate for Bovine CA and Human CAII is para-nitrophenyl acetate (p-NPA) because of short and few acyl groups. p-NPA (Fig. 2.3) is bound as neutral species to the CA active site, allowing the strong nucleophilic ($Zn^{2+}(OH)^-$) attack, without any electrostatic repulsions, thus effectively hydrolyze it (Innocenti *et al., 2*008). Table 2.3 shows the kinetic constants of CA for different ester form of substrates.

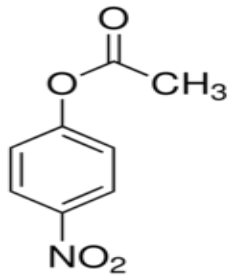


Fig. 2.3: para-Nitrophenyl Acetate.

Table 2.3. Rate constants for the CA-catalyzed hydrolysis (Thorslund *et al.*, 1967)

Substrate	**Rate Constant (k_{cat}/K_M; $M^{-1}s^{-1}$)**
Phenyl acetate	3
o-nitrophenyl acetate	100
m-nitrophenyl acetate	240
p-nitrophenyl acetate	960
p-nitrophenyl propionate	68
p-nitrophenyl butyrate	6
p-nitrophenyl caproate	2

2.3.2.2 Mechanism of Action of Carbonic Anhydrase, Effect of pH and Zinc

pH affects carbonic anhydrase in a sigmoidal fashion. The higher the pH, the more active the enzyme is (since it is in the optimal conditions for deprotonation). Fig. 2.4 describes the mechanism of CA action.

1. The binding of zinc lowers the pKa of water from 15.7 to 7, generating a hydroxide ion (OH^-) to attack carbon dioxide. Zinc releases a proton from a water molecule to generate this hydroxide ion. pH decreases as a result from the decrease in the pKa. According to Le Chatelier's principle, this drives the reaction towards deprotonation.

2. The carbon dioxide substrate binds to the enzymes active site and is positioned for optimal interaction.

3. The hydroxide ion (being a great nucleophile) attacks the carbonyl of carbon dioxide, converting it to bicarbonate ion through the nucelophilic attack. Oxygen on the carbon dioxide molecule forms an intermediate bond with the Zn metal during the conversion process.

4. The enzyme is regenerated and the bicarbonate ion is released. The enzyme is ready for another reaction to occur. This regenerative ability of this enzyme allows for this reaction to be highly efficient and kinetically fast to constantly process carbon dioxide within the blood cells.

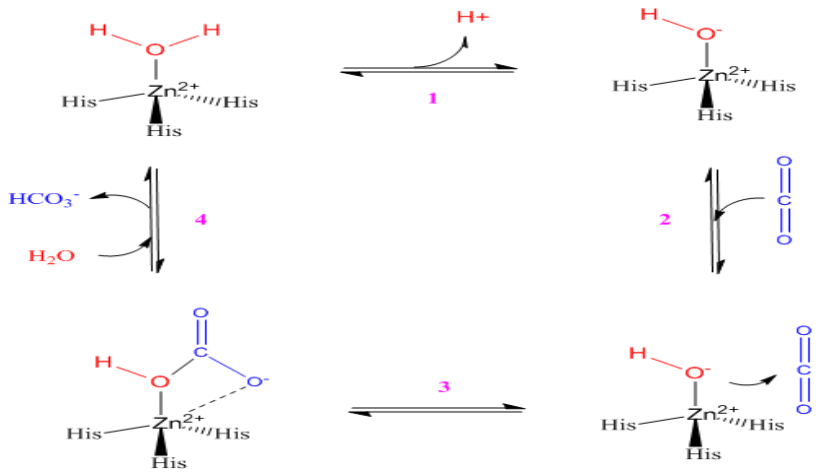


Fig. 2.4: Detailed mechanism of action of CA

2.3.2.2 Role of Zinc

Zinc's role in carbonic anhydrase is to facilitate the water to create a proton H^+ and a nucleophilic hydroxide ion. The nucleophilic water molecules attack the carbonyl group of carbon dioxide to convert it into bicarbonate. This is obtained through the +2 charge that the zinc ion has, which attracts the oxygen of water, deprotonates water, thus converting it into a better nucleophile so that the newly converted hydroxyl ion can attack the carbon dioxide. Water naturally deprotonates itself, but is it's a rather slow process and not in large quantities. Zinc deprotonates water by providing a positive charge for the hydroxide ion. Zinc alone cannot deprotonate water fast enough to reach the 10^6 per second rate that it has been measured, however, the proton is donated temporarily to the surrounding amino acid residues, which will later be given to the environment, while allowing the reaction to continue and not slowing down the process. Metal ions are good because it increases the reactivity of the chemicals and can create strong bonds. Zinc is able to help the deprotonation of water by lowering the pk_a of water. Binding of water to zinc lowers the pka of water from about 15.7 to 7. This means more water molecules are now able to deprotonate at a lower pH than normal, and this makes it easier for water to turn into a hydroxide ion which is a better nucleophile.

2.4 Activity Staining

Zymography or Activity Staining is an electrophoretic technique for the detection of hydrolytic enzymes based on the substrate repertoire of the enzyme. Three types of zymography are used; *in gel* zymography, *in situ* zymography and *in vivo* zymopgraphy.

Samples are prepared in a standard, non-reducing loading buffer for SDS-PAGE. No reducing agent or boiling are necessary since these would interfere with refolding of the enzyme. A suitable substrate (commonly gelatin or casein) is embedded in the resolving gel during preparation of the acrylamide gel. Following electrophoresis, the SDS is removed from the gel (or zymogram) by incubation in unbuffered Triton X-100, followed by incubation in an appropriate digestion buffer for an optimized length of time at 37°C. The zymogram is subsequently stained and areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme. Ramanan *et al., 2*007 modified the earlier protocol of Hou *et al* (2000) to carry out Carbonic anhydrase activity staining by using 15% SDS–PAGE gels followed by

colour change with bromothymol blue stain. CA activity revealed yellow bands against the blue background of bromothymol blue.

2.5 Polymorphism of Carbonate Crystals

Biomineralization of calcium carbonate results in the production of different phases of calcium carbonate as anhydrous polymorphs**:** calcite, aragonite and vaterite or two hydrated crystalline phases, monohydrocalcite ($CaCO_3.H_2O$) and ikaite ($CaCO_3.6H_2O$) (Rodriguez Navarro *et al.*, 2012; Dhami *et al.*, 2013b) (Fig. 2.5). Studies suggested that phase, amount and morphology of calcium carbonate minerals depend on supersaturation, temperature, pH and $[Ca2^{+}]$ / $[CO_3^{2-}]$ ratio. The saturation index, SI = log Ω= log IAP/Ks; where Ω is the saturation state of the system, IAP is the ion activity product and Ks is the thermodynamic solubility product of the relevant phase. Calcium carbonate precipitation in microbial systems typically occurs when the saturation index (with respect to calcite) is above 1 (Arp *et al*, 2001; Mitchell *et al.*, 2006). As these carbonate polymorphs play important role in various technical applications, there is growing need to characterize these polymorphs.

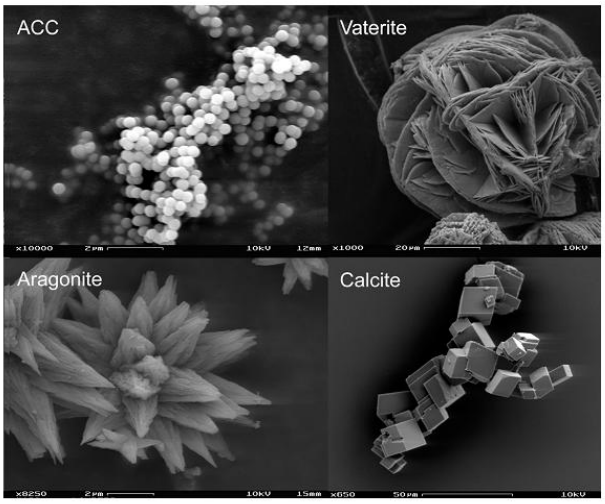


Fig. 2.5: Polymorphs of Calcium Carbonate precipitated by MICCP (Source: A. Niedermayr, A. Immenhauser).

2.6 Optimum conditions for MICCP

The type of bacteria, bacterial cell concentration, initial urea concentration, reaction temperature, the initial Ca^{2+} concentration, ionic strength, and the pH of the media are

some factors that control the activity of the urease enzyme, and may have a significant impact MICCP. Sharma *et al.*, (2008) reported Citrate and Urea to maximally increase enzyme production from all the four isolates (*Enterobacter gergoviae*, *Enterobacter taylorae, Aeromonas hydrophila*, *Aeromonas caviae*). Wei Li *et al.*, 2013 reported that the intracellular and extracellular CA could promote $CaCO_3$ precipitation. The Ca^{2+} ions in the enzymatic systems at initial pH 8.0 were completely deposited at 48 h, which were respectively 21 h, 15 h and 14 h earlier compared with that at initial pH 6.0, pH 6.5 and pH 7.0, indicating that higher pH favored $CaCO_3$ precipitation in the experimental pH range, and was beneficial to the catalytic action of microbial CA on $CaCO_3$ precipitation.

Chapter 3:
Materials and Method

CHAPTER 3

MATERIALS AND METHOD

Screening of efficient bacteria for the production of Carbonic anhydrase

Sample collection

Bacterial isolates were collected from sea water and cultures were maintained at 4°C until their use.

Screening of CA producing bacterial species

Five halophilic bacterial isolates were checked for the presence of CA production on sea water media (SWM) plates supplemented with 1mM pNPA. The cultures were inoculated on SWM + 10µM $ZnSO_4$ + 1mM pNPA plates and incubated at 37°C for overnight for growth of CA producing isolates. To select the efficient CA producers, the intensity of yellow color formation on pNPA plates was checked.

After confirming CA production qualitatively on plates, the selected 2 bacterial isolates were inoculated (1%) into SWM broth supplemented with 10 µM $ZnSO_4$ upto 5 days. *Bacillus pumilus* was taken as positive control and for negative control, no bacterial inoculation was done. Samples were taken from the culture flasks after interval of 24 hrs, centrifuged at 8000rpm for 10 mins and checked for quantitative production of CA.

Table 3.1 Composition of sea water (3.0 % w/v) media

Component	Quantity(g/l)
NaCl	30.0
$MgCl_2$	5.0
$MgSO_4$	2.0
KCl	0.5
$FeSO_4$	0.001
Peptone	5.0
Yeast extract	1.0
pH	7.5

Carbonic anhydrase assay

The carbonic anhydrase activity was determined for both the bacterial isolates by measuring the amount of p-nitrophenol produced according to spectrophotometric assay of Smith and Ferry, 1999. The culture filtrate (200 µl) was added to a mixture containing 1.80 ml of 100 mM phosphate buffer, pH 7.0 and 1 ml of 3 mM p-nitrophenyl acetate solution. The increase in A_{348} nm was recorded for 5 minutes. The $A_{348\ nm}$ minute was computed using the maximum linear rate for both the test and blank. One unit of carbonic anahydrase activity is defined as the amount of enzyme required to form 1 µmole of p-nitrophenol per minute.

Screening of Urease Activity

For estimating the production of urease, both the CA producing halophilic bacterial isolates were checked for qualitative production of urease on Urea Agar Base plates. This was followed by quantitative production of Urease activity in phenol hypochlorite assay.

Both the bacterial cultures (1%) were inoculated into SWM broth supplemented with 2% urea and kept at 37°C upto 5 days. Samples were taken after an interval of 24 hours from

the culture flasks for estimation of Urease production. Positive controls were made with *Bacillus pasteurii* which is a known producer of Urease. For negative control, no bacterial inoculation was done.

Urease activity was determined for all the bacterial isolates by measuring the amount of ammonia released from urea according to the phenol-hypochlorite assay method (Natarajan, 1995) at different time intervals. Ammonium chloride (50-1000 µM) was used as the standard. The culture filtrate (250 µl) was added to a mixture containing 1 ml of 0.1M potassium phosphate buffer (pH 8.0) and 2.5 ml of urea (0.1M). The mixture was incubated at 37°C for 5 minutes followed by addition of phenol nitroprusside and alkaline hypochlorite, 1 ml each and incubated at 37°C for 25 minutes. Optical density was measured at 626 nm. One unit of urease is defined as the amount of enzyme hydrolyzing one µmole urea per min. The urease activity was calculated with reference to a caliberation graph plotted from the results obtained by standards.

Physiological characterization of halophilic bacteria

Morphological and biochemical studies of bacterial isolates

To characterize both the bacterial isolates conventional physiological and biochemical characterization tests were carried out as described in Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994).

Gram staining

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

Fermentation of carbon substrate by bacterial isolates

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

Effect of varying Carbon and Nitrogen Sources on CA enzyme production

Effect of Carbon source on Enzyme production

The effect of various Carbon source (2% w/v) on enzyme production was determined by substituting carbon source: Maltose; Fructose; Dextrose; Glucose; Sucrose; Lactose; Starch and Citric Acid individually in BSS medium. The samples were removed after 3rd day after being incubated at 37°C pH 8 and enzyme activity was determined under standard assay conditions following spectrophotometric assay as described earlier.

Effect of Nitrogen source on Enzyme production

The effect of various Nitrogen source (2% w/v) on enzyme production was determined by substituting nitrogen source: Urea; Peptone; Beef Extract; Yeast Extract; Potassium Nitrate and Ammonium Sulphate individually in BSS medium. The samples were removed at 4th day after being incubated at 37°C pH 8 and enzyme activity was determined under standard assay conditions following sepctrophotometric assay as described earlier.

Characterization of CA enzyme

Preparation of crude enzyme extract

Both the bacterial isolates grown (A_{600} = 0.50) in Sea Water Media containing 10 µM $ZnSO_4$ for 3 days were centrifuged at 8000rpm for 5 minutes at 4°C. Supernatant was used at crude enzyme extract.

Effect of pH on Enzyme stability

The stability of the enzyme was monitored by incubating the crude enzyme extract for 4 hrs with respective buffers ranging from pH 6 to 10 (Citric Acid Buffer pH 6; Sodium Phosphate Buffer pH 7-8; Glycine-NaOH Buffer pH 9-10). The residual enzyme activity was estimated under standard assay conditions following the spectrophotometric assay as described earlier.

Effect of temperature on Enzyme stability

The thermostability of the enzyme was monitored by incubating crude enzyme extract at a temperature range of 25°C to 55°C for 30 mins with respective buffer at optimized pH for each isolate and then the residual enzyme activity was determined under standard assay conditions following the sepctrophotometric assay as described earlier.

Enzyme kinetics

Crude enzyme extract was taken directly for estimation of K_m and *Vmax* but for lyophilized enzyme, 50 ml of the crude extracts were lyophilized to 5ml by using lyophilizer (LyoLab 3000). K_m and V_{max} values of crude and lyophilized CA were determined by measuring the activity of CA in the presence of varying concentrations of p-NPA (substrate) concentrations (1, 2, 3, 4 and 5 mM). Michaelis–Menten constant (K_m) values and the maximum velocities (V_{max}) were determined using the Lineweaver–Burk double reciprocal plot (Lineweaver and Burk 1934).

Activity staining

Carbonic anhydrase activity staining of crude enzyme extracts of both the isolates was performed in 15% SDS–PAGE gels followed by color change with bromothymol blue stain. CA activity will reveal yellow bands against the blue background of bromothymol blue according to the procedure from Ramanan *et al* (2002) with slight modifications. The samples were mixed with sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 1% bromophenol blue and 10% glycerol) and kept at 37°C overnight. After electrophoresis, the gel was washed in Buffer A containing 25% isopropanol in 10 mM of Tris–HCl buffer, pH 8.0, to remove SDS ahead of activity staining. The gel was washed again in

Buffer B containing 100 mM Tris-HCl buffer, pH 8.3, and stained overnight using 0.25% bromothymol blue and destained using Buffer B.

Role of Carbonic anhydrase in Microbially Induced Calcium Carbonate Precipitation

Calcium carbonate precipitation induced by CA

For $CaCO_3$ precipitation assay, CA induced bacterial cultures grown for 3 days were supplemented with 25mM $CaCl_2$ and 25mM $NaHCO_3$. Controls consisted of uninoculated bacterial cultures media supplements. The flasks were incubated at 37° C for 24 hours and the precipitated carbonates were collected and estimated by EDTA titration method.

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

Effect of various parameters on calcium carbonate precipitation

Effects of varying pH, varying concentrations of $CaCl_2$, $NaHCO_3$ and $ZnSO_4$ were investigated on MICCP by measuring Calcium carbonate estimation in each case.

Effect of various calcium sources on MICCP

The effect of various Caicium source (25 mM) on MICCP was determined by substituting calcium source: Calcium acetate; Calcium nitrate; Tricalcium acetate individually in Sea Water Media. The samples were removed at 4^{th} day after being incubated at 37°C pH 8 and precipitation kinetics were studied by measuring Calcium carbonate estimation as described earlier.

Crystal morphology

Bacteria around and inside carbonate crystals were detected using Loeffler's methylene blue stain (Fisher Scientific, United Kingdom). Carbonate crystals mounted on glass microscope slides were 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 1,000 magnification and immersion oil.

Polysaccharides associated with carbonate crystals were detected using alcian blue–periodic acid-Schiff stain (PAS). Crystals on glass microscope slides were stained with alcian blue-PAS (Fisher Scientific, United Kingdom) by applying 100% ethanol for 5 min, rinsing with dH_2O for 5 min, applying alcian blue for 5 min followed by 1% Schiff's reagent for 5 min, and, finally, rinsing with dH_2O for 5 min.

Statistical analysis

Data were statistically analyzed by an analysis of variance (ANOVA) and when observed differences were significant, the means were compared by Tukey's honestly significant difference test. All the experiments in this study were performed in triplicates. Graph pad prism (5.0) software was used for all analysis.

Chapter 4:
Results and Discussion

CHAPTER 4

RESULTS AND DISCUSSION

Enzymes are a focus of intense research worldwide due to their applications in various industries such as baking, detergent, food and beverages, textile, environmental sciences and many more. However, only in fast few decades, microbial enzymes have been used economically in environmental issues.

Carbonic anhydrase has been widely studied by many researchers for CO_2 sequestraion studies in the recent times (Ramanan *et al., 2*009; Zhang *et al., 2*011; Anjana Sharma *et al., 2*011). Though the role of bovine CA in CO_2 sequestrations has been widely studied but bacterial CA are a new class in this category which have fetched wide attention. Because of its role in biomimetic carbon sequestrations, its applications have been investigated for calcium carbonate precipitations and biomineralization studies in recent time(Li *et al.,* 2011). Bacterial CA are also been focus of study these days. Initial works on bacterial CA was carried out by Smith and Ferry (2000). Several bacterial isolates capable of producing CA have now been isolated from different sources (Prabhu *et al.,* 2011; Alber and Ferry 1994; 1997; Braus *et al.,* 1997; Yagawa *et al.,* 1984; Sharma *et al., 2009*) but not much has been investigated from halophilic bacteria. Halophilic bacteria are an important class of bacterial diversity as they have several commercial applications and also impart to evolutionary studies.

In the present study, two halobacterial isolates from Sea Water Sample (pH 11.0) were studied for their ability to produce extracellular Carbonic anhydrase. Out of five isolates screened initially, two were found to be positive. Appearance of bright yellow colour on pNPA plates indicated the presence of Carbonic anhydrase by both the isolates (Fig. 4.1a). These cultures were also checked for the production of Urease by plate assay method on Urea Agar Base plates. Formation of pink colour confirmed the presence of Urease in both the isolates (Fig. 4.1b). Both cultures showed efficient ureolytic and CA producing ability on their specific plates.

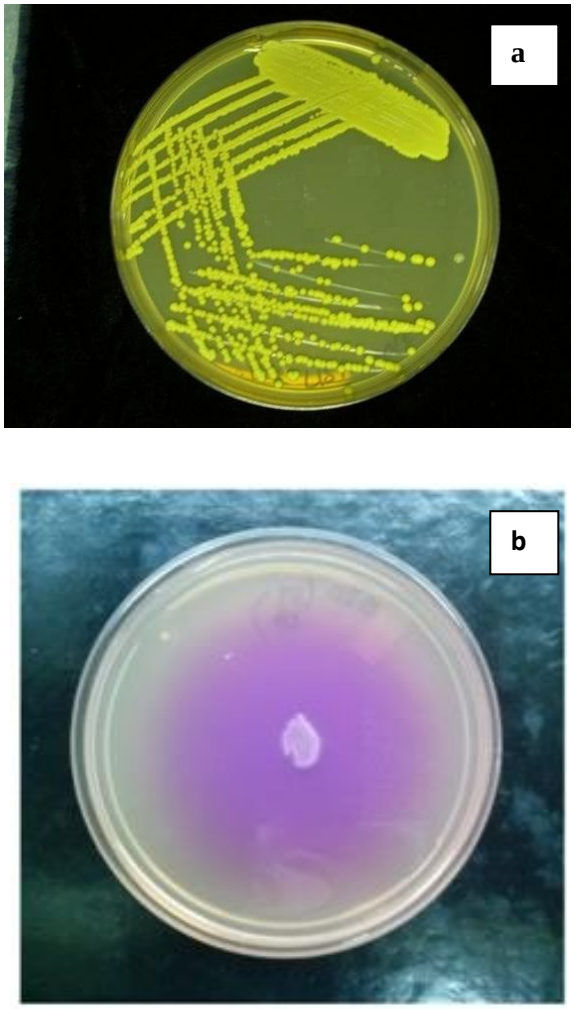


Fig. 4.1a. Carbonic anhydrase production on p-NPA plate
b. Urease production on Urea Agar plate

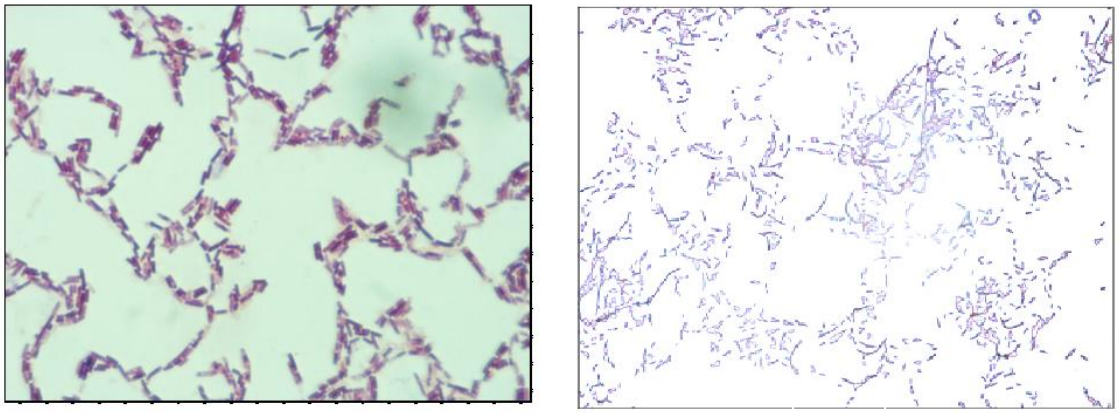
BACTERIAL CHARACTERIZATION

The historic way to characterize bacteria is to describe quantitatively as many phenotypic traits as possible, such as morphology, structure, cultivation, nutrition, biochemical metabolism etc. These phenotypic characteristics are important for declineation of taxa (Busse *et al.*, 1996). In order to characterize these ureolytic and CA producing bacteria, gram staining, morphological studies and fermentation studies were carried out. It was revealed that both the isolates are Gram positive bacilli (Table 4.1; Fig. 4.2). This character was further confirmed by MacConkey Agar test which is an enrichment medium on which only Gram negative bactreria can grow. No growth on MacConkey Agar Plates was an indication that the isolates are Gram positive.Both the bacterial

isolates were found to be rod shaped, Gram positive and formed pinkish colonies on sea water agar media containing urea.

Table 4.1: Bacterial Characterization of bacterial strains A3-5 and A1-4

Sample	Mc Conkey Agar	Morphological		Biochemical		
		Gram Character	Shape	Fermentation of		
				Glucose	Mannitol	Sucrose
A3-5	No growth	+ve	Bacilli	+	-	+
A1-4	No growth	+ve	Bacilli	+	+	+



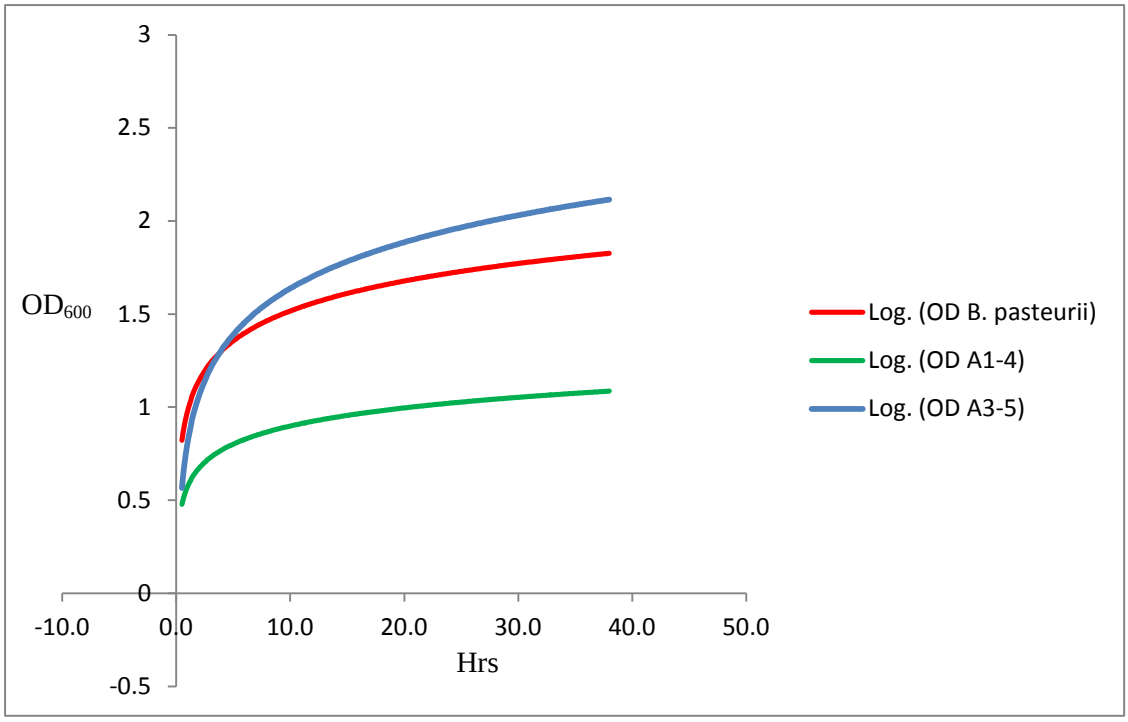
a **b**

Fig. 4.2: Gram staining of bacterial strains A3-5 (a) and A1-4 (b)

GROWTH CURVE

The growth curves of both the isolates were also studied over a span of 38 hrs in Sea Water Broth (Fig. 4.3). It was noticed that both the isolates showed increasing growth till 24 hours while after that it was found to be static. Bacterial growth studies reveal s sigmoidal graph in the later phases as after stationary phase, due to deprivation of nutrients, the bacterial cells start with the death phase. In our study the stationary phase was found upto 38 hours.

Fig. 4.3: Growth Curve of bacterial strains A3-5 and A1-4 taking *Bacillus pasteurii* as reference strain



ENZYME PRODUCTION PROFILE

Sea Water Media was inoculated with freshly prepared 12 hr old culture of both the isolates and incubated for different time intervals (24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs) at 37°C. The enzyme activity of the extracellular Carbonic anhydrase secreted in the supernatant was studied by carrying out p-NPA assay while the urease activity was studied by Sodium hypochlorite assay. For urease assay, *Bacillus pasteurii* (Bp) was taken as reference strain while for CA, *Bacillus pumilus* (Bpm) was the reference culture. It was found that Carbonic anhydrase activity increased with time in both the isolates till 3rd day but after that it declined. Maximum activity of CA from both the isolates was found to be 108 U/ml for A3-5 and 153 U/ml for A1-4on 3rd day. Sharma *et al* (2010) also found efficient CA production by *Enterobacter taylorae, E. gergoviae, A. hydrophila* and *Aeromonas caviae* while Prabhu *et al* (2010) found CA activity of *B. pumilus*, *P. fragii* and *M. lylae* to be 6840 U/g, 4890 U/g and 4400 U/g respectively in case of purified and lyophilized CA. In case of urease also, the activity was found to increase till 3rd day while after that it declined gradually (Fig 4.4). A3-5 showed maximum activity with 556 U/ml while A1-4 gave maximum with 656 U/ml on 3rd day.

Stocks – Fisher *et al* (1991) reported the activity of *Bacillus pastuerii* to be 0.11mM / min while De Muynck *et al* (2011) reported Urease activity in the range of 0.22 – 0.12 mM/min. It was found that the halophilic bacterial isolates of the present study were more efficient than previously reported bacterial isolates on basis of urease production while less efficient on the basis of CA produing strains.

From the above activities, it was observed that both the activities coincided on the basis of production time. Previous reports also suggested that urease and CA activity might be correlated with each other (Achal *et al.,* 2010; Dhami *et al.,* 2013). Hydrolysis of urea results in accumulation of both bicarbonate and ammonia in the cell, which favours the physiological and regulatory links between urea and bicarbonate metabolism (Stahler *et al.,* 2005). The incorporation of nickel into the active site of urease is dependent on CO_2/ HCO_3^- metabolism which is in turn regulated by carbonic anhydrase (Park and Hausinger, 1995). So, both Urease as well as CA might have some relationship in bacterial systems of ureolytic isolates.

Table 4.2: Production profile of CA by bacterial strains A3-5 and A1-4. Values are mean ± SD (n = 3). Means followed by the same letter within the column are not significant at P<0.05.

	CA activity (U/ml)					
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
B.pumilis	82.0 ±0.5^a	124.0±6.5^a	164.0±7.2^a	116.0±9.4^a	64.6±6.4^a	44.3±3.7^a
A3-5	50.0 ±2.0^b	74.6±5.0^b	108.0 ±5.9^b	81.7± 9.2^b	42.1±3.6^b	36.4±5.8^a
A1-4	75.1±6.3^b	117.0 ±7.1^a	153.0± 8.3^a	85.6±7.5^b	39.0±4.2^b	32.3±7.7^a

Table 4.3: Production profile of Urease by bacterial strains A3-5 and A1-4. Values are mean ± SD (n = 3). Means followed by the same letter within the column are not significant at P<0.05.

	Urease activity (U/ml)					
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
B.pasteurii	330.3 ±9.2[a]	472.0±4.5[a]	964.6±9.1[a]	586.0±3.4[a]	468.6±6.7[a]	420.2±3.7[a]
A3-5	314.2 ±2.0[a]	331.8±5.4[c]	556.0 ±8.4[b]	340.6±7.8[b]	264.1±4.6[b]	222.7±5.8[d]
A1-4	197.8±6.3[b]	317.3 ±3.4[b]	656.0± 8.5[a]	340.4±9.2[b]	315.0±3.2[a]	276.3±7.7[c]

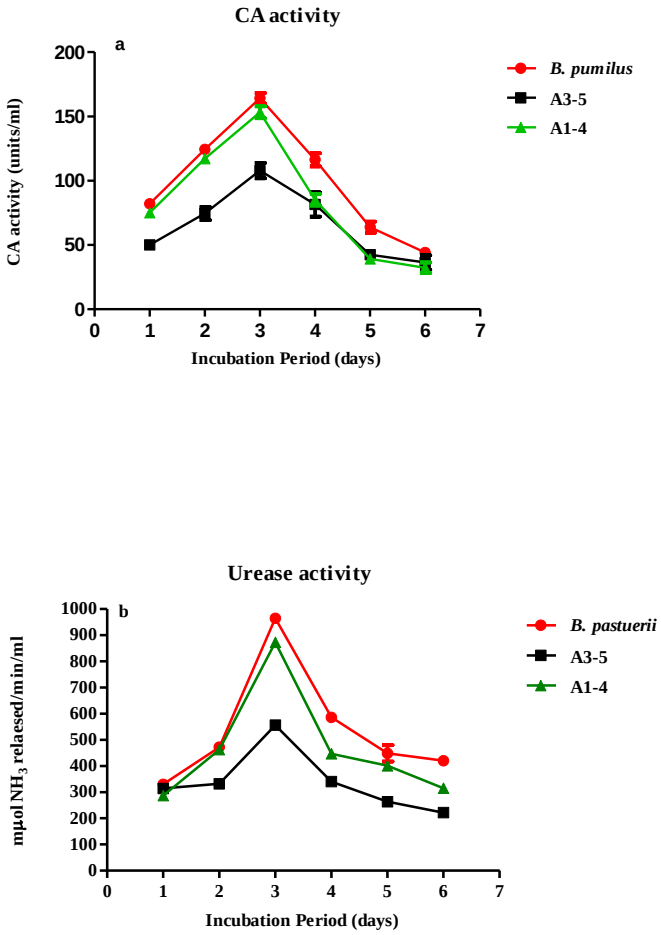


Fig. 4.4a. CA activity of bacterial strains A3-5 and A1-4 along with B. *pumilus*
b. Urease activity of bacterial strains A3-5 and A1-4 along with B. *pasteurii*

OPTIMIZATION OF CULTURE CONDITIONS

The basic factors for efficient production of Carbonic anhydrase are the selection of potential species, optimization of appropriate growth conditions and an appropriate substrate in a well defined medium. To find out suitable media for Carbonic anhydrase production, different substrate concentrations were tried in Basal Salt Solution (BSS).

BSS of different Carbon (Maltose, Fructose, Dextrose, Glucose, Sucrose, Lactose, Starch Citric Acid) and Nitrogen (Urea, Peptone, Beef Extract, Yeast Extract, Potassium Nitrate and Ammonium Sulphate) sources were inoculated with freshly prepared 12 hr old cultures of A3-5 and A1-4 isolates and incubated for 96 hrs at 37°C (Table 4.1 and 4.2).

In present work, maximum CA activity of 202.6 U/ml was observed with Fructose and yeast extract as carbon and nitrogen source in case of A3-5 while A1-4 showed maximum activity of 249.5 U/ml with glucose and peptone (Table 4.4 and 4.5). For Urease, maximum activity of 202.6 U/ml was observed with glucose and peptone in case of A3-5 where as A1-4 showed maximum activity of 249.5 U/ml with sucrose and beef extract as Carbon and Nitrogen Source respectively (Fig. 4.5 and 4.6). Similar studies were carried out by Sharma *et al* (2010) and Balan *et al* (2012) in case of CA and UA. CA production was found to be maximum with citrate and urea along with peptone by Sharma et al (2010) while glucose and peptone was found to be best for maximum urease production. However Ghasemi *et al* (2004) recorded yeast extract as the best nitrogen source for urease production by fungi *Aspergillus niger*.

Table 4.4: Effect of Carbon source on CA and Urease activity of bacterial strains A3-5 and A1-4. Values are mean ± SD (n = 3). Means followed by the same letter within the column are not significant at P<0.05.

Carbon Source	Residual CA Activity (%)		Residual Urease Activity (%)	
	A3-5	A1-4	A3-5	A1-4
Maltose	47 ± 1.5^{de}	93.00 ± 2.5^{b}	59.1 ± 2.1^{e}	78.9 ± 3.5^{c}
Fructose	100.00 ± 1.0^{a}	53.33 ± 3.3^{de}	29.4 ± 1.3^{f}	77.00 ± 2.5^{c}
Dextrose	58.1 ± 3.61^{c}	59.00 ± 6.5^{d}	52.3 ± 3.1^{e}	45.59 ± 2.2^{e}
Glucose	71.6 ± 2.5^{b}	100.00 ± 0.0^{a}	100.00 ± 1.5^{a}	66.55 ± 3.6^{cd}
Sucrose	46.5 ± 4.01^{cd}	73.66 ± 2.7^{b}	43.67 ± 1.4^{e}	100.00 ± 1.5^{a}
Lactose	38.1 ± 1.94^{e}	67.00 ± 1.5^{c}	74.64 ± 2.3^{c}	84.89 ± 1.5^{b}
Starch	37.0 ± 2.53^{ef}	53.33 ± 2.50^{de}	50.32 ± 1.3^{e}	66.59 ± 2.7^{cd}
Citric Acid	39.6 ± 3.2^{e}	64.33 ± 1.6^{cd}	47.60 ± 6.2^{e}	46.94 ± 1.5^{e}

Table 4.5: Effect of Nitrogen source on CA and Urease activity of bacterial strains A3-5 and A1-4. Values are mean ± SD (n = 3). Means followed by the same letter within the column are not significant at P<0.05.

Nitrogen Source	CA Activity (U/ml)		Urease Activity (U/ml)	
	A3-5	A1-4	A3-5	A1-4
Urea	39.22 ± 2.5^{c}	117.80 ± 4.5^{b}	44.30 ± 1.2^{b}	45.60 ± 1.6^{c}
Peptone	-33.90 ± 1.1^{e}	211.16 ± 3.3^{a}	73.60 ± 2.6^{a}	65.00 ± 2.0^{c}
Beef extract	-14.64 ± 1.2^{e}	21.36 ± 1.5^{e}	33.00 ± 1.8^{c}	178.67 ± 3.7^{a}
Yeast Extract	77.42 ± 3.3^{a}	148.09 ± 3.1^{b}	-23.60 ± 1.0^{f}	5.75 ± 0.3^{e}
Potassium Nitrate	23.96 ± 2.1^{cd}	2.76 ± 0.3^{f}	14.0 ± 0.5^{d}	44.00 ± 1.2^{c}
Ammonium Sulphate	18.24 ± 1.1^{d}	10.09 ± 0.4^{e}	-51.30 ± 1.5^{f}	-26.00 ± 1.5^{f}

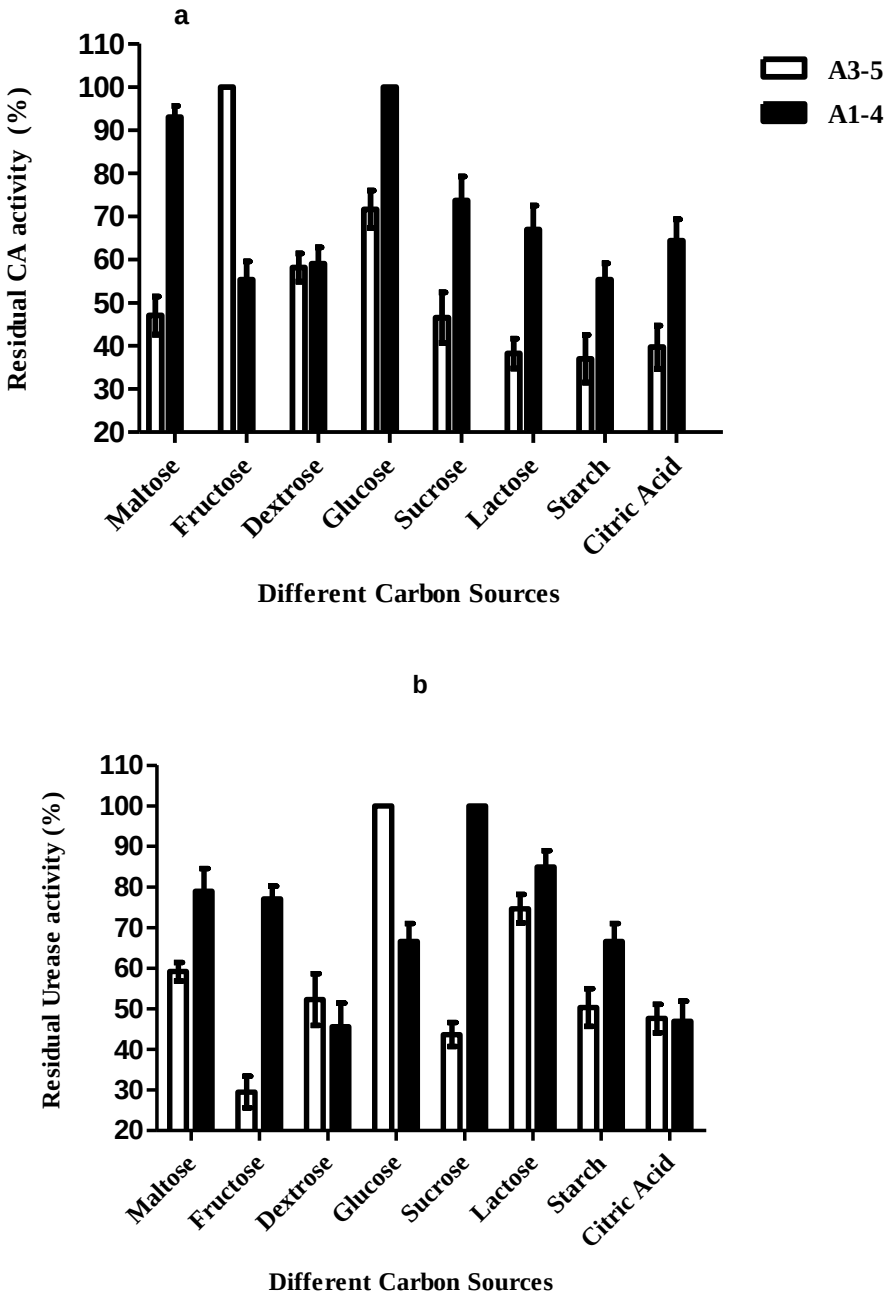


Fig 4.5a. Effect of Carbon Sources on Carbonic anhydrase activity of A3-5 and A1-4

b. Effect of Carbon Sources on Urease activity of A3-5 and A1-4

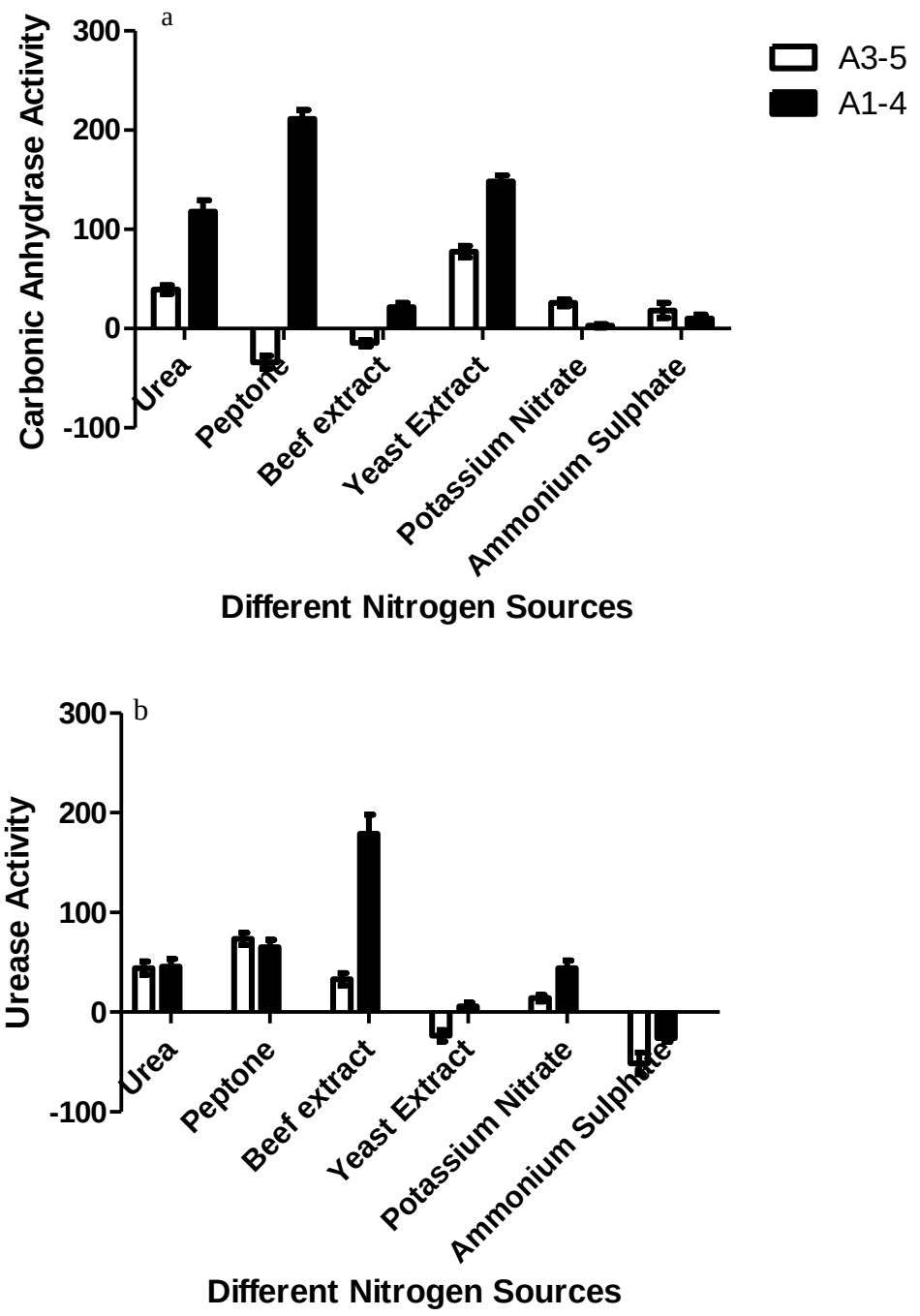


Fig 4.6a. Effect of Nitrogen sources on Carbonic anhydrase activity of A3-5 and A1-4

b. Effect of Nitrogen sources on Urease activity of A3-5 and A1-4

ENZYME CHARACTERIZATION

For obtaining crude CA enzyme supernatant, bacterial cells were inoculated into SWM supplemented with 10µM $ZnSO_4$ for 3 days and centrifuged to get the supernatant while for characterizing the urease from halophilic bacterial isolates, the cultures were inoculated into SWM with 2% and cultures were centrifuged to separate the supernatant. Effect of different parameters (temperature, pH, substrate concentration) were investigated on both CA and Urease activity. The optimal values of these parameters were, thus, obtained.

Effect of pH and Temperature on Enzyme Activity

pH and Temperature play vital role in activity of an enzyme. Being proteinaceous in nature, they get denatured at very high and very low temperatures and pH. For studying effect of pH on stability of carbonic anhydrase and urease, the crude supernatants were incubated in varying pH buffers for 4 hrs and the residual activity was checked afterwards. where as to study effect of temperature on their stability, enzyme extracts were incubated at varying temperatures for 30 minutes.

In present study, results showed that maximum CA activity was observed at pH 7 for isolate A1-4 while at pH 8 for A3-5. In case of temperature, maximum stability of CA was found at 55°C for both the isolates (Table 4.6 and 4.7). In case of UA, pH 9 and 40°C was found to be optimum for both the isolates (Fig 4.7 and 4.8). Similar studies have been done by many researchers. Sharma *et al* (2010) reported pH 8.5 as optimum pH for CA production and stability whereas for Urease, Balan *et al* (2010) reported its maximum stability at pH 8.

Table 4.6: Effect of Temperature on CA and Urease activity of bacterial strains A3-5 and A1-4. Values are mean ± SD (n = 3). Means followed by the same letter within the column are not significant at P<0.05.

	Residual CA Activity (%)		**Residual Urease Activity (%)**	
Temperature (°C)	A3-5	A1-4	A3-5	A1-4
25	78 ± 3.0^{b}	63.67 ± 7.1^{c}	78 ± 7.0^{b}	63.6 ± 5.0^{d}
37	75.33 ± 4.0^{b}	83.00 ± 7.6^{b}	75.33 ± 5.8^{b}	83.00 ± 5.6^{b}
40	73.00 ± 5.5^{b}	76.65 ± 6.5^{bc}	100 ± 0^{a}	100 ± 4.5^{a}
55	100 ± 4.5^{a}	100 ± 3.5^{a}	73.00 ± 7.0^{b}	76.6 ± 9.0^{bc}

Table 4.7: Effect of pH on CA and Urease activity of bacterial strains A3-5 and A1-4. Values are mean ± SD (n = 3). Means followed by the same letter within the column are not significant at P<0.05.

	CA Residual Activity (%)		**Residual Urease Activity (%)**	
pH	A3-5	A1-4	A3-5	A1-4
6	93 ± 4.5^{b}	63 ± 7.6^{d}	56 ± 6.6^{e}	48 ± 3.1^{ed}
7	93 ± 3.5^{b}	100 ± 5.5^{a}	67 ± 7.7^{d}	44 ± 4.7^{e}
8	100 ± 3.5^{a}	94 ± 3.5^{b}	95 ± 5.2^{b}	51 ± 9.5^{e}
9	87 ± 4.0^{b}	92 ± 3.5^{b}	100 ± 2.5^{a}	100 ± 2.50^{a}
10	69 ±d 5.69	67.0 ± 7.5^{d}	52 ± 6.02^{e}	66 ± 4.7^{d}

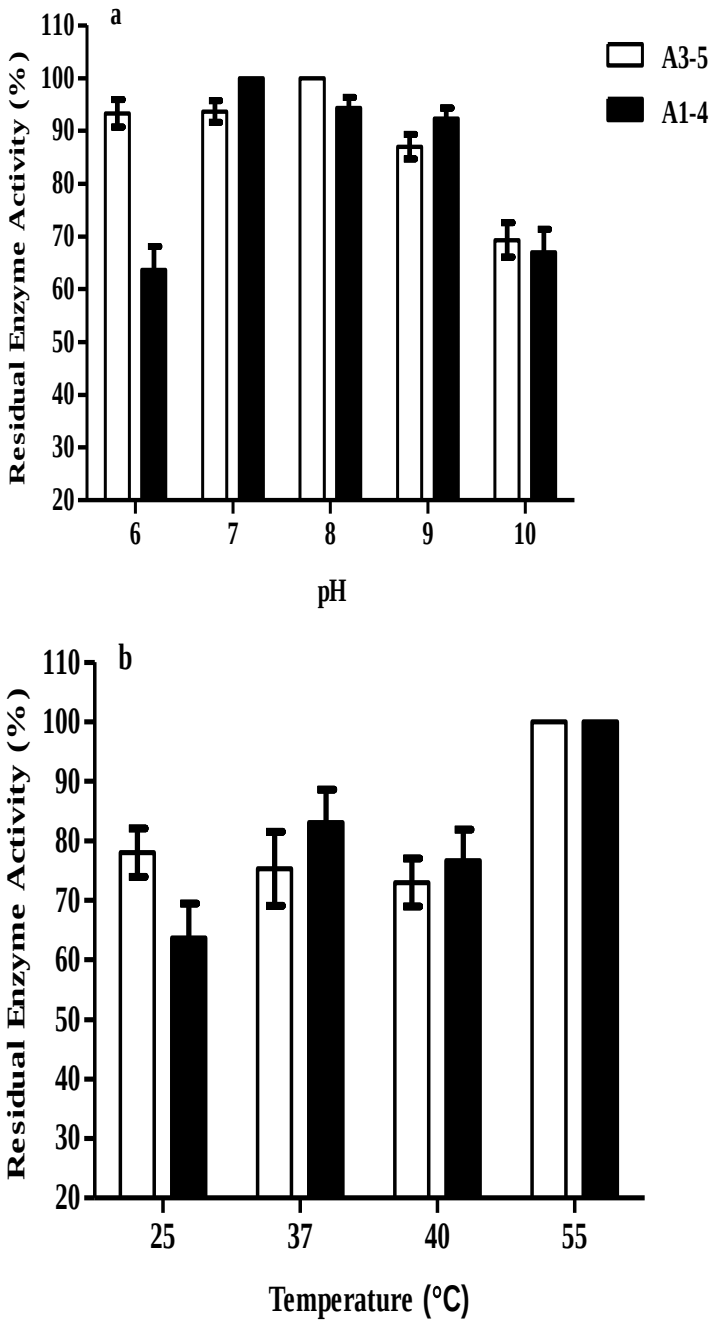


Fig 4.7a. Effect of pH on Carbonic anhydrase activity of A3-5 and A1-4

b. Effect of Temperature on Carbonic anhydrase activity of A3-5 and A1-4

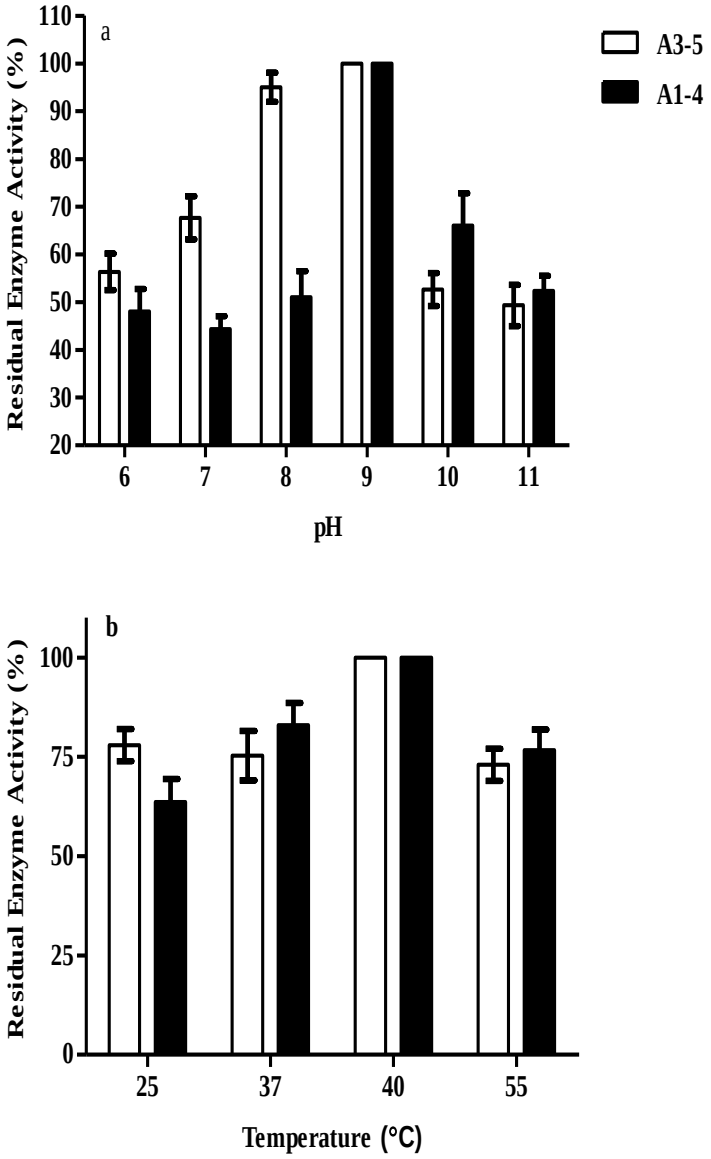


Fig 4.8a. Effect of pH on Urease activity of A3-5 and A1-4

b. Effect of Temperature on Urease activity of A3-5 and A1-4

Enzyme Kinetics

Enzyme kinetics play vital role in determining the properties of enzymes. The mechanism of enzyme catalyzed reactions is often studied by making kinetic measurements on enzyme-substrate reaction systems. These studies include measuring rates of the enzyme-catalyzed reactions at different substrate and enzyme concentrations. Kinetic information is useful for examining possible mechanisms for the reaction. This is true for all types of reactions; kinetic principles are used to understand both catalyzed and non-catalyzed reactions. For enzymes, kinetic information is useful for understanding how and at what conditions enzyme works to its full potency. It is impossible to understand enzymes without understanding the kinetic principles of the reactions they mediate.

In the present study, the kinetic constants (K_m and V_{max}) for free and lyophilised enzyme were determined by using Lineweaver–Burk plots as shown in Fig 4.9. K_m and V_{max} values of crude and lyophilised CA were calculated from the intercepts on x and y axes of the Lineweaver–Burk plots, respectively. The K_m and V_{max} values for crude and lyophilised enzyme are summarized in Table 4.8. The K_M, and V_{max} values for crude enzyme were found to be 0.31mM and 1.88 µmoles/min and 0.67mM and 1.49 µmoles/min respectively for A3-5 and A1-4 using p-NPA as substrate. The lyophilized enzyme showed an increased K_M value of 0.20 mM and 0.37 mM for A3-5 and A1-4 respectively. Whereas V_{max} value is slightly lower for A3-5 (1.14 µmoles/min) but increased in case of A1-4 (1.69 µmoles/min) at 25°C. Similar results were reported by Wanjari *et al (*2011) who reported K_M and V_{max} for purified CA from *B. pumilus*. K_m and V_{max} in case of free CA was 0.87 mM and 0.93 µM/min respectively, whereas in case of immobilized CA they were 2.30 mM and 0.54 µM/min respectively.

Table 4.8: Enzyme Kinetics of Crude and Lyophilized Carbonic anhydrase from bacterial strains A3-5 and A1-4

Samples	**Extracellular Carbonic Anhydrase**			
	Crude enzyme		**Lyophilised enzyme**	
	K_M (mM)	**V_{max} (µmoles/min)**	**K_M (mM)**	**V_{max} (µmoles/min)**
Bacillus pumilus	0.21	2.5	0.14	2.89
A3-5	0.31	1.88	0.20	2.14
A1-4	0.67	1.49	0.37	1.69

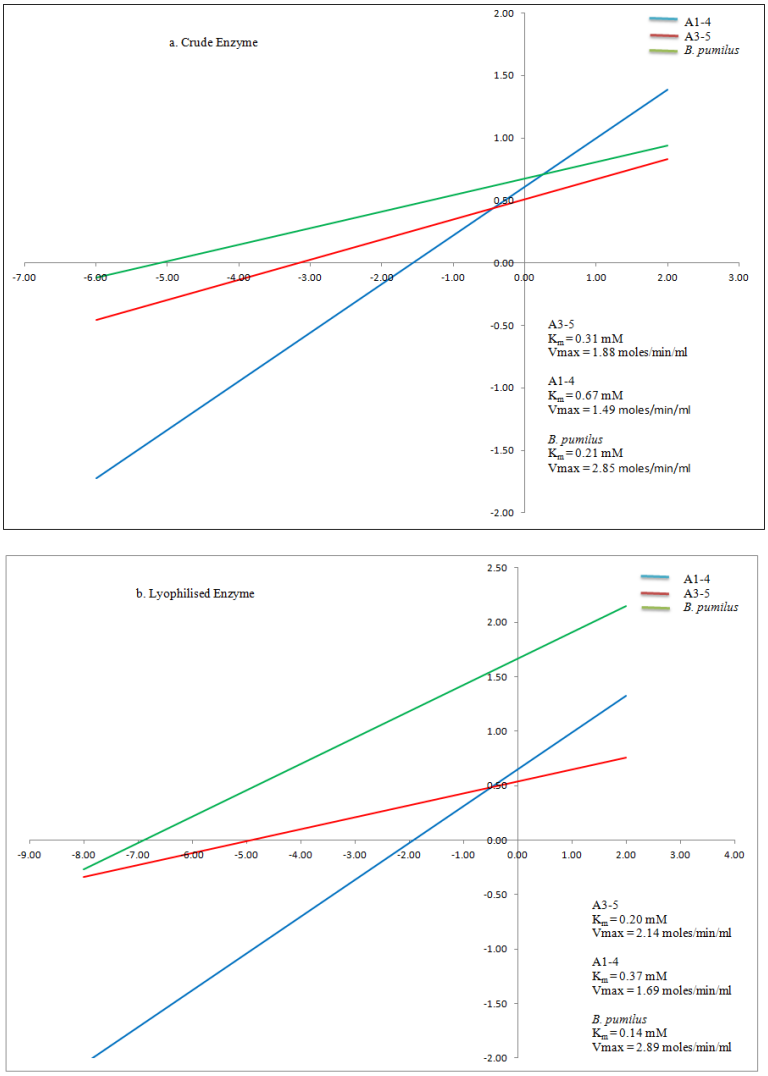


Fig. 4.9: Lineweaver-Burk plots for Crude (a) and Lyophilized (b) CA Enzyme by bacterial isolates A3-5 and A1-4.

Activity staining

Carbonic anhydrase activity staining of enzyme extract was performed in 15% SDS–PAGE gels followed by color change with bromothymol blue stain. CA activity revealed yellow bands against the blue background of bromothymol blue in both the enzyme extracts from A3-5 and A1-4 according to the procedure from Ramanan *et al* (2002) (Fig 4.10). The formation of distinct yellow bands evidenced the presence of CA in both the halophilic bacterial isolates. The size of the enzyme was estimated to be around 27kDa.

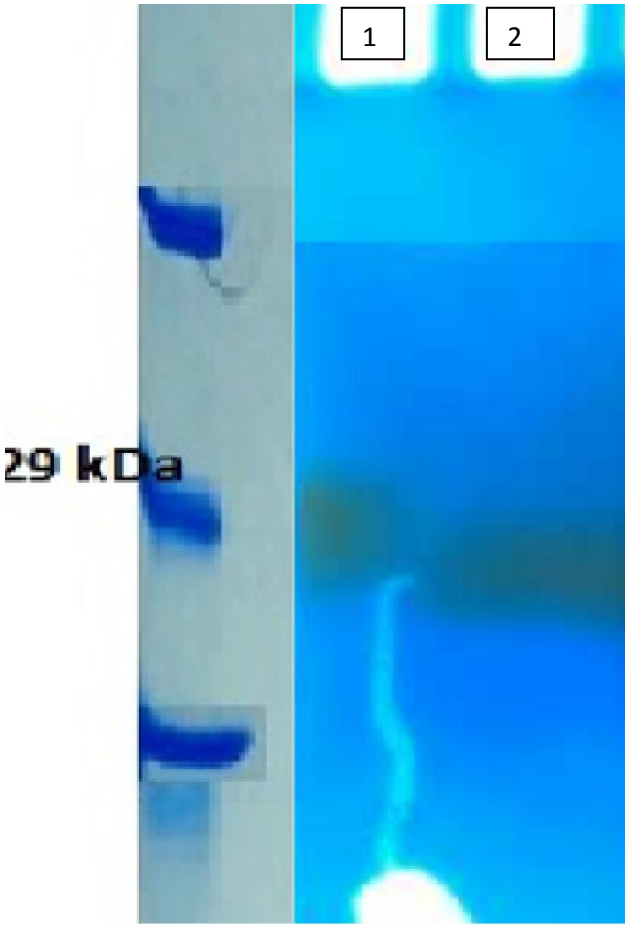


Fig. 4.10: Activity Staining of Carbonic anhydrase from A3-5 (Lane 1) and A1-4 (Lane 2) crude enzyme extracts

Ramanan *et al (*2009) also got positive bands by CA activity staining in *Proteus vulgaris* RS7; *Citrobacter freundii* SW3; *Bacillus subtilis* SA3; *Enterobacter sp*., RS1. Sharma *et al* (2010) also isolated CA from *Enterobacter taylorae, Aeromonas hydrophila, Aeromonas caviae, Pseudomonas fragii* which was reported to be 29 kDa. In our study crude CA from both the isolates were clearly produced and isolated.

MICROBIALLY INDUCED CALCIUM CARBONATE PRECIPITATION

Microbially induced calcium carbonate precipitation (MICCP) studies have been carried out by various researchers around the globe who have reported different bacterial isolates or different conditions for precipitation of carbonates by bacteria while few have even tried to optimize the conditions for efficient production of bacterial carbonates (Banfield & Hamers 1997; Douglas & Beveridge 1998; Ehrlich 2002; Ferrer *et al.*, 1988; Rivadeneyra *et al.*, 1991, 1993, 1994). Most of the previous works have been carried out via ureolytic pathway but not much has been reported on biomineralization studies via bacterial carbonic anhydrases. In the present work, the precipitation of carbonates has been done by activating the Carbonic anhydrase enzyme and the role of this enzyme in biomineralization of carbonates has been investigated. Sea water media containing 25 mM $CaCl_2$ + 25mM $NaHCO_3$ + 10uM $ZnSO_4$ was inoculated with freshly prepared 12 hr old inocula of both the cultures (grown in SWM + $ZnSO_4$) and incubated at 37°C for precipitation of carbonates. Control set was also prepared without bacterial inoculation and another control set was prepared by inoculating *B. pasteurii* in the media (Table 4.9). Precipitated carbonates in the pellets of all sets were collected and $CaCO_3$ content was measured by EDTA titration method (AHPA 1989) after 5 days. It was observed that efficient carbonate precipitation occurred in all bacterial inoculated sets while very small amount of precipitation was seen in uninoculated set. Maximum precipitation was found in *B. pasteurii* followed by A1-4 and A3-5. Though both halophiles produced lesser carbonate precipitates compared to *B. pasteurii* but still, efficient precipitation was noticed (Fig. 4.11, 4.12). This proved that bacterial inoculation is requisite for carbonate precipitation and Carbonic anhydrase plays significant role in bacterially induced $CaCO_3$ precipiation. Similar results have been shown by previous researchers (Stocks-Fisher *et al* 1999; De Muynck *et al;* Li *et al 2012*).

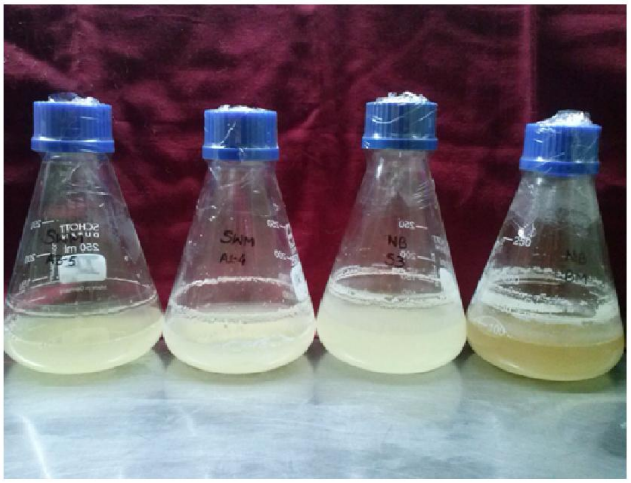


Fig. 4.11: Calcium carbonate precipitation by bacterial strains A3-5 and A1-4

Table 4.9: Precipitation Kinetics of $CaCO_3$ by bacterial strains A3-5 and A1-4 taking *B. pasteurii* as reference

mg $CaCO_3$ / 25 ml media			
Control	***B. pasteurii***	**A3-5**	**A1-4**
4.2 ± 0.21^{d}	36 ± 0.14^{a}	28 ± 0.61^{b}	34 ± 0.45^{a}

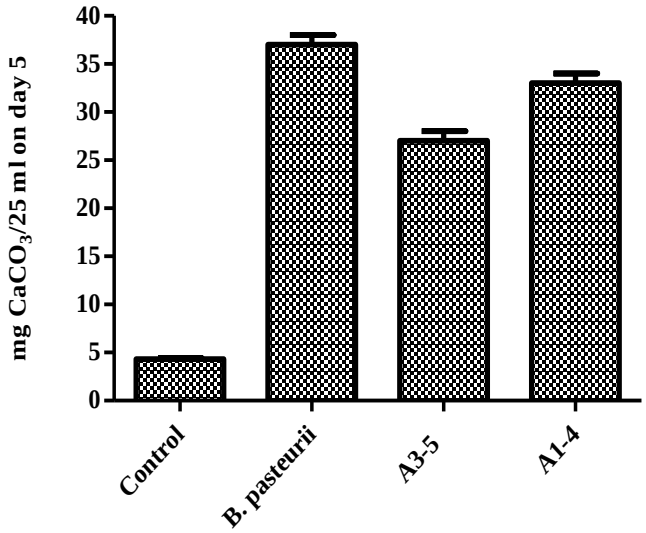


Fig. 4.12: Precipitation Kinetics of $CaCO_3$ by bacterial isolates A3-5 and A1-4.

Influence of different parameters on Calcium carbonate precipitation

Various parameters affect $CaCO_3$ precipitation. Among those, concentration of Ca, $NaHCO_3$, $ZnSO_4$ and pH plays important role. Experiments were designed based on some of these factors to determine the optimum conditions that take into consideration the influence of CA and other parameters on MICCP giving economic advantage while at the same time giving quality results. Bacterial isolates were grown at constant temperature (37 °C) with varying concentrations of $NaHCO_3$, $ZnSO_4$, pH and $CaCl_2$ to optimize the best conditions for Calcium carbonate precipitation.

Firstly in case of varying $CaCl_2$ concentrations, it was found that in the initial stages of increasing Ca concentration, the carbonate precipitation increased from 10 mM to 100 mM but after 100mM, it was found to decrease (Table 4.10 and Fig.4.13a). Maximum precipitation was seen to occur at 100mM concentration. Our results suggest that beyond a limit, merely increasing the concentration of Ca does not affect precipitation of carbonates. Nemati and Voordouw (2003) also established that increasing Ca^{2+} concentration beyond 90 g L^{-1} respectively do not increase the amount of Calcium carbonate precipitation in MICCP. Okwadha *et al* (2010) in their studies found 250mM concentration to be optimum for carbonate precipitation.

Table 4.10: Effect of varying $CaCl_2$ concentrations on calcium carbonate precipitation by bacterial strains A3-5 and A1-4. Means followed by the same letter within the column are not significant at $P<0.05$.

	mg $CaCO_3$ / 25ml		
$CaCl_2$ (mM)	**Control**	**A3-5**	**A1-4**
10	1.9 ± 0.2^{d}	28.8 ± 3.1^{d}	21.3 ± 1.05^{e}
25	5.4 ± 0.9^{cd}	32.4 ± 1.7^{d}	29.3 ± 2.4^{d}
50	7.7 ± 1.5^{b}	46.4 ± 2.2^{cd}	48.0 ± 2.2^{d}
100	13.6 ± 2.5^{b}	80.8 ± 2.7^{a}	89.5 ± 3.7^{a}
150	11.5 ± 1.2^{a}	64.06 ± 2.2^{b}	72.6 ± 2.41^{b}
200	14.36 ± 3.6^{b}	53.03 ± 3.1^{b}	53.50 ± 3.3^{cd}

Precipitation studies were also checked out at varying pH as it plays a major role in $CaCO_3$ precipitation (6-10) (Table 4.11, 4.13b). In this case, it was noticed that there was increase in precipitation with increase in pH from 6 to 10. Maximum precipitation was observed at pH 9 in A3-5 while in case of A1-4, it was highest at pH 10. These results indicated that pH influenced the rate of $CaCO_3$ precipitation significantly. Higher the pH of the solution, more the precipitation of $CaCO_3$. Similar results were reported by Li *et al* (2013). At lower pH, more carbonic acid is produced (Li *et al.,* 2013) while at a high pH (>8), more CO_3^{2-} ions are mainly produced favouring more precipitation. Therefore, high initial pH of solution favors the precipitation of $CaCO_3$. Stocks-Fischer *et al* (1999) also demonstrated that higher pH favored $CaCO_3$ precipitation.

Table 4.11: Effect of varying pH on calcium carbonate precipitation by bacterial strains A3-5 and A1-4. Means followed by the same letter within the column are not significant at P<0.05.

	mg $CaCO_3$/25ml		
pH	**Control**	**A3-5**	**A1-4**
6	0.9 ± 0.62^{e}	11.9 ± 2.6^{c}	16.3 ± 2.0^{c}
7	4.4 ± 0.9^{b}	17.2 ± 2.1^{c}	17.6 ± 2.8^{c}
8	5.1 ± 0.9^{b}	31.0 ± 2.5^{b}	35.7 ± 2.5^{b}
9	7.6 ± 1.5^{a}	47.9 ± 2.9^{a}	52.7 ± 2.1^{a}
10	6.1 ± 1.2^{b}	42.3 ± 1.4^{bc}	54.10 ± 1.6^{bc}

When precipitation studies were carried out with varying $ZnSO_4$ concentration (5 µM – 100 µM), it was seen that precipitation increase with increasing zinc. Though not much has been done on studying the effect of zinc in biomineralization but researchers have reported that optimum concentration of Zinc is prerequisite for activity of carbonic anhydrases (Li *et al.,* 2011; Ramanan *et al.,* 2009). In our study, the optimum concentration of zinc for maximum carbonate precipitation was found to be 100 µM (Table 4.12, Fig. 4.13c).

Table 4.12: Effect of varying $ZnSO_4$ concentrations on calcium carbonate precipitation by bacterial strains A3-5 and A1-4. Means followed by the same letter within the column are not significant at P<0.05.

	mg $CaCO_3$/25ml		
$ZnSO_4$(µM)	**Control**	**A3-5**	**A1-4**
5	3.4 ± 0.98^{c}	8.41 ± 1.2^{d}	5.7 ± 1.10^{d}
10	3.90 ± 1.2^{c}	9.51 ± 0.9^{d}	9.9 ± 0.80^{d}
20	5.75 ± 1.5^{b}	19.20 ± 0.41^{c}	29.9 ± 1.07^{bc}
50	6.43 ± 0.8^{b}	20.63 ± 0.1^{c}	31.3 ± 1.2^{bc}
100	7.36 ± 0.6^{a}	53.2 ± 4.3^{a}	54.7 ± 1.3^{a}

Precipitation studies were also carried out with varying $NaHCO_3$ concentrations (10 mM – 200 mM). Though not much has been reported on effect of $NaHCO_3$, but as the concentration of CO_3^{2-} ions increases, carbonate precipitation increases upto 75mM while above that, there was decline in the precipitation (Table 4.13, Fig. 4.13d). These results were in the similar lines of Li *et al* (2013) who reported that calcium carbonate precipitation is equally dependent on Ca as well as carbonates. With a fixed number of bacterial cells, maximum carbonate precipitation is achieved at an optimum concentration of Ca as well as carbonates. So, the optimal range of both these need to be standardized for a given experimental work.

Table 4.13: Effect of varying $NaHCO_3$ concentrations on calcium carbonate precipitation by bacterial strains A3-5 and A1-4. Means followed by the same letter within the column are not significant at P<0.05.

	mg $CaCO_3$/25ml		
$NaHCO_3$(mM)	**Control**	**A3-5**	**A1-4**
10	4.9 ± 0.2^{d}	44.2 ± 2.5^{b}	37.5 ± 1.8^{b}
25	9.76 ± 1.2^{c}	38.3 ± 2.15^{c}	34.23 ± 1.1^{b}
75	6.43 ± 0.4^{d}	50.03 ± 2.8^{a}	48.63 ± 1.5^{a}
150	14.3 ± 1.6^{b}	25.90 ± 1.30^{d}	34.83 ± 1.2^{b}
200	16.8 ± 2.1^{a}	37.3 ± 2.56^{c}	21.66 ± 2.50^{c}

From the above results, 100mM $CaCl_2$ + 75mM $NaHCO_3$ + 100 µM $ZnSO_4$ and pH 9-10 are the optimum conditions for carbonate precipitation via CA by halophilic bacterial isolates. Okwadha and Li (2010) found that the optimum conditions for MICCP via ureolytic pathway were 250 mM Ca^{2+}, 666 mM Urea and bacterial cell concentration of 2.3 x 10^8.

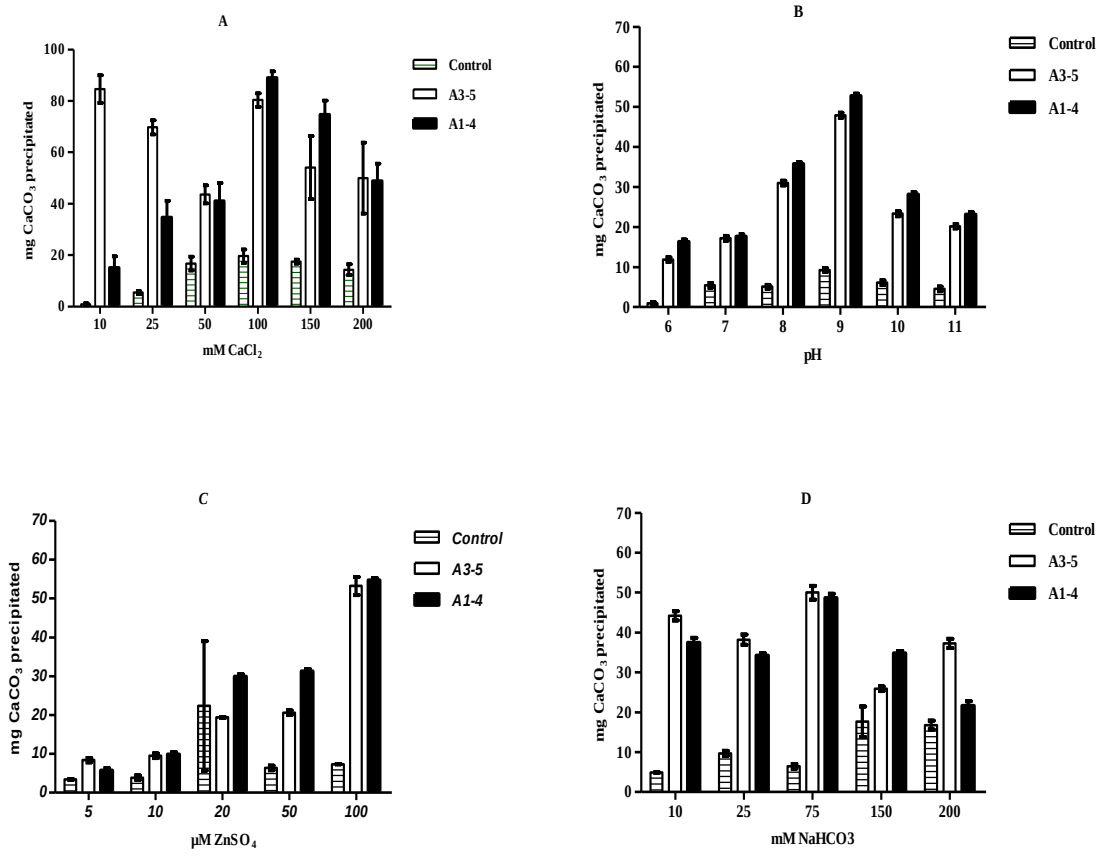


Fig. 4.13a. Effect of varying $CaCl_2$ concentration on calcium carbonate precipitation by bacterial isolates A3-5 and A1-4 in comparison to control

b. Effect of varying pH on calcium carbonate precipitation by bacterial isolates A3-5 and A1-4 in comparison to control

c. Effect of varying $ZnSO_4$ concentration on calcium carbonate precipitation by bacterial isolates A3-5 and A1-4 in comparison to control

d. Effect of varying $NaHCO_3$ concentration on calcium carbonate precipitation by bacterial isolates A3-5 and A1-4 in comparison to control

Effect of Varying Calcium Sources on Calcium Carbonate Precipitation

Different $CaCO_3$ are being widely used for various technical applications. They are now a days used in industries such as paper, pigment, paint, plastic, medicine, biomimatic CO_2 sequestration and bioremediation (Xue *et al.*, 2003; Wang 2008; Warren 2001). Besides its scientific interest $CaCO_3$ polymorphism selection has many important technical implications (Meldrum and Colfen, 2008). Bacterial conservation of building materials require formation of coherent, durable, $CaCO_3$ cement, i.e., calcite. This is not fully achievable if metastable Vaterite is formed. In the present studies, methyl blue staining of all the crystals showed the formation of rhombohedral crystals by both the isolates which are depicted to be calcite. Formation of these calcite crystals pave way for application of these bacterial for biocalcification purposes.

In case of crystals formed at varying pH, it was seen that as the pH increase, the size of the crystals decreased. At lower pH of 6 and 7 both the isolates formed very big sized crystals while at higher pH, tiny crystals were formed (Fig 4.14 and 4.15). These results were in agreement with Hammes (2004) who reported that as the pH increases, size of crystals decreases after 8.

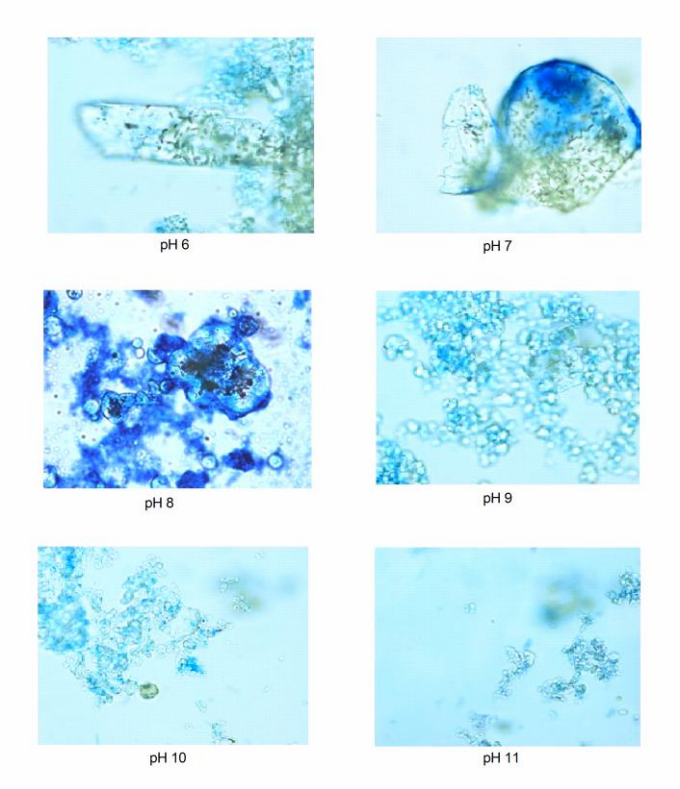


Fig. 4.14: Effect of varying pH on $CaCO_3$ crystal morphology by bacterial isolate A1-4

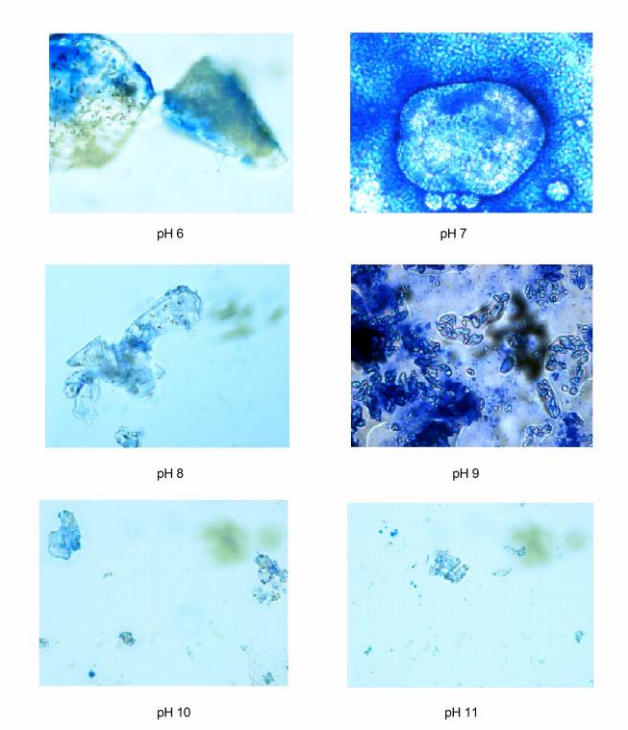


Fig. 4.15: Effect of varying pH on $CaCO_3$ crystal morphology by bacterial isolate A3-5

In case of varying Ca sources also, varying size of $CaCO_3$ polymorphs were formed from both the isolates. Calcium carbonate precipitation and Crystal morphology for different sources of Calcium chloride like Calcium acetate; Calcium chloride; Calcium nitrate and Tricalcium phosphate were assayed. These results also suggested that source of Calcium also plays a significant role in determining the carbonate polymorph formed by a bacterial isolate. Table 4.14 comprises of yield of calcium carbonate precipitates. Maximum yield of crystals was obtained with calcium chloride and calcium nitrate (Fig. 4.16). These results also signified that source of calcium also determines the yield of calcium carbonates in MICCP.

Table 4.14: Effect of Varying Calcium sources on Carbonate Precipitation by bacterial isolates A3-5 and A1-4 . Means followed by the same letter within the column are not significant at $P<0.05$.

	Control (mg $CaCO_3$/25ml)	**A3-5 (mg $CaCO_3$ /25ml)**	**A1-4 (mg $CaCO_3$ /25ml)**
Calcium Acetate	2.8 ± 0.26^{b}	4.7 ± 0.89^{b}	5.9 ± 0.72^{b}
Calcium chloride	2.9 ± 0.8^{b}	6.4 ± 0.35^{a}	6.8 ± 0.65^{a}
Calcium nitrate	4.8 ± 0.85^{a}	6.7 ± 0.49^{a}	7.3 ± 0.55^{a}
TCP	4.73 ± 0.56^{a}	6.9 ± 0.9^{a}	5.8 ± 0.60^{b}

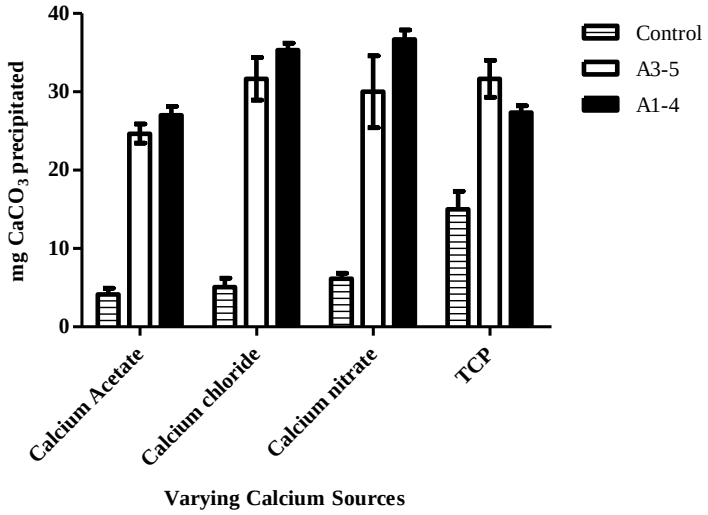


Fig 4.16. Effect of varying Calcium Sources on Calcium carbonate precipitation of bacterial isolates A3-5 and A1-4

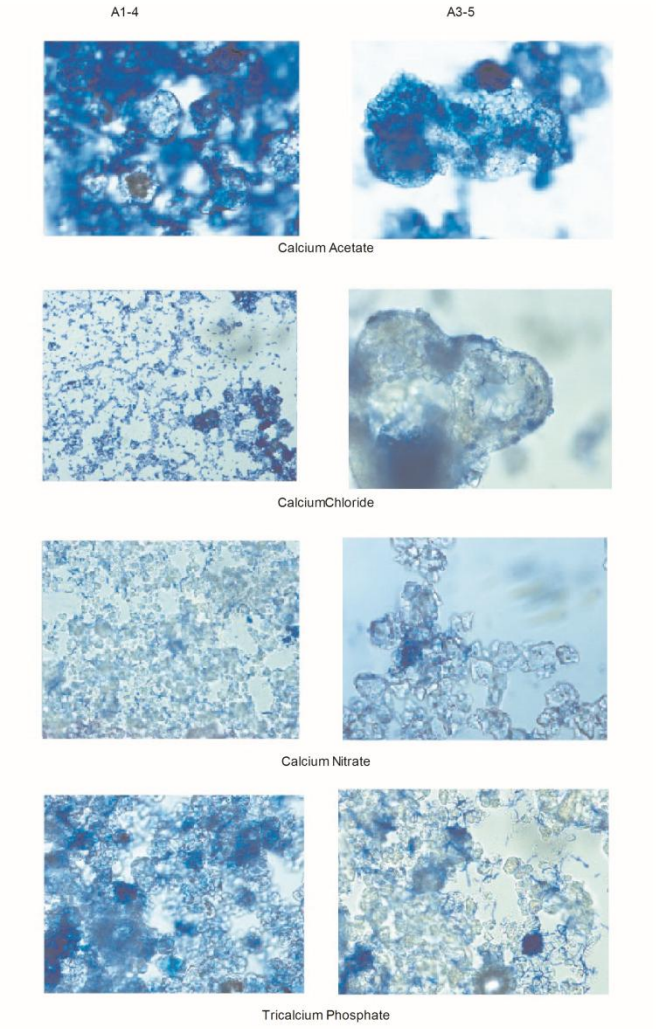


Fig. 4.17: Effect of varying calcium sources on $CaCO_3$ crystal morphology in bacterial isolates A1-4 and A3-5

Chapter 5: Conclusion

CHAPTER 5

CONCLUSION

In the present study, successful evaluation of halophilic bacterial CA was performed with respect to its characterization and role in MICCP. Two halophilic strains A3-5 and A1-4 were selected and studied. The CA activity of both the isolates showed efficient production by both the halophiles. The CA production was maximum on 3rd day in both the isolates which also coincided with the urease activity. This showed that some synergism might exist in both the enzymes in calcium carbonate precipitation. Crude enzyme preparation of extracellular CA showed activity over a wide range of pH (6-10) and temperature (35°C - 55°C), with maximum activity at pH 9 and 50°C temperature. In case of varying Carbon and Nitrogen sources, A3-5 showed maximum activity of 202.6 U/ml with fructose and yeast extract while in case of A1-4, maximum activity of 249.5 U/ml was achieved with glucose and peptone respectively. Kinetic studies revealed that the K_m and V_{max} for crude enzyme is 0.31 mM and 1.88 µmoles/min for A3-5 while 0.67 mM and 1.49 for A1-4. The lyophilized enzyme showed an increased Km value of 0.20 mM. and V_{max} value of 1.14 µmoles/min in case of A3-5 while 0.37mM and 1.69 µmoles/min for A1-4. The zymography study also confirmed the presence of CA in the gel by both the halophiles.

In study of MICCP via CA producing bacterial isolates, it was found that efficient carbonate production occurred by both the isolates in comparison to the control sets which proved the potential of bacterial CA in calcium carbonate precipitation. Various parameters were investigated for finding the optimum conditions for carbonate precipitation and it was found that CA producing halophiles produced maximum carbonates with 100mM $CaCl_2$ + 75mM $NaHCO_3$ + 100 µM $ZnSO_4$ at pH 9. This study has confirmed the role of CA in MICCP and also standardized the conditions to achieve highest $CaCO_3$ precipitation via MICCP by the CA producing halophilic bacterial isolates.

Chapter 6:
Annexure

CHAPTER 6

ANNEXURE

BUFFER COMPOSITIONS

PHOSPHATE–CITRATE BUFFER; PH 2.2–8.0, PK_A = 7.20/6.40

Add the following to create 100 ml of phosphate/citrate buffer solution. Stock solutions are 0.2 M dibasic sodium phosphate; 0.1 M citric acid (Pearse, 1980).

0.2 M Na_2HPO_4 (ml)	**0.1 M citrate (ml)**	**pH**
5.4	44.6	**2.6**
7.8	42.2	**2.8**
10.2	39.8	**3.0**
12.3	37.7	**3.2**
14.1	35.9	**3.4**
16.1	33.9	**3.6**
17.7	32.3	**3.8**
19.3	30.7	**4.0**
20.6	29.4	**4.2**
22.2	27.8	**4.4**
23.3	26.7	**4.6**
24.8	25.2	**4.8**
25.7	24.3	**5.0**
26.7	23.3	**5.2**
27.8	22.2	**5.4**
29.0	21.0	**5.6**
30.3	19.7	**5.8**
32.1	17.9	**6.0**
33.1	16.9	**6.2**
34.6	15.4	**6.4**
36.4	13.6	**6.6**
40.9	9.1	**6.8**
43.6	6.5	**7.0**

Preparation of Phosphate Buffer (pH 5.8–8.0 at 25°C)

To prepare 100ml of a 0.1M phosphate buffer, mix sodium phosphate, dibasic dihydrate and sodium phosphate monobasic monohydrate, as given below, and dilute to 100ml with water.

Solution A: 0.2M sodium phosphate, dibasic dehydrate ($Na_2HPO_4 \cdot 2H_2O$)

Solution B: 0.2M sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)

Solution A (ml)	Solution B (ml)	pH 25 °C
4.0	46.0	**5.8**
6.15	43.85	**6.0**
9.25	40.75	**6.2**
13.25	36.75	**6.4**
18.75	31.25	**6.6**
24.5	25.5	**6.8**
30.5	19.5	**7.0**
36	14	**7.2**
40.5	9.5	**7.4**
43.5	6.5	**7.6**
45.75	4.25	**7.8**
47.35	2.65	**8.0**

GLYCINE– NAOH BUFFER; PH 8.6–10.6, $PK_A = 9.78$

To prepare 100ml of a 0.2M glycine -NaOH buffer, mix 0.2 M glycine and 0.2 NaOH as given below, and dilute to 100ml with water.

Combine 25 ml glycine stock solution with *x* ml 0.2 M NaOH and dilute with DI to make a 100 ml solution (Pearse, 1980).

0.2 M NaOH	**pH**
2.0	**8.6**
3.0	**8.8**
4.4	**9.0**
6.0	**9.2**
8.4	**9.4**
11.2	**9.6**
13.6	**9.8**
19.3	**10.4**
22.75	**10.6**

STANDARD GRAPHS

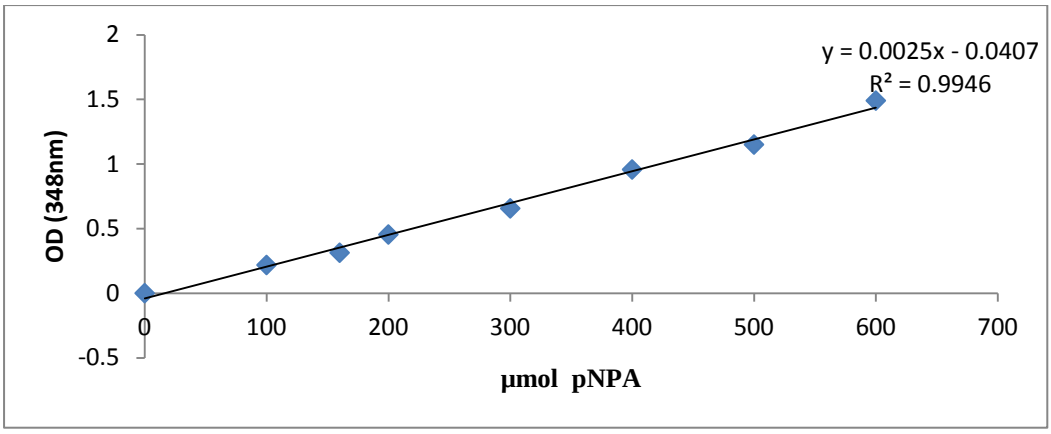


Fig 6.1 represents the standard graph of p-NPA for Carbonic anhydrase estimation

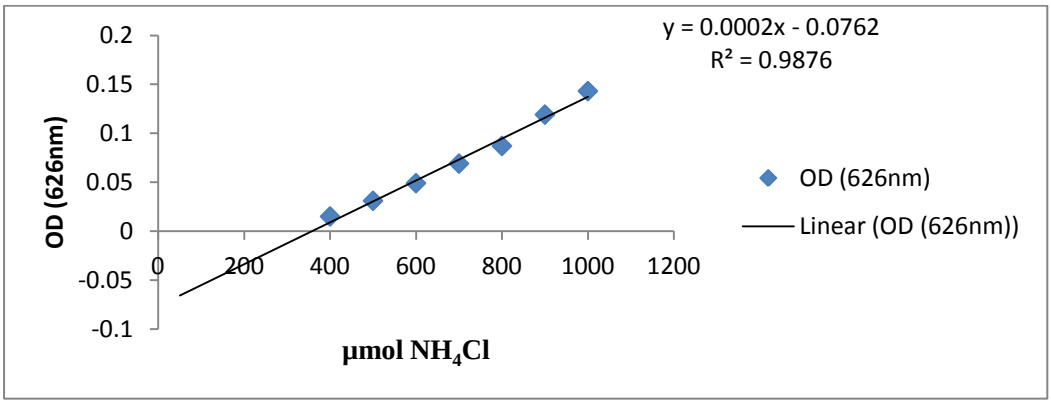


Fig 6.2 represents the standard graph of Ammonim chloride for Urease estimation

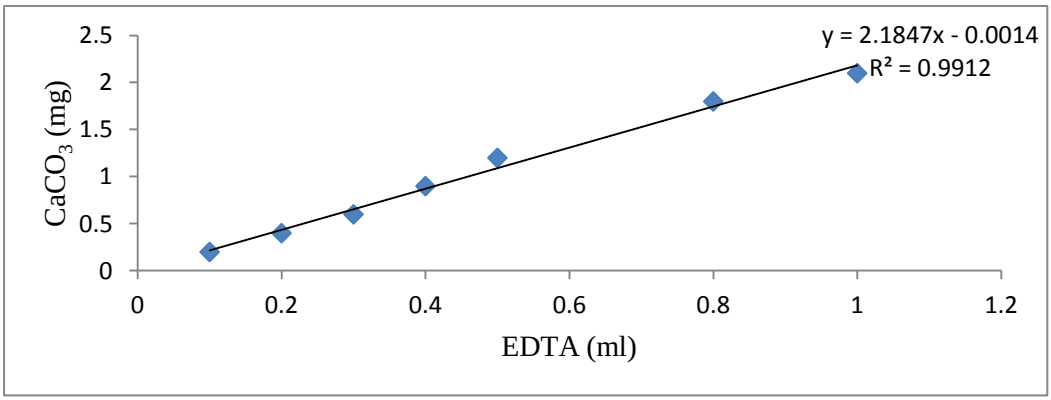


Fig 6.3 represents the standard graph of Calcium chloride for Urease estimation

Chapter 7: References

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