STABILITY AND ACTIVITY OF ENZYMES FROM HALOPHILIC AND HALOALKALIPHILIC BACTERIA IN THE PRESENCE OF ORGANIC SOLVENTS

A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

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REGISTRATION NO: 3300 DATE: SEPTEMBER 12, 2005

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SEPTEMBER, 2011

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I take pleasure in forwarding the thesis entitled "**STABILITY AND ACTIVITY OF ENZYMES FROM HALOPHILIC AND HALOALKALIPHILIC BACTERIA IN THE PRESENCE OF ORGANIC SOLVENTS"** of **Mr. Sandeep Pandey** for the acceptance of the degree of Doctor of Philosophy in **Microbiology**. Thesis presented here embodies records of original results and investigations carried out by him.

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ACKNOWLEDGEMENT

I always knew that one day, I would take this road but till yesterday; I did not know today would be the day! "Thank God". One of the few pleasures that come from writing a treatise of this kind is that it gives me a chance to state publically how much I have been helped by others, and gladly seize the opportunity to acknowledge my numerous indebtednesses.

I would like to express my sincere gratitude to my supervisor Prof. Satya P. Singh, Head, Department of Biosciences , Saurashtra University, for his continuous support to my PhD study and research, for his patience, motivation, ideas, immense knowledge and valuable suggestions. I consider myself highly privileged to be trained in his guidance. His ideals and concepts have had a remarkable influence on my entire career.

I wish to express my deep sense of gratitude to Prof. V. C. Soni, Prof. A. N. Pandey, Prof. V. S. Thaker, Dr. R. Kundu, Dr. S.V. Chanda, Dr. B.R.M. Vyas, Dr. N. Panchal, Ms. V. M. Trivedi and Ms. J. Patel for their valuable instruction and suggestion pertaining to my work.

I thank all the non teaching staff members of our Department, Dr. S. Bhatt, Mr. R. Purohit, Mr. Bhatt, Mr. Joshi, Mr. R. Solanki and others for the support they provided during my work.

I express my sincere gratitude to my colleagues Dr.Mital Dodia, Dr. Rupal Joshi, Dr. Jignasha Thumar, Mr. Chirantan Raval, Mr. Himanshu Bhimani, Ms. Megha Purohit, Ms. Sangeeta Gohel, Ms. Viral Akabari, Mr. Vikram Rawal, Mr. Bhavtosh Kikani, Mr. Rushit Shukla and Mr. Amit Shrama for their humble support.

I will be failing in my duty if I do not express my deepest gratitude to those microbes, who sacrificed their life during my experiment.

I am highly grateful to my family for their unflagging love and support throughout my life; they have provided the best possible environment for me to grow up physically and mentally. I am indebted to my father, Shree Prem Sagar Pandey and mother Smt. Aruna Pandey for their care, love and silent support. I cannot ask for more from my parents as they are simply perfect.

I always owe my sister, Nootan for her motivation and care, and remember her endless confidence on me whenever I encountered difficulties.

I express my innermost feeling of gratitude towards my Wife Mrs. Ira Tripathi whose support, love and persistent confidence in me has taken the load of my shoulder.

I am very much thankful to my Friends for their constant help and encouragement during the various stages of this period. A special mention is to Arif, Ashwini and Vishal for making everything worthwhile. Time spent with all of them is really unforgettable.

I feel paucity of words when it comes to express my gratitude for all the respondents who formed the basis of the present study. In last but not least I express my deep sense of gratitude to almighty GOD, who selected me for this short of work.

- *Sandeep Pandey*

CONTENTS

Chapter 1

General Introduction

General Introduction

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Microbes are truly omni-present organisms and contribute more than half of the living biomass on the planet. Among various microbes, extremophiles can grow and thrive in extreme environments, which were formerly considered too hostile to support life. The extreme conditions may be high or low temperature, high or low pH, high salinity, high metal concentrations, very low nutrient content, very low water activity, high radiation, high pressure and low oxygen tension. Some extremophiles are subjected to multiple stress conditions. Extremophiles are structurally adapted at the molecular level to withstand these harsh conditions.

In view of the imminent role in human society, extremophiles have been the center of attraction for researchers and during the last several decades, turning points and milestones were established. Early environmental conditions were extreme compared to today's, but might still be common beyond our planet. What we previously thought of as in surmountable physical and chemical barriers to life, are now seen as yet another niche harboring "Tiny creature" known as "Extremophiles". The term extremophiles was first used by Mac Elroy in 1974.

Extreme conditions can relate to physical (temperature, pressure or radiation), nutritional (low and high concentrations of nutrients) and geochemical extremes (such as salinity and pH). Most of the extremophiles identified to date belong to the domain Archaea (Woese *et al.,* 1990; Rothschild and Mancielli, 2001, Orange *et al.,* 2011). However, many extremophiles from the eubacterial and eukaryotic kingdoms have also been identified and characterized during the recent years (Anton *et al.,* 2000; Vreeland *et al.,* 2000; Sanchez-porro *et al.,* 2003; Pikuta *et al.,* 2003).

Haloalkaliphiles are salt and alkaline pH loving organisms that inhabit hyper saline and alkaline environments. They can be found at saturated brine concentration. Some of the natural and man-made habitats are highly saline and alkaline in nature. Therefore,

microbes dwelling in such environments are adapted to both high alkalinity and high salinity. Among the adaptation strategies production of large amounts of an internal solute or retention of a solute extracted from outside are key points. For instance, *Halobacterium salinarum*, an archaea, concentrates potassium chloride in its interior. As might be expected, the enzymes in its cytoplasm will function only if a high concentration of potassium chloride is present. But proteins in *H. salinarum* cell structures that are in contact with the environment require a high concentration of sodium chloride.

Many prokaryotic and eukaryotic microorganisms balance the osmotic pressure of the environment and resist the denaturing effects of salts and alkaline pH. Among halophilic microorganisms, there are variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes. Among the well-adapted and widely distributed extremely halophilic microorganisms; Archaeal *Halobacterium* species, Cyanobacteria, such as *Aphanothece halophytica* and the green alga *Dunaliella salina* are prominent ones.

The biocatalysts, called extremozymes, produced by these microorganisms, are proteins that function under extreme conditions. Due to their extreme stability, extremozymes offer new opportunities for biocatalysis and biotransformation. Examples of extremozymes include cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases,esterases, catalases, peroxidases and phytases, which have great potential for application various biotechnological processes. Currently, only 1–2 % of the microorganisms have been commercially exploited and amongst them there are only a few extremophiles. However, the renewed interest that is currently emerging as a result of new developments in the cultivation and production of extremophiles and success in the cloning and expression of their genes in mesophilic hosts will increase the biocatalytic applications of extremozymes.

Of particular interest are the enzymes that help extremophiles to function in brutal circumstances and therefore, they have reserved their significant position in market. The

primary reason of selecting enzymes from extremophiles is their high stability and activity under extreme conditions. The enzymes from these extremophiles are usually referred as "Extremozymes". They have potential to eliminate the need for added steps, thereby increasing efficiency and reducing the costs. They can also form the basis of entirely new enzyme-based processes. This would be possible due to their ability to maintain catalytic power at un-conventional conditions.

Recent studies have revealed that non-archaeal bacteria can also be extremely halophilic in nature, being able to grow at salinity 30-37 % (Anton *et al.,* 2000). One of such bacteria belonging to *Flavobacterium- Bacteroides* phylum could grow at saturated NaCl, a finding that is against the accepted norm that extreme halophiles belong to Archaea. Halophilic archaea isolated from Soda Lake in Tibet were reported having gram negative, pleomorphic, flat and non- motile features with strict oxygen requirement (Xu *et al.,* 1999). Growth required at least 12 % NaCl between pH 8- 11 with an optimum pH at 9-9.5. On the basis of r-RNA phylogenetic tree and DNA-DNA hybridization these isolates were grouped as *Natrorubrum tibetenes* sp. Polar lipid composition in addition to 16S rRNA analysis have proved particularly useful in the classification of the Halobacteria (Ross and Grant 1985; Xu *et al.,* 1999).

An extreme haloalkalophilic bacterium HAM-2 with pleomorphic rods was isolated from the soda lakes of inner Magnolia (Tian *et al.,* 1997). HAM-2 could grow at salt levels 12-30 % having pH range 7.8-10.4, optimum 9-9.5. Similarly, an extremely haloalkalophilic, chemoorganotrophic, homoacetogeneic bacterium was isolated from the bottom mud of the soda lake in Magadi, Kenya. The bacteria was obligate anaerobe, motile, Gram positive spore forming rod able to grow at pH range 8.1-10.7 and optimally in the range of 9.7-10. The optimum salt concentration was 12-15 %.

Usually enzymes do not function in organic solvents. Kim(Kim *et al.,* 1997), has isolated enzymes from halophilic and mesophilic bacteria and converted them into extremozymes. These extremozymes function in a variety of organic solvents and are thermostable. Enzymes isolated from halophiles and mesophiles were freeze-dried in the presence of

high salt concentration and then the powdered form of freeze-dried enzymes was added into organic solvents which showed considerable activity. Salt dehydrates the enzyme and sticks to it, shielding it from water. A sufficient amount of water is present to retain suitable charge distribution at the active site and the conformation of the enzyme.

In organic solvents the thermostability was also increased due to the rigid nature of enzymes. Some industrially important enzymes, such as lipases, proteases, lysozymes and ribonuclease have also been made to function in a variety of organic solvents.

In recent years a new class of solvent tolerant microbes having unique ability to sustain under non-aqueous system has drawn considerable attention. Such organisms are attractive for applications in solvent bioremediation and biotransformation in non-aqueous media (Sardessai and Bhosle, 2004; Gupta *et al*., 2005, Rahaman *et al.,* 2006 and Thumar *et al.,* 2009). Some of these microbes are rich source of the solvent stable enzymes. Such biocatalysts are increasingly being used to assist in synthetic routes to complex molecules of industrial interest. Particular interest in the use of biocatalysis to create new routes to lower value chemicals, the biggest role for biocatalysis still remain in the pharmaceutical sector , where its exquisite regioselective and stereoselective properties enable difficult syntheses (often requiring multiple protection and deprotection). Only limited reports are available in literature on the screening of microorganisms, which produce organic solvent-stable proteases. With particular reference to halophiles and haloalkaliphilic bacteria, such studies are further restricted.

Toxicity is considerably changeable among organic solvents. Solvent toxicity correlates inversely with its log Pow, the logarithm of its partitioning coefficient between defined octanol-water mixture (log Pow) (Sikkema *et al.,* 2002).

Organic solvents with lower log Pow values are more toxic than those with higher log Pow values. The organic solvent with the lowest logPow in which target microorganisms can grow is called the index solvent, and the logPow value of the index solvent is called the index value.

Solvents with a log Pow below 4.0, e.g., benzene (log Pow 2.13), toluene (log Pow 2.69), octanol (log Pow 2.92), xylenes (log Pow 3.12–3.2), and styrene (log Pow 2.95), are extremely toxic for microorganisms because they accumulate in the cytoplasmic membrane of bacteria and disrupt the cell membrane structure. Solvent toxicity depends not only on the inherent toxicity of the compound but also on the intrinsic tolerance of the bacterial species and strains.

Proteases being one of the most explored enzymes have attracted considerable attention over the last few decades. They are the most variable group with respect to origin, mechanism of action and specificity. Haloalkaline proteases, which catalyze protein hydrolysis under alkaline conditions in the presence of salt, have recently attracted attention of scientists from academic institutions and industries. This is because of their vital role in leather, food and detergent industries. In India, however, the use of industrial enzymes in general and extremozymes in particular is still quite limited. Great emphasis has to be put on efficient production of the enzymes at large scale and vast market potential has to be realized.

Biocatalysis in non-conventional (non-aqueous) media has potential to expand the spectrum of applications of proteases to the reactions that cannot precede effectively in aqueous environments, for instance, the synthesis of peptide bonds instead of their hydrolysis (Illanes and Barberis, 1994; Quiroga *et al.,* 2000a). The reaction media include organic solvents (Clark *et al.,* 2004; Gupta and Roy, 2004), supercritical fluids (Kamat *et al.,* 1992; Mesiano *et al.,* 1999), eutectic mixtures (Gill and Vulfson, 1994), solid-state (Halling *et al.,* 1995; Erbeldinger *et al.,* 1998) and ionic liquids (Park and Kazlauskas, 2003; Van *et al.,* 2003; Lou *et al.,* 2004; Machado and Saraiva, 2005).

These reactions offer other potential advantages as well, such as, the possibility of using poorly water soluble substrates; the modification of the equilibrium of reaction as a consequence of the alteration of the partition coefficients of substrates and products in the case of biphasic systems, the reduction of inhibitory effects by substrates and products, the easiness of biocatalyst, product recovery, the increase in the thermostability of the

biocatalyst and, in some cases, the variation in substrate specificity and the increase in the stereo and enantiospecificity in the resolution of racemic mixtures (Kawashiro *et al.*,1997; Klibanov 2001). As compared to chemical synthesis, a most important advantage of biocatalysis is the specificity of the reaction, which reduces the requirement of side-chain protection.

Within the last decade there has been dramatic increase in the need for bioactive compounds with novel activities. Advances in microbiological techniques and enzyme technology in the 1960's and 1970's lead to the development of several industrial enzyme applications. Production of therapeutic proteins made by the discovery of new technologies has in 2001, generated sales exceeding 25 billion dollars. This combined with other commercial applications of the enzymes such as the synthesis of various fine chemicals have necessitated the need for wider sources of the biocatalysts (Gupta and Roy, 2002). The enzymes from haloalkaliphilic bacteria and archaea have many specific functions coming into the realization (Margesin and Schinner, 2001, Diego *et al.*, 2007, Dodia *et al.*, 2008).

In view of the increasing emphasis on the solvent tolerant microbes and possible biotechnological potential of Haloalkaliphilic bacteria for non aqueous enzymology, the present study aims at the following objectives:

- Diversity of organic solvent tolerant halophilic / haloalkaliphilic bacteria and assessment of the effect of various solvents on the growth of these bacteria
- Studies on the production of extracellular enzymes (protease and amylases) as a function of their tolerance against organic solvents
- Effect of organic solvents on the enzyme activity and stability with crude, partially purified and purified enzymes

The chapters in this thesis have been organized in the following manner:

Chapter 1: **General Introduction**

Chapter 2: Review of Literature

Chapter 3: Diversity of organic solvent tolerant haloalkaliphilic bacteria

Chapter 4: Optimization of Amylases catalysis in the presence of organic solvent from haloalkaliphilic bacteria

Chapter 5: Optimization of Alkaline Protease catalysis in the presence of organic solvents from haloalkaliphilic bacteria

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Chapter 2

Review Of Literature

REVIEW OF LITERATURE

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Organic solvents are among the prominent toxicants for microbial flora. Only a low concentration can disrupt the structural and functional viability of cell (Inoue *et al*., 1989 and Sikkema *et al*., 1994). However, some microbes nullify the toxic effect of solvents. The toxicity of solvent for microorganism is equally distributed on natural toxicity of particular solvent as well as on innate tolerance level of the species and strain of microbes.

For long, it was assumed that microorganisms can not survive with organic solvent (Aono etal., 1991 and Inoue *et al*., 1989). In 1989, Inoue published first paper about a solvent tolerant bacterium which could actively grow and multiply in the presence of 50% (v/v) toluene (Inoue *et al*., 1989). Later, some other findings were reported, explaining and validating solvent tolerant microorganism (Cruden *et al*., 1992, Kim. *et al*., 1998, Zhang *et al*., 1998, Ramos *et al*., 1995) and efforts were focused on the mechanism of this interesting phenomenon.

Pseudumonas strains especially *P. putida* was extensively reported as solvent tolerant bacterium. Earlier, it was supposed that gram negative bacteria are more tolerant to organic solvents as compared to gram positive bacteria. This is arguably due to the fact that the Gram negative bacterial cytoplasmic membranes are made up of phospholipid and lipopolysacharide (Inoue *et al*., 1991 and paje *et al*., 1997). In later years some organic solvent tolerant gram positive strains, such as *Bacillus*, *Rhodococcus* and *Archi*

bacteria were reported (Abe *et al*., 1995, Kato *et al*., 1996, Moriya *et al*., 1995 and Page *et al*., 1997)

2.1 Physiological basis of solvent toxicity and concept of organic solvent tolerance

Cell membrane is foremost site for solvent interaction. Cytoplasmic membrane is made up with bi-layer of phospholipids which consist of various enzymes and embedded transporter proteins. It crucially participate in transportation of solute, regulation of intracellular metabolism, protecting energy of cell, signal transduction, energy transducing process and turgor pressure. Solvents create segment in cell membrane and disturb the lipid bilayer and affect the cell viability (Inoue *et al*., 1989, Sikkema *et al*., 1995 and Sikkema *et al*., 1994). It is not the chemical structure of the solvent, but its concentration accumulated in the cell membrane that plays a crucial role in causing the toxicity (Bont *et al*., 1998 and Isken *et al*., 1998).

Physiological investigation of microbes has revealed a correlation between solvent toxicity and its logPow value. The parameter log P is defined as the partition coefficient of the given solvent in an equimolar mixture of octanol and water (Inoue *et al*., 1989). Greater the polarity, lower the log P value and greater the toxicity of the solvent is observed. Generally, solvents with log P values below 4 are considered extremely toxic as their degree of partitioning in aqueous layer (which contains cell) and from there into the lipid membrane bilayer is high. The greater the degree of accumulation of the solvent in the membrane, the higher its toxicity (Bont *et al*.,1998 and Isken *et al*., 1998).

Each organism has its own intrinsic tolerance level for organic solvent, which is determined genetically and is also influenced by environmental factors (Koyabashi *et al*., 1998). Organic solvent tolerance is believed as strain specific (Huertas *et al*., 1998). The tolerance of microorganism is represented by two terms, the index solvent and index value. The index value is the log P value of the most toxic organic solvent (index solvent) among those that can be tolerated by the organism. Each bacterium can grow on agar media overlaid with any one of the organic solvents having a logP value greater than indx value. However, under such condition, the growth of bacteria is suppressed by organic solvent having log P value near the index value (Aono *et al*., 1991).

2.2 Microbial adoption to tolerate organic solvent

Specific permeabilization of the cell membrane get affected due to solvent accumulation, causing leakage of ATP, potassium and other ions , RNA, phospholipids and protein (Heipieper *et al*., 1991; Ramos *et al*., 1997; Woldringh *et al*.,1973). Additionally organic solvent interrupt the fluidity of the membrane (Sikkema *et al*., 1994). In response to the toxicity of the solvent, the tolerant bacteria adapt some alteration, as reflected by several studies (Heipieper *et al*., 2007; Isken and de Bont, 1998; Mohammad *et al*., 2006; Ramos *et al*., 2002 Weber and de Bont, 1996). The emerged adaptive features are described as below:

2.2.1 Strengthening of the cell membrane

During long time exposure, there appears to be a shift in the ratio of saturated to unsaturated fatty acid in cell membrane (Mohammad *et al*., 2006; Pinkart *et al*., 1996;

Weber *et al*., 1994). Isomerization of the naturally formulated cis-isomer of an unsaturated fatty acid was changed to trans-isomer by an energy-independent periplasmic isomerase (Heiper *et al*., 1995; Mohammad *et al* 2006., Nielsen *et al*., 2005; Weber *et al*., 1994). In context of tolerance, some bacteria exhibit changes in fatty acid compositions along with the alteration of phospholipids (Nielsen *et al*., 1994). While in some bacteria, change in composition of lipoploysachirdes (LPS), lipid protein ratios and outer membrane protein are also associated with the solvent tolerance (Pinkart *et al*., 1996; Ramos *et al*., 1997). These adoptions change the fluidity of the membrane and thus suppress the effects of the solvent.

2.2.2 Degradation and biotransformation of organic solvents

In some bacteria, such as *E. coli* and *Rhodococcus* sp. (Ferrante *et al*., 1995; Paje *et al*., 1997), metabolism of organic solvent converts toxic hydrocarbon into simpler non-toxic compounds (Ferrante *et al*., 1995; Paje *et al*., 1997).

2.2.3 Solvent-efflux pumps

During the last decade, many bacteria were reported which utilized solvent efflux pumps to sustain with organic solvents and majority of them fall in RND (resistance/ nodulation/ cell division) family. Only a few efflux pumps for organic solvent, namely tolC, mar, rob SoxS and acrAB have been identified in *Pseudomonas* sp. (Kieboom *et al*., 1998; Li *et al*., 1998; Ramos *et al*., 1998) and *E. coli*. (Asako *et al*., 1997; Kobayashi *et al*., 2001).

2.2.4 Enlargement of cell size

P. *Putida* and *Enterobacter sp.* tolerated organic solvents by growing their cell size to reduce the relative surface for attaching organic solvent. It is clear that performance of solvent efflux pump will be better if over all membrane is reduced. This escort to a reduction in the area that allows diffusion and partitioning of solvents into the membrane where they are recognized and excluded by the efflux –pump proteins (Neumann *et al*., 2005)

2.3 Enzymes of solvent tolerant bacteria

Solvent-tolerant microbes have been less studied from the perspective of non-aqueous enzymology. Lately, enzymatic studies under non-aqueous conditions have emerged as interesting filed of research and efforts are focused on to optimize reaction conditions for synthetic applications of enzymes in the presence of solvents. As non-aqueous enzymology has generated possibilities to synthesize biologically active compounds, it is obvious that the enzymes display striking novel features and attain higher catalytic activity in organic solvents (Ogino and Ishikawa, 2001). Some of the industrially important enzymes such as lipase, protease and amylases have been studied from solvent tolerant microbes (Doukyu *et al*., 2003; 2007; Geok *et al*., 2003; Ghorbel *et al*., 2003; Gupta *et al*., 2005; kardzic *et al*., 2004; ogino and Ishikawa,2001, Thumsr *et al*., 2009). Some halophiles have also been studied to exhibit the properties of solvent-tolerant enzymes; such as amylase from an extremely halphilic archaea, *Haloarcula* sp. strain S-1 (Fukushima *et al*. 2005) and protease from moderately halophilic bacterium *Sainiovibrio* sp. Strain Af-2004 (Haidari *et al* 2007, Thumsr *et al*., 2009). These studies

have opened possibilities of new enzymatic potential with the enzymes having tolerance and ability to function under multitude of extreme conditions.

Enzymes of solvent tolerant microbes were explored specially for reverse reaction demand of biotechnological industries or in conditions of comparatively poor solubility of desired substrate in aqueous medium. Such enzymes are mainly classified as proteases (Gupta *et al*., 2005; Ogino *et al*., 1999 a, b), lipases (Ogino *et al*., 2000), cholesterol oxidase (Doukyu and Aono, 1998) and recently cholesterol esterase (Takeda *et al*., 2006). One of the common features of enzymes from solvent tolerant sources is better solubility in hydrophobic solvents especially alkenes. Generally they are monomeric proteins of molecular weight 20-80kDa with hydrophobic surface and number of disulfide bonds. In literature, ion exchange chromatography and hydrophobic interaction chromatography (HIC) are mainly applied for enzyme purification. Most of such enzymes are from *Pseudomonas* and few *Bacillus* sp. (Geok *et al*., 2003; Ghorbel *et al*., 2003; Gupta *et al*., 2005; Ogino *et al*., 1994). In most of the cases, there were similarity among the enzymes from different *Pseudomonas* and others. They are stable in alkanes and majority of them are stable in long chain aliphatic hydrocarbons, benzene, toluene and alcohols.

2.3.1 Proteases

Pharmaceutical industries significantly need variety of solvent stable proteases to satisfy emerging demand of various type of enzymaticaly synthesized peptides as protease-catalyzed synthesis of peptide has several advantages over chemical catalysis, e.g. region- and stereo-selectivity, absence of racemization, lack of requirement of side chain

production and milder non-hazardous reaction conditions (Gill *et al*., 1996, Jakubke *et al*., 1985; Kilbanov *et al*., 1986; Rahaman *et al*., 2007). Number of peptides like analgesic dipeptide Kyotorphin (tyr-arg) (Jonsson *et al*., 1996; Sareen *et al*., 2004) as well as aspartame (Eichhorn *et al*., 1997) has been produced in aqueous or non aqueous media using protease. Successful synthesis of peptide by Subtilisin, thermolysin and other proteolytic enzymes has been accomplished in organic solvents (Isowa and Ichikawa, 1979; Oka and Morihara, 1978, 1980; Pauchon *et al*., 1993). However, inactivation of enzyme in organic solvents is negative aspects of enzymatic peptide synthesis which leads the low rate of peptide synthesis (Ogino *et al*., 1999a; Vulfson *et al*., 2001). For example, ά-chemotrypsin could not catalyze peptide synthesis in the presences of 50% (v/v) ethanol, DMF, DMSO, acetone or Acitonitrile (Jakubke *et al*., 1985). Ogino and his group reported (Ogino *et al* 1995; 1999a) first solvent stable protease from solvent tolerant *Pseudomonas* stain; *P. areuginosa* PST-01. This bacterial strain was quiet stable in cyclohexane, toluene, ethanol and acetone. In water-miscible and immiscible organic solvents, it's protease had higher stability compared to commercially available known protease, thermolysin, subtilisin Carlsberg and ά-chymotrypsin (Ogino *et al*., 199a).The PST-01 protease was successfully employed for peptide synthesis in the presence of organic solvents, such as in the synthesis of dipeptides; Cbz-Arg-Leu_Nh2, equilibrium yields of more than 60% in the presence of 50%(v/v) DMF and 50mM sodium Phosphate buffer (pH 7.0). The equilibrium yield of Cbz-Arg-leu-NH2 synthesized from Cbz-Arg and Leu-NH_2 using the PST protease was similar to thermolysin and trypsin and considerably higher than that using papain, elastase, pepsin, ά-chymotrypsin or subtilisin (Ogino *et al*., 1999b).

Another attractive protease for peptide synthesis in aqueous acetonitrile media by a mutant strain (*B. licheniformis* RSP-09-37) was reported by Sareen et at., 2004. The performance of this protease for synthesis of a Kyotorphin precursor was quiet better compared to commercially available ά-chymotrypsin. As the *B. licheniformis* RSP-09-37 protease tolerated higher concentrations of acetonitrile , this resulted in higher conversion rates, less hydrolysis and increased stability, and thus makes this biocatalyst suitable for the synthesis of peptides.

Protease from PST-01 reported by Ogino (Ogino *et al*. (2001)) has two internal disulfide bond which enhances it's stability in the presence of organic solvents. On the basis of some current studies, it's clear that amino acid residues on the surface of enzyme play important role in stability of protein in organic solvents (Gupta *et al*., 2007; Ogino *et al*., 2007). Most of the reported solvent tolerant isolates were form soil samples, while few were from fishing industry wastewater (Ghorbel *et al*., 2003) and cutting oil used in industrial metal-working processes (Karadzic *et al*., 2004). Majority of the solvent stable proteases belonged to *Pseudomonas* Sp., and very few are from *Bacillus* species (Ghorbel *et al*., 2003; Sareen *et al* ., 2004).

Common trend for both, *Pseudomonas* and *Bacillus* proteases were recorded for the catalysis in solvents, especially alkanes (Gupta and khare,2006; Ogino *et al*.,1995; Rahaman *et al*.,2006; 2007). In some cases, activity in the presence of alcohol was also reported (Ghorbel *et al*., 2003; Karadzic *et al*., 2004; Sana*et al*.,2006). Anion exchange and /or hydrophobic –interaction choromatography were mainly used to purify these solvent tolerant protease.

2.3.2 Amylases

Starch is one of the most abundantly presented polymers in nature and amylases play central role in the utilization of this natural polymeric substrate. Amylases are produced broadly by all the three domains of life, Eucarya, prokaryotes, and Archaea (Upadek *et al*., 1997, kikani *et al*., 2011). Enzymes utilized for the hydrolysis of starch are greatly diverse and broadly useful for many industrial processes, such as starch liquefaction, pulp process and in detergent (Kadziola *et al*., 1998, Machius *et al*., 1995). With involvement in vast range of biotechnology based industry, amylases are now one of the most demanding enzymes. Food and starch-processing industries (Vihinen *et al*., 1994) require a huge quantity of amylases. However, amylases are comparatively less attended enzymes for the study of solvent tolerance. Solvent tolerant amylases can open the new avenues for bioremediation and transformation of solvent polluted starch waste of industry as well as improvement of detergent for acting at low water conditions. Need of starch hydrolysis under non-aqueous conditions is another reason to explore a solvent stable amylases.

A solvent tolerant amylase, having molecular mass of 70 kDa, from a halophilic archaeon, *Haloarcula* sp. strain S-1 was reported by Fukushima (Fukushima *et al*., 2005). This amylase was quiet active and stable in various organic solvents; benzene, toluene and choloroform, while the enzyme was not active at all in ethyl alcohol and acetone. Further, this enzyme maintained high activity with methanol at low ionic strength.

Morita reported (Morita *et al*., 1995) an alpha-amylase which greatly accelerated the conversion of soluble starch to malto-oligosaocharides with two phase system of water and dodecane. However, a rapid inactivation of the enzyme was observed in this system. Addition of surfactants, such as Tween 60 or bis (2-ethylhexyl) sodium sulfosuccinate (AOT), was effective for the enzyme stability. Effect of enzyme immobilization on the stability of α-amylase, using Ca-alginate and chitosan beads, was also reported. The stability of immobilized enzyme was clearly enhanced in a 5–10% (v/v) water content two-phase system, whereas the free enzyme was inactivated within 41 h (Morita *et al*., 1995).

An attractive amylase AmyA from the hyperthermophilic bacterium, *Thermotoga maritime* was able to hydrolyze internal 1,4-glycosidic bonds in various glucans at 85°C as the optimal temperature (Almazo *et al*., 2005). It was found that when methanol or butanol was used as the nucleophile instead of water, AmyA was able to catalyze alcoholysis reactions. This capability has been evaluated in the past for some amylases, with the finding that only the saccharifying fungal amylases from *Aspergillus niger* and from *Aspergillus oryzae* present measurable alcoholysis activity (Santamaria *et al*., 1999). Replacement of residue His222 by glutamine generated an increase in the alkyl glucoside yield as a consequence of a higher alcoholysis/hydrolysis ratio. The same change in specificity was observed for the mutants H222E and H222D, but instability of these mutants toward alcohols decreased the yield of alkyl glucoside.

2.3.3 Lipases

Lipases are among the most sought-after enzymes from the view point of solvent stability (Khare *et al*., 2000a; Sharma *et al*., 2001; Tasi *et al*., 2006). The reason being that lipases have industrial potential for exploitation of esterification and trans esterification reaction in non-aqueous medium and are being used for the production of various types of flavor esters, cocoa butter equivalent, the human milk fat substitute 'betapol' structured lipids and biodiesel (Bosley *et al*.,1997; Gaur *et al*.,2008; Jager and Reetz.,1998; Khare *et al*.,2000b). In addition, the substrates; fats and oil and products of lipase-catalyzed reaction are often insoluble in aqueous solutions, while the enzyme is insoluble in organic solvents, thus necessitating the presence of organic-aqueous two phase media. The solvent stable lipases have mainly been isolated from *Pseudomonads* and *Bacillus* spp. *Fusarium heterosporum*, a fungal strain, produced a solvent stable lipase (Shimada *et al*., 1993). The lipases have been purified by using combination of various chromatographic techniques. An organic solvent – stable lipase from *P. aeruginosa* LST-03 was purified

by ion exchange and hydrophobic interaction chromatography (Ogino *et al*., 2000). Lin *et al*., (1996) purified an alkaline lipase from *P. Pseudoalcaligenes* F-111 by acetone precipitation, gel filtration and hydrophobic interaction chromatography. The molecular weights are 30-36 kDa or 54-60kDa, indicating that there may be groups of solvent – stable lipase. Despite organic solvent stability, these lipases are also alkaline in nature except from *P. aeruginosa* LST-03 and *F. heterosporum* (Ogino *et al*.,2000; Shimada *et al*.,1993).

2.3.4 Esterase

Industrial applications of esterases (carboxylesterases) are mainly transesterification of industrially used reactions. It comprises the resolution of racemic mixture, enantio- and region-selective hydrolysis, synthesis of natural and non-natural drugs, detergents, polymers, and additives in organic solvent (Bornscheuer *et al.,* 2002; Vulfson *et al*., 2001). Mostly fungi, yeast and bacteria (Fojan *et al*., 2000; Gupta *et al.*, 2000; Perrone *et al*., 1999) are reported sources for microbial esterase to resolute racemic mixtures, enantio- and regioselective hydrolysis, and synthesis of drugs and polymers. However, poor reaction rates and low product yields are great limitation of microbial esterase (Claon and Akoh, 1994).

A thermo-resistant and alcohol tolerant bacterium, *Bacillus licheniformis* S-86, was reported (Torres *et a.,l* 2005), which produced a stable esterase in high organic solvent concentration. Solvent-tolerant *B. licheniformis* S-86 displayed an approximately two fold higher specific activity of esterase in culture supplemented with C3-C5 alkanols (2,3-butanediol,propan-2-ol,butan-1-ol,and 3-methylbutan-1-ol) than the control (without

alcohol). Crude extracts of *B. lichenformis* S-86 displayed high esterase activity in 50% hydroxylic-water-solvent mixtures, and an optimum enzyme activity between 65ºC and 70ºC. These properties make this enzyme quite attractive for the use in organic synthesis and industrial biocatalysis.

2.4 Application of solvent-tolerant microbes and their enzyme

Bioremediation/ biotransformation and valuable enzyme are beneficial out come of solvent tolerant microbes. The primary necessity for microbial transformation of hydrocarbons, soil remediation and waste-stream purification, is the sustainability and growth of microbes in toxic effluent (Kieboom *et al*., 1998). The presence of solvents in contaminated sites is indicative of the lack of natural system that can efficiently degrade these compounds.

Due to toxic effect of solvent, it is quiet difficult to apply biological system for solvent rich- pollute sites (Mohammad *et al*., 2006). Organic solvent tolerant bacteria can be quite useful for such processes. For biphasic systems, the enzymes can be a good candidate. Exploration of solvent tolerant bacteria for biotransformation in biphasic system has been recently reviewed by Heipieper *et al*., 2007. Several examples of whole cell bioconversions in organic media are presented by Leon *et al*.,1998 and Salter and Kell (1995).

The potent environmental pollutants are sulfur, nitrogen and oxygen hetrocycles. Recently, a review on microbial degradation of these toxic compounds by Xu *et al*., (2006) has been reported. *Pseudomonas* sp. strain St-200 and *Arthrobacter* sp. ST-1

showed high cholesterol degradation in the presence of mixed organic solvents (p-xylene and p-diphenyl methane3:7, V/V), and n-decane and n-dodecane, respectively (Aono *et al*., 1994; Moriya *et al*., 1995). Two *Bacillus* strains have been reported for the transformation of cholesterol to cholest-4-ene-3,6-dione in the presence of chloroform as the organic phase (Sardessai and Bhosle, 2003).

Bioremediation of crude-oil-polluted sea water by an immobilized bacterial strain have been reported by Gentili *et al*., (2006). This isolate immobilized on chitin and chitosan flakes, was able to remove 60% of the hydrocarbon, while in case of non-immobilized form 13% removal of hydrocarbon was recorded. A mixed consortium, isolated from oil-contaminated soil, was capable of degrading propanol and isopropanol (Bustard *et al*., 2000). In another report, Mohammad *et al*., (2006) have described the mineralization of a high concentration of isopropanol by a solvent- tolerant strain of *S. mizutae*. This isolate was able to utilize comparatively higher concentration of isopropanol as the sole carbon source, with mineralization occurring via an acetone intermediate, into the central metabolism (Mohammad *et al*., 2006).

Butanol and other volatile solvent are health hazard features and influence on photochemical smog. Therefore, it's highly desirable to efficiently transform it into non toxic form. Biodegradation of solution-phase 1-butanol by solvent-tolerant *Enterobacter* sp. VKGH12(NCIM 5221) was reported by Veerengouda *et al*., (2006). This isolate utilized 1-butanolas as a sole source of carbon. Paje *et al*., (1987) isolated a *Rhodococcus* sp. strain 33 from a contaminated site in Sydney, Australia. This strain tolerated and degraded high concentration of benzene, and therefore, could be useful in clean-up

operations. In another interesting case, a solvent-tolerant desulfurizing bacterium (*P. putida* A4), was constructed by introducing the biodesulfurizing gene cluster dszABCD, from *R. erythropolis* XP, into the solvent-tolerant strain *P.Putida*. The strain degraded dibenzothiophene in the presence of various organic solvents. This study was significant step in the exploration of the biotechonological potential of novel biocatalysts for developing an efficient biodedesulurization process in biphasic reaction mixtures containing toxic organic solvents (Tao *et al*., 2006).

2.5 Conclusions and future perspectives

Last decade has added impressive growth in enzymatic reaction under low water and solvent media. Many new reactions for synthetic purpose were discovered. Maintenance of enzymatic stability and efficacy in organic solvents is essential for such applications. New strategies and effort has been incorporated to obtain solvent tolerant enzyme. So in this perspective, solvent tolerant microbes seem to have ample scope with great probability of innate stability with solvent. For cost effective bioremediation and conversion of organic solvent, solvent tolerant bacteria will prove a prominent option.

The effective mineralization of high concentration of solvent by microbes raise the possibility to replace traditional physical and chemical techniques, which do not involve a pretreatment step for effluents so as to render them suitable for 'normal' biological condition. These possibilities represent a future avenue of research for both microbiologists and enzymologists.

Diversity of Organic solvent tolerant Haloalkaliphilic Bacteria

3.1 INTRODUCTION

On account of the industrial revolution, the production and use of chemicals have been constantly increasing. As a consequence, many kinds of products are synthesized and released into the environment. Certain products such as organic solvents or fuels reach the biosphere through losses during production, storage, accidents and solvent evaporation. There is now growing awareness concerning the possible toxic or even carcinogenic effects of these chemicals. Although the release of many of them is restricted by legislation, a number of pollutants have already reached the biosphere and thus need to be eliminated. The biological treatments to remove toxic chemicals seem quite promising (Ramos *et al.,* 1994).

Chemical toxicity adversely affects microorganisms in the removal of pollutants from waste streams and dump sites. Therefore, this is a serious problem with microbial bioremediation in reactors, biofilters, and soils (Heipieper et *al.,* 1994, Segura *et al., 2001,* Sikkema *et al., 1992*), particularly when organic solvents are in high concentrations. Solvent toxicity correlates with its log Pow, the logarithm of the partitioning coefficient of a solvent in a defined octanol-water mixture (log Pow) (Sikkema *et al., 1995*). Solvents with a log Pow below 4.0, e.g., benzene (log Pow 2.13), toluene (log Pow 2.69), octanol (log Pow 2.92), xylenes (log Pow 3.12–3.2), and styrene (log Pow 2.95), are extremely toxic for microorganisms because they accumulate in the cytoplasmic membrane of bacteria disrupting the membrane structure.

Organic solvents damage the cell membrane by impairing vital functions (loss of ions, metabolites, lipids, and proteins; dissipation of the pH gradient and electrical potential) or by inhibiting membrane protein functions. This damage is often followed by cell lysis and death (Desmet *et al.,*1978, Sikkema *et al., 1995*). Solvent toxicity depends not only on the inherent toxicity of the compound but also on the intrinsic tolerance of the bacterial species and strains. For example, certain strains of *Escherichia coli* are tolerant to cyclohexane (log Pow 3.44), while others are sensitive (Aono *et al.,* 1991). Most of

microorganisms are highly sensitive to aromatic solvents with a log Pow between 2.0 and 3.3 (Sikkema *et al., 1995*); nevertheless, there exist several *Pseudomonas* species that grow in high concentrations of toxic organic solvents, such as toluene, styrene, and p-xylene (Kim *et al.,* 1998, Ramos *et al.,* 1995 , Weber *et al.,*1994).

For the tolerance to aromatic hydrocarbons, a number of elements are involved in the response to these toxic chemicals: (a) metabolism of toxic hydrocarbons, which can contribute to their transformation into nontoxic compounds; (b) rigidification of the cell membrane via alteration of the phospholipids composition; (c) alterations in the cell surface that make the cells less permeable; (d) efflux of the toxic compound in an energy-dependent process; and(e) formation of vesicles that remove the solvent from the cell surface .

Only fractions of microbes have been explored as it is not practically possible to grow majority of them. This realization emphasizes on the investigation of microbes in general and extremophiles in particular. Haloalkaliphiles are an interesting domain of extremophilic organisms that have adapted to harsh, hyper saline and alkaline conditions, and are not attended for organic solvent tolerance. With some native feature; as active efflux pump to maintain osmotic pressure, quick cis to trans isomerization of unsaturated fatty acid of cell membrane and comparative more hydrophobic amino acid on surface of enzyme, haloalkaliphilic bacteria would be of particular interest as model system for the study on tolerance against organic solvents .

With innate adaptation and adaptive alteration for organic solvent tolerance of haloalkaliphiles, there are ample possibilities that they can be easy source for valuable solvent stable enzymes for biotechnological stand point. The use of enzymes in organic media (with low water content) has been one of the most exciting facets of enzymology in recent times. It is an area in which applications and phenomena preceded the understanding of catalysis at the molecular level.

In the light of above scenario, the study in this chapter has focused on the screening and diversity of organic solvent tolerant haloalkaliphilic bacteria isolated from Gujarat Coast in Western India. The emphasis has been on the effect of varying concentrations of range of organic solvents on growth and protease production profile of haloalkaliphilic bacteria. Many potent isolates were subjected for identification and phylogenetic analysis, based on 16S rRNA sequences. By Scanning Electron Microscopy (SEM) adaptive morphological alteration were also assessed.

3.2 MATERIAL AND METHOD

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3.2.1 Sample Collection, isolation and preservation of haloalkaliphilic bacteria

The haloalkaliphilic bacteria were isolated from salt enriched soil and sea water samples were collected from 7 different sites along the Coast of Gujarat (Somnath Diu, Okha, Mithapur, Jodiya and two sites of Veraval). The samples were collected in sterile plastic bottles and bags; the pH and temperature of all the samples were measured manually at the time of the sample collection, and processed within four days after the sample collection. The water samples were stored at 4°C for further work. The work on the isolation of these haloalkaliphilic strains were carried out by Dr. Mital Dodia and Dr. Rupal Joshi as part their doctoral research in the laboratory of Prof. S.P. Singh, Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India.

For the isolation, 2.0ml of the sea water sample was inoculated into the 100ml of the enrichment medium. The bacteria were isolated by using enrichment culture techniques in Complex Medium Broth (CMB) consisting, (g/liter): Glucose, 10; Peptone, 5; Yeast extract, 5; KH2PO4, 5; with varying concentration of NaCl (10-20%, w/v) at different pH 8-10. The pH of the medium was adjusted by adding separately autoclaved Na2CO3 (20%, w/v). After inoculation, flasks were incubated on environmental shaker at 37°C with regular monitoring on the turbidity of the enrichment media. After 48-72h of growth, a loop full culture was streaked on the CMB agar (3%, w/v) plate and incubated at 37°C. After 48h of the incubation, on the basis of colony characteristics, various isolated colonies were selected and pure cultures were obtained by subsequent streaking on the CMB agar plate

The pure cultures were preserved on the CMB agar media (10% w/v NaCl; and pH 8- 10) and stored at 4°C. After screening for the extra cellular enzymes, the protease producers were preserved on gelatin agar medium respectively. The cultures were subsequently transferred on fresh CMB agar at 3 months intervals.

3.2.2 Effect of organic solvents on growth and extra cellular protease production

The effect of solvents on growth and protease production haloalkaliphilic bacteria was assessed. The inoculum was prepared by adding a loop full of pure culture into 10 ml sterile CMB medium ((g/liter): Glucose, 10; Peptone, 5; Yeast extract, 5; KH_2PO4, 5; NaCl, 10 % w/v and pH9) and incubated at 37oC on a environmental shaker for 24 hour. Thereafter, 5 % inoculum from the culture (At A540; 1.0) was inoculated into 250 ml flask, containing 50 ml CMB medium.

Organic solvents; n-hexane, methanol, propanol and butanol at 1- 5 % (v/v) were added separately. Cultures were incubated under shaking condition (140 rpm) at 37ºC. Controls (without solvents) were also included for individual isolates. The culture aliqouts were withdrawn aseptically up to 96 hour at the interval of 24 hour and the growth was measured at A540. The cultures were centrifuged at 5,000 rpm for 10 min at 4^0C and the cell free extracts were used as crude preparation to measure protease activity.

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3.2.3 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopic (SEM) analysis was carried out isolate Kh-10-9_1 from Okha. SEM was performed for the cultures grown with organic solvent. Culture was centrifuged at 5000 RPM to get the pellet of the pure culture. The homogenous suspension of pure culture was prepared in HPLC grade water. The copper grid (10mm) was covered with the bacterial suspension and allowed to air dry on the Whatman filter paper. After drying, the preparation was mounted and viewed on Philips Tecnai 20 Electron Microscope.

3.2.4 16 S rRNA amplification and nucleotide sequencing

Genomic DNA was isolated from the pure culture of 14 potent isolates. The ~1.5 kb rDNA fragment was amplified through high-fidelity PCR polymerase by using consensus primers. The PCR product was bi-directionally sequenced by using the forward; 5'-AGAGTTTGATCATGGCTCAG-3' and Reverse primer; 5'-TACGGTTACCTTGTTACGACTT-3'

3.2.5 Phylogenetic analysis of the 16 S rRNA sequences

The published 16S rRNA gene sequences were obtained in aligned form from the Ribosomal Database Project (RDP) prokaryote ssu rRNA database (WWW site: http;//rdpwww.life.uiuc.edu/ (Maidak *et al,*. 1996) using the 'subalign' service. The Rt3 sequence was added to this alignment and manually aligned in accordance with RDP "align sequence" report, using the alignment editor AE2 (Larsen Likelihood (ML). The phylogeny of the aligned sequence was obtained using the RDP 'suggest tree' service from fast DNAml program (version 1.08).

3.3 RESULT

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3.3.1 Sites for sample collection

In order to obtain a pool of the haloalkaliphilic bacteria, six different locations along the costal region of Gujarat (India) were selected for the isolation. The sites of the isolation were Jodiya (22° 43' 0" N / 70° 17' 0" E) , Okha (22° 28' 9" N / 69° 3' 38" E) , Mithapur (22°25′N 69°00′E / 22.41°N 69.00°E / 22.41; 69.00[1]), Diu (20° 42’ N, 71° 01’ E), Somnath (20° 54’ N & 70° 23’ E) and Veraval (20° 53’ N & 70° 26’ E).

3.3.2 Isolation of the organisms

Total 82 haloalkaliphilic bacteria isolates from 6 different sites were obtained using different enrichment conditions of salt and pH in the medium. Twenty five strains were isolated from Mithapur; 13 from Okha; 8 from Jodiya, 14 from Veraval, 9 from Somnath and 13 from Diu (Figure 3.1).

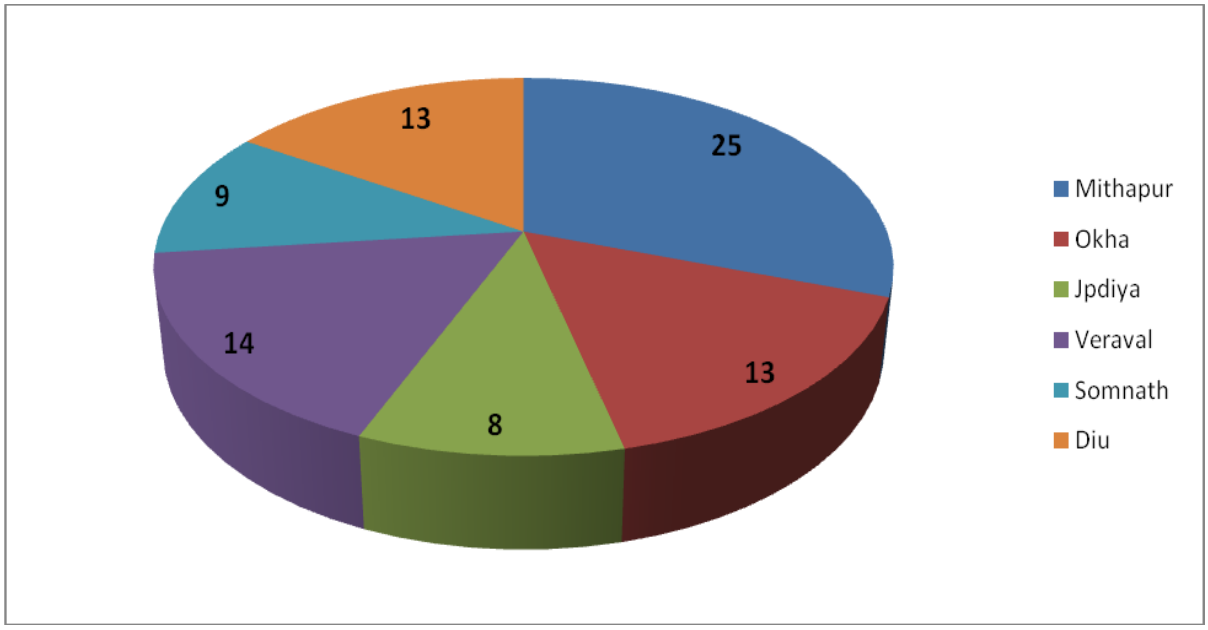

Figure 3. 1- Isolates from all the six sites obtained by enrichment

3.3.3 Cell morphology and Gram reaction

The isolates were examined for their cell morphology and Gram reaction. Microscopic observations revealed that majority of the organisms form Mithapur were Gram positive, with only 2 displaying Gram negative features and 3 being Gram variable. Majority of the isolates were rod shaped; however, their size varied from long thin rod to short thick rod. The cells were arranged singly, in pair or in chain. Curved rod cells formed both, chain as well as clusters.

Organisms isolated form Okha showed maximum variation in cell shape and arrangement. Size and shape of the cells varied from long thin rod to short thin rod, amongst them few were curved. Eight isolates among 13 were Gram negative and 5 were Gram positive. Gram-positive isolates dominated (9) over Gram negative ones (6). None of the isolates were Gram variable. The cell arrangements were similar to that of the Mithapur and Okha isolates. Maximum numbers of Gram-positive isolates were isolated form Mithapur, while Gram negative character dominated by Okha isolates and the isolates from Mithapur were largely Gram variable in nature

The isolates from Veraval were: Gram positive (6), Gram negative (6) and few Gram variables (2). All the isolates of the Veraval were small to large rod and arranged singly and/or in pair and among all, the number of the Gram positive isolates (8) dominated over the Gram variable isolate (1). From 9 isolates of Somnath, 6 were small thin to thick rod shaped arranged singly and in pair. Compared to other sites, maximum numbers of the Gram positive isolates were obtained from Diu. Most of the isolates appeared as short thick rod arranged singly and/or in pair and rest of with cocci shaped arranged in pair and tetrad (Table 3.1).

Isolates	Size and Shape	Arrangement	Gram Reaction
Mithapur			
Mi10-3_1	Thin long rod	Singly and in chain	Positive
Mi10-3_2	Thick short rod	Singly and in chain	Positive
Mi10-3_3	Thick short rod	Singly	Positive
Mi10-3_4	Long rod	Singly and in chain	Positive
Mi10-3_5	Thick short rod	Singly	Positive
Mi10-3_6	Thin long rod	Pair and in chain, clear gap between	Positive
Mi10-3_7	Very small rod	Pair and in chain	Positive
Mi20-3_1	Short rod	Single, Pair, Chain	Positive
Mi20-3_2	Very Thin Long rod	Curved long chain, some of in single and pair	Variable
Mi20-3_3	Long rod mostly curved	Single, Pair and in chain	Positive
Mi30-3	Short rod	In bunch	Variable
Mi10-4_1	thick long rod	Pair and in chain	Positive
Mi10-4_2	Long thick rod	Singly	Negative
Mi20-4_1	Short thick rod	Mostly in single,	Positive
Mi20-4_2	Short thin rod	Chain	Positive
Mi20-4_3	Short thick rod	Mostly single and in pair	Positive
Mi25-4_1	Medium rod	Chain, pair and single	Positive
Mi25-4_2	Very short rod	Coiled chain	Positive
Mi25-4_3	Long thin rod	In pair	Negative
Mi10-5_1	Long rod	Chain	Positive
Mi10-5_2	Very small rod	Single and in chain	Positive
Mi10-5_3	Short rod	Chain	Positive
Mi10-5_4	Short thick rod	Singly and pair	Positive
Mi10-5_5	Short curved thick rod	Pair and in chain	Positive
Mi20-5_1	Short very thick rod	Mostly single and in pair	Variable

Isolates	Size and Shape	Arrangement	Gram Reaction
Okha			
Kh10-8_1	Long thin rod	Mesh like structure	Negative
Kh10-8_2	Short thick rod	Chain, single	Negative
Kh10-8_3	Short thick rod	Chain which is in coil form	Positive
Kh10-8_4	Thin rod	Single	Negative
Kh10-9_1	Very thin long rod	Single	Negative
Kh10-9_2	Cocci	Cluster	Positive
Kh10-10_1	Short thin rod	In chain	Positive
Kh15-8_1	Short thick rod	Pair	Negative
Kh15-9_1	Short rod, curved	In pair	Negative
Kh15-9_2	Short thin rod	In chain	Positive
Kh20-8_1	Short thin rod	In chain	Negative
Kh25-8_1	Cocci	Cluster	Positive
Kh25-9_1	Very thick short rod	Singly	Positive

Isolates	Size and Shape	Arrangement	Gram Reaction
Jodiya			
AH-6	Long thin rod	Cluster, single	Positive
AH-10	Short thin rod	Single, chain	Negative
AH-11	Long rod	Chain	Negative
AH-12	Thin long rod	Pleomorphic	Negative
CM-12	Thick long rod	Pair and in bunch	Positive
CM-6	Long rod	Single and in chain	Positive
Sj-1	Short thin rod	Cluster	Positive
Sj-2	Thin long rod	Cluster	Positive

Isolates	Gram reaction	Size and Shape	Arrangement
Somnath			
S-10-8_1	Positive	Short rod	Single and pair
S-10-8_2	Positive	Small cocci	Single
S-10-8_3	Positive	Small thin rod	Single and some in
S-10-9_1	Positive	Short thick rod	Single
S-10-9_2	Positive	Very short thin rod,	Single and most of
S-10-9_3	Variable	Small thin rod	Single and in pair
S-10-10	Positive	Short thick rod	Single
S-15-9	Positive	Small cocci	Single
S-20-9	Positive	Small oval shape	In tetrad only

Isolates	Gram reaction	Size and Shape	Arrangement
Veraval			
Ve1-10-8_1	Negative	Short thin rod	Single and in pair
Ve1-10-8_2	Negative	Very short thick rod	Single and in chain (2-3)
Ve1-10-8_3	Variable	Long thin rod	Single
Ve1-10-9_1	Negative	Small thin rod with terminal spores	Single
Ve1-10-9_2	Negative	Medium thin rod	Single and in pair
Ve1-10-9_3	Negative	Small thin rod	Single and in pair,
Ve2-10-8_1	Negative	Long thin rod	Single and chain,
Ve2-10-8_2	Positive	Short thick rod	Single and in pair
Ve2-10-9	Variable	Short thick rod	Single and in pair
Ve2-10-10	Positive	Small cocci	Single and in pair
Ve2-15-9_1	Positive	Small cocci	Single
Ve2-15-9_2	Positive	Small cocci	Single
Ve2-20-9_1	Positive	Very small cocci	Single and in
Ve2-20-9_2	Positive	Very small cocci	Single

Isolates	Gram reaction	Size and Shape	Arrangement
Diu			
D-10-8_1	Positive	Very Short thick rod	Single
D-10-8_2	Positive	Small thin rod	Single
D-10-9	Positive	Short thick rod	Mostly single but some in pair
D-10-10_1	Positive	Long thin rod	Single
D-10-10_2	Positive	Small thick rod	Single and in pair
D-15-9	Positive	Small cocci	Single
D-20-9_1	Positive	Small cocci	In pair and clustered
D-20-9_2	Positive	Very small cocci	Single
Dj10-8_1	Large Cocci	Singly and cluster	Positive
Dj10-8_2	Long thin rod	Chain	Negative
Dj20-8_1	Short thin rod	Chain and in clusters	Positive
Dj30-8_1	Long very thick	in clusters	Positive
Dj30-8_2	Thick long rod	Chain	Negative

Table 3.1- Cell morphology and gram reaction of all the isolates

3.3.4 Effect of solvents on growth of Haloalkaliphilic bacteria

As microbial life can be found in the most of extreme conditions and due to their quick multiplication rate they are the best tool for remediation of particular extreme environment pollutants. Focusing on organic solvent pollution, capability of organic solvent degradation by any bacteria can be enumerated based on its ability to grow on organic solvent mixed media.

For screening of organic solvent tolerant haloalkaliphilic bacteria, 1 to 5 %(v/v) of four Organic solvents methanol (log P_{ow} 0.82) Propanol (log P_{ow} 0.25), n-hexane (log P_{ow} 3.5) and butanol (log P_{ow} 0.9) were supplemented in growth medium.

Figure 2 illustrates tolerance of haloalkaliphilic Bacteria in presence of varying concentrations of solvents. Growth was reduced with increasing solvent concentrations and in general, better growth was observed in solvents of higher log $P_{o/w}$ values. However, in the presence of immiscible solvents of low log $Po_{/w}$, such as Butanol, growth was severely inhibited.

Most of Jodiya isolates were tolerating all tested concentration of solvent. Majority of Jodiya isolates were able to tolerate 3-4 % (v/v) butanol while AH-10 and CM-10_1 were sustaining in presence of 1% (v/v) butanol. CM-10_2 did not show any growth in presence of butanol. With rest of solvent, decreasing growth rate were observed with enhancement in solvent concentration.

The extent of solvent tolerance by mithapur isolates specialy for methanol, propanol and n-hexane did not vary extensively. Except Mi10-51 and Mi10-52 Majority of isolates were tolerating all tested solvent. These isolates were poorly tolerating all examined solvents. They were not even retaining 50% growth density of control. Mi10-5_4 and Mi10-5_5 growth was totally inhibited in presence of butanol.
Some isolates like Mi10-5_5, Mi10-5_2 , Mi20-4_1, Mi10-4_1, Mi30-3, Mi20-3_2 Mi10-3_6 were displaying growth either better or equal to control in presence of particular

solvent . In presence of 4% (v/v) n-hexane after 96 hour cell density was equal to control.

Solvent tolerance among Diu isolates were widely divers again. Except in presence of butanol, some isolates as Dj10-8_2, Dj20-8_1, Dj30-8_1 were potently tolerating rest of examined solvent. Reduced growth in presence of butanol is a obvious trend as butanol is highly hydrophobic and toxic to living flora. Remaining isolates as D-10-9, D-10-10_1, D-10-10_2, D-15-9, D-20-9_1, D-20-9_2 and Dj10-8_1 were exhibiting mixed trend of growth which was varying according to solvents.

Somnath isolates were extensively diversified on the basis of the solvent tolerance. Isolate S-10-10 was not tolerating any solvent at all. Growth of S-15-9 was totally blocked in presence of butanol while with rest of solvent it's performance was quiet well. S-10-9_3 denoted maximum tolerant against methanol while many of them were tolerating n-hexane adequately.

The extent of organic solvent tolerance was varied for Okha isolates. Kh-10-9_1 was highly tolerant for all tested solvent even it was growing very well even in presence of 5% (v/v) butanol. Isolate was attending almost similar cell density in presence of all tested solvent (Figure 3.2).

Figure 3.2- Growth Pattern of haloalkaliphilic Bacteria in presence of 0(♦), 1(■) ,2(▲), 3(+), 4(×) and 5(●) % (v/v) solvent

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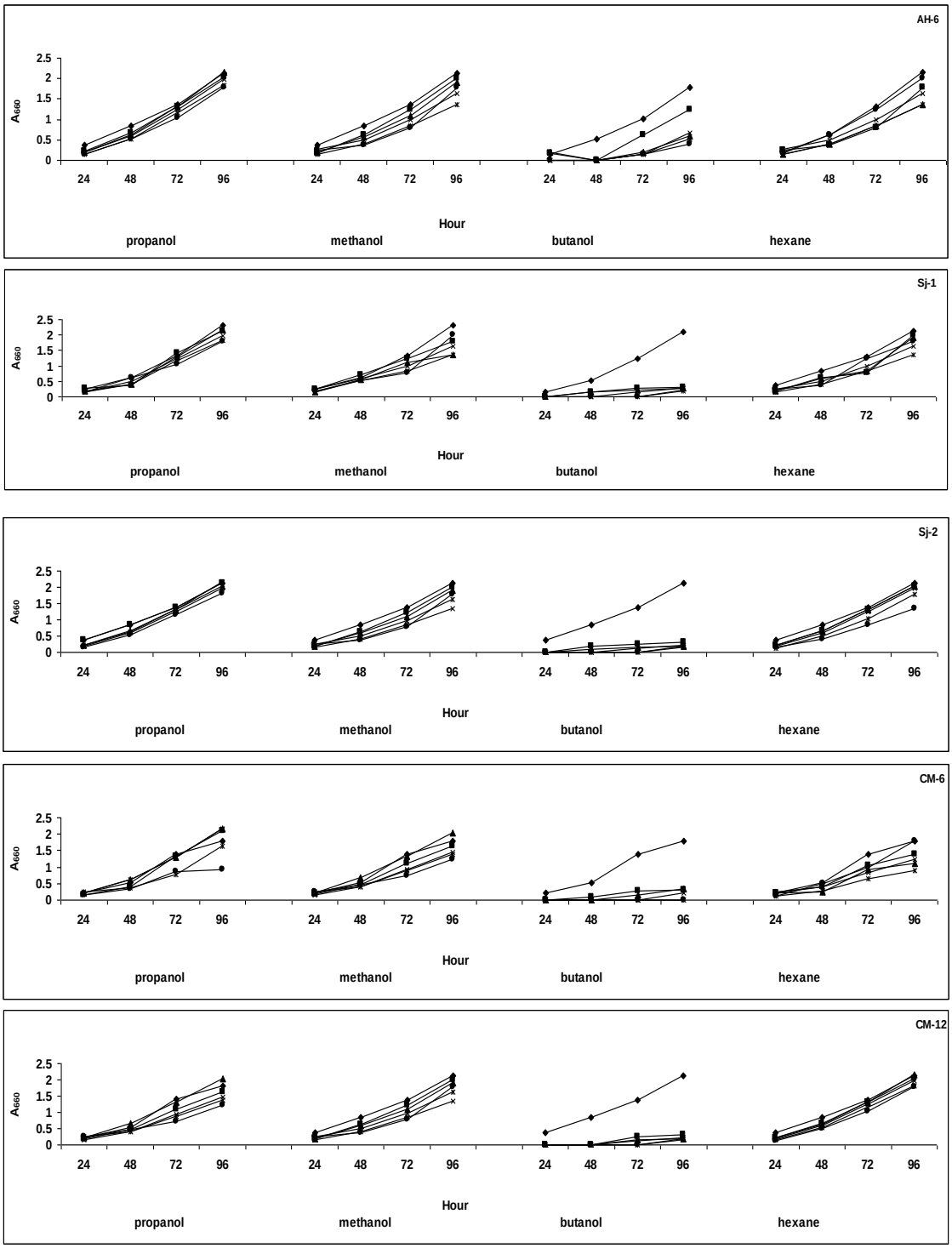
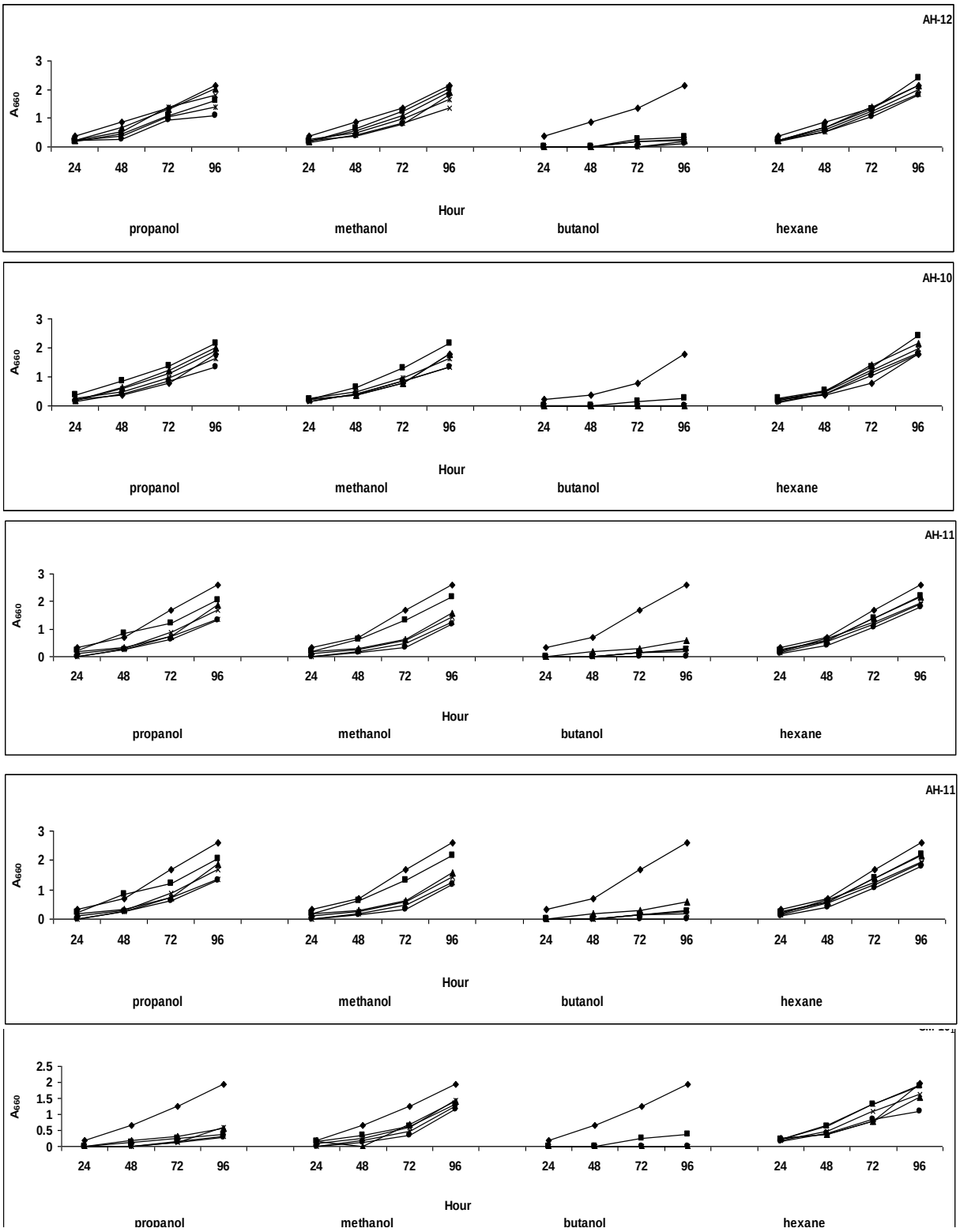
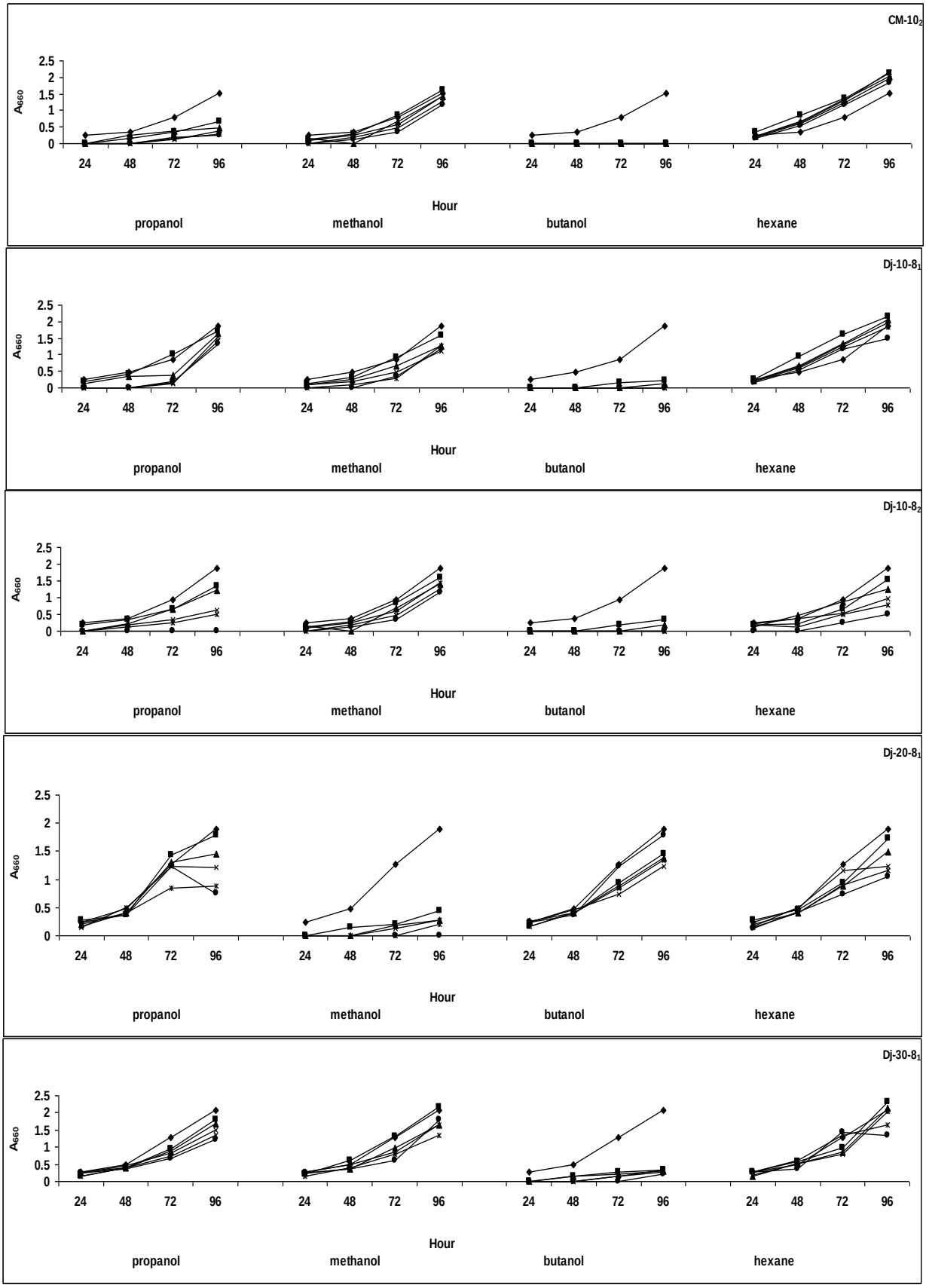
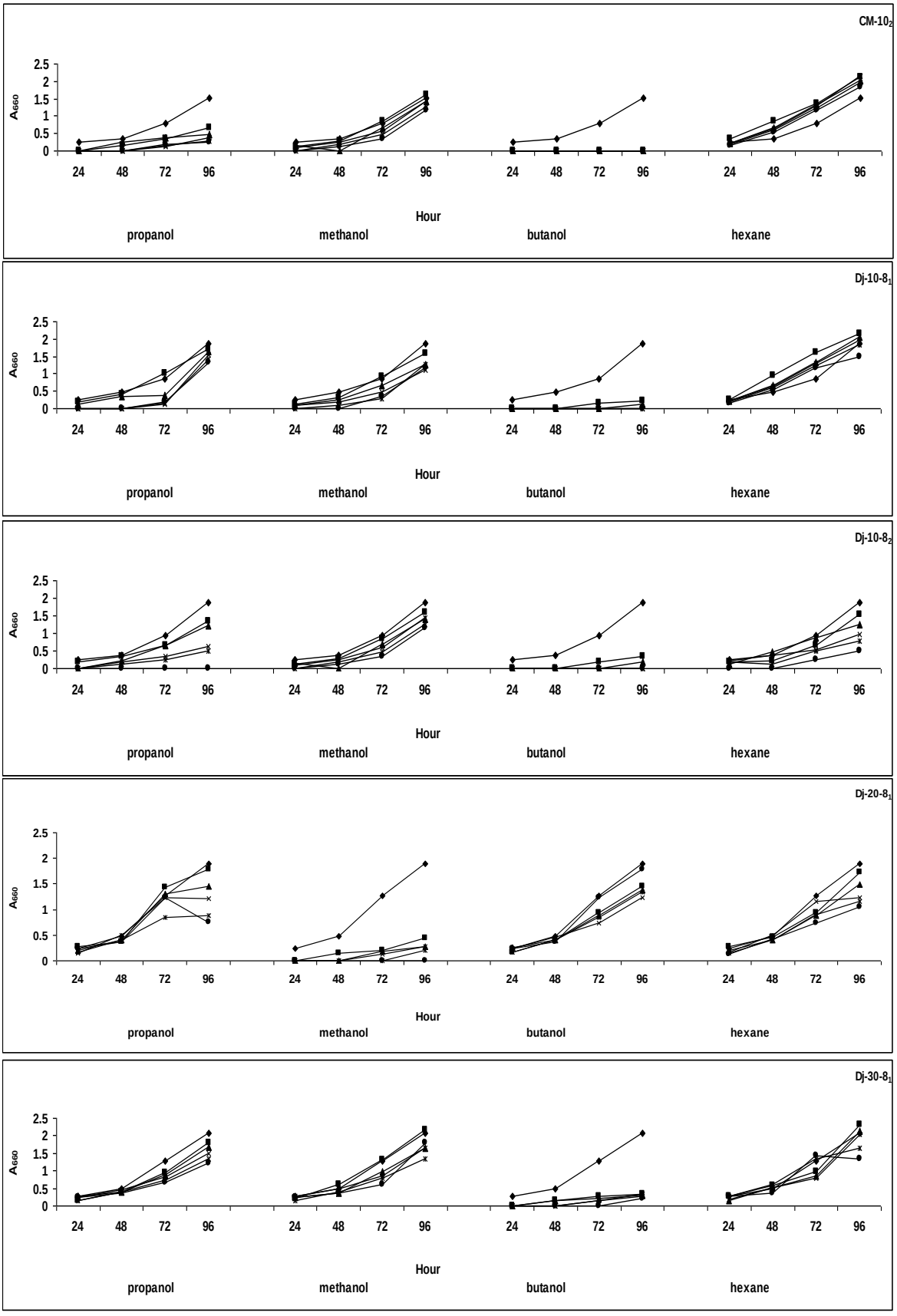



AH-12
AH-10
AH-11
AH-11
A_{660}
Hour
propanol
methanol
butanol
hexane
24
48
72
96


CM-10$_2$
Dj-10-8$_1$
Dj-10-8$_2$
Dj-20-8$_1$
Dj-30-8$_1$
A_{660}
2.5
2
1.5
1
0.5
0
24
48
72
96
Hour
propanol
methanol
butanol
hexane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Dj-10-8$_1$
Dj-10-8$_2$
Dj-20-8$_1$
Dj-30-8$_1$
A_{660}
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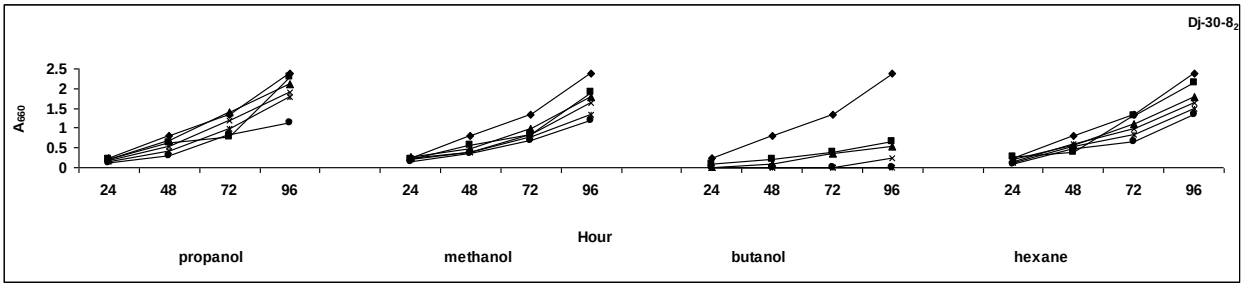
2
1.5
1
0.5
0
24 48 72 96
Hour
propanol
methanol
butanol
hexane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A_{660}
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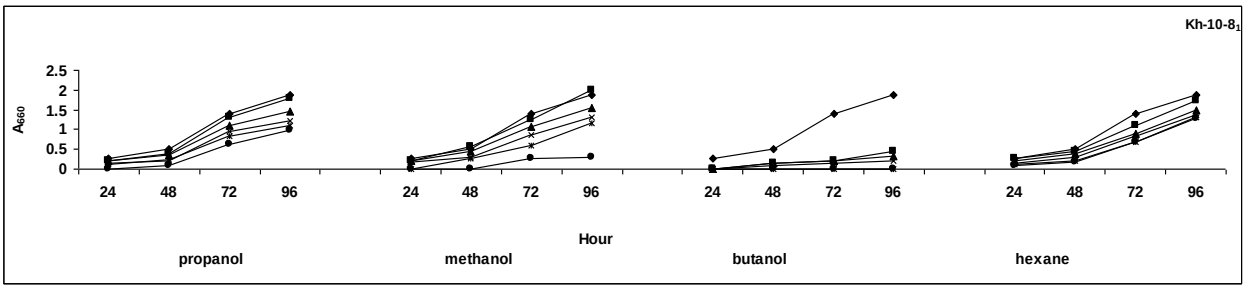
2
1.5
1
0.5
0
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Hour
propanol
methanol
butanol
hexane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A_{660}
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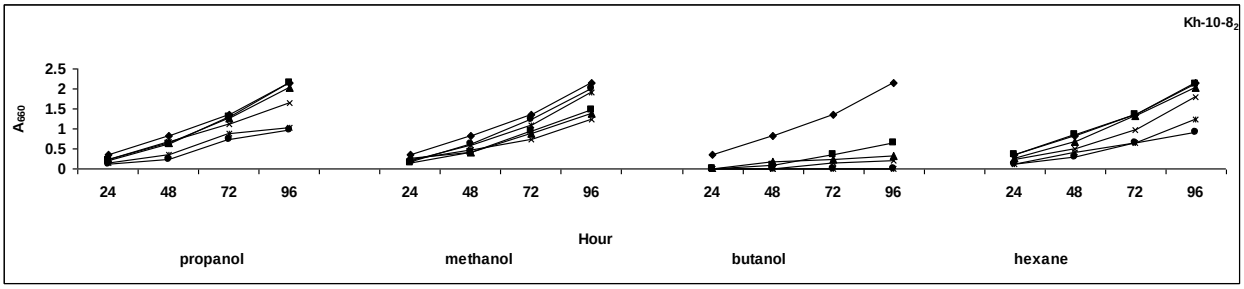
2
1.5
1
0.5
0
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Hour
propanol
methanol
butanol
hexane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1
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Hour
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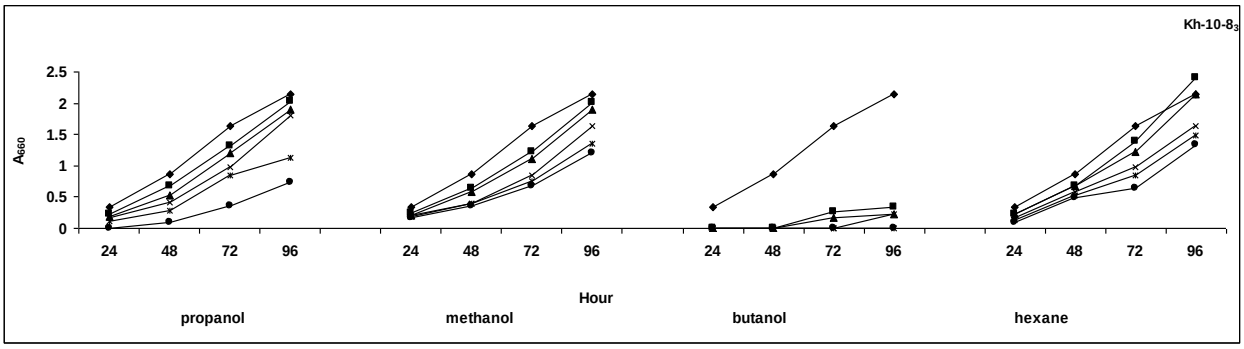
methanol
butanol
hexane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1
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0
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Hour
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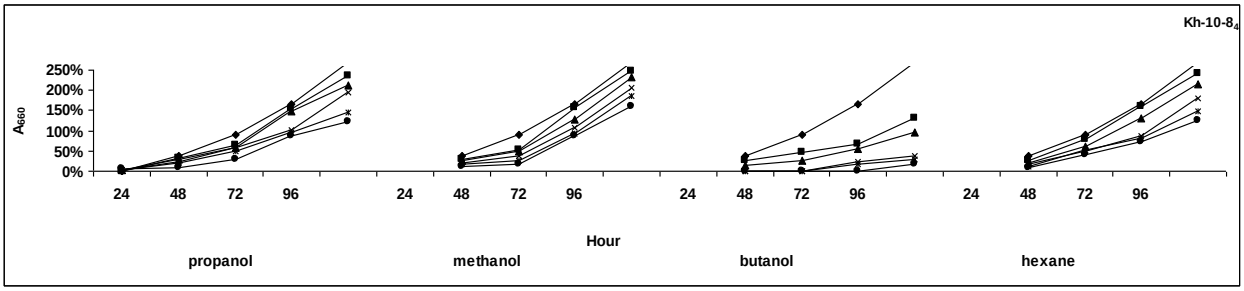
methanol
butanol
hexane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200%
150%
100%
50%
0%
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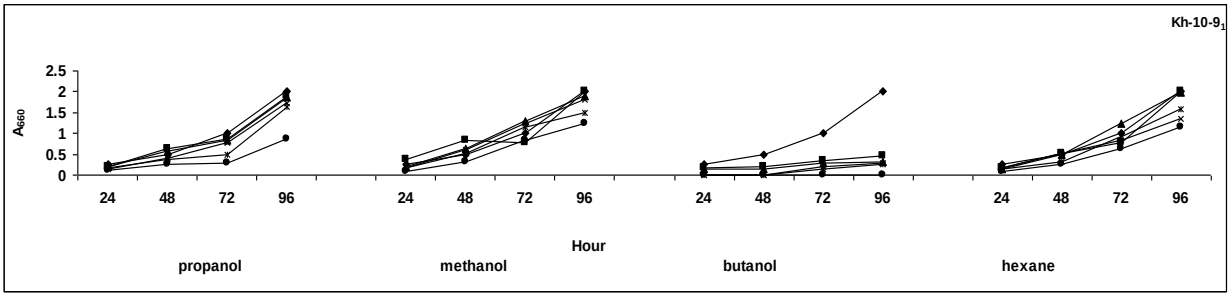
Hour
propanol
methanol
butanol
hexane


Kh-10-9$_1$
A_{660}
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2
1.5
1
0.5
0
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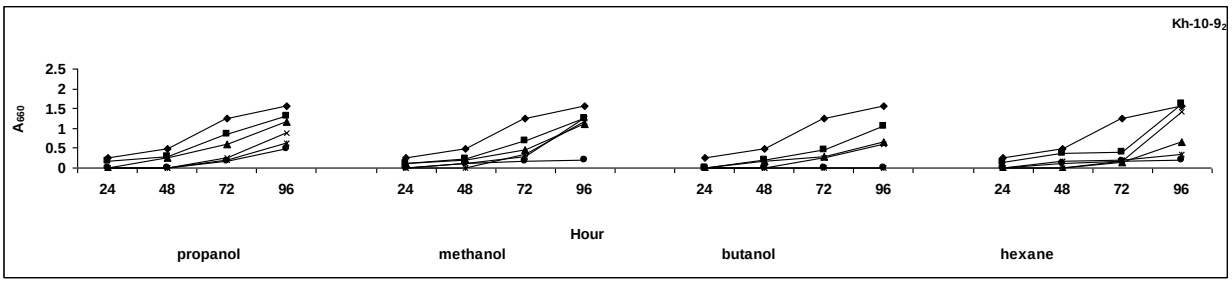
Hour
propanol
methanol
butanol
hexane


Kh-10-9$_2$
A_{660}
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2
1.5
1
0.5
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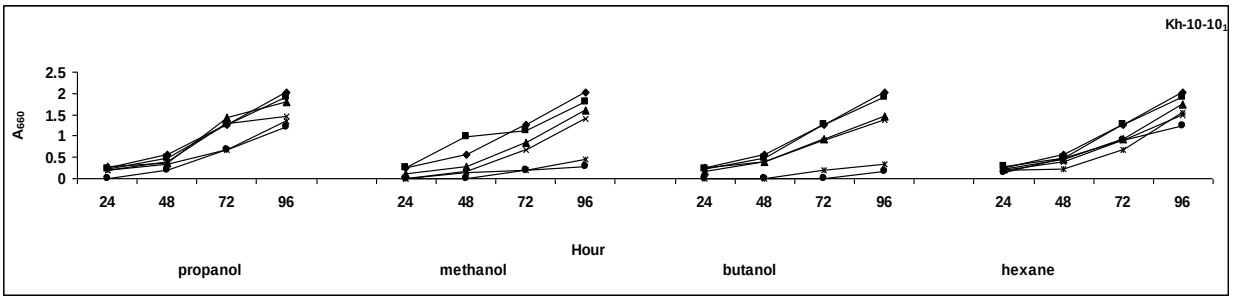
Hour
propanol
methanol
butanol
hexane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1
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Hour
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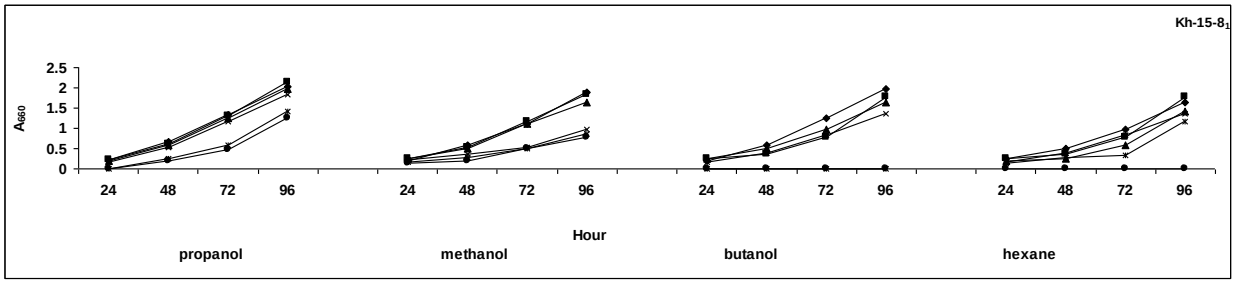
methanol
butanol
hexane


Kh-15-8$_1$
A_{660}
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0
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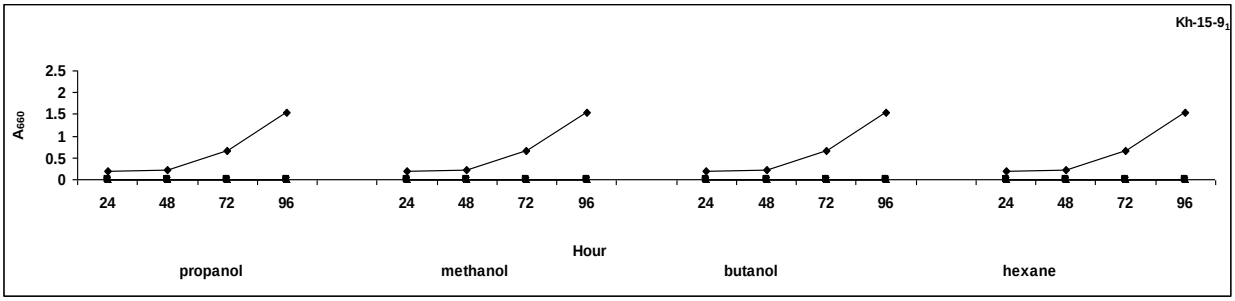
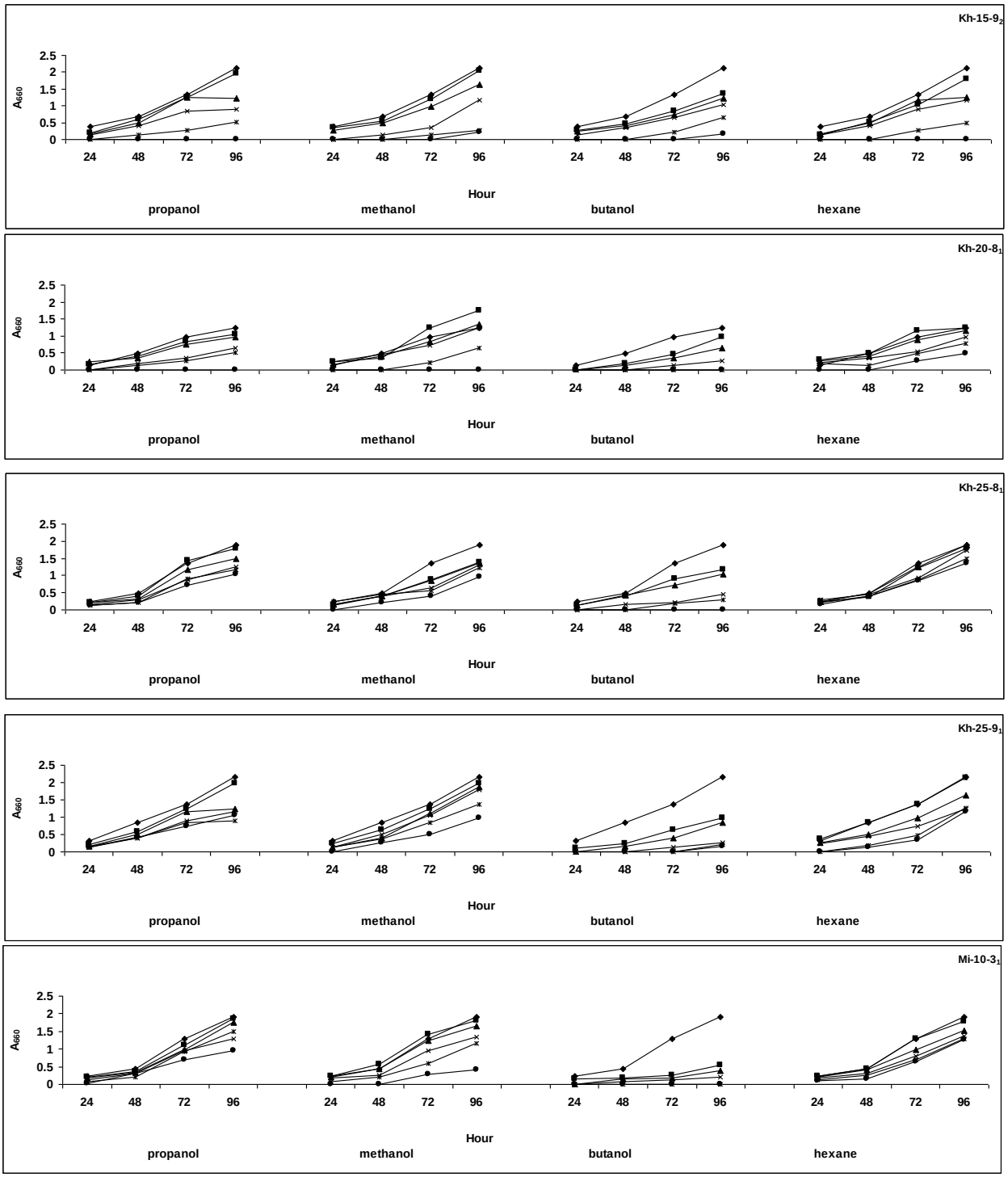
Hour
propanol
methanol
butanol
hexane


Kh-15-9$_1$
A_{660}
2.5
2
1.5
1
0.5
0
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Hour
propanol
methanol
butanol
hexane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Kh-20-8$_1$
Kh-25-8$_1$
Kh-25-9$_1$
Mi-10-3$_1$
A_{660}
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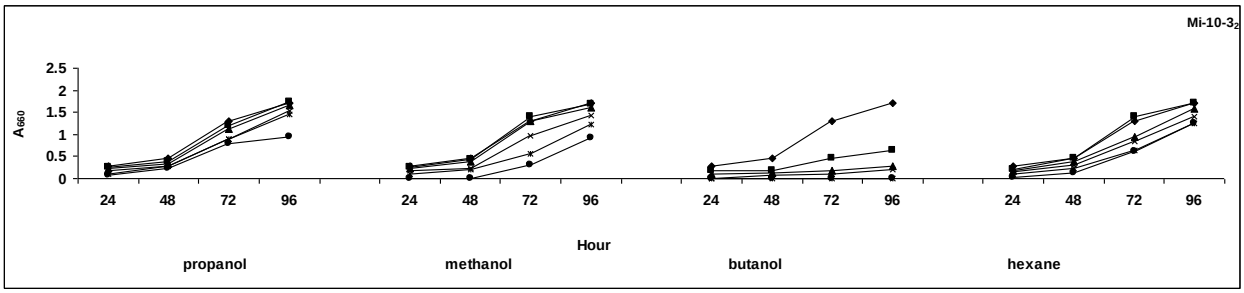
2
1.5
1
0.5
0
24
48
72
96
Hour
propanol
methanol
butanol
hexane


Mi-10-3$_2$
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1.5
1
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0
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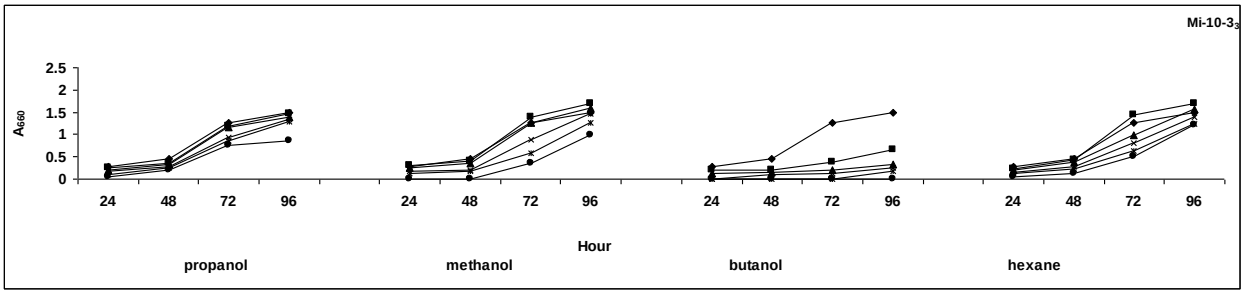
Hour
propanol
methanol
butanol
hexane


Mi-10-3$_3$
A_{660}
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1.5
1
0.5
0
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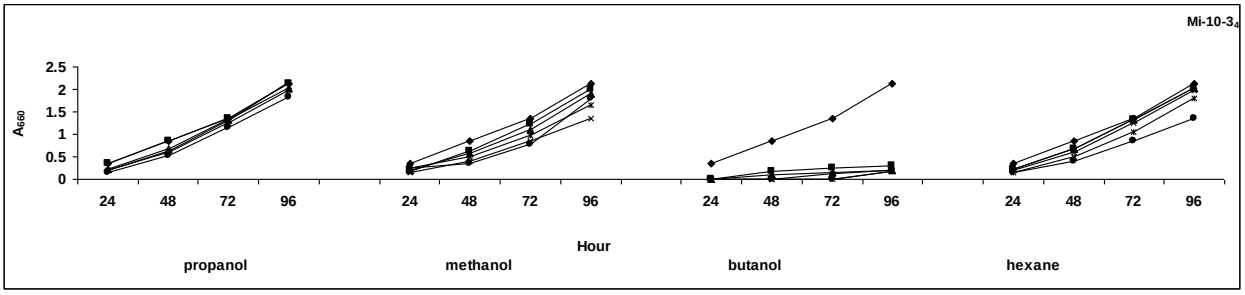
Hour
propanol
methanol
butanol
hexane


Mi-10-3$_4$
A_{660}
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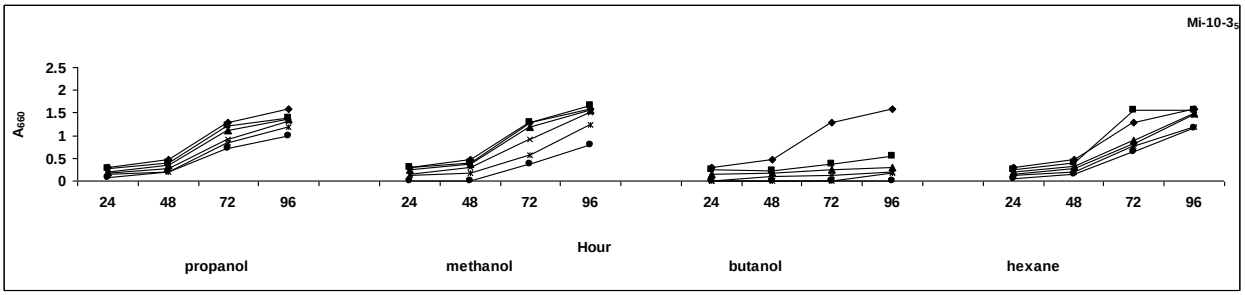
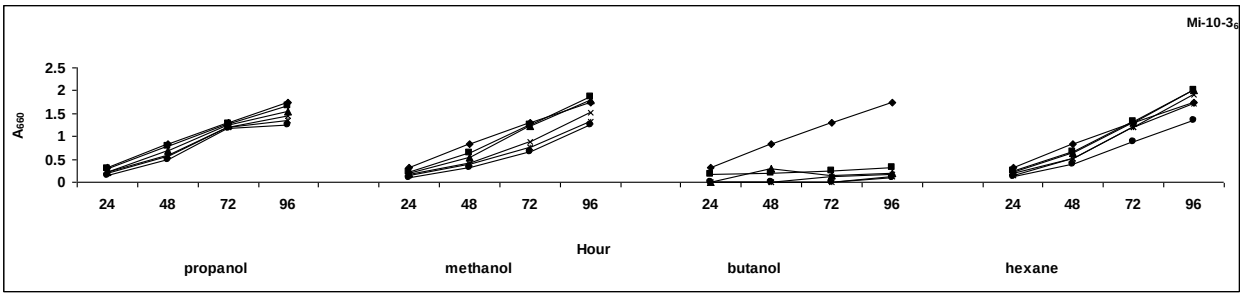
2
1.5
1
0.5
0
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Hour
propanol
methanol
butanol
hexane


Mi-10-3$_5$
A_{660}
2.5
2
1.5
1
0.5
0
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Hour
propanol
methanol
butanol
hexane


Mi-10-3$_6$
A_{660}
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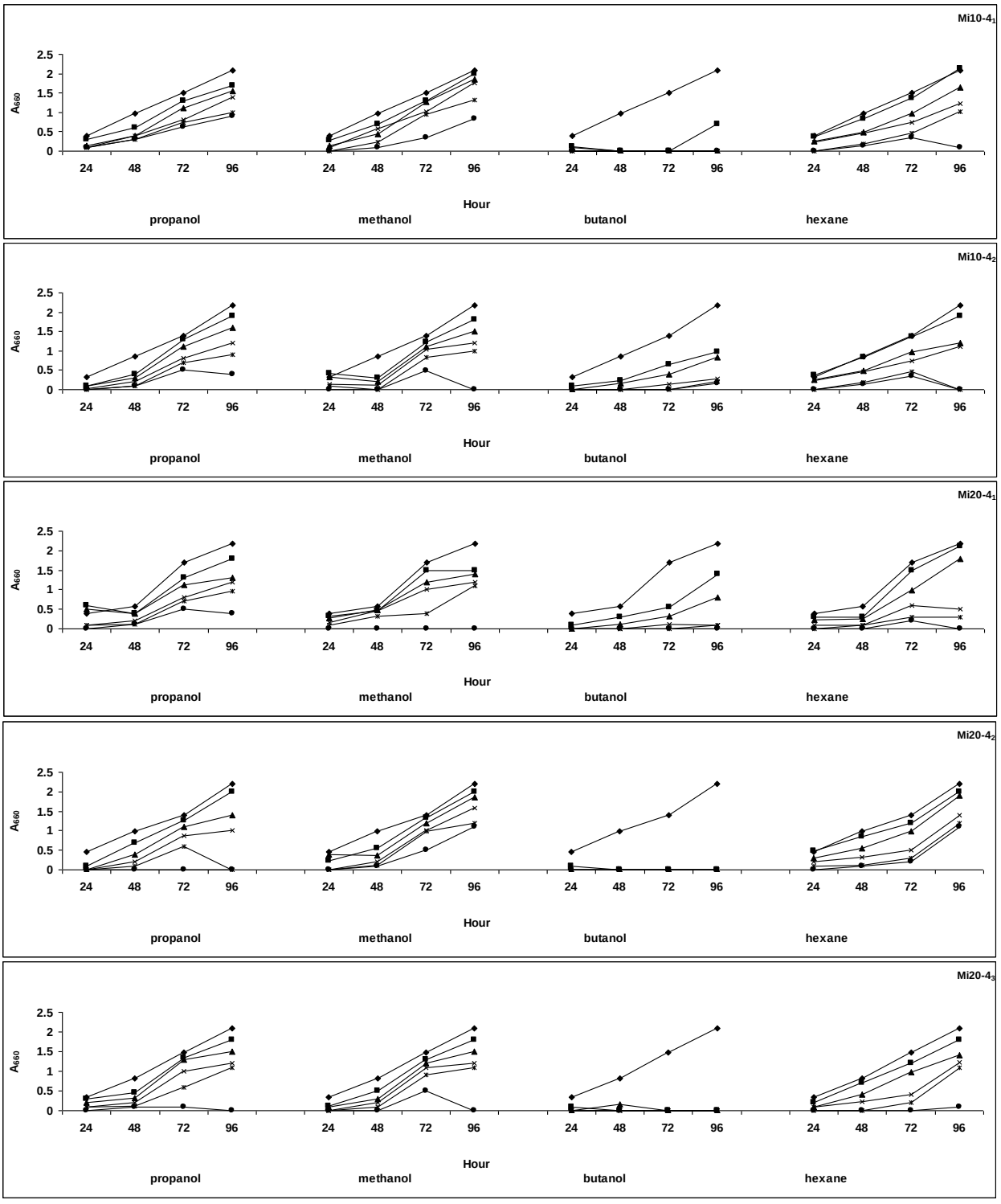
2
1.5
1
0.5
0
24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Hour
propanol
methanol
butanol
hexane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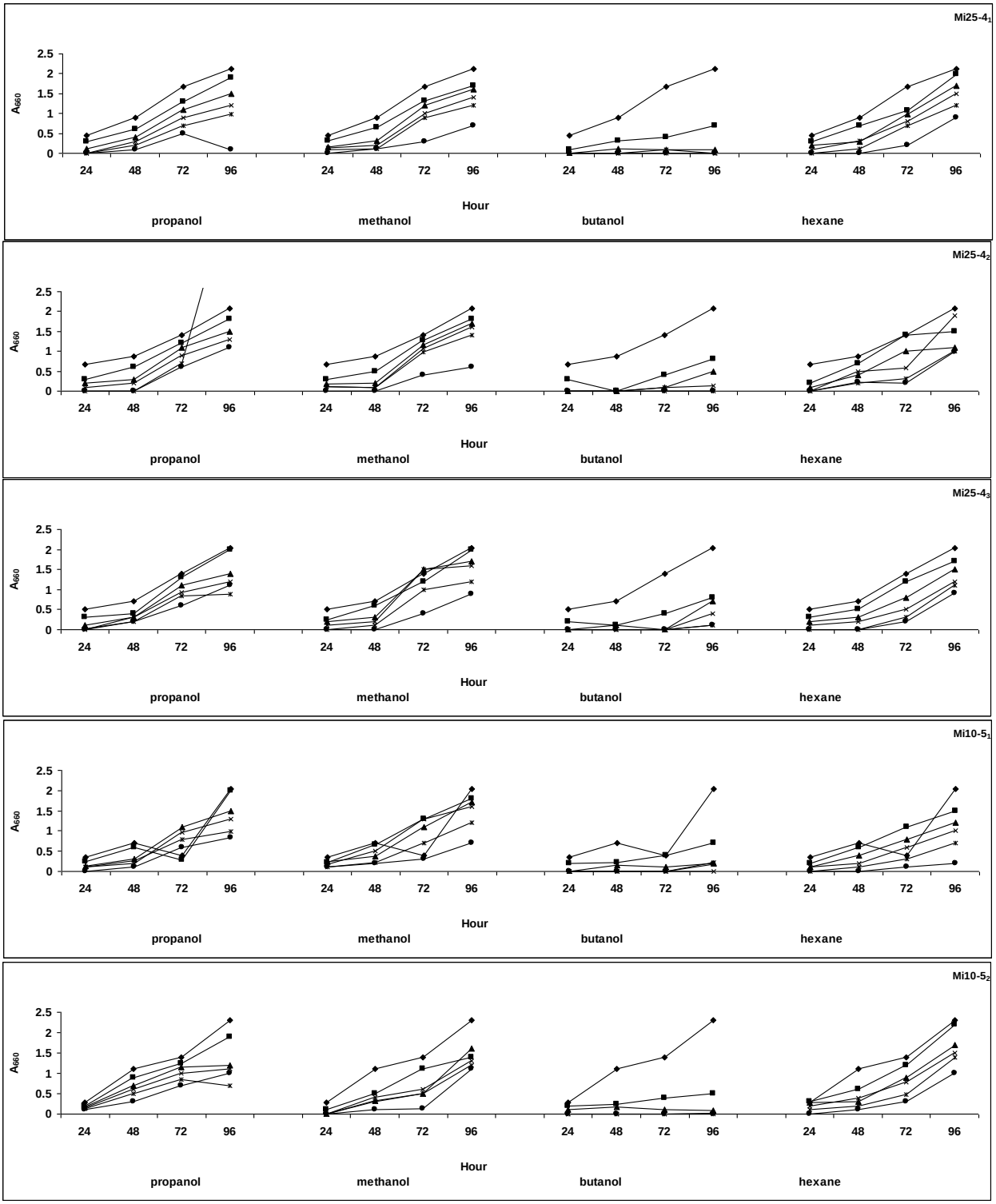
Mi10-4$_2$
Mi20-4$_1$
Mi20-4$_2$
Mi20-4$_3$
A_{660}
0
0.5
1
1.5
2
2.5
24
48
72
96
Hour
propanol
methanol
butanol
hexane


Mi25-4$_1$
Mi25-4$_2$
Mi25-4$_3$
Mi10-5$_1$
Mi10-5$_2$
A_{660}
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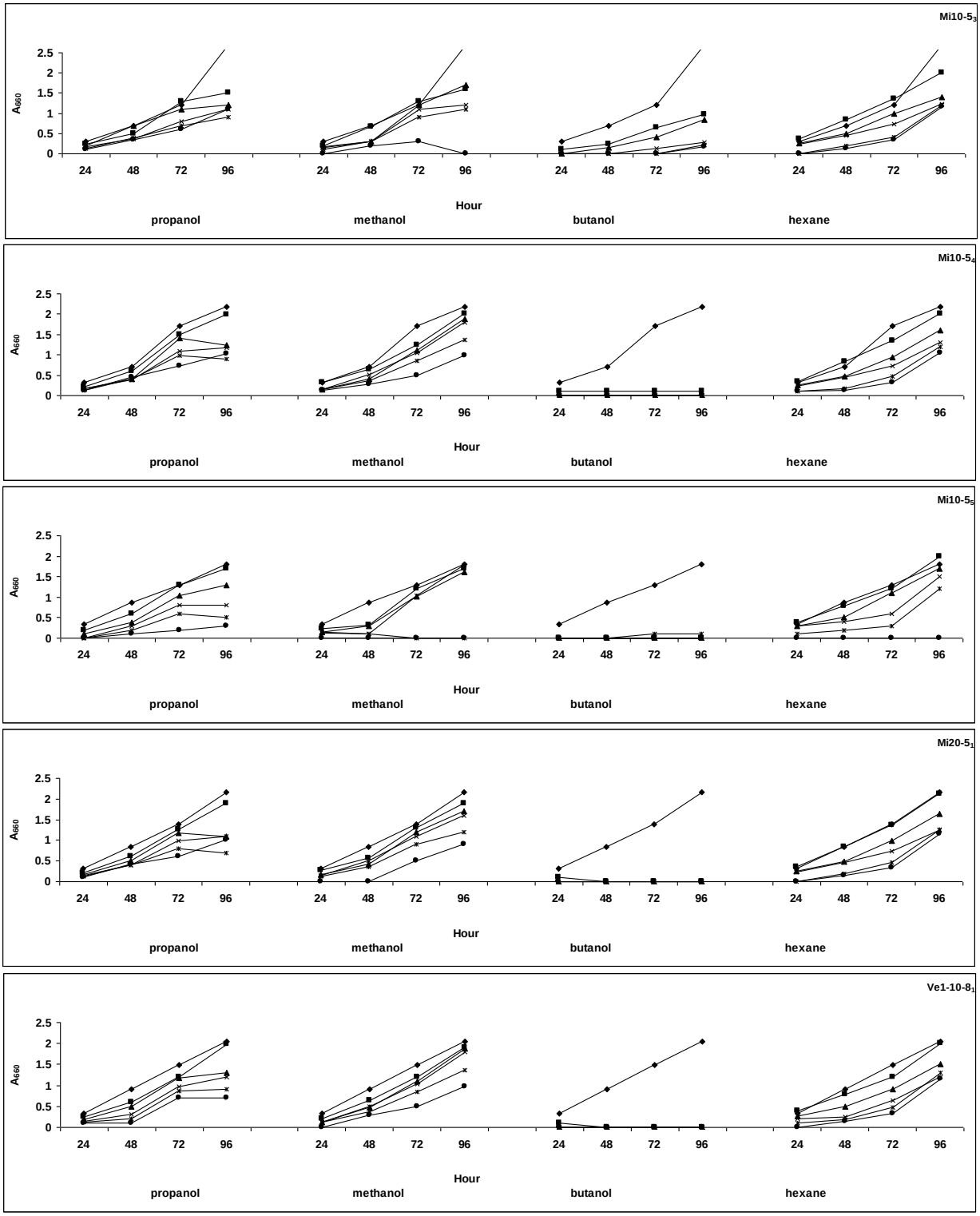
1
1.5
2
2.5
24
48
72
96
Hour
propanol
methanol
butanol
hexane


Mi10-5$_3$
Mi10-5$_4$
Mi10-5$_5$
Mi20-5$_1$
Ve1-10-8$_1$
A660
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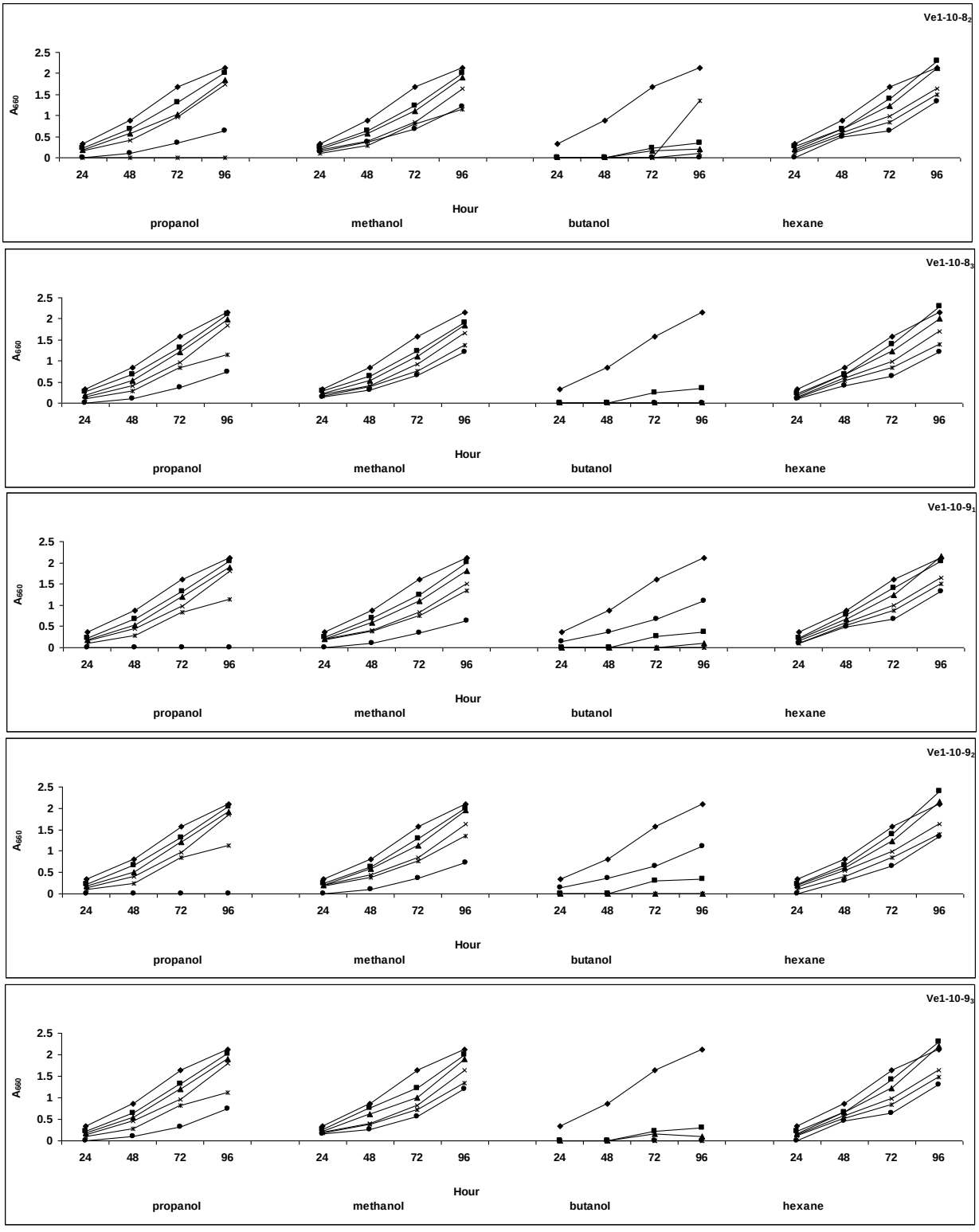
1.5
1
0.5
0
24
48
72
96
Hour
propanol
methanol
butanol
hexane


Ve1-10-8$_2$
Ve1-10-8$_3$
Ve1-10-9$_1$
Ve1-10-9$_2$
Ve1-10-9$_3$
A_{660}
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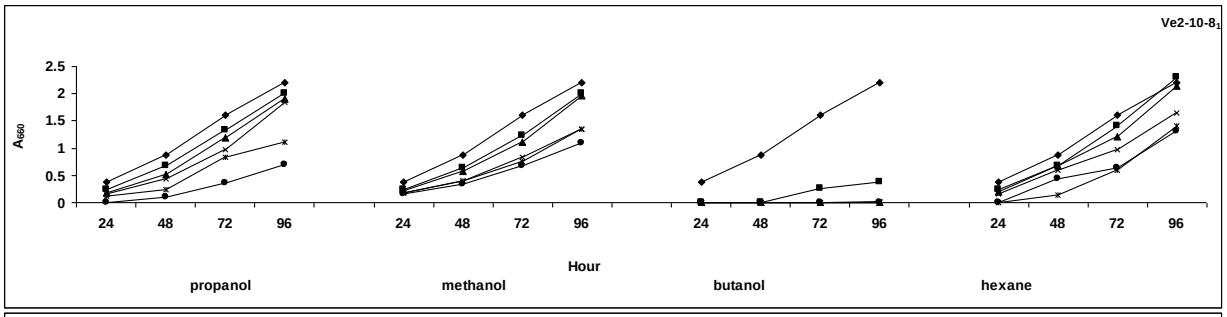
1.5
1
0.5
0
24
48
72
96
Hour
propanol
methanol
butanol
hexane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Hour
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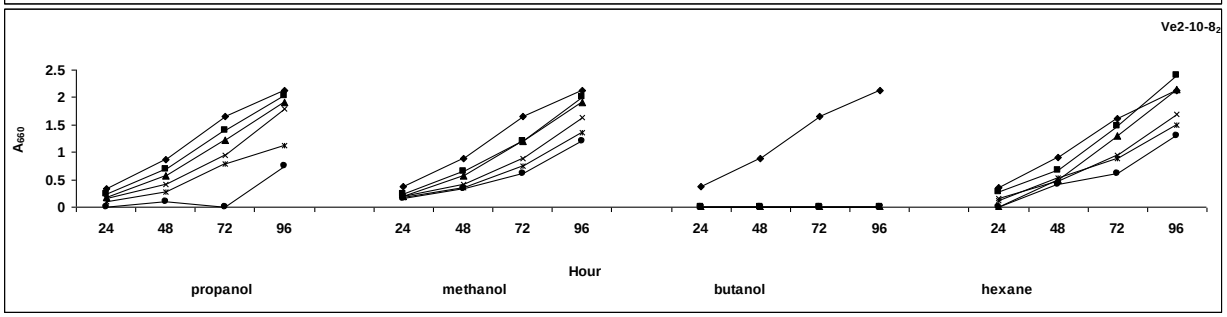
methanol
butanol
hexane


Ve2-10-8$_2$
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1.5
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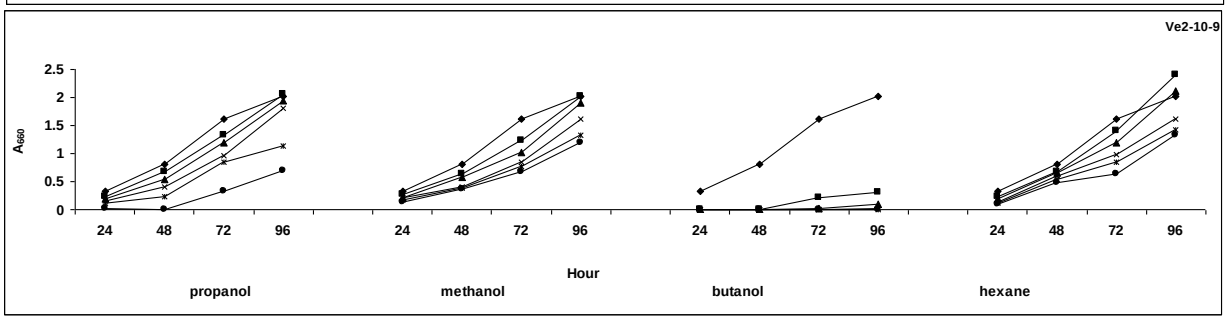
Hour
propanol
methanol
butanol
hexane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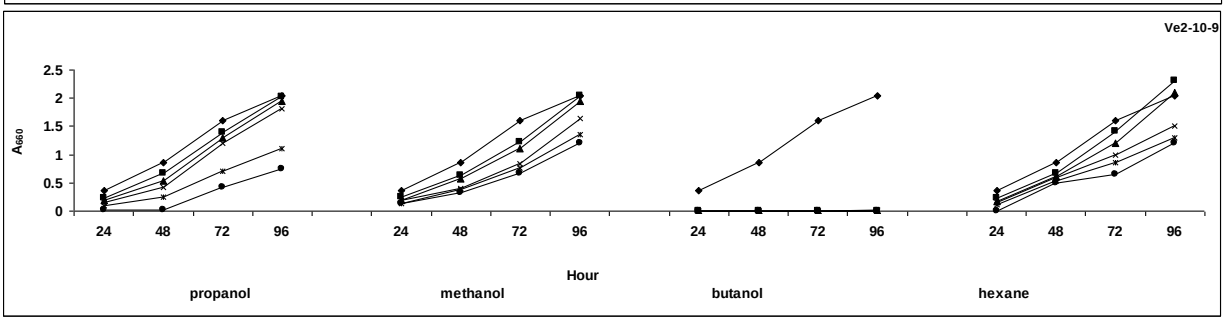
methanol
butanol
hexane


Ve2-10-9
A_{660}
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1.5
1
0.5
0
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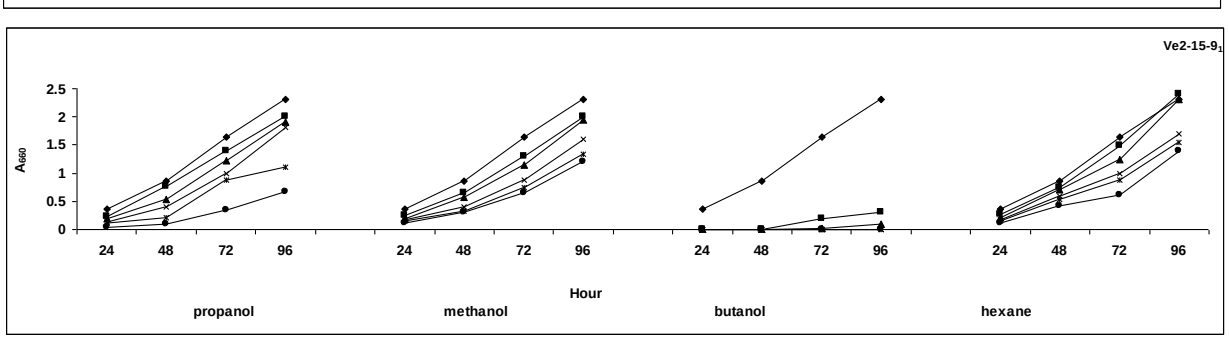
Hour
propanol
methanol
butanol
hexane


Ve2-15-9$_1$
A_{660}
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24 48 72 96 24 48 72 96 24 48 72 96 24 48 72 96
Hour
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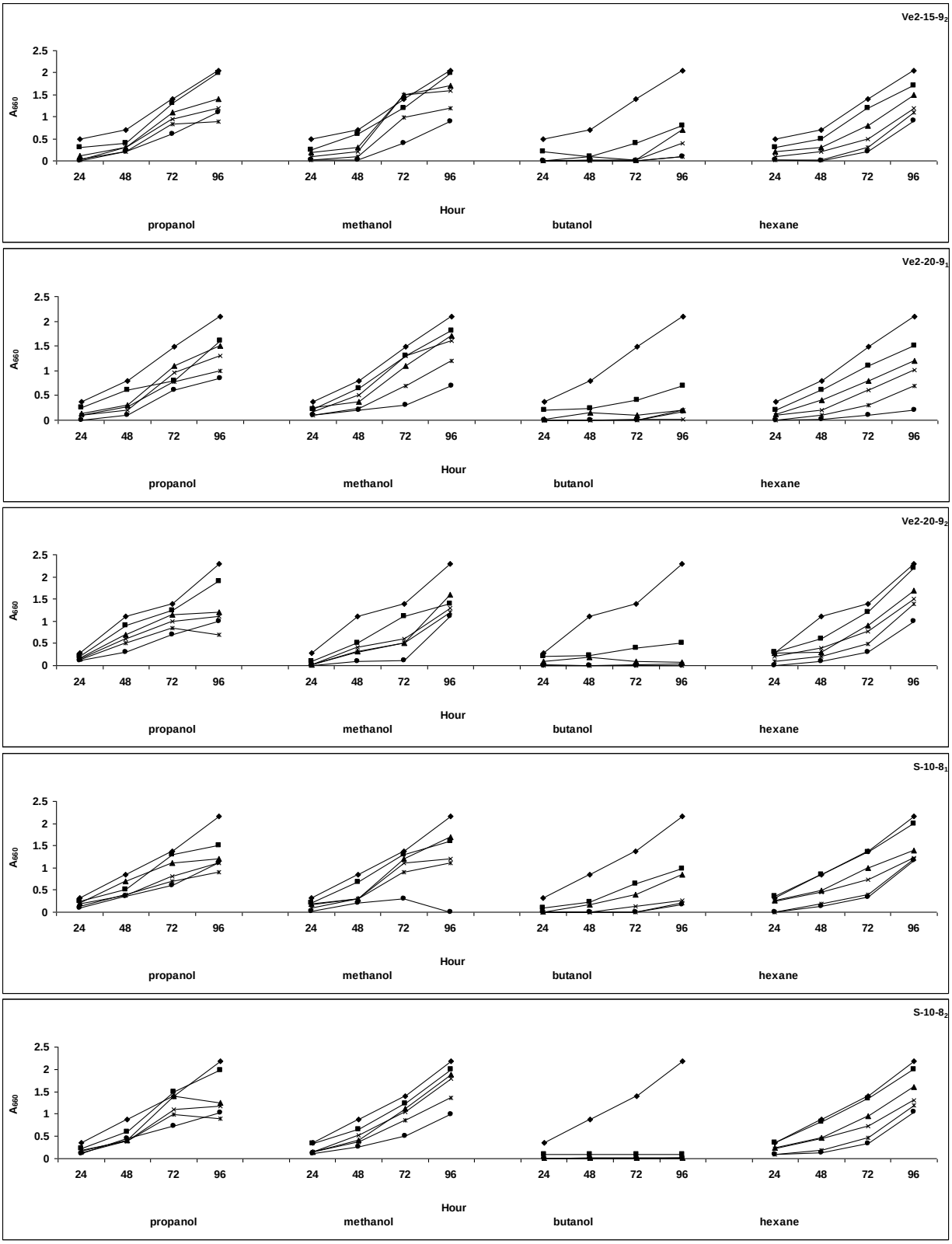
methanol
butanol
hexane


Ve2-15-9$_2$
Ve2-20-9$_1$
Ve2-20-9$_2$
S-10-8$_1$
S-10-8$_2$
A660
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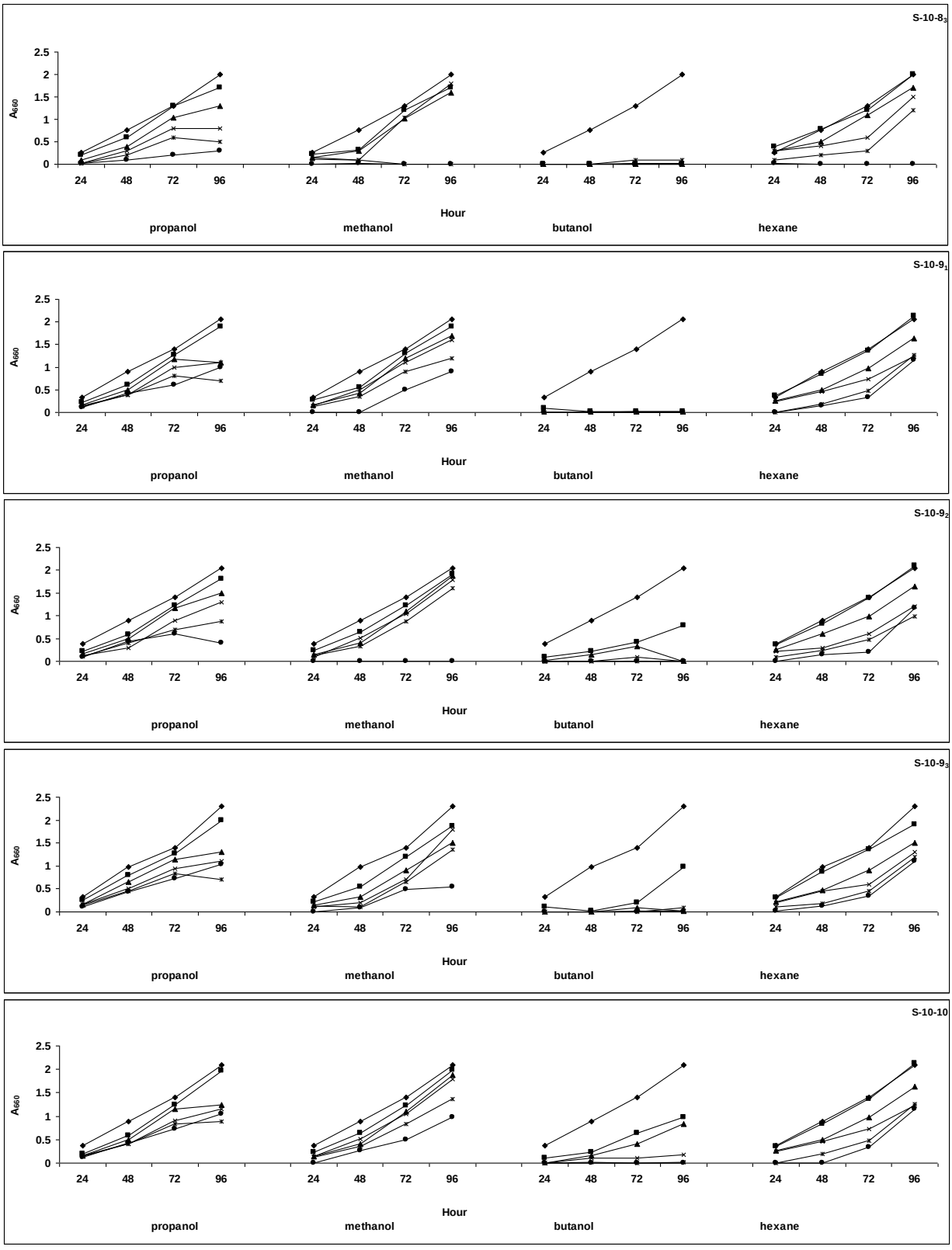
1.5
1
0.5
0
24
48
72
96
Hour
propanol
methanol
butanol
hexane


S-10-8$_3$
S-10-9$_1$
S-10-9$_2$
S-10-9$_3$
S-10-10
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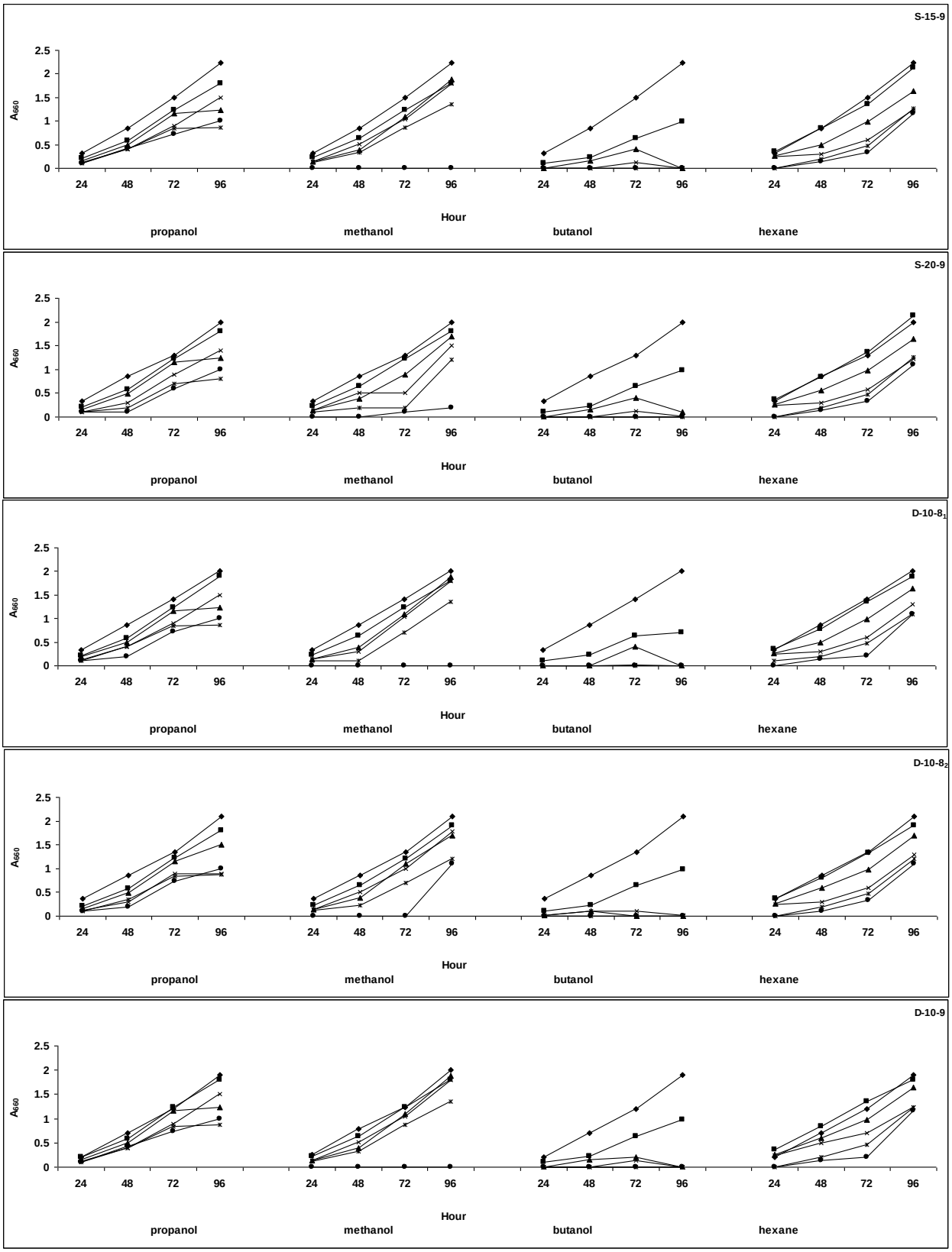
1
1.5
2
2.5
24
48
72
96
Hour
propanol
methanol
butanol
hexane


S-15-9
S-20-9
D-10-8$_1$
D-10-8$_2$
D-10-9
A660
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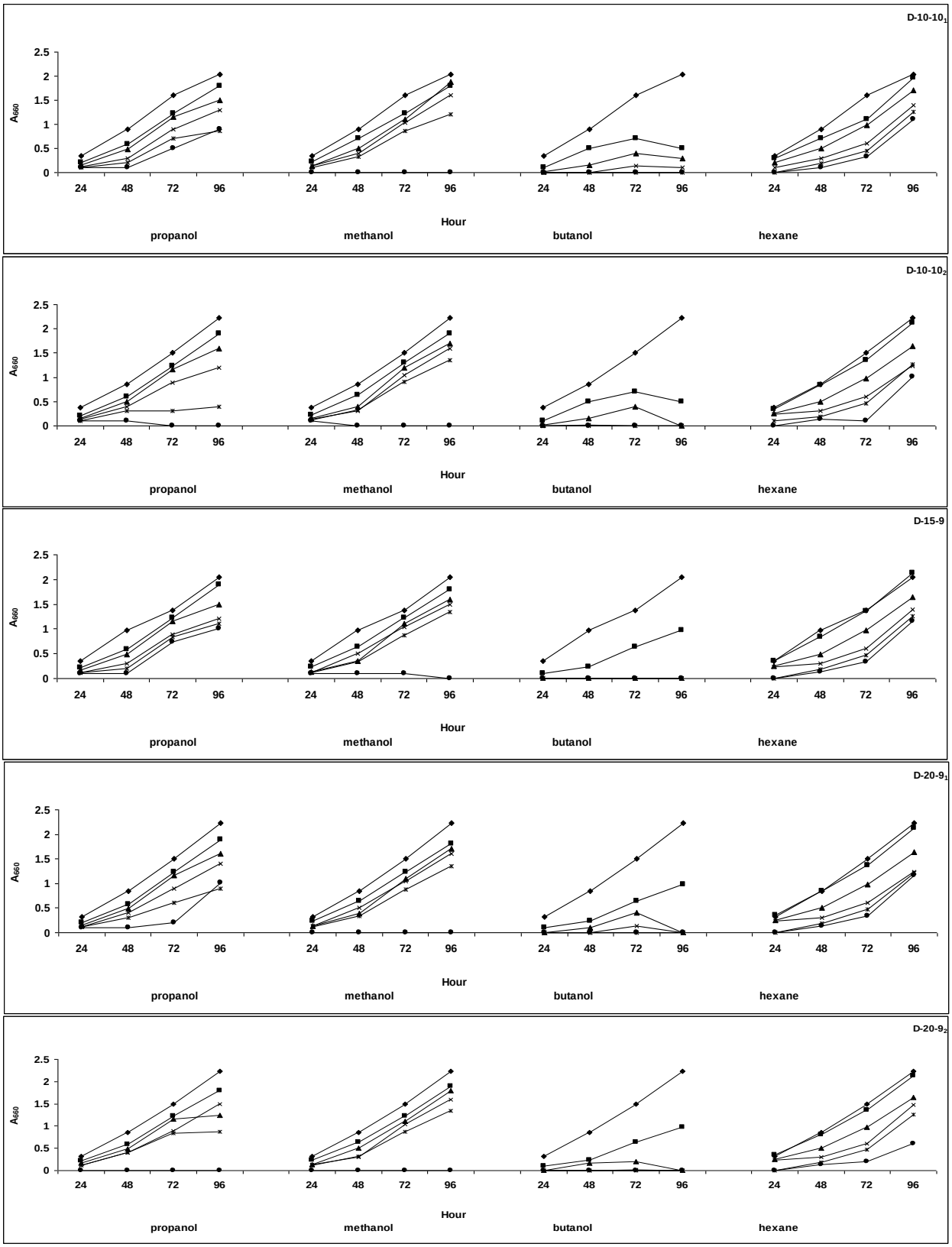
2
1.5
1
0.5
0
24
48
72
96
Hour
propanol
methanol
butanol
hexane


D-10-10$_1$
D-10-10$_2$
D-15-9
D-20-9$_1$
D-20-9$_2$
A_{660}
0
0.5
1
1.5
2
2.5
24
48
72
96
Hour
propanol
methanol
butanol
hexane

3.3.5 Diversity of organic solvent tolerant haloalkaliphilic isolates

The extents of solvent tolerance varied among the isolates from the same site as well as those from different sites**.** Among Okha isolates, 92% of the isolates were tolerating methanol, propanol and n-hexane, whereas 84% isolates tolerated butanol as additional supplement in CMB medium. In case of methanol 46 , 61, 76, 84 and 92 % isolates tolerated 1-5, 1-4, 1-3, 1-2 and 1 % (v/v) methanol, respectively . Better tolerance was recorded for propanol, where 53, 76, 69, 92 and 92% isolates of Okha sustained in the rage of 1-5 , 1-4, 1-3, 1-2 and 1 % (v/v) propanol, correspondingly. Among the Okha isolates, most effective tolerance was observed for n-hexane. With 1-5 , 1-4, 1-3, 1-2 and 1 % hexane , 69, 76,92,92 and 92 % isolates were able to maintain there growth.

For butanol, only 23% Okha isolates were tolerating 1-5% (v/v) solvent. For lower range of butanol, comparatively better tolerance was observed; 46 and 84% isolates were able to grow with 1-4 and 1-3 %(v/v) butanol, while in the presence of 1-2 and 1% butanol, 92% isolates exhibited their sustainability (Figure- 3.3A(a) and 3.3A(b)).

In Case of Jodiya isolates, all the isolates were able to tolerate hexane and propanol while 75 and 87% isolates were able to tolerate butanol and methanol respectively. At front of percent solvent tolerance, a broad diversity was observed. 62.62,75,100 and 100 % isolates were sustaining in presence of 1-5 , 1-4, 1-3, 1-2 and 1 % (v/v) methanol respectively. Best tolerances were noted down for solvent hexane, 100 % isolates maintained their growth at all 5 tested concentration of hexane. Jodiya isolates were effectively tolerant to propanol too. 62 and 76% bacteria were able to grow in presence of 1-5 and 1-4%(v/v) Propanol. Whereas at other tested concentration 100% isolates sustained nicely (Figure -3.3 B(a) and 3.3 B(b)).

A more diverse spectrum was observed in case of somnath's haloalkaliphilic isolates. At this site 88, 88, 75 and 88% isolates were able to grew in presence of methanol, propanol, butanol and hexane.

Here 85,91, 96, 100 and 100% haloalkaliphilic isolates were able to tolerate 1-5 , 1-4, 1-3, 1-2 and 1 % (v/v) propanol correspondingly. In case of butanol extra diversity were recorded as in presence of 1-5 , 1-4, 1-3, 1-2 and 1 % (v/v) butanol , 62,70,89, 85 and

100% isolates were able to sustain. Most of Isolate of Somnath were tolerant all examined concentration of methanol and hexane (Figure-.3.3 C (a) and 3.3 C (b))

Among Diu isolates, 91% isolates were able to tolerate butanol. With rest of tested solvent all the haloalkaliphilic bacteria of same site were able to maintain their growth. None of isolates of Diu were able to tolerate 1-5% (v/v) butanol. In presence of 1-4, 1-3,1-2 and 1 % butanol 16,61,84 and 100% isolate were able to tolerate. For tested concentration of propanol 33, 50, 66,100 and 100 % isolates exhibited tolerance. Comparatively better sustainability was observed in presence of methanol and hexane. Against the lower concentration of these two solvent all the isolates displayed growth (Figure- 3.3 D(a) and 3.3 D(b))

Maximum numbers of isolates were from Mithapur site. Almost 100% isolates of this site were able to sustain in presence of methanol, propanol, hexane and butanol. Eighty four , 87, 100, 100 and 100 % isolates were able to grow in presence of 1-5,1-4, 1-3, 1-2 and 1 % (v/v) hexane. In case of methanol 72, 66, 62,100 and 100 % haloalkaliphilic bacteria of Mithapur site were able to sustain their growth. Consideable level of tolerance were observed in presence of propanol too. Where as poor growth were recorded in presence of higher concentration of butanol , only 8 and 32% isolates were exhibiting growth in presence of 1-5 and 1-4% (v/v) butanol (Figure 3.3 E(a) and 3.3 E(b)). Comparatively less diversity for solvent tolerance were recorded for Veraval isolates. Except 93% for butanol, all the isolates of this site were maintaining their growth with rest of three solvents. In presence of 1-5 and 1-4% (v/v) methanol 85% isolates were able to grow and with remaining concentration of same solvent 100% bacteria exhibited tolerance. With hexane, 100% bacteria of Veraval site were exhibiting growth except in slot of 1-5% (v/v), 94% isolates were able to grow at this concentration. Propanol was most friendly solvent for the isolates of this site, all the bacteria were tolerant every tested concentration pf propanol (Figure . 3.3 F (a) and 3.3 F (b))

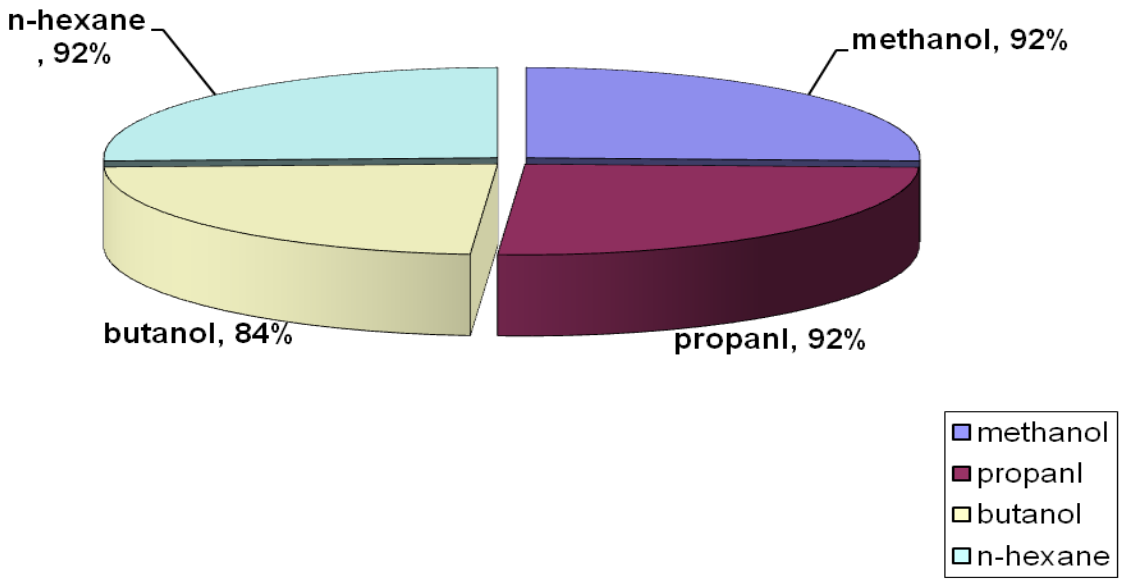


Figure 3.3 A- (a) Percent isolates of Okha site, tolerating particular solvents

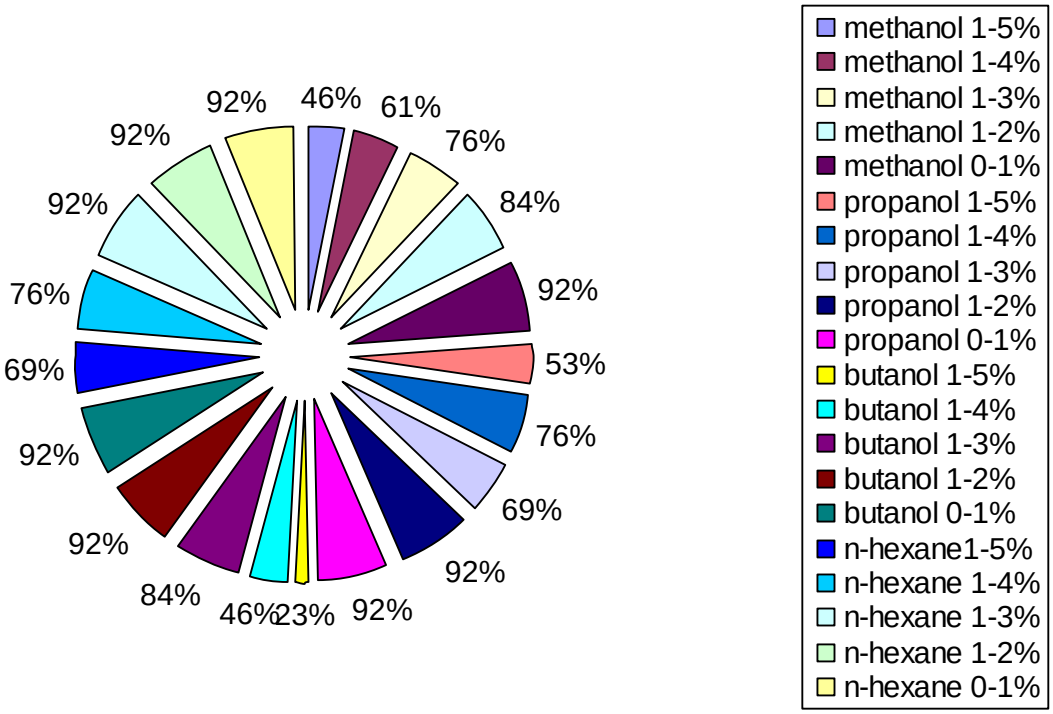


Figure 3.3 A(b)- Percent isolates of Okha , tolerating range of solvents

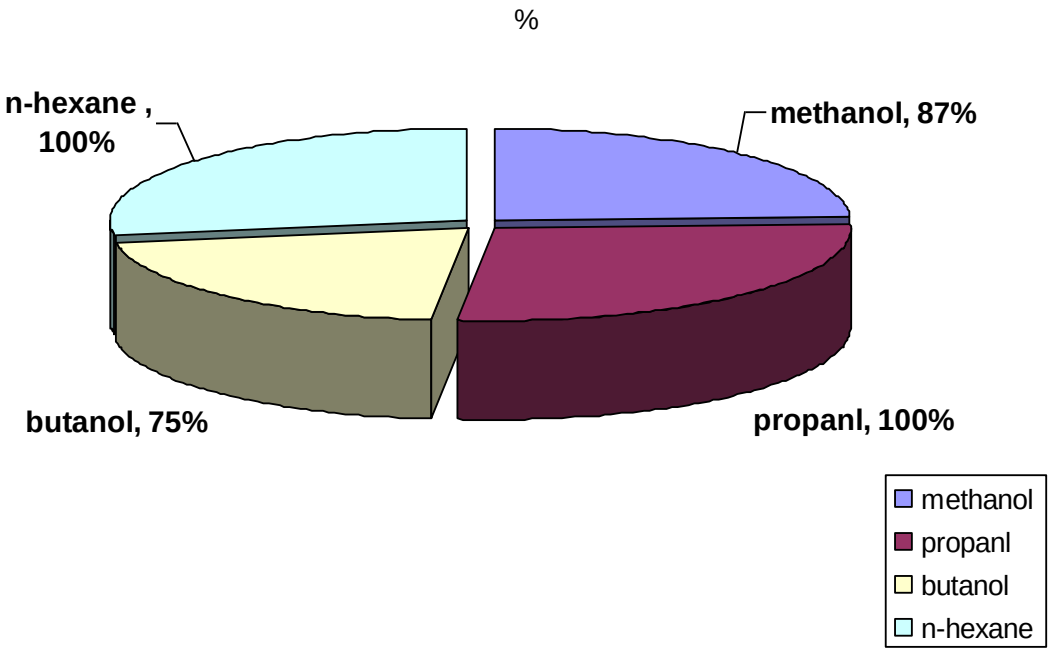


Figure 3.3 B (a)- Percent isolates of Jodiya site, tolerating particular solvents

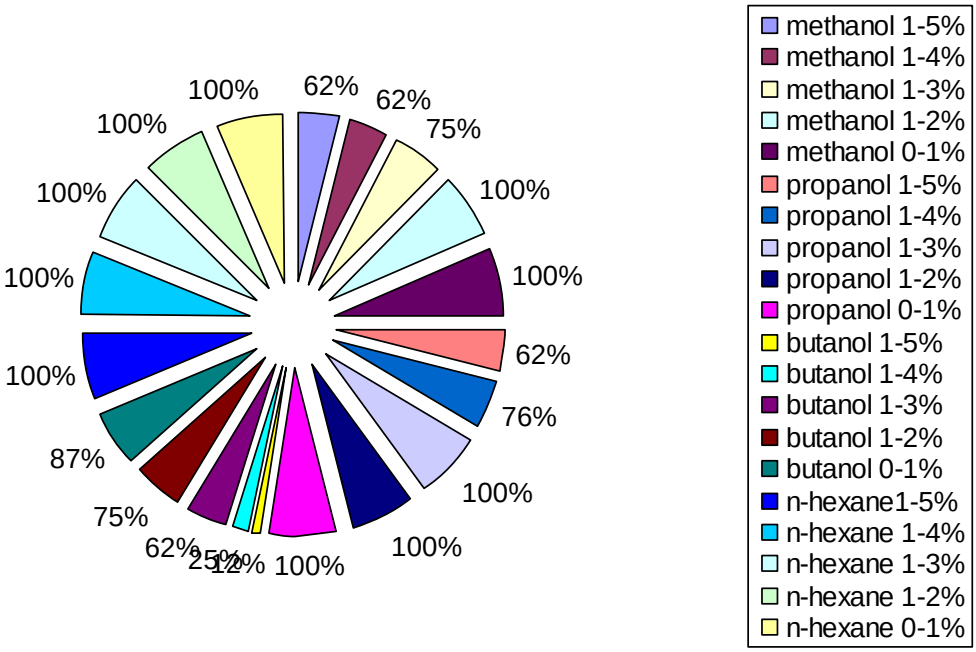


Figure 3.3 B (b)- Percent isolates of Jodiya, tolerating range of solvents

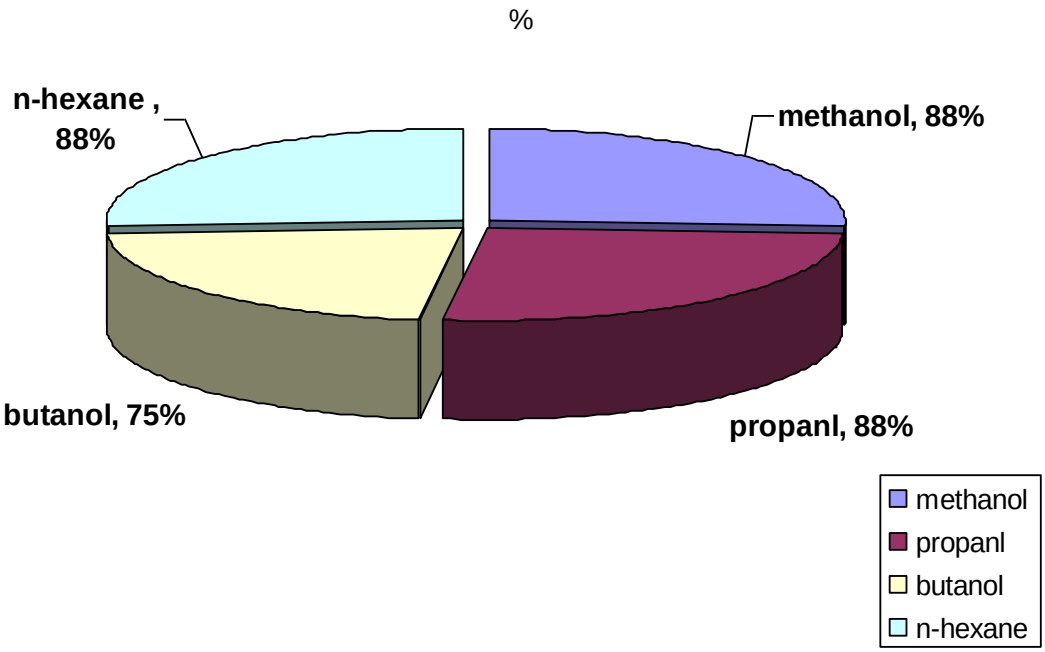


Figure 3.3 C (a)- Percent isolates of Somnath site, tolerating particular solvents

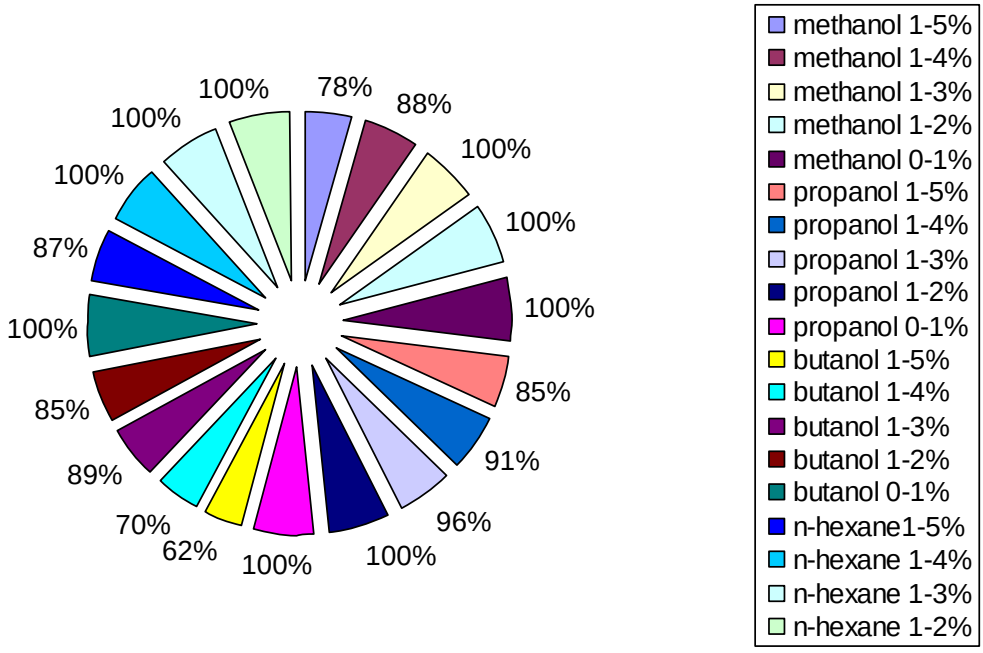


Figure 3.3 C (b)- Percent isolates of Somnath, tolerating range of solvents

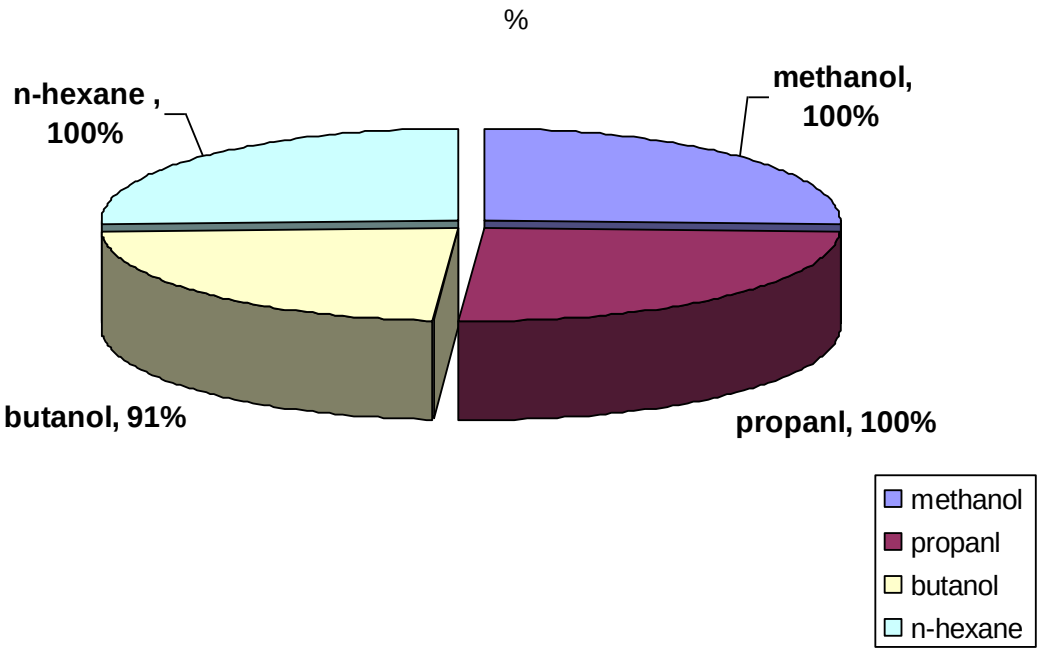


Figure 3.3 D (a)- Percent isolates of Diu site, tolerating particular solvents

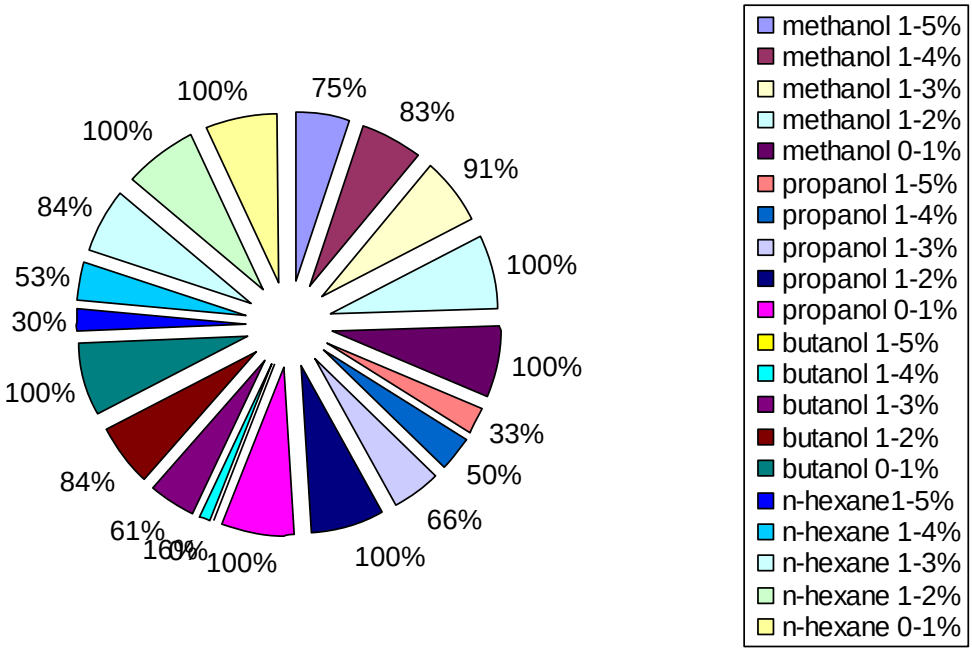


Figure 3.3 D (b)- Percent isolates of Diu, tolerating range of solvents

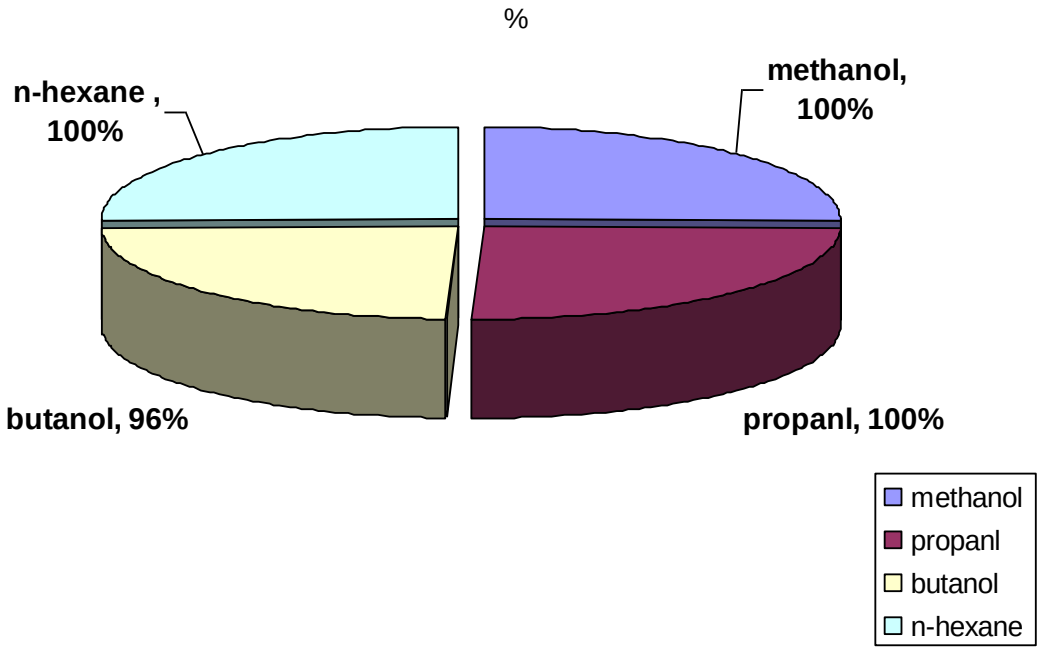


Figure 3.3 E (a)- Percent isolates of Mithapur site, tolerating particular solvents

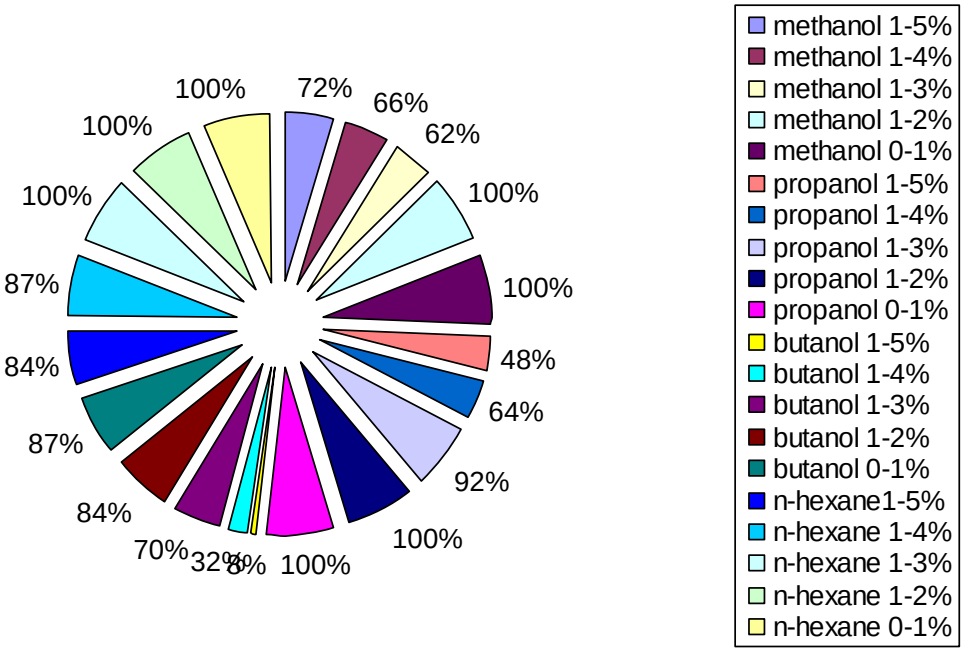


Figure 3.3 E (b)- Percent isolates of Mithapur , tolerating range of solvents

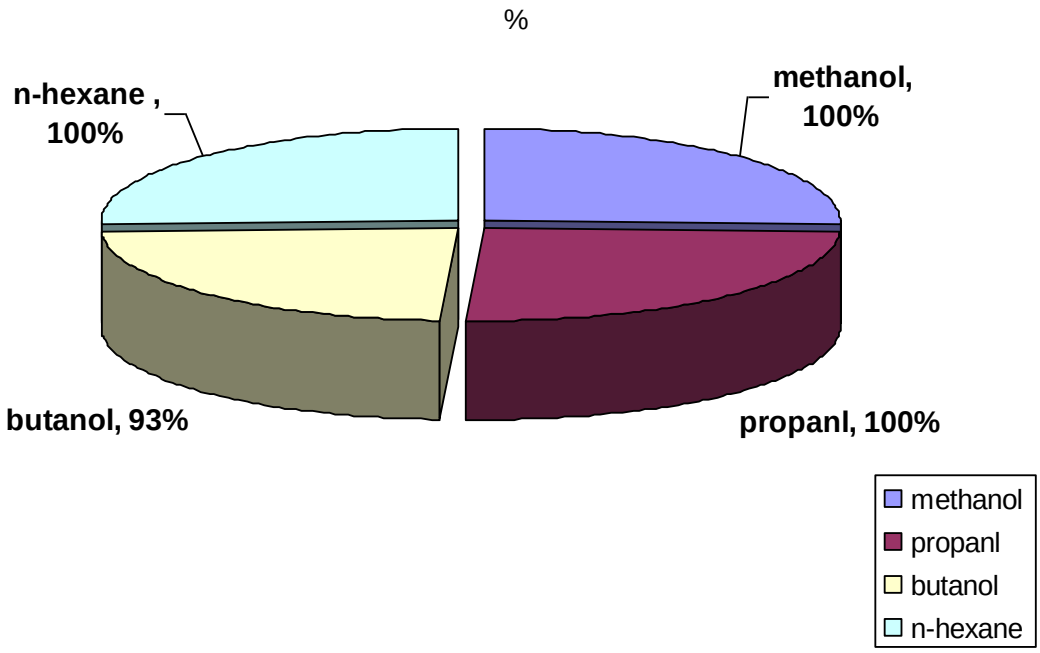


Figure 3.3 F (a)- Percent isolates of Veraval site, tolerating particular solvents

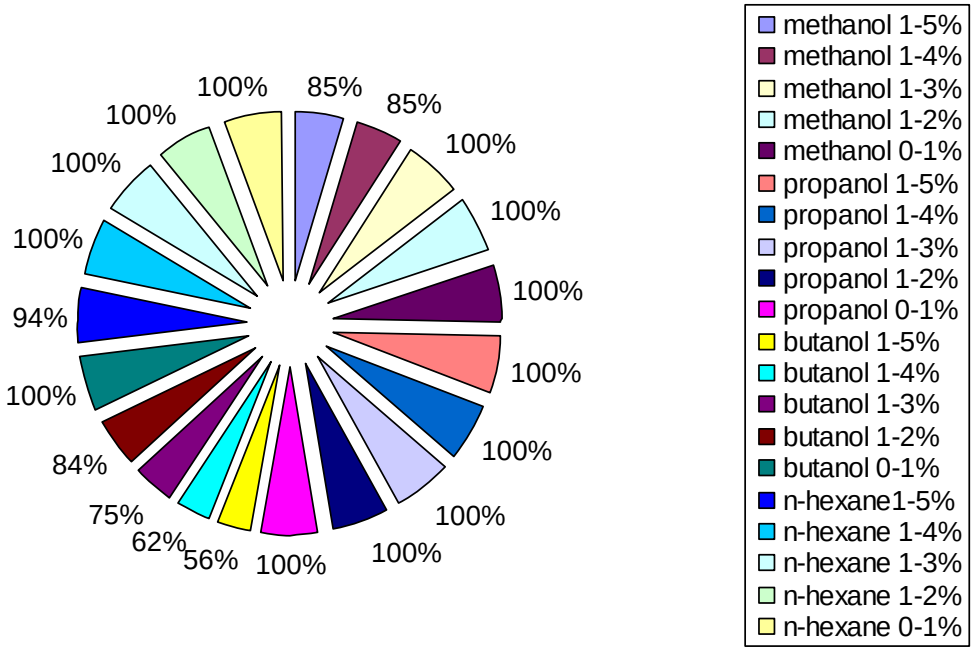


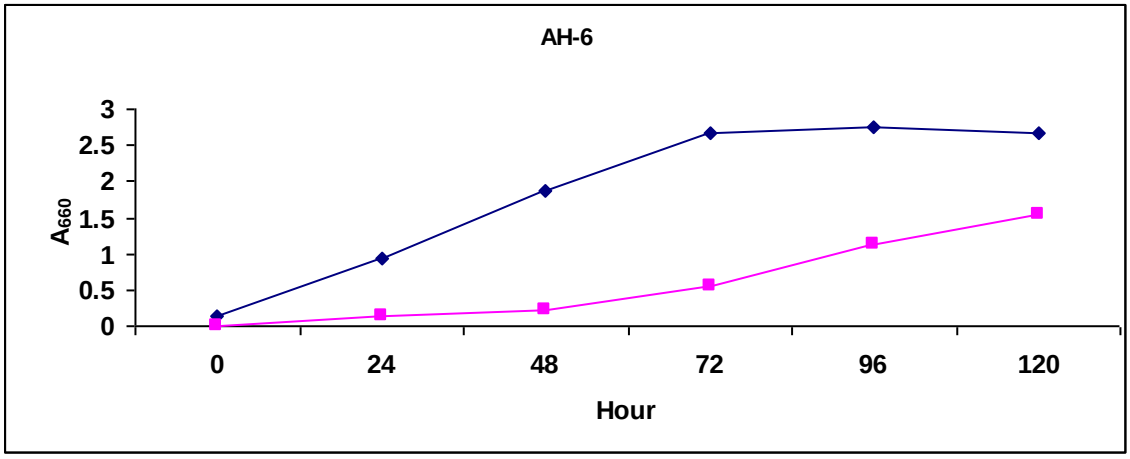
Figure 3.3 F (b)- Percent isolates of Veraval, tolerating range of solvents

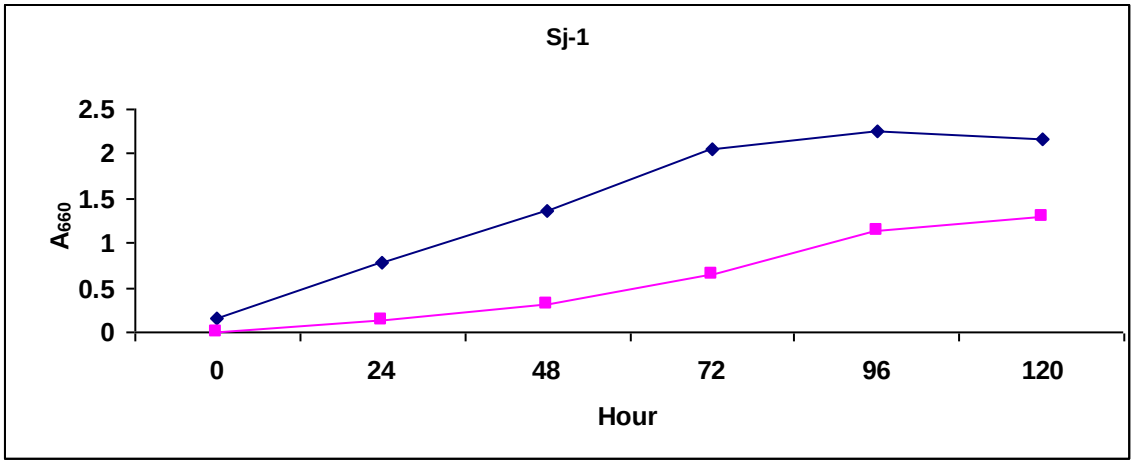
3.3.6 Growth pattern of haloalkaliphilic bacteria in presence of organic solvent

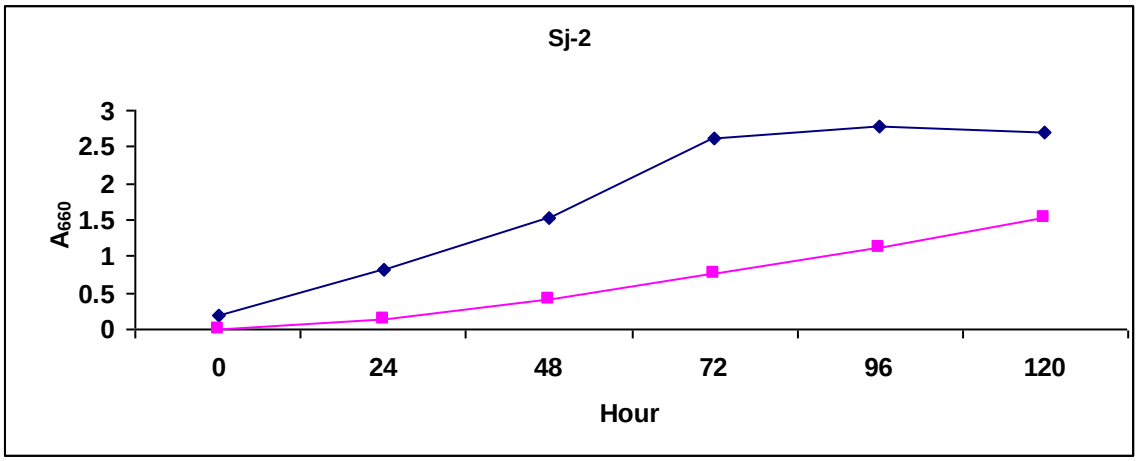
Twelve 16S r RNA sequenced isolates, which were expressing potent tolerance against organic solvent, were objected for growth pattern study. Growth of isolates AH-6, Sj-1, Sj-2, S-20-9, S-15-9, Ve2-10-8_2, Kh-10-10_1, Kh-10-9_1, Ve1-10-8_2, Ve1-10-8_3, Mi-10-6_2 were measured periodically in the presence of 20% (v/v) n-hexane. Two different type of growth pattern were observed. In first pattern the growth rate at exponential phas was considerably lower in the presence of n-hexane, but finally cell density reached above 80% of that in absence of n-hexane.

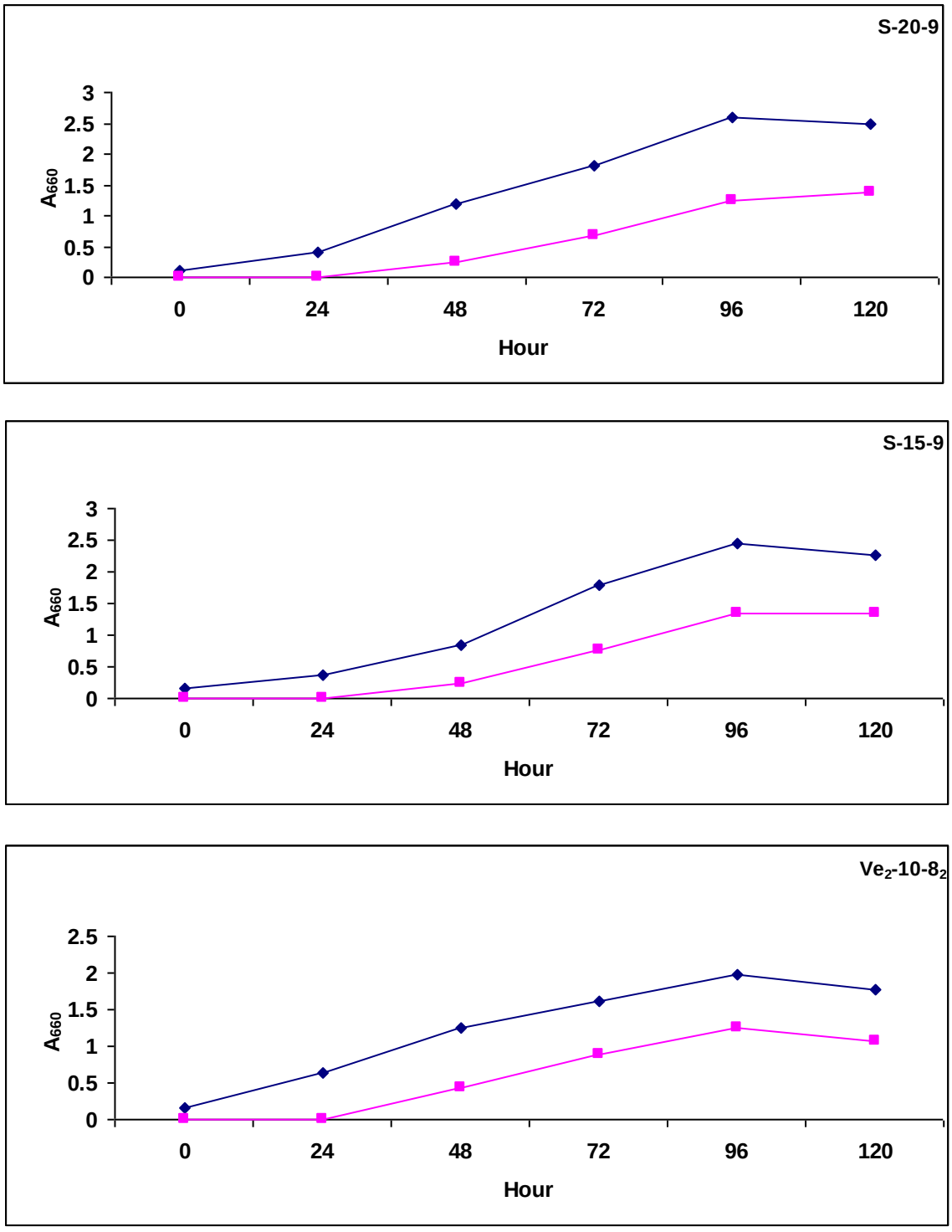
In second pattern both growth rate and finally cell density were low in the presence of n-hexane. In this type of growth pattern cell density in presence of n-hexane was not exceeding to 55 to 65% of control. Isolate Kh-10-10_1, Kh-10-8_2, Kh-10-9_1, Ve1-10-8_2, Ve1-10-8_3 and Mi-10-6_2 were fallowing first type of growth pattern. In exponential phase comparatively lower growth rate and cell density were recorded in presence of n- hexane and up to decline phase in presence of n-hexane, cell density was almost equal to control (Figure 3.4 A)

Isolates AH-6, Sj-1, Sj-2, S-20-9, S-15-9 and Ve2-10-8_2 were following the growth pattern 2. Reduced growth rate and cell density of these isolates were recorded in presence of n-hexane. With solvent exponential phase was starting somewhat delayed and final cell density of these isolate was not exceeding more than 65% of control (Figure 3.4 B).

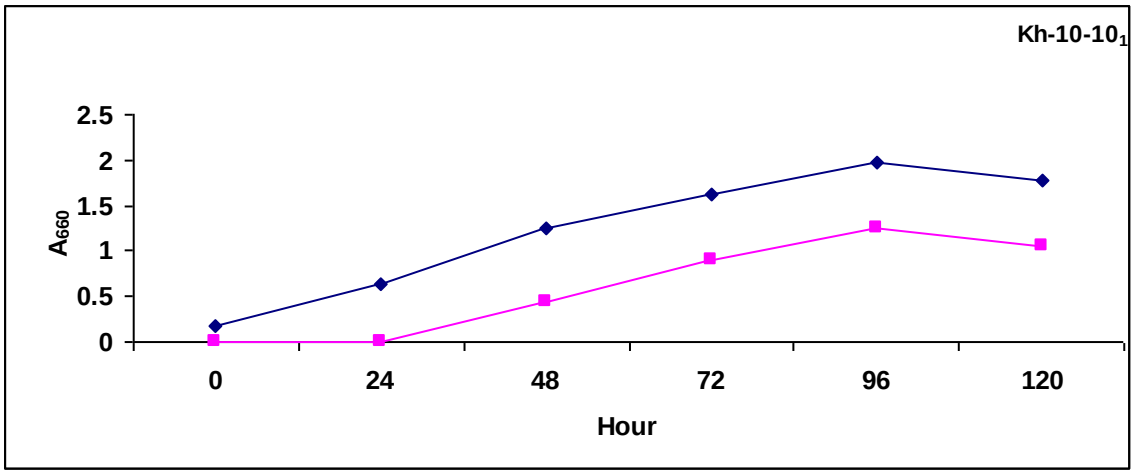

AH-6
A660
3
2.5
2
1.5
1
0.5
0
0
24
48
72
96
120
Hour

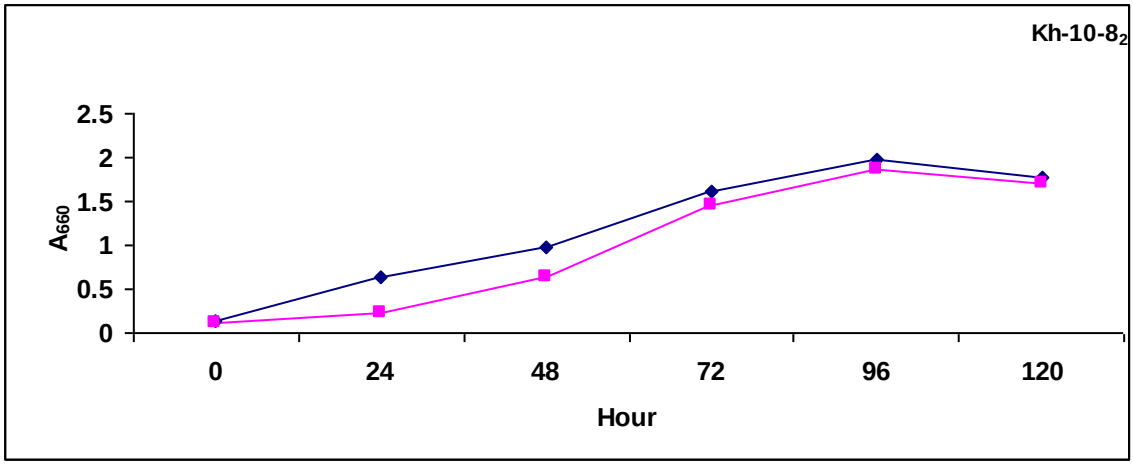

Sj-1
A660
2.5
2
1.5
1
0.5
0
0
24
48
72
96
120
Hour

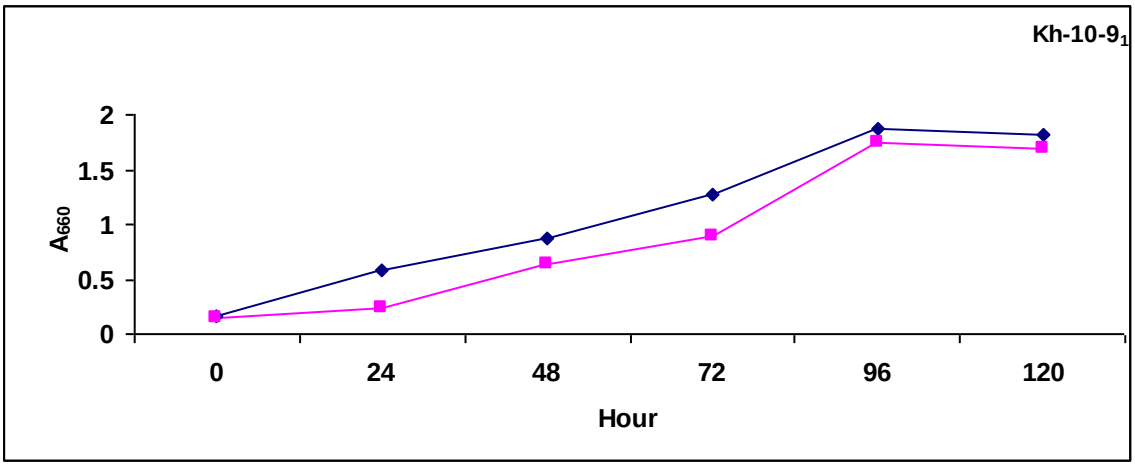

Sj-2
A660
3
2.5
2
1.5
1
0.5
0
0
24
48
72
96
120
Hour



Figare 3.4 A- Growth pattern 1. Growth of control (◊) and growth in presence of 20%(v/v) n-hexane(■)


Kh-10-10$_1$
2.5
2
1.5
1
0.5
0
A_{660}
0
24
48
72
96
120
Hour


Kh-10-8$_2$
2.5
2
1.5
1
0.5
0
A_{660}
0
24
48
72
96
120
Hour


Kh-10-9$_1$
2
1.5
1
0.5
0
A_{660}
0
24
48
72
96
120
Hour

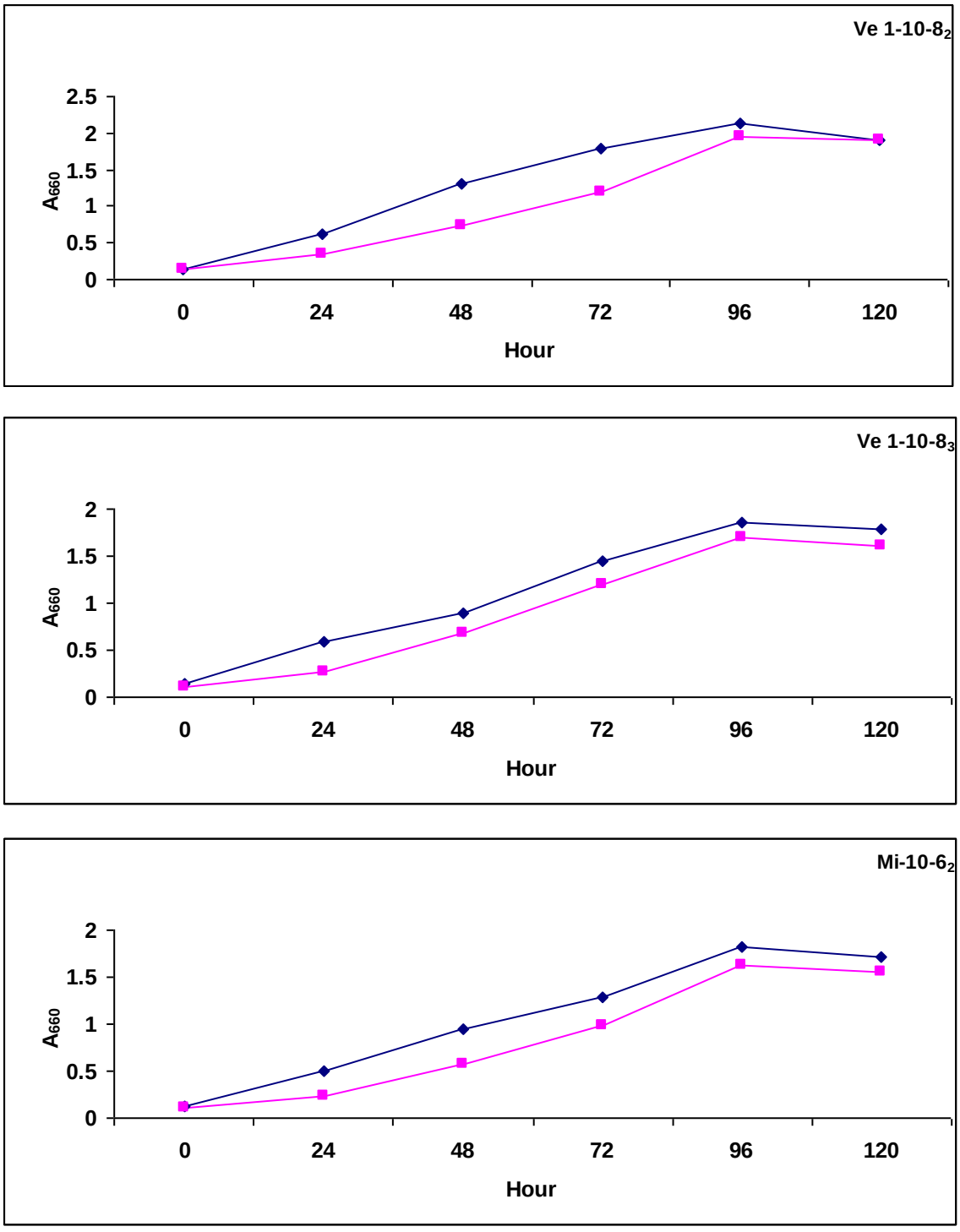
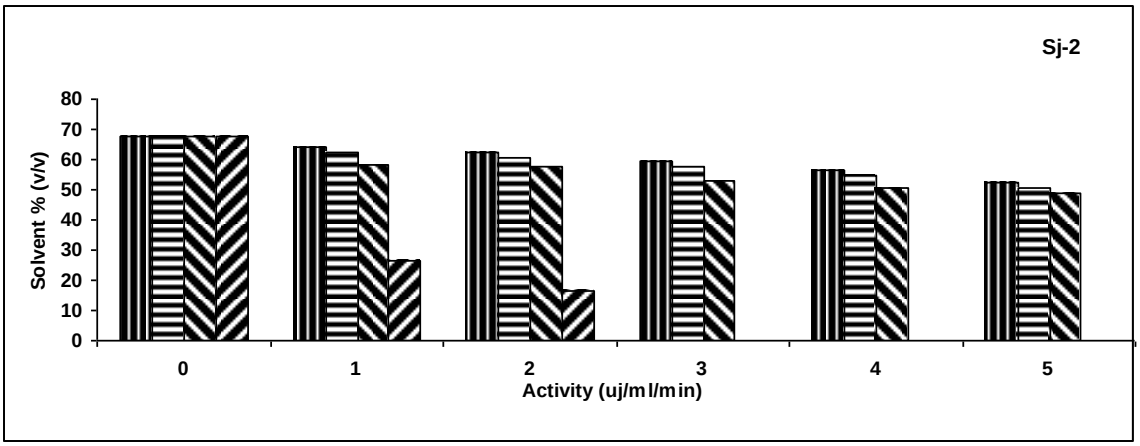


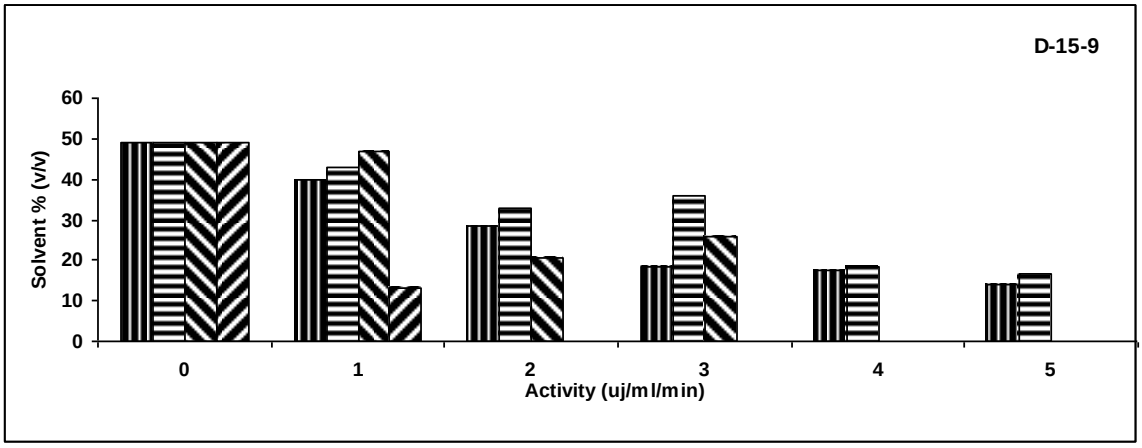
Figure 3.4 B – Growth pattern 2 . Growth of control (◊) and growth in presence of 20%(v/v) n-hexane(■)

3.3.7 Effect of organic solvent on protease production

Protease production profile was not varying much in presence of organic solvent. In presence of all tested solvents reduced Production were observed. In presence of butanol , production was poor for all examined isolates. D-15-9 was able to secret considerable protease in presence of methanol and propanol. While In case of n-hexane, secretion was observed only up to 3% (v/v).

Isolates of Jodiya Sj-1, Sj-2 and AH-6 were potent producer of protease. Significant protease productions were recorded in presence of methanol, propanol and n-hexane. Protease Production was inhibited in presence of butanol, (Figure 3.5.)





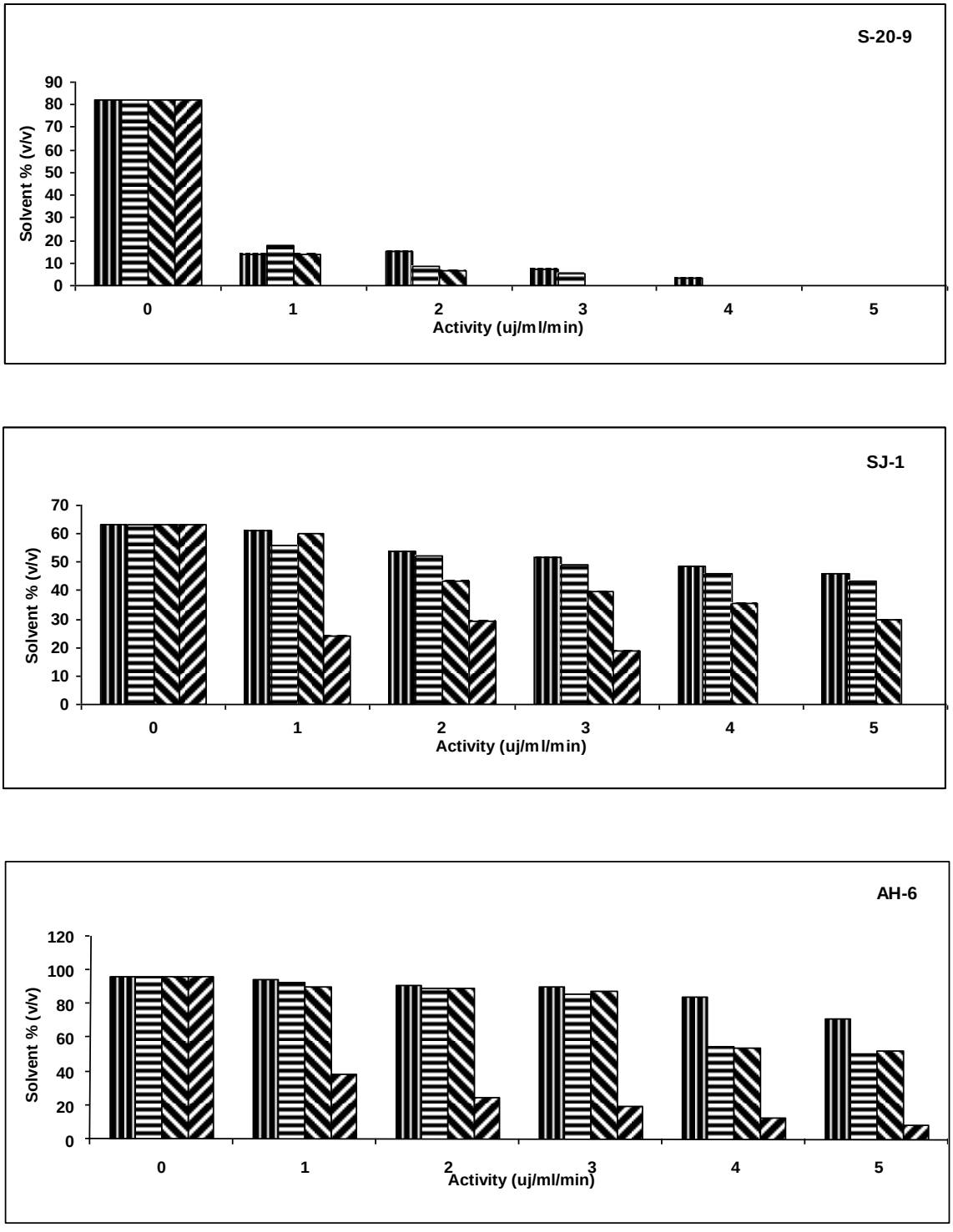


Figure 3.5- Effect of methanol(▥)), propanol (▤), n-hexane (▧) and butanol(▨) on Protease production

3.3.8 Phylogenetic analysis of the 16S rRNA sequences

On the basis of 16S rRNA gene sequencing, 18 strains were classified in kingdom Bacteria. Their identifications on the basis of gene homology and distance matrix along with their accession number are listed in Table 3.2.

No.	Isolate	accession no. and Identified strain
1	S-15-9	*GU059918 Haloalkaliphilic bacterium*
2	Sj-1	*GQ162111 Oceanobacillus sp.*
3	Sj-2	*EU090232 Bacillus pseudofirmus strain*
4	AH-6	*EU118361 Haloalkaliphilic bacterium*
5	S-20-9	*EU118360 Haloalkaliphilic bacterium*
6	Ve2-10-10	*EU118360 Haloalkaliphilic bacterium*
7	Ve2-15-91	*HM047796 Oceanobacillus onchorynchy strain*
8	Ve2-20-92	*HM047797 Oceanobacillus iheyensis*
9	D-15-9	*HM047795 Halophilic and alkaliphilic*
10	D-20-91	*HM047798 Oceanobacillus onchorynchy*
11	Ve_2-10-8_2	*EU604320 Haloalkaliphilic bacterium*
12	Kh-10-10_1	*DQ026060 Bacillus okhensis*
13	Kh-10-91	*EU684463 Halomonas venusta*
14	D-10-102	*GU059919 Haloalkaliphilic bacterium*
15	Ve1-10-82	*GQ121034 Oceanobacillus oncorhynchi*
16	Ve1-10-83	*EU6484464 Halomonas aquamarina strain*
17	Ve2-10-91	*HM047794 Haloalkaliphilic bacterium*
18	Mi-10-62	*GQ121032 Bacillus agaradhaerens*

Table 3.2 Phylogenetic relatedness and Gene accession of 16S r RNA sequenced isolates

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While performing CLUSTAL W for our 18 isolates, total eleven minor and 3 major clusters were obtained. Briefly describing the clusters, the first top cluster belonged to genera haloalkaliphile. In this cluster, 5 roots (species) were observed and the organisms were able to tolerate organic solvents. The second major cluster included 3 genera: *Ocenobacillus, Bacilli* and Haloalkaliphilic group. Significant variation was evident from geographical point of view. Most potent isolates on front of solvent tolerance and protease production in presence of solvents were from this cluster. Third and last cluster had very few species which belonged to halobacilli and haloalkaliphilic sps. In this cluster, all the species were able to produce protease (Figure 3.6).

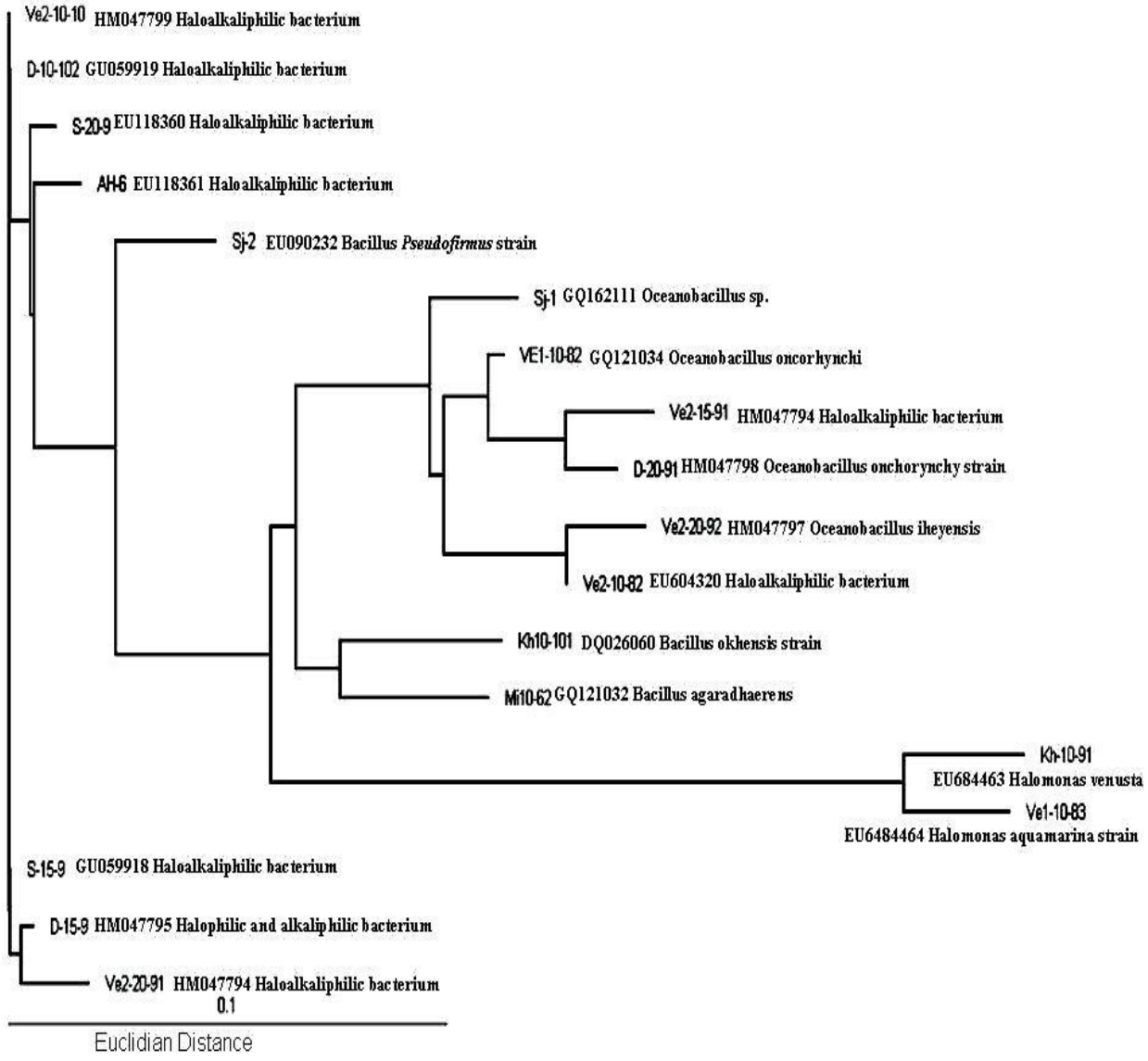

Figure 3.6- Phylogram for our 16S r RNA sequence constructed by CLUSTAL W

3.3.9 Scanning Electron Microscopy (SEM) of solvent tolerant bacteria

SEM analysis was carried out for isolates: Kh-10-9_1 S- from Okha. Freshly grown Isolate in presence of 30% actone and control were subjected for SEM Analysis. From SEM analysis it was confirmed that the organism was rod shaped. After growing in the presence of 30% acetone, enlarged cell size was observed. In control, Kh-10-9_1 cell size was in the range of 896 nm to 2.88 um (Figure 3.7 A), while after growth in the presence of acetone, the cell size ranged 5.51 to 7.11 um (Figure 3.7B).

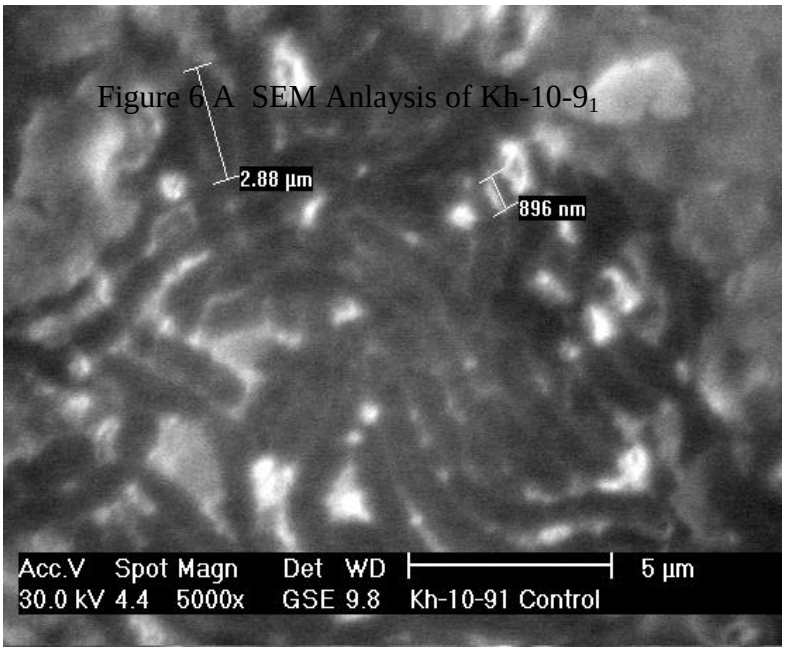

Figure 6 A SEM Anlaysis of Kh-10-9_1

Figure 3.7A - SEM Anlaysis of Kh-10-9_1 grown with out solvent

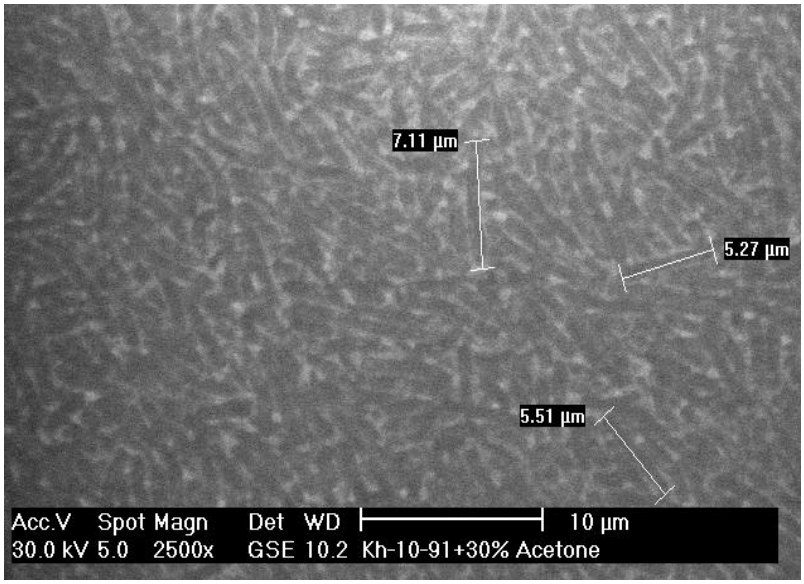

Figure 3.7 B- SEM Anlaysis of Kh-10-9_1 grown with 30 % Acetone

3.4 DISCUSSION

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In the present study, possibilities of a range of applications for haloalkaliphilic bacteria were highlighted in biotechnology and bioremediation. The degradation or transformation of organic solvents as pollutants and the production of alternative energy are some of the recent and important fields of extremophiles. The biodegradation (transformation or mineralization) of a wide range of hydrocarbons, including aliphatic, aromatic, halogenated and nitrated compounds, has been shown to occur by some halophilic and haloalkaliphilic microorganisms (Moriya and Horikoshi, 1993b; Abe *et al.,* 1995). Therefore, study of organic solvent tolerance in the presence of high salt concentrations at alkaline pH appears to be quite attractive preposition for the bioremediation of oil-polluted salt marshes and industrial wastewaters, contaminated with aromatic or chlorinated hydrocarbons (Ushami *et al*., 2003).

Organic solvents are regularly utilized in many industries and can be toxic to the microbes, Based on the inherent toxicity of the solvents and the basic tolerance of the bacterial species and strains, their log *Pow* symbolize the toxicity. Greater the polarity of a solvent, the lower its log *Pow* value and the greater its toxicity. In general, solvents with log *Pow* values below 4 are considered extremely toxic. Solvent tolerance is a strain-specific property and every microorganism has a limiting log *Pow* value below which it is unable to grow.

In general, the concentrations of the solvents supplied to these microorganisms are extremely low (Ooyama and Foster, 1965; Yamada *et al*., 1965; Arai and Yamada, 1969; Bean and Perry, 1974; Stiriling and Watkinson, 1977; Kanemitu, 1980; de Carvalho *et al*., 2004; Fukushima *et al*., 2005). However, during the last several years, many highly organic solvent-tolerant microorganisms have been isolated and described (Inoue and Horikoshi, 1989; Cruden *et al*., 1992; Moriya and Horikoshi, 1993a, 1993b; de Carvalho *et al*., 2004; Schneiker *et al*., 2006). These strains are capable of growing in the presence of a large volume of p-xylene and toluene.

Gujarat (Western India) posses the major portion of coastline covering 1600 Km long shore, with industrial activities of many projects, huge amount of organic solvents is released into the atmosphere. Existence of halotolerant, haloalkalitolerant and haloalkaliphilic bacteria clearly indicated the wide spread distribution of such organisms in natural saline environment beyond the conventionally described habitats of Salt Lakes, solar salt evaporation ponds and salt deserts.

Extensive work has been done on the organic solvent tolerance of other domain of microbes, but only limited information are available on the solvent tolerance of haloalkaliphilic bacteria. In order to explore the haloalkaliphilic microbes for bioremediation of organic solvent pollutants, 1 to 5 % (v/v) of 4 solvents: methanol, propanol, n-hexane and butanol were added in growth medium of 82 haloalkaliphilic bacteria isolated from the saline habitats of Coastal Gujarat.

Most of isolates displayed growth in the tested solvents. In complex medium, Kh-10-9_1 tolerated butanol along with methanol, propanol, methanol and n-hexane. These result are quiet interesting as with 0.8 Log $P_{ow,}$ butanol is one of the most toxic solvents and the results suggested it's potential for bioremediation. The findings resembled with a toluene resistant strain *Pseudomonas putida.* The bacteria tolerated 30% toluene (Inoue and Horikoshi, 1989). In another study, *Mycobacterium* sp.NRRL B-3805 displayed tolerance to butanol (de Carvalho *et al*., 2004). Except Kh-10-$9_{1,}$ none of the isolates effectively tolerated butanol. This trend was similar to earlier work by Gimenez *et al.,* 2000).

Isolates of all 6 isolation sites: Somnath, Veraval, Okha, Mithapur, Diu and Jodiya grew well with n-hexane. The results resembled with the work of Ogino (Ogino *et al.,* 1995) in which *P. aeruginosa* PST-01 tolerated solvents of similar range of log P_{ow}. Mixed trends were recorded for methanol and propanol. A halotolerant *Streptomyces* sp, isolated from an oil field in Russia, has been reported to degrade crude petroleum (Nicholsan, *et al*., 2003). It did not grow in the presence of organic solvent with log *Pow* values equal or less than 2.5. However, the tolerance of *Pseudomonas aeruginosa* PST-01 was relatively

less than that of earlier described strain *Pseudomonas aeruginosa* LST-03 (Ogino *et al*., 1994). *Escherichia coli* strains, in general, are highly sensitive to organic solvents and can only survive in the presence of solvents with a log *Pow* greater or equal to 4 (Aono and Horikoshi, 1997).

Enzyme production was greatly inhibited by tested solvents. Poor Protease productions were recorded by many potent isolates as S-20-9 and D-15-9. Ogino and coworkers (1995) reported the delayed and reduced production of proteolytic enzymes in the presence of cyclohexane in *Peudomonas aeruginosa* PST-01, where delayed enzyme production was due to direct effect of cyclohexane on the bacterium.

Our some isolate were able to produce protease potently in the presence of all 4 solvents planned for screening. AH-6, Sj-1 and Sj-2 are potent protease producers. Production was reduced with organic solvents. The trends are quite similar to earlier reports (Ogino *et al.*, 1995; Gupta *et al.*, 2006). In another report (Geok *et al.,2002*), protease from *Pseudomonas aeruginosa* strain K was not active in organic solvents, with log P values, equal to or lower than 4.0. As mentioned earlier, the lower the log P, the greater the polarity value and the greater the toxicity of the solvent to the enzyme. Generally, solvents with log P values below 4 are considered extremely toxic as their degrees of partitioning into the aqueous layer are higher (de bont *et al.*, 1998). In comparision to some of these reports, the haloalkaliphilic bacteria in the present study sustained the production of protease with lower log P ow solvents.

In context to growth in the presence of solvents, two different types of growth pattern were recorded. In first type, growth rate and cell density were lower in the presence of solvent, while final cell density reached more than 80% of control. In the second type of growth pattern, the cell density and growth rate both did not more than 65% of the control. Difference in growth pattern can be related with genes responsible for organic solvent tolerance.

Analysis of the 16S rRNA gene sequences provides a strong footing and is currently being used as a powerful tool in molecular chronology. On the basis of 16S rRNA

nucleotide sequencing, 18 haloalkaliphilic isolates were analyzed and diversity was judged.

Isolates Kh-10-9_1 grew in the absence and presence of 30% (v/v) acetone were subjected for scanning electron microscopy to access structural alteration in cell. Cell size was enlarged by 2 to 2.5 fold, which clearly indicated the formation of vesicles for the storage and elimination of entered organic solvents into the cell.

Formation of vesicles for storage of toxic solvent has been reported by Cruden (Cruden *et al.,* 1992) and Gupta (Gupta *et al.,* 2005) for *Pseudomonas* sp. cells grown in *p*-xylene and *Enterobacter* sp. grown in the presence of cyclohexane, respectively. Solvents are reported to damage the integrity of cell membrane structure. This causes loss of permeability regulations. In extreme cases, leakage of cell RNA, phospholipid and protein also takes place (Sikkema *et al.,* 1995). Solvent tolerant cells adapt by making changes in fatty acid composition and protein/lipid ratio in cell membrane to restore the fluidity (Isken and debont, 1998).

To emphasize, the haloalkaliphilic bacteria and their extracellular haloalkaliphilc enzymes may tolerate organic solvent and thus can be subjected to bioremeadation where high salt and concentrations along with pH and organic solvents are present. The organic solvent tolerant haloalkaliphilic bacteria may be able to bioremeade at organic solvent pollution and their solvent stable protease may be proved valuable for peptide synthesis under non-aqueous conditions, which otherwise would be thermodynamically unfavorable in water. Further, the study as a whole appears quite interesting as haloalkaliphilic organisms have rarely been explored for their tolerance under organic solvent.

Optimization of Amylase catalysis in the presence of organic solvent

4.1 INTRODUCTION

Starch is one of the most abundant polymers in nature and amylases play central role in its utilization. Amylases are produced by eukaryotes, prokaryotes and archaea displaying its diverse nature (Upadek *et al.,* 1997). The amylases are significant in many industrial processes, such as starch liquefaction, pulp process and in detergent. (Kadziola *et al.,*1998 and Machius *et al.,* 1995).

Amylases have increasingly become one of the most valuable enzymes in biotechnology, particularly in the food and starch processing industries . Besides, food and starch processing industries require a huge quanity (Vihinen *et al.,* 1994). Therefore, with the involvement in many industries, amylases have emerged as the key enzymes of biotechnological significance. Haloalkaliphiles are an attractive group of Extremophiles, having ability to survive under saline and alkaline conditions. With these feature, such organisms provide unique system for investigating biocatalysis under multitude of extremities.

Haloalkaliphiles are a class of extremophilic organisms adapted to saline and high pH conditions (Dodia *et al.,* 2008 and Gupta *et al.,* 2005). Haloalkaliphilic proteins are stable at high salt concentrations due to their innate habitat at saline environment and special arrangement of amino acids (Lanyi *et al.,*1974, Eisenberg *et al.,* 1992, Madern *et al.,* 2000 and Mevarech *et al.,* 2000). With comparatively larger number of negatively charged amino acids on surface, halophilic proteins display hydrophobic characteristics in contrast to non-halophilic proteins. This feature is beneficial to avoid precipitation of enzyme as well as maintains structural flexibility with organic solvents (Mevarech *et al.,* 2000). Noticeably, haloalkaliphic proteins need high salt for activity and stability and majority get unfolded and inactivated at less than 1–2 M NaCl or KCl (Madern *et al.,* 2000). High salt creates a hydrophobic environment and thus, haloalkaliphilic enzymes are suitable for biocatalysis under non-aqueous conditions.

While many amylases are reported from microbial sources, only a few haloalkaliphilc bacteria are known in this context. In particular, studies on the organic solvent tolerance among haloalkaliphilc amylases are nearly non-existent.

Due to exceeding boundaries of biotechnology, requirement of variety of organic solvent tolerant enzymes has enhanced. Demand of organic solvent tolerant amylases is greatly enhanced due to their significance in clinical, medicinal and analytical sectors. Present work focused on the catalytic potential of a haloalkaliphilc amylase in the presence of organic solvents.

In the view of above perspectives, optimization of the haloalkaliphilic amylases with reference to various organic solvent is of utmost significance. Studies presented in this chapter, deal with the solvents effect on haloalkaliphilic amylases isolated from costal belt of Gujarat. We believe that results would be highly significant in scaling up the process to commercial level

4.2 MATERIAL AND METHODS

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4.2.1 Microorganism and culture conditions

For the isolation of amylase producing haloalkaliphilic bacteria, mud and sea water samples were collected from Okha (22°28′0″N 69°4′0″E), Diu (20°43′N 70°59′E20.71°N 70.98°E) and Veraval (20°54′N 70°22′E20.9°N 70.37°E) along the Costal Gujarat. Samples were subjected to enrichment culture techniques in Complex Medium Broth (CMB) consisting, (g/liter): Glucose, 10; Peptone, 5; Yeast extract, 5; KH_2PO_4, 5; with varying concentration of NaCl (10-20%, w/v) at different pH 8-10.

The pH of the medium was adjusted by adding separately autoclaved Na_2CO_3 (20%, w/v). After inoculation, flasks were incubated on environmental shaker at 37°C with regular monitoring on the turbidity. After 48-72 hour of growth, a loop full culture was streaked on the CMB agar (3%, w/v) plate and incubated at 37°C. After 48 hour of the incubation, on the basis of colony characteristics, various isolated colonies were selected and pure cultures were obtained by subsequent streaking on the CMB agar plate.

For screening of amylase producing isolates, actively growing cultures were prepared as mentioned above and inoculated on starch agar plates (g/liter: Starch, 2; Yeast extract, 3; Peptone, 5; NaCl, 100, pH 8-10; Agar, 30) as spot. The plates were incubated for 24-48 hour at 37°C and after sufficient growth, amylase producing bacteria appeared on the starch agar plate were picked and preserved at 4°C.

4.2.2 Amylase production and assay

From activated culture of amylase producing isolate ($A_{540;}$ 1.0), 5% was inoculated into Starch medium (g/liter: Starch, 2; Yeast extract, 3; Peptone, 5; NaCl, 100, pH 9). Culture was harvested after 12 hour, which was pre standardized for maximum amylase production. The culture was centrifuged at 8,000 rpm for 10 min at 4°C and the cell free extract was used as crude enzyme preparation. Amylase was measured by

estimating reducing groups released from starch, by the reduction of 3, 5-dinitrosalicylic acid (DNS) with slight modification of Bernfeld (12) method. 0.5ml enzyme was added to 1ml (2%, w/v) starch prepared in NaOH-Borax buffer (20mM, pH 10) and incubated at 37°C for 20min. One ml of DNS reagent (g/liter: DNS, 10; Sodium potassium tartarate, 300; Sodium hydroxide, 16) was added to the mixture and kept in boiling water bath for 10min. After cooling, the mixture was diluted with 8 ml distilled water and absorbance measured at 540nm. Unit of amylase activity was defined as 1µg of maltose liberated by enzyme from starch per minute.

4.2.3 Organic Solvents

Methanol, butanol, propanol and n-hexane were selected for non-aqueous studies on amylase, based on their Log P_{ow} values (0.9, 0.25, 3.9 and 0.8), hydrophobicity and hydrophilicity.

4.2.4 Screening of amylase for nonaqueous condition

Amylases of 8 bacterial isolates; Mi-10-6_2, Kh-10-101, Dj-30-8_1, Ve1-10-8_1, Ve1-10-8_2, Ve1-10-8_3, Ve1-10-9_1, Ve1-10-9_2 and Ve1-10-9_3 were selected for screening of solvent tolerance. Kh-10-9_1, Dj-30-8_1, Mi-10-6_2 were isolated from Okha (22°28′0″N 69°4′0″E) , Diu (20°43′N 70°59′E20.71°N 70.98°E / 20.71; 70.98) , Mithapur (22°30′0″N 68°4′0″E) respectively, while Ve1-10-8_1, Ve1-10-8_2, Ve1-10-8_3, Ve1-10-9_1 , Ve1-10-9_2 and Ve1-10-9_3 were from Veraval (20°54′N 70°22′E20.9°N 70.37°E). Amylase activity was measured in a reaction mixture of 0.5 ml enzyme and 1 ml starch solution (2%, w/v) prepared in NaOH-Borax buffer (20mM, pH10) with 5, 10 and 20% (v/v) of methanol, propanol, n-hexane and butanol. The enzyme estimation was performed as describe above. Controls of each set were also included.

4.2.5 Effect of pH on activity and stability of Amylase

Amylase catalysis in the presence of organic solvents was assessed at different pH, using buffers (20mM); Sodium Phosphate (pH 5.5 - 8), Tris-HCl (pH 8 – 9.5), NaOH-Borax (pH 9.5 - 10) and Glycine - NaOH (pH 8 - 12). The enzyme was incubated with 5, 10 and 20% (v/v) of methanol and butanol along with respective buffer. To investigate solvent's effect on stability of the enzyme at different pH, the pH adjusted to 5-13 with above

buffers in the presence of 20% methanol. After incubation for 30-180 minute, residual activities were estimated.

4.2.6 Effect of NaCl on activity and stability of Amylase

The effect of NaCl and organic solvent in conjunction on enzyme activity was assessed by supplementing the reaction mixture with 0.5- 4M NaCl. Amylase assay was carried out at 37°C with 5, 10 and 20% (v/v) of the tested solvents.

For the study of the stability of amylase in response to salt and solvent, the enzyme was incubated with NaCl in the range of 0- 4M NaCl and the aliquots were withdrawn at regular time intervals for monitoring residual activity. The amylase activity in the absence of NaCl was considered as a 100%.

4.2.7 Effect of Temperature activity and stability of Amylase

The temperature profile for amylase activity was examined by incubating the assay reaction mixture at different temperatures in the range of 37-80°C. The amylase activity was determined as mentioned above. The temperature stability was studied by incubating the enzyme at different temperatures (37-80°C). The aliquots were withdrawn at 30, 60, 90, 120 and 180 min and reaction mixture was incubated at optimum temperature. The residual enzyme activities were measured.

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4.3 RESULTS

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Amylase catalysis in the presence of organic solvents

Amylases of all isolates were able to catalyze the reaction in presence of all tested solvents. Amylases from Dj-30-$8_{1,}$ Ve1-10-$8_{2,}$ Ve1-10-8_3 and Ve1-10-9_3 were quiet active with 20% (v/v) of methanol, propanol, n-hexane and butanol (Figure- 4.1). Compared to control, with 5% (v/v) solvents, there was almost similar catalysis. However, at higher concentrations of solvents, varying patterns emerged, as reflected in Figure-4.1.

On the basis of relative production of amylases, Ve1-10-8_2 and Mi-10-6_2 were selected for further study. Based on 16S rRNA gene sequencing, Ve1-10-8_2 was phylogenetically nearest to *Oceanobacillus oncorhynchi* and hence the isolate was designated as *Oceanobacillus oncorhynchi* Ve1-10-8_2 (the accession number of 16S rRNA gene sequence-GQ121034) Mi-10-6_2 was phylogenetically nearest to *Bacillus agaradhaerens*. The accession number of the submitted 16S rRNA gene sequence from Mi-10-6_2 is GQ121032.

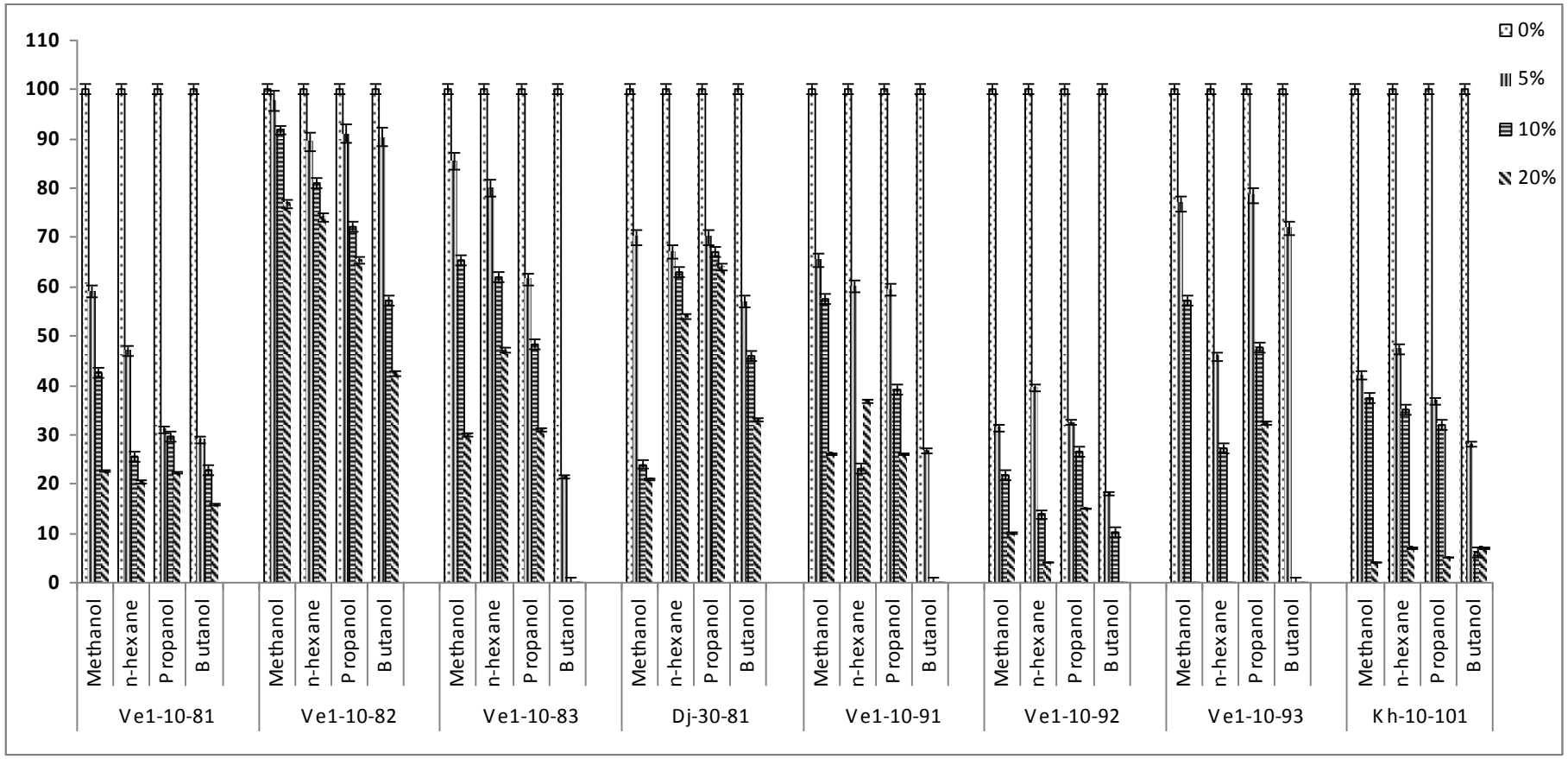


Figure 4.1- Catalysis of various Amylases in presence of organic solvents

4.3.1 Mi-10-6_2

Partial purification of the enzyme was achieved by ammonium sulphate precipitation with 3.9 fold purification, specific activity of 1246.6 U/mg and 34.87% yield.

4.3.1.1 Amylase catalysis in the presence of organic solvents

The crude and partially purified preparations of Mi-10-6_2 amylase were quiet active in the presence of the solvents. The enzyme was noticeably active up to 30 % (v/v) of propanol, hexane, heptane, decane and dodecane. With alcohols and lower alkenes, however, relatively reduced activity was evident in partially purified enzyme.

Catalysis in the presence of 10 % (v/v) hexane, heptane, deacne and dodecane was comparable to control. While with 10% (v/v) dodecane, the activity was nearly the same as control, the enzyme retained 50% activity in the presence of 10 % (v/v) Butanol. With 0.8 log Pow value, butanol is highly toxic for living organisms and their macromolecule and therefore, its quite interesting feature of this enzyme to be substantially active in its presence.

Partially purified amylase with 30% (v/v) dodecane, decane heptane, hexane, methanol and propanol exhibited 80, 75, 72, 71, 40 and 35% residual activities, respectively. However, with 30% (v/v) butanol, the amylase activity was totally lost. Amylase in crude form retained 80, 78, 71, 61, 56, 47 and 42% residual activities with dodecane, decane, heptane, hexane, propanol, methanol and butanol, correspondingly (Figure-4.2). Activity of control was considered as 100% for calculating residual activity. The residual activities of crude and partially purified enzyme are summarized in Table 4.1.

Mi-10-6_2 Amylases residual activity with Organic solvent														
Solvent % (v/v)	**Methanol**		**n-hexane**		**Propanol**		**Butanol**		**Heptane**		**Decane**		**Dodecane**	
	P..P. Enzyme	Crude Enzyme	P..P. Enzyme	Crude Enzyme	P..P. Enzyme	Crude Enzyme	P..P. Enzyme	Crude Enzyme	P..P. Enzyme	Crude Enzyme	P..P. Enzyme	Crude Enzyme	P..P. Enzyme	Crude Enzyme
10%	68.24	80.86	87.4	83.04	65.67	82.24	48.82	62.5	91.58	85.94	95.89	89.3	104.9	91.73
20%	50.08	58.11	81.17	78.1	48.85	64.92	23.71	44.3	80.42	75.79	85.78	87.53	88.03	86.15
30%	39.85	46.59	70.99	61.3	34.56	56.23	0	41.58	71.93	70.65	74.87	77.89	79.45	79.27

Table 4.1- Residual activities of Mi-10-6_2 Amylase in crude and partially purified form with different solvents

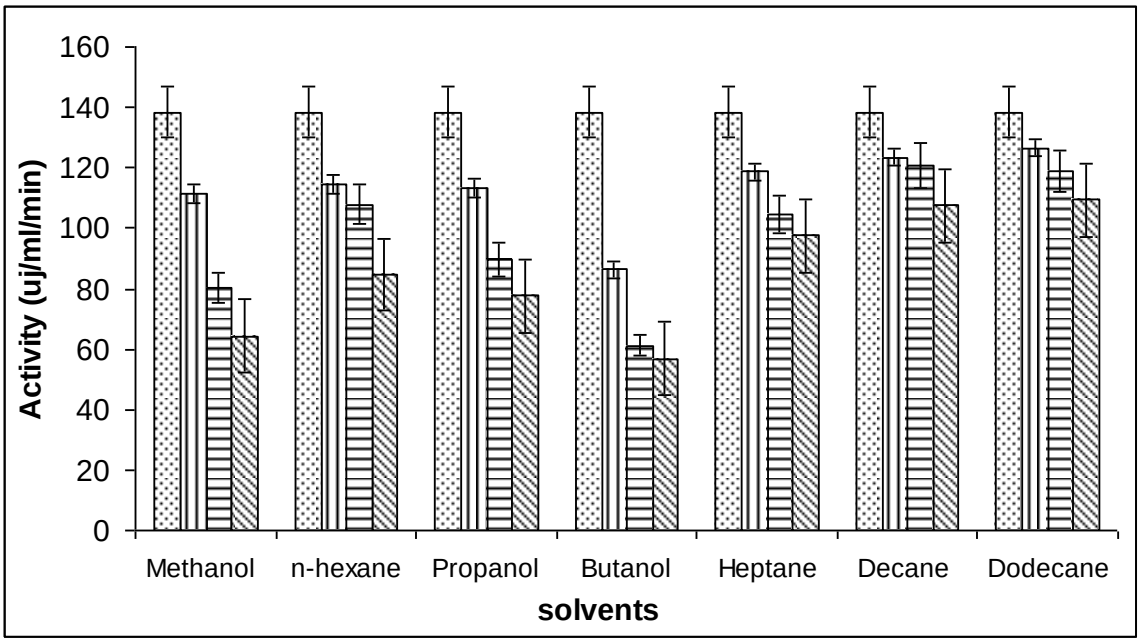


Figure 4.2A- Catalysis of Mi-10-6$_2$ crude Amylases in presence of organic solvents

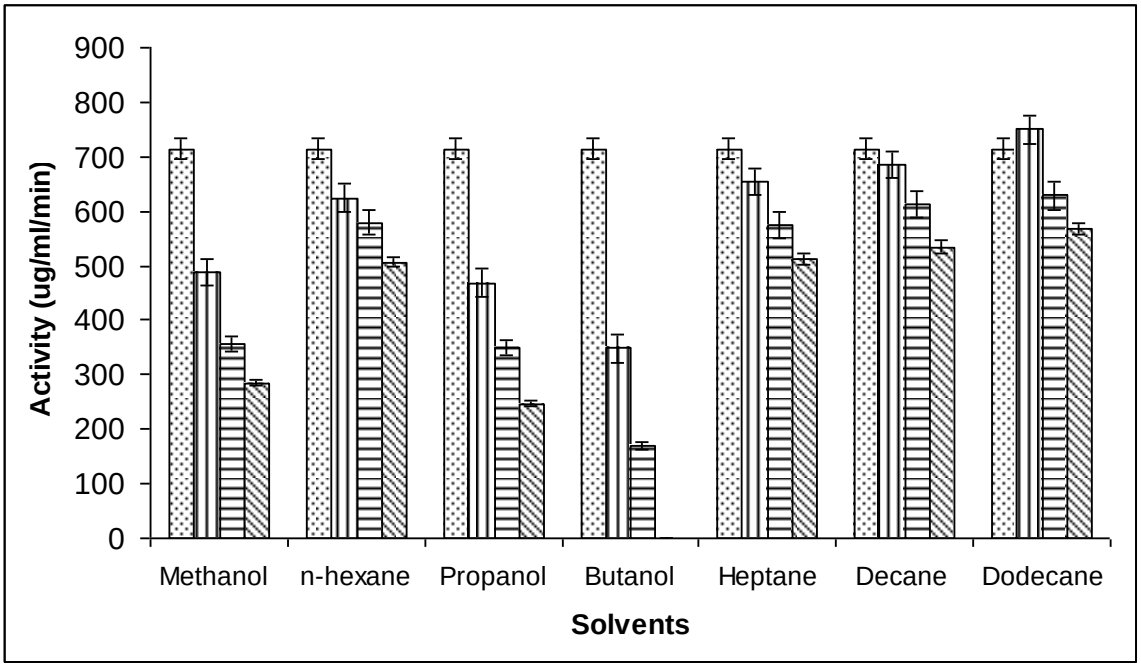


Figure 4.2B- Catalysis of Mi-10-6$_2$ partially purified Amylases in presence of organic Solvents

Figure 1- Effect of organic solvents; 0 %(▨) 10 %(▥) 20 %(▤) 30 %(▧), V/V, on the catalysis of Mi-10-6$_2$ Amylase in crude (4.2A) and partially purified preparations (4.2B)

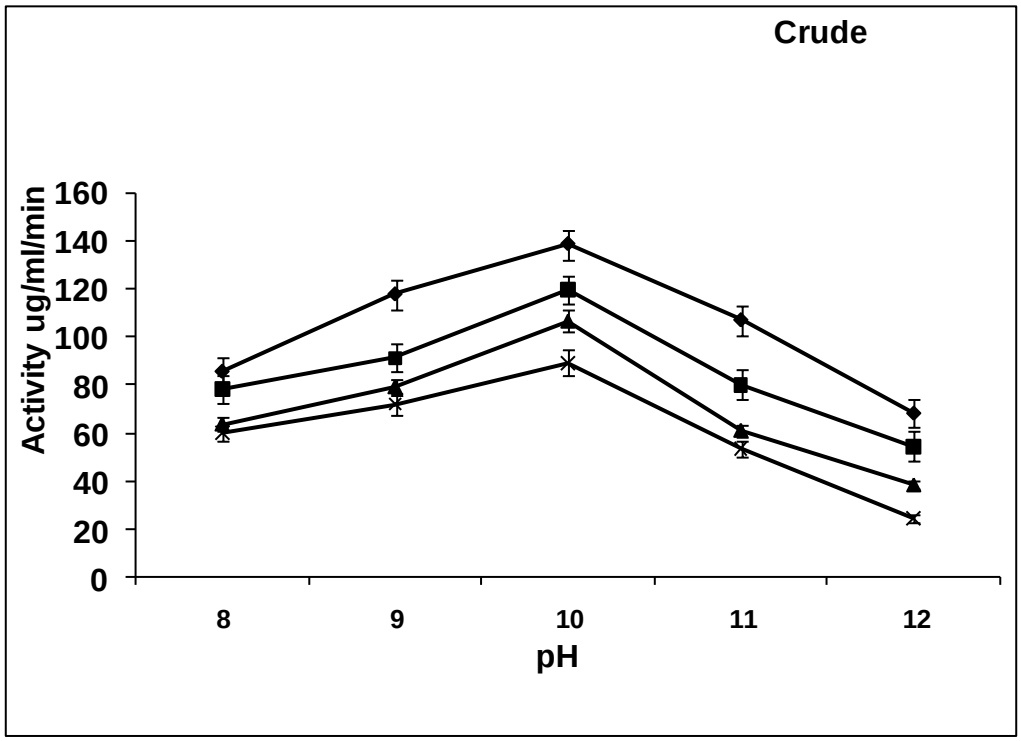
4.3.1.2 Effect of pH on the catalysis of amylase

Effect of pH on amylase was assessed in the presence of popanol and dodecane, where enzyme was active in alkaline pH, 8 to 12. The activity at pH 10, without any solvent, was considered as 100%. At several combinations of pH and solvent concentrations, the residual activities were monitored. Crude amylase retained 56, 46 and 45 % residual activities with 10, 20 and 30% (v/v) propanol at pH 8. The loss of activities of partially purified enzyme under similar conditions of pH and solvents were quite comparable to those of crude preparation (Figure 4.3 A). Enzyme had 83, 70 and 63% residual activities in crude form and 46, 42 and 36% in partially purified preparation with the tested concentrations of dodecane (Figure 4.3B).

The crude amylase exhibited 57, 50 and 30 % residual activities, while the partially purified enzyme retained 73, 62 and 40% activities in the presence of 10, 20 and 30% (v/v) of propanol. At the same concentrations of dodecane, the crude and partially purified enzymes had 75, 60, 55 and 55, 60, 40 % residual activities, respectively.

At optimum pH 10; 86, 77 and 64% activities for crude and 79, 68 and 62 % activities for partially purified enzymes were obtained with tested concentrations of propanol. However, in the presence of dodecane, comparatively higher residual activities 90, 85, 78 and 80, 70, 63% were recorded for crude and partially purified enzymes, respectively.

Amylase was quiet efficient at pH 11 with Dodecane. At pH 11, the enzyme activities for crude were 70, 58 and 42% while partially purified enzyme had 57, 43 and 32 % activities in the presence of 10, 20 and 30% (v/v) solvent, respectively. At pH 12, the activities were quite negligible for both tested solvents. Effect of pH on amylase catalysis in the presence of solvents is presented in (Figure 4.3 A and 4.3B).



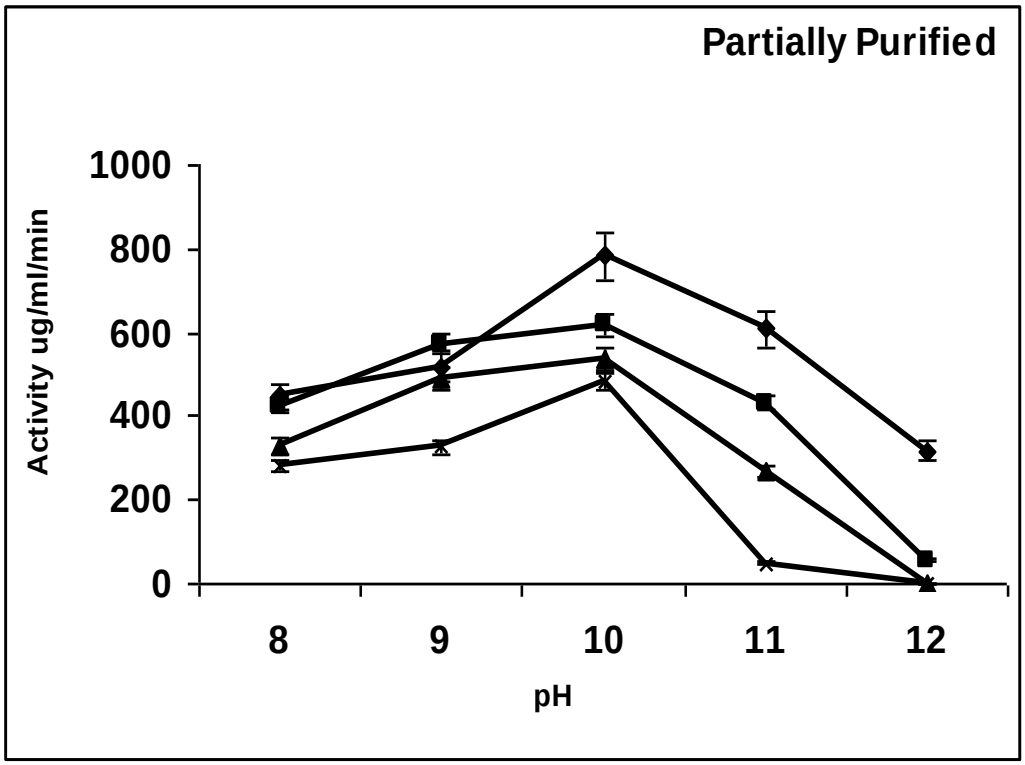
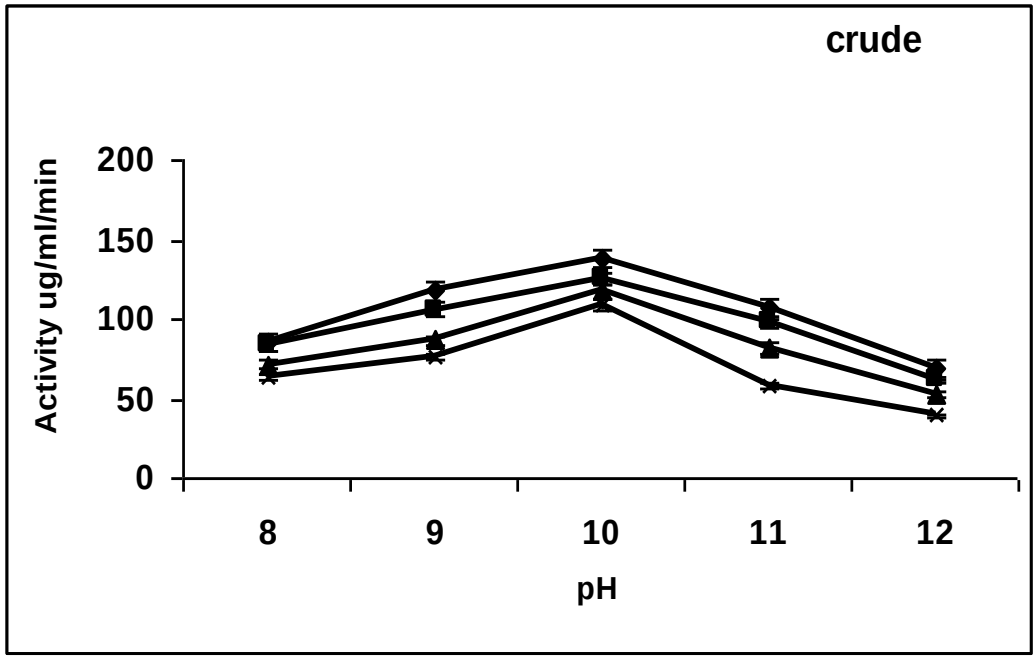


Figure 4.3A-Effect of pH on Mi-10-6$_2$ Amylases in presence of propanol



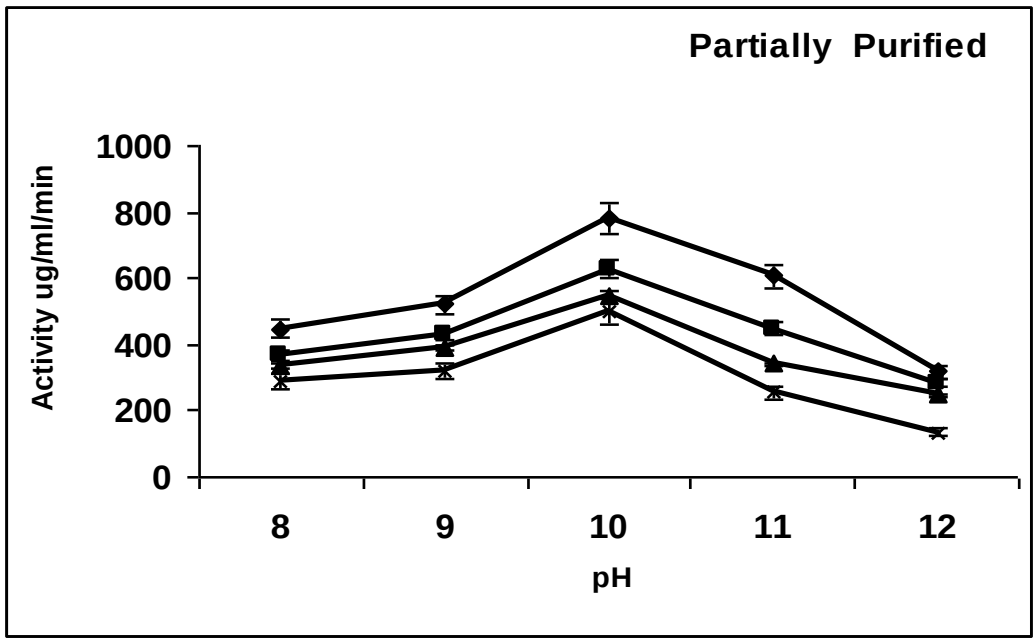


Figure 4.3B- Effect of pH on Mi-10-6$_2$ Amylases in presence of dodecane.

Figure 4.3- Effect of pH on Mi-10-6$_2$ Amylase activity with 0% (♦), 10%(■), 20%(▲) and 30%(×), (v/v), propanol (4.3A) and dodecane (4.3B)

4.3.1.3 Effect of Salt on catalysis of Mi-10-6_2 Amylases

Effect of 0.5-4M NaCl on Mi-10-6_2 amylase catalysis was examined in the presence of 10-30% (v/v) propanol and dodecane. With both solvents, a change in the pattern of salt profile was evident. For crude and partially purified enzyme, the salt optima were 3 and 2M NaCl, respectively. Crude amylase with 0.5 M salt and 10, 20, and 30 % (v/v) propanol and dodecane had 58, 50 43, and 52, 47, 40 ug/ml/min respectively. For partially purified enzyme, compared to control (358 ug/ml/min), the activities were 325, 300 and 285 with Propanol and 335, 315 and 290 ug/ml/min with dodecane, respectively.

At 1 M salt, better enzyme activity was observed with both tested solvents. As compared to activity at 0.5 M salt, nearly two fold enzyme activities were recorded at 1 M salt. With 1 M salt, the activities of crude enzyme were 100, 98 and 86 at tested concentrations of propanol and 100, 97 and 62ug/ml/min with dodecane.

With the same concentrations of salt and propanol, activities of partially purified enzyme were 560, 535 and 485, while with dodecane; 600, 575 and 500ug/ml/min activities were recorded. Salt at 2M was optimum for partially purified enzyme; exhibiting 710, 670, 595 ug/ml/min activities with Propanol and 735, 698 and 657 ug/ml/min activities with the tested concentrations of dodecane. With further increase in salt, partially purified enzyme resulted in loss of activity (Figure 4.4 A and 4.4B). NaCl at 3M was optimum for crude amylase, resulting in 150, 130, 100 and 140, 130, 120 ug/ml/min activities with Propanol and dodecane, respectively. At 4 M salt, decreased activities were evident with both solvents.

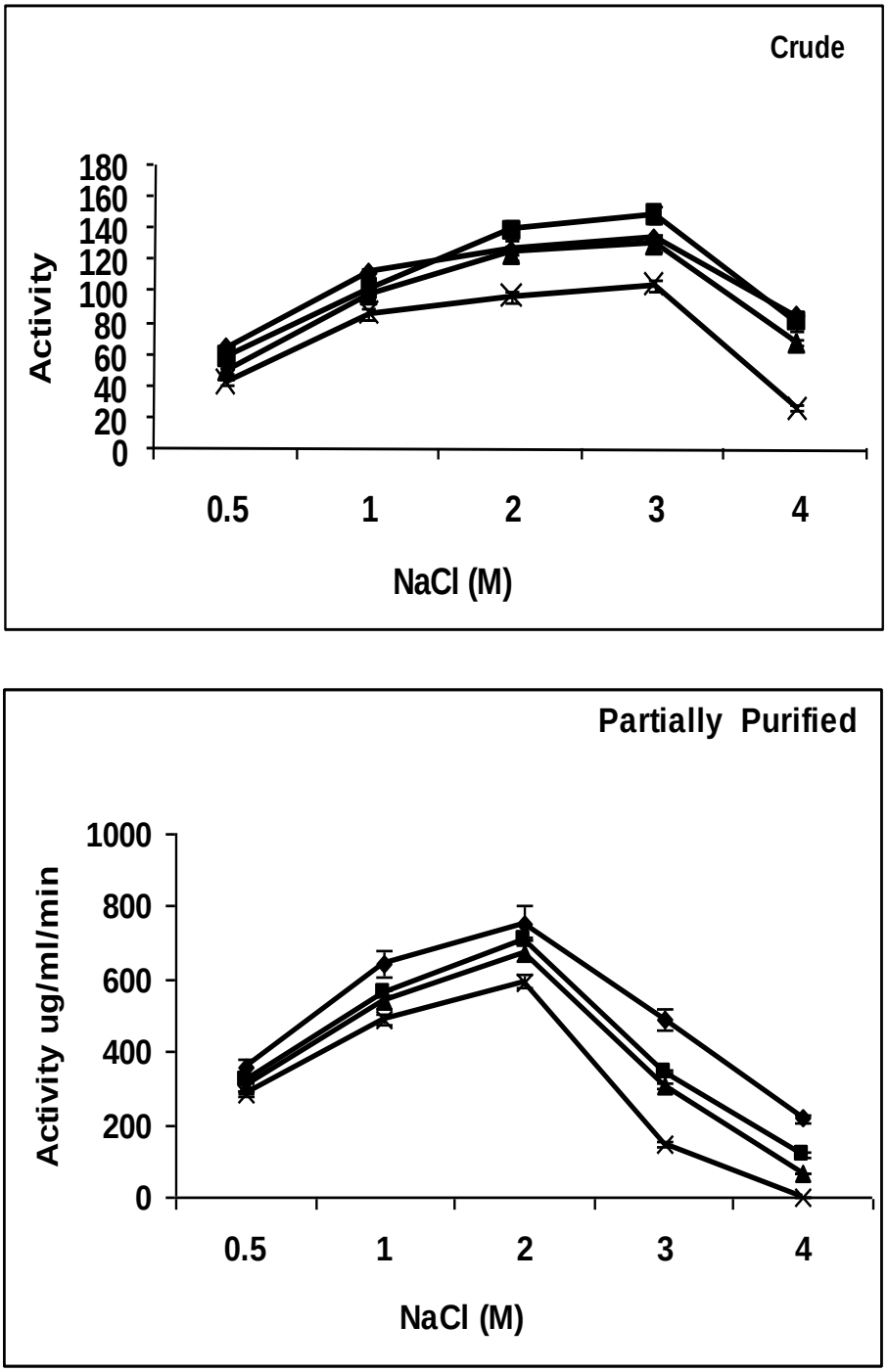


Figure-4.4A- Effect salt on Mi-10-6_2 Amylases in presence of propanol

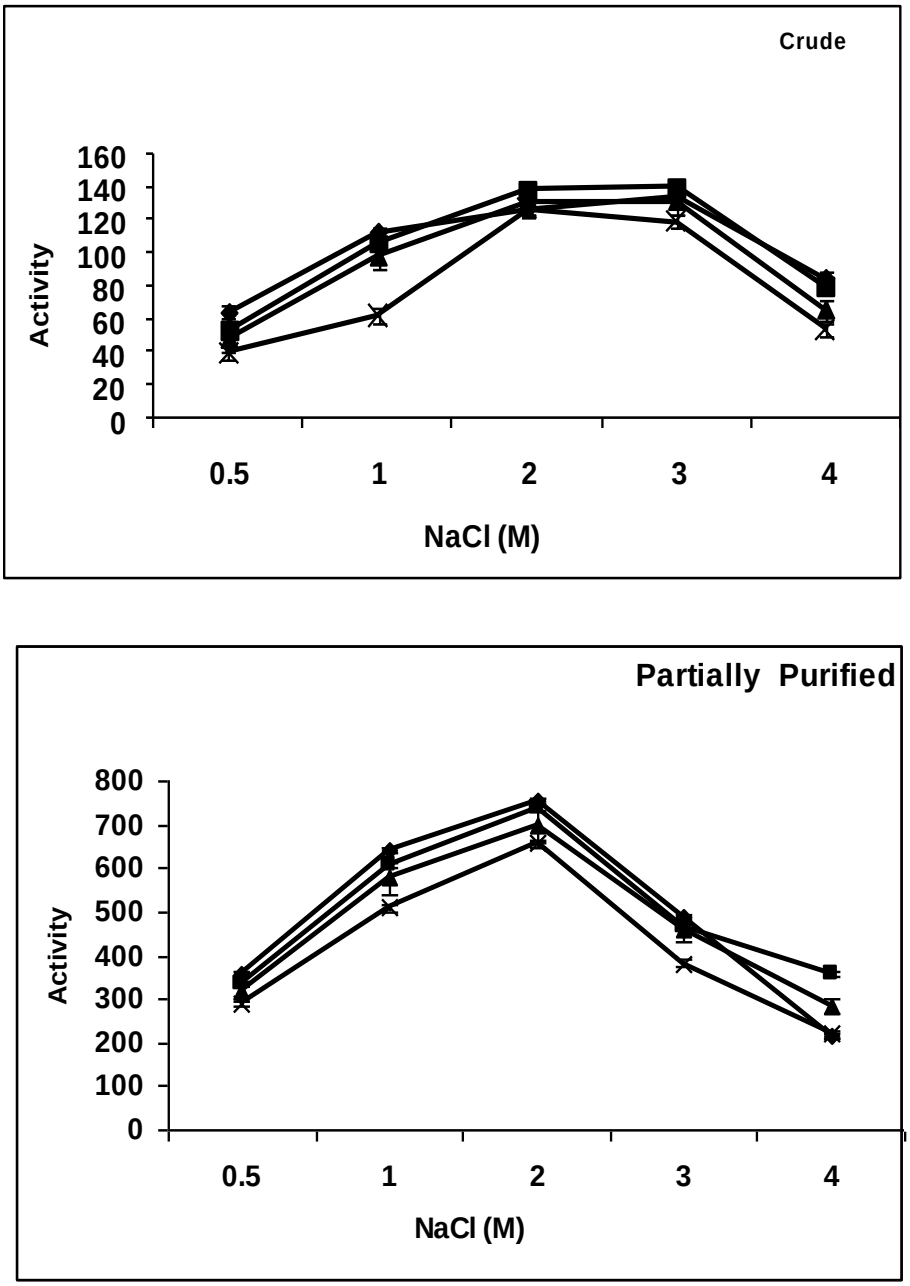


Figure- 4.4 B- Effect salt on Mi-10-6_2 Amylases in presence of dodecane

Figure 4.4- Effect of salt NaCl on Mi-10-6_2 amylase activity in the presence of 0% (♦) , 10%(■), 20%(▲) and 30%(×) (v/v) Propanol (4.4A) and dodecane (4.4B)

4.3.1.4 Temperature optima of Mi-10-6_2 in presence of Propanol

Figure 4.5 displays the effect of temperature on the catalysis of Mi-10-6_2 amylase in the presence of propanol. Optimum temperature for crude amylase was 50°C, which shifted to 60°C in the presence of propanol. At 20 % (v/v) propanol, the partially purified enzyme retained comparable activity as control with the enhanced temperature optima. At 30% (v/v) solvent, the enzyme retained nearly 50% of the residual activity.

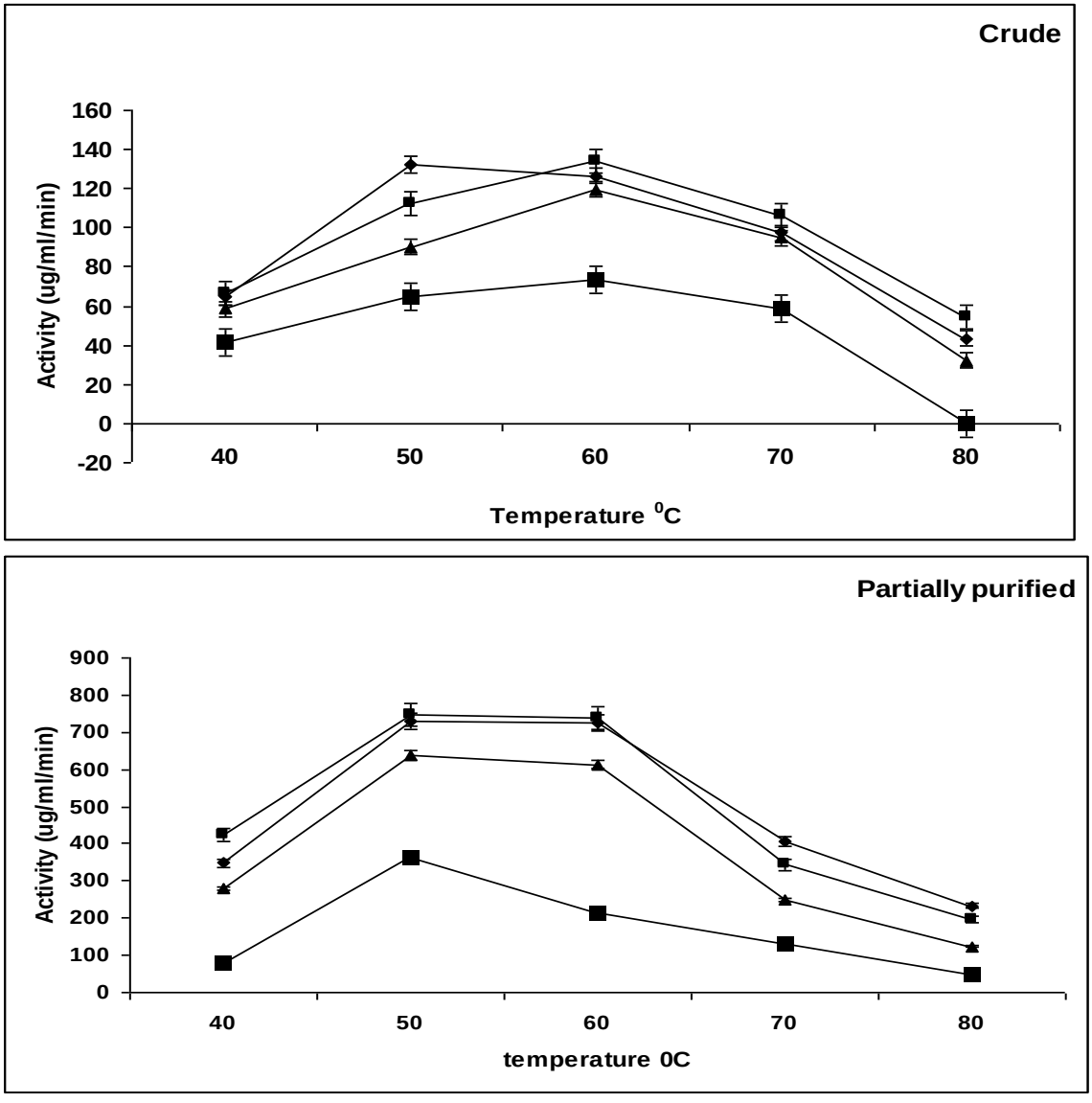


Figure 4.5- Temperature optima of Mi-10-6_2 amylase with propanol

Figure 4.5- Effect of propanol on temperature optima of enzyme; 0% (♦), 10%(■), 20%(▲) and 30%(×) (v/v) Propanol

4.3.2 Ve1-10-8_2

4.3.2.1 Effect of pH on catalysis of Ve1-10-8_2 Amylase

Effect of pH on enzyme catalysis was judged in the presence of methanol and butanol. The enzyme was quiet active at broader range of acidic and alkaline pH. Activity at pH 10, without any solvent was assumed as 100%. With many combinations of pH and concentrations (v/v) of solvents, enhanced activities were recorded. At pH 5, relative to control; 92, 108 and 57% activities with 5, 10 and 20% (v/v) of methanol and 40, 30 and 20% activities at the same concentrations of butanol, respectively, were evident.

Enzyme performance was quiet better at pH 6. The activities were 106, 100 and 82% of control at 5, 10 and 20% (v/v) of methanol and 100, 80 and 96% with the same concentrations of butanol. At neutral pH, however, the activities declined in the presence of methanol, while it remained unaltered with butanol. At pH 7, the residual activities were 50, 75 and 50% at 5, 10 and 20% of methanol, respectively. At same pH with butanol, the residual activities were 92, 108 and 74% at the tested concentrations of the solvent.

In alkaline pH range better activities were recorded. At pH 8 enzyme exhibited 61, 73 and 87% residual activities at 5, 10 and 20% (v/v) methanol. At pH 9; 70, 90 and 85 of the residual activities with 5, 10 and 20% (v/v) methanol, while 92, 87 and 82% activities with the corresponding concentrations of butanol were observed. However, at pH 10, the residual activities were 125, 115 and 90 % of control with 5, 10 and 20% of methanol. With the same concentrations of butanol, at pH 10; the residual activities were 90, 68 and 52%. pH 11 was quiet favorable for enzyme activity with methanol , although butanol at the same pH was not as favored. At pH 11, the activities were 116, 92 and 85% with methanol and 60, 52 and 50% with 5, 10 and 20% v/v of butanol. The residual activities were significantly reduced with both solvents at pH 12.

The enzyme exhibited 70, 67 and 57% residual activities with methanol and 36, 18 and 13% with butanol. At pH 13, with 5, 10 and 20% v/v methanol; highly reduced residual activities at 35, 30 and 20% were recorded. While with butanol at pH 13, the

activities were totally lost at higher solvent concentrations. At pH 13, with 5% (v/v) butanol, 30 %residual activity was detected (Figure 4.6A and Figure 4.6 B).

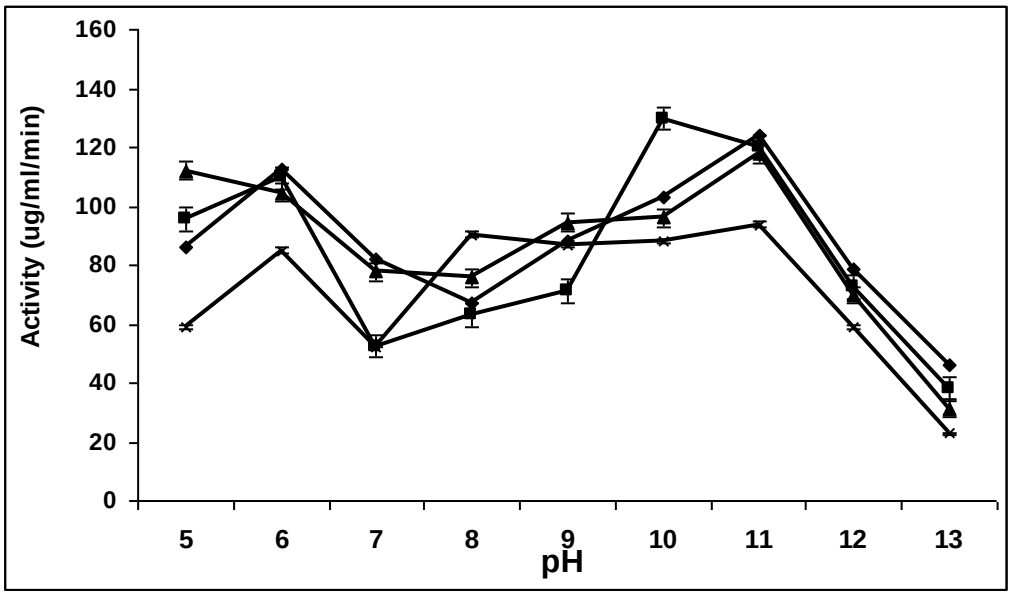


Figure- 4.6 A- Effect of pH on Ve1-10-8_2 Amylases in the presence of methanol

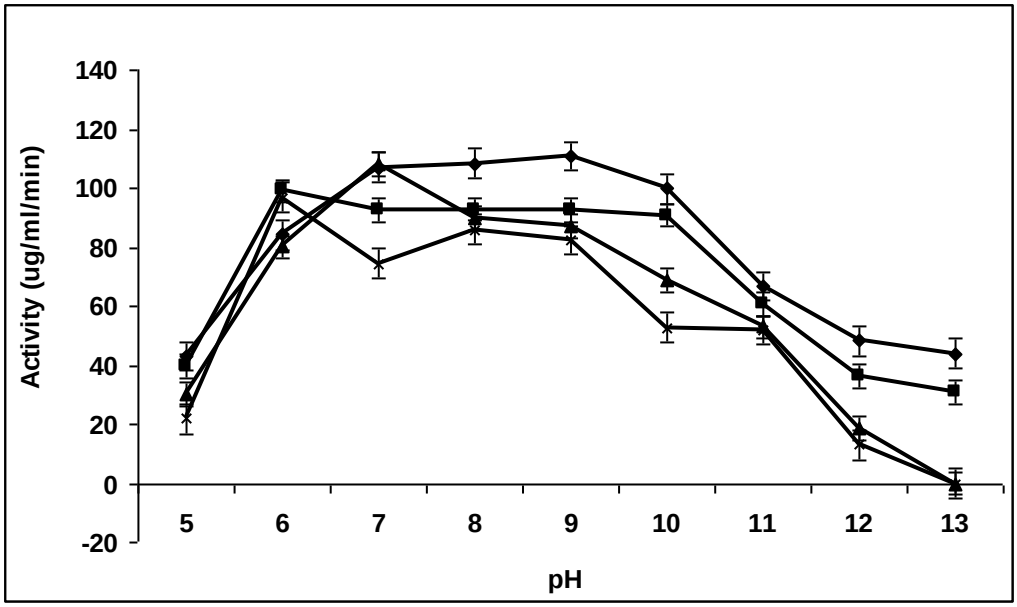


Figure- 4.6B- Effect of pH on Ve1-10-8_2 Amylases) in presence of butanol

Figure 4.6- Effect of pH on Ve1-10-8_2 Amylase activity with 0% (♦) , 5% (■), 10%(▲) and 20% (×) (v/v) methanol (4.6A) and butanol (4.6B)

4.3.2.2 Effect of salt on the enzyme catalysis

Effect of NaCl (0.5 - 4M) on enzyme catalysis was investigated in the presence of 5, 10 and 20% (v/v) methanol and butanol. With 0.5 M Salt, in presence of 5, 10 and 20% (v/v) methanol and butanol 31, 25, 39 and 46, 44, 39 (µg/ml/min) activities were recorded. With 1 M salt, comparatively better activities were observed at the tested concentrations of both solvents. In case of butanol, there were slightly enhance activities at 46, 48 and 42 µg/ml/min, while for methanol, the enhancement in activities was nearly threefold of the value at 0.5M salt.

At 1 M salt, the activities were 100, 84 and 130 µg/ml/min, correspondingly. Salt at 2 M was optimum for catalysis in presence of both solvents. At this salt concentration; 80, 73 and 48 µg/ml/min activities with butanol and 113,105 and 100 µg/ml/min activities with methanol were observed. On further increase in salt concentrations, the activities were reduced for both tested solvents. At 3 M salt; 63, 52, 47 and 122, 113, 93 µg/ml/min activities were observed in the presence of 5, 10 and 20% butanol and methanol, respectively. At 4 M salt; 48, 54, 33 and 70, 68, 60 µg/ml/min activities were evident with butanol and methanol, correspondingly (Figure 4.7A and 4.7B).

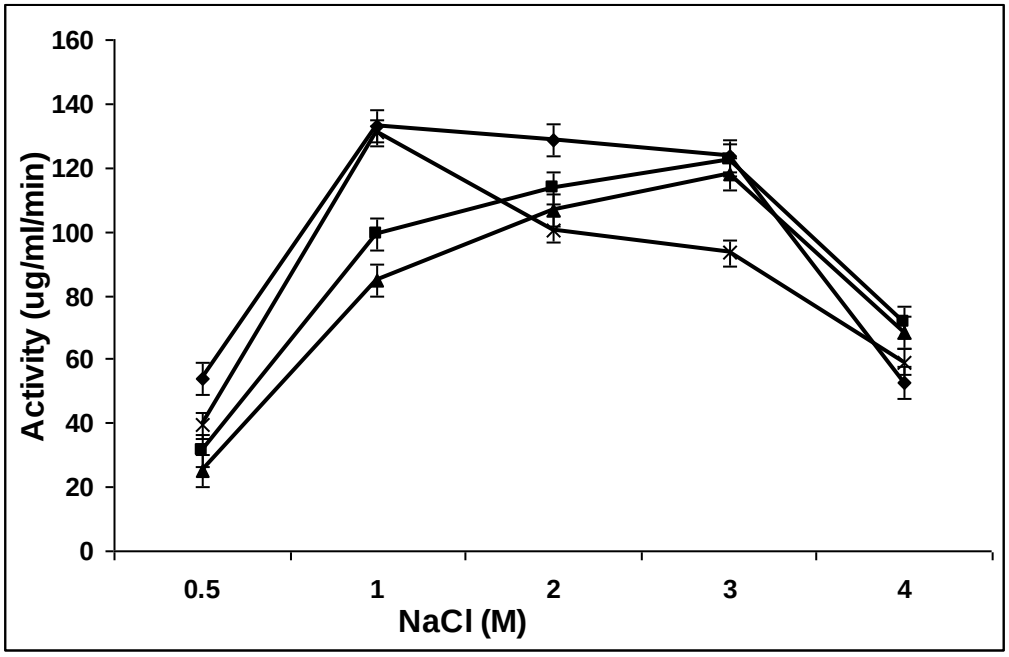


Figure 4.7A- Effect salt on Ve1-10-8$_2$ Amylases in the presence of methanol

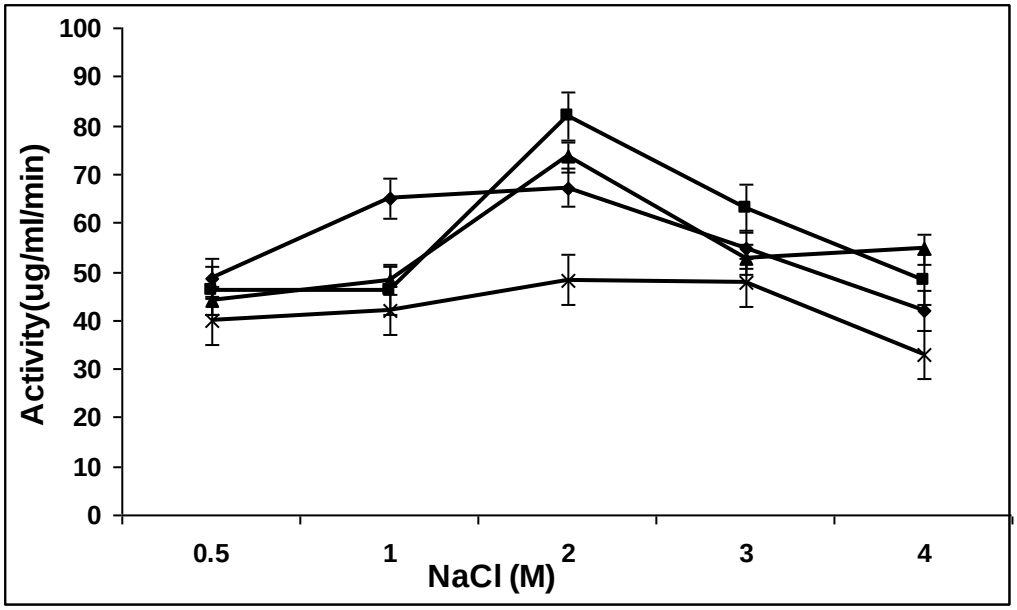


Figure 4.7 B- Effect salt on Ve1-10-8_2 Amylases in the presence of butanol

Figure 4.7 - Effect of salt (NaCl) concentration on Ve1-10-8_2 amylase activity in the presence of 0% (♦) , 5% (■), 10% (▲) and 20% (×) (v/v) methanol (4.7A) and butanol (4.7B)

4.3.2.3 Stability of Ve1-10-8_2 in presence of methanol

Amylase of Ve1-10-8_2 was quiet stable with methanol up to 180 minute, at 50 and 60^{o}C. While at 70^{o}C, the enzyme was active only up to 90 minute(ure 4.8). Residual activities of at various methanol concentrations are shown in Table-4.2. Amylase was stable and active at acidic and alkaline pH range. While, in acidic range (pH 5-6), the enzyme was stable up to 90 minute , at neutral pH with 5 and 10% (v/v) methanol, it was stable up to 180 minute with reduced activity (Figure 4.8). In alkaline range, at pH 9-11, the enzyme was stable up to 180 minute retaining good activity, the pH at 11 being optimum for activity and stability. At pH 12, the activities and stability decreased significantly. The residual activities with different concentrations of methanol at varying pH are presented in Table-4.3.

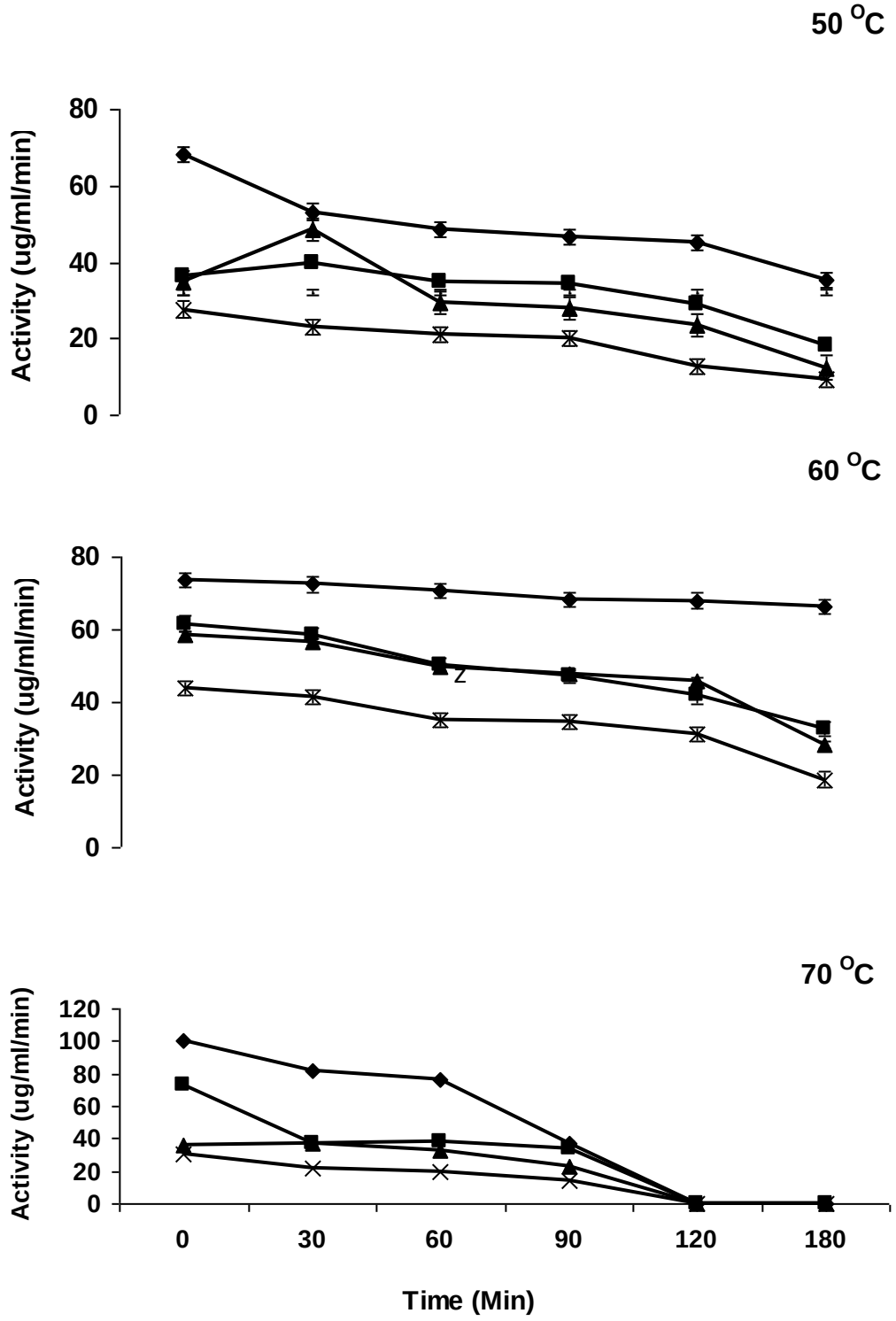


Figure 4.8- Thermo stability of Ve1-10-8$_2$ Amylase

Figure 4.8- Effect of methanol on thermo stability of Ve1-10-8$_2$ amylase with 0% (♦) , 5% (■), 10% (▲) and 20% (×) (v/v) methanol

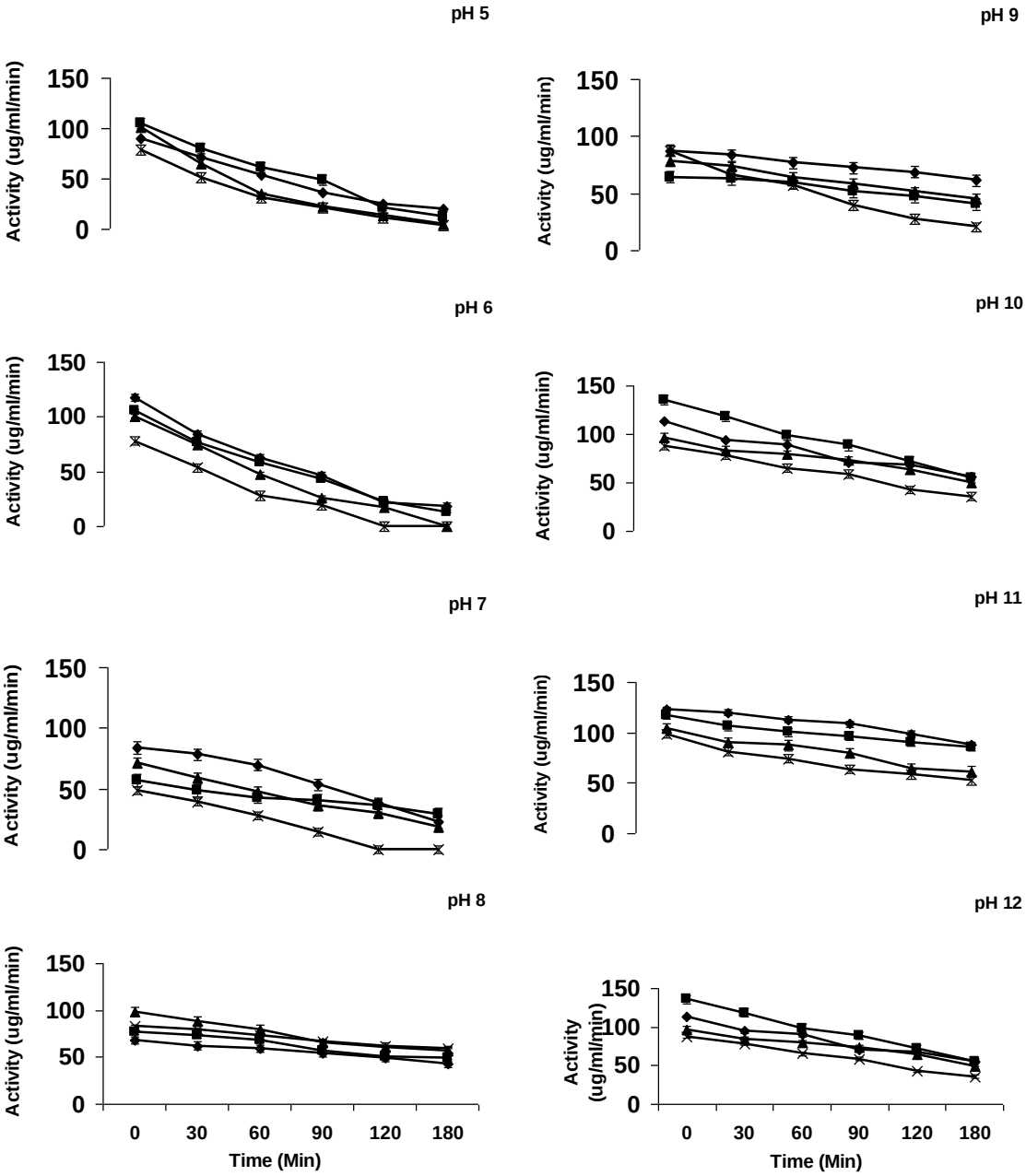


Figure 4.9 Stability of Ve1-10-82 Amylase at different pH

Figure 4.9- Stability of Ve1-10-8$_2$ amylase at various pH in the presence of 0% (♦), 5 %(■), 10 %(▲) and 20 % (×) (v/v) methanol

Temp °C	**% Methanol (v/v)**	**Residual activity at different Time interval (Minute)**					
		0 (Min)	**30(Min)**	**60(Min)**	**90(Min)**	**120(Min)**	**180(Min)**
50 °C	0	100	78.20	71.43	68.70	66.24	52.14
	5	53.70	58.14	50.94	50.61	42.38	26.80
	10	50.92	71.19	43.20	41.03	34.75	18.32
	20	40.74	33.77	30.86	29.27	18.63	13.93
		0 (Min)	**30 (Min)**	**60 (Min)**	**90 (Min)**	**120(Min)**	**180(Min)**
60 °C	0	100	98.22	95.63	92.50	92.24	89.95
	5	83.59	79.46	68.13	64.27	56.58	44.46
	10	79.50	76.83	67.70	64.87	61.88	38.50
	20	59.51	56.39	47.58	46.77	42.08	25.42
		0 (Min)	**30 (Min)**	**60 (Min)**	**90 (Min)**	**120(Min)**	**180(Min)**
70 °C	0	100	82.33	76.69	37.59	0	0
	5	73.30	37.59	38.34	34.15	0	0
	10	35.71	36.84	32.36	22.48	0	0
	20	30.82	21.66	19.81	14.39	0	0

Table 4.2- Residual activities of Ve1-10-8_2 amylase in the presence of methanol at various temperatures (°C)

pH	**% Methanol (v/v)**	**Residual Activity at different Time interval (Minute)**					
5		**0 (min)**	**30(min)**	**60(min)**	**90(min)**	**120(min)**	**180(min)**
	0	100	79.77	59.70	41.01	27.97	21.87
	5	116.9	89.82	68.81	54.06	24.37	13.79
	10	113.59	71.98	39.50	25.58	15.23	5.02
	20	87.61	56.89	34.59	23.34	11.91	3.57
6		**0 (min)**	**30 (min)**	**60 (min)**	**90 (min)**	**120 (min)**	**180 (min)**
	0	100	71.81	52.88	39.30	18.08	15.70
	5	89.64	65.20	49.63	37.01	19.64	11.24
	10	85.62	63.11	40.74	22.07	14.73	0
	20	66.40	45.50	23.68	16.04	0	
7		**0 (min)**	**30 (min)**	**60 (min)**	**90 (min)**	**120 (min)**	**180 (min)**
	0	100	93.54	83.37	63.87	45.21	27.27
	5	67.94	58.13	51.31	48.31	43.52	34.56
	10	85.59	70.21	57.24	43.31	35.35	22.24
	20	58.49	43.54	33.01	17.41	0	0
8		**0 (min)**	**30 (min)**	**60 (min)**	**90 (min)**	**120 (min)**	**180 (min)**
	0	100	91.21	87.26	79.25	72.76	63.39
	5	113.47	107.40	100.29	83.74	73.79209	71.15
	10	143.19	129.98	116.54	95.08	88.05	83.16
	20	121.81	116.39	107.61	98.38	89.70	87.52

pH	% Methanol (v/v)	Residual Activity at different Time interval (Minute)					
9		**0(min)**	**30(min)**	**60(min)**	**90(min)**	**120(min)**	**180(min)**
	0	100	95.56	87.90	82.92	78.24	70.07
	5	73.75	71.33	67.78	58.75	53.84	46.29
	10	89.49	84.01	73.73	67.32	58.64	52.12
	20	99.96	76.10	65.84	45.26	31.55	23.37
10		**0 (min)**	**30 (min)**	**60 (min)**	**90 (min)**	**120 (min)**	**180 (min)**
	0	100	83.59	79.26	62.45	60.21	49.27
	5	119.90	104.81	87.27	78.23	63.55	48.30
	10	85.50	73.81	70.24	64.50	56.30	43.87
	20	77.60	68.98	57.68	52.12	37.75	31.73
11		**0 (min)**	**30 (min)**	**60 (min)**	**90 (min)**	**120 (min)**	**180 (min)**
	0	100	97.79	92.08	88.65	80.57	71.34
	5	96	86.77	82.04	78.66	73.92	69.87
	10	85.06	73.87	71.42	65.14	52.81	50.04
	20	80.48	66.13	60.57	51.83	47.73	42.77
12		**0 (min)**	**30 (min)**	**60 (min)**	**90 (min)**	**120 (min)**	**180 (min)**
	0	100	78.48	64.79	39.89	14.64	4.69
	5	95.69	64.66	38.46	24.11995	6.25	0
	10	88.39	63.36	29.15	18.59	0	0
	20	64.66	47.58	24.11	0	0	0

Table 4.3 - Residual activities of Ve1-10-8_2 amylase at different pH with % (v/v) methanol

4.4 DISCUSSION

Extremozymes have attracted considerable attention due to their potential to meet industrial demand for enzymes with multitude of extremities. High salinity, alkaline conditions and non-aqueous medium are some of the examples of extremity for biocatalysis. However, only limited literature is available on the enzymes from haloalkaliphilic bacteria with respect to non-aqueous biocatalysis (Herbert *et al.,* 1992. Madigan *et al.,* 1997, Niehaus *et al.,* 1999 and Eichler *et al.,* 2001).

Some intra and extra cellular enzymes from extremely and moderate halophilic and haloalkaliphilic bacteria have been isolated and characterized, which might have potential applications in food, chemical, pharmaceutical, leather, tanning, paper pulp and waste-treatment industries (Costa *et al.,* 1998, Patel *et al.,* 2005, Jogi *et al.,* 2005 , Patel *et al.,* 2006 and Wejse *et al.,* 2003).

The studies on haloalkaliphilc amylases with respect to their tolerance against organic solvent have not been investigated in great deal. However, some haloalkaliphilic archaea and their relationship with organic solvents have been investigated during the recent years (Tadamasa *et al.,* 2005 and Saraiva *et al.,* 1996). It is well reported that enzymes are inactivated in the presence of organic solvents, and catalytic activities in non-aqueous environment are generally lower than those in aqueous system (Ogino *et al.,* 2001 and Ru *et al.,* 1999). Therefore, it was quite interesting to study an amylase from haloalkaliphilic bacteria in non-aqueous medium.

The haloalkaliphilic bacteria in the present report were screened against 4 organic solvents; methanol, propanol, n-hexane and butanol. The isolates displayed varying diversity with respect to catalysis in these solvents. With quiet less Log Pow value, butanol is extremely toxic for living cell and their macromolecules. Therefore, the catalytic of Ve1-10-8_2 even in the presence of butanol reflected a unique feature of the enzyme.

Enzyme catalysis with 5% (v/v) water miscible and immiscible alcohols and alkane, similar to control, signify its robust nature at lower solvent concentration. At higher concentrations, varying results were evident.

Mi-10-6_2 amylase reported in this study was screened against 7 organic solvents; Methanol, Propanol, n-hexane, Butnol, Heptane, Decane and Dodecane. The amylase displayed varying responses against these solvents. Catalysis of Mi-10-6_2 amylase in the presence of Butanol was an interesting feature of the study. Catalysis with 20% (v/v) water miscible and immiscible alcohols and alkane, indicated the robust nature of the enzyme. At concentrations above 20% (v/v), varying effects were observed.

Mi-10-62 amylase was active over wide range of pH, 8-11; the optimum being at 10. These values are marginally higher than those reported for an amylase from an alkaliphilic Bacillus sp. (Igrashi *et al.,* 1998) and significantly higher than those reported for another amylase from Halobacterium salinarum (Good *et al.,* 1970). Other organisms, such as thermophilic and halotolerant bacteria, Halothermothrix orenii are reported to have amylases with optimal activities in the similar range of pH (Mijts *et al.,* 2001).

Amylase from Ve-10-82 was active at both acidic and alkaline pH, while at pH 7 it lost activity. The optimal pH for the catalysis was 10-11, which appeared to be higher than an amylase from alkaliphilic *Bacillus sp*. (pH 8.0-8.5) (Igrashi *et al.,* 1998). The Ve-10-82 enzyme retained stability over a wide range of pH; 6.0-10.0, which is quiet higher than an amylase from *Halobacterium salinarum* (Good *et al.,* 1970). Other prokaryotes, such as a thermophilic and halotolerant bacterium, *Halothermothrix orenii* was reported to have optimal amylase activity in range of pH 6.0-9.5 . Regarding the stability at different pH, the enzyme was quite active over acidic and alkaline range. At pH 5 and 6 with 5 and 10% (v/v) methanol, the enzyme was stable up 90 min, while it was highly stable at pH 10-11. While the enzyme retained activity over a wide pH range of 5-11, enhanced activities were observed at alkaline pH range. Therefore, the alkaliphilic nature of the enzyme was highly pronounced.

Halophilic enzymes, in general, are not stable in low salt concentrations, because of ionic charges and salt dependent structural stability (Madern *et al.,* 2000, Danson *et al.,* 1997, Bonnete *et al.,* 1994 , Martınez *et al.,* 2009). Therefore, an increase of activity with salt concentrations is a common feature of the halophilic enzymes (Dym *et al.,* 1995). The amylase from Mi-10-6_2 in the present study displayed an upward shift in activity with salt from 0.5 to 2 M. Salt affects the binding between the enzyme and substrate .

Most of the halophilic and haloalkaliphilic enzymes are inactivated at NaCl or KCl concentrations below 2 M (Camacho *et al.,* 1995). As described earlier, the amylase activity in Mi-10-6_2 increased with increasing salt concentrations, indicating an overall effect of salt on the reaction. The enzyme was quiet active with 1M NaCl even in the presence of solvents. Optimum catalysis at comparatively low salt and its behavior to retain activity with broader range of salt concentrations in the presence of solvents is quite relevant to haloalklaiphilic bacteria. The findings are quite comparable to a moderately halophilic and aerobic bacterium, *Halomonas meridiana* (Coronado *et al.,* 1995). The Mi-10-6_2 amylase had differential effects in response to salt when crude and partially purified enzymes were compared. While 2M salt was optimal for partially purified enzyme, the crude preparation required 3M for maximal activity.

Normally structure of halophilic enzymes are not stable in low salt concentrations because of ionic charge required for the stability of the enzyme structure (Joshi *et al.,* 2008 and Madern *et al.,* 2000). Therefore, decrease in the salts required by halophilic enzymes may lead to the loss of their structure and function (Danson *et al.,* 1997, Bonnete *et al.,* 1994, Rosa *et al.,* 2009). The amylase reported here appears to be adapted to the high NaCl concentrations. The increase of activity with salt is a common feature of halophilic enzymes (Dym *et al.,* 1995). Amylase of Ve1-10-82 highlights its halophilic character with upward shifting of enzyme activity from 0.5-1M NaCl.

Higher concentrations of salt affect the binding mechanism between the enzyme and substrate (starch). However, as described earlier, the activity increased at higher salt concentrations, indicating that salt had an overall positive effect on the reaction rate. Most of the halophilic and haloalkaliphilic enzymes studied are inactivated when the

NaCl or KCl concentration decreases to less than 2 M (Madern *et al.,* 2000). In contrast to this general acceptance, amylase in the present report was optimally active at 1M NaCl with methanol. While with butanol, the enzyme required 2M NaCl concentration for its maximal activity. Optimal performance at comparatively low salt concentrations and ability to retain activity with border range of salt concentrations was evident in a moderately halophilic and mesophilic aerobic bacteria, *Halomonas meridiana* (Coronado *at al.,* 2000). There were substantial changes in salt profile of the enzyme. In the presence of butanol, the optimum activity shifted from 1M to 2M salt concentration.

The optimal temperature (55 to 60 ^{0}C) for Mi-10-62 amylase was quite comparable with the enzyme from *Halobacterium salinarum* (Good *et al.,* 1970). However, the enzyme was active at higher temperatures, and with 20% v/v propanol, it retained significant activity up to 70^{0}C. Comparable to our studies, the enzyme from an alkaliphilic *Bacillus sp*. also exhibited the optimal temperature at 60^{0}C (Igrashi *et al.,* 1998). Other Halophilic enzymes, such as NAD and NADP glutamate dehydrogenases from Halobacterium salinarum displayed maximal activity at 70^{0}C, with higher temperature stability (Bonete *et al.,* 1987). The temperature profiles and stability were quite comparable to a thermophilic amylase from *Thermus sp*. AMD33, which had optima at 70^{0}C (Nakamura *et al.,* 993), or with a halophilic and thermophilic bacteria *Halothermothrix orenii*, with an optima at 65 ^{0}C (Mijts *et al*., 2001). The thermophilic nature of our enzyme was also reflected by a shift in temperature optima to higher range. The high optimal temperatures for enzymatic catalysis in halophilic organisms may be considered an adaptive feature as these enzymes have to endure in their natural salt environments, such as, slatterns exposed to intense sunlight. The thermophilic nature has been further reported for several halophilic enzymes (Camacho *et al.,* 1995 and Marhuenda *et al.,* 2002).

The optimal temperature for the Ve1-10-8_2 amylase was in the same range (55-60 ^{0}C) as reported for the enzyme from *Halobacterium salinarum*. However, it retained activity at higher temperatures. Similar to Ve1-10-8_2 amylase, an enzyme from alkaliphilic *Bacillus sp*. had optimal temperature at 55^{0}C (Igrashi *et al.,* 1998). Other Halophilic enzymes such as NAD and NADP glutamate dehydrogenases from Halobacterium salinarum displayed maximal activity at 70^{0}C, with high temperature

stability (Bonete *et al.*, 1987), which quite are comparable to an amylase from *Thermus sp*. AMD33 (34). Along the similar lines, a halophilic and thermophilic bacterium, *Halothermothrix orenii*, had temperature optima at 65^0C (Mijts *et al.*, 2001).

Ve1-10-82 amylase displayed thermophilic character in its stability as well. The thermal stability, however, decreased sharply at 80^0C. In the literature, there are some reports on the thermostable amylases from mesophilic and halophilic organisms (Mijts *et al.*, 2002).

High optimal temperature may be considered as an adaptive response to the high temperatures these enzymes have to endure in their natural salt environments, i.e., salterns exposed to intense sunlight. The thermophilic nature has been reported for some other halophilic enzymes (Camacho *et al.*, 1995 and Marhuenda *et al.*, 2002). In conclusion, the enzyme described in the present report reflected several features close to those reported for halophilic enzymes. However, the effect of solvents on amylase from moderately halophilic bacteria reflected towards the new findings. The action of various solvents in combination with varying conditions of pH, salt and temperatures further highlighted the significance of the enzymatic studies under non-aqueous conditions. The residues implied in the chemical mechanism seem to share some common features related to the halophilic nature of the enzyme and its ability to function in combination of other extreme conditions. Findings on haloalkaliphilic amylases and its performance with organic solvent will enrich the data base of non-aqueous enzymology.

In conclusion, the enzyme described in the present report highlighted several features quite similar to those found in other halophilic enzymes, including salt-dependent activity. Further, the temperature profile and thermal stability closely resembled to features reflected in thermophilic organisms. The findings on the haloalkaliphilic extracellular amylase with respect to its catalysis and enzymatic stability under multitude of extremities; salt, temperature and organic solvents would enrich the knowledge on non-aqueous enzymology, broadening the prospects of biocatalysis.

Chapter 5

Optimization of Protease catalysis in the presence of organic solvent

5.1 INTRODUCTION

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The haloalkaliphilic isolates are valuable sources of novel enzymes and possess unique metabolic machinery able to produce novel molecules (Eichler *et al*., 2001; Rozzell *et al*., 1999; Herbert *et al*., 1992). Most of the halophiles and haloalkaliphiles produce extracellular hydrolytic enzymes which are salt and thermo tolerant, such as protease, amylase, lipase, xylanases and pullulanase can be utilized in detergent, food and leather industries, waste water treatment, biotransformation of uranium compound and in textile industries. Because of the extreme nature of their enzymes, they can execute the current requirement of industries. However, only few extracellular enzymes have been characterized from Halophiles and haloalkaliphiles.

Microorganisms served as an important source of proteases mainly due to their shorter generation time, the ease of bulk production and the ease of genetic and environmental manipulation. Proteases are the most important kind of industrial enzymes (Joo *et al.*, 2002) and account for about 65% of the total worldwide sale of industrial enzymes in the world market (Johnvesly and Naik, 2001). Application of proteases in detergent, leather, silk, bakery, soy processing, meat tendering and brewery industries is well documented. However, its application in the production of peptide synthesis in organic media is limited by the specificity and the instability of enzyme in the presence of organic solvents. Enzymes can be stabilized in the presence of organic solvents (Desantis and Jones, 1999; Kamiya *et al.,* 2000). Having naturally stable and highly active enzymes in organic solvents are of greater advantage.

Bacterial resistance to organic solvents has attracted attention from a number of laboratories due to the significant potential of resistance in non aqueous medium. (Heipieper *et al.,* 1995). Apart from the biotechnological interest, the study of solvent resistance should enrich our understanding of the adaptive mechanisms, when challenged by extreme environmental conditions. An organic solvent appearing in a microbial habitat

from either a natural or man-made source is one of the harshest stress factors microorganisms can come across, and yet they have developed organic solvent resistance (Aono., *et a.,l* 1995).

In recent years a new class of solvent tolerant microbes having unique ability to sustain under non-aqueous system has drawn considerable attention. Such organisms are attractive for applications in solvent bioremediation and biotransformation in non-aqueous media (Isken and de Bont, 1998; Pieper and Reineke, 2000; Sardessai and Bhosle, 2004; Gupta and Khare, 2005). Some of these microbes are reported to be rich source of the solvent stable enzymes. Very few reports are available in literature concerning the screening of microorganisms, which produce organic solvent-stable proteases. Protease from solvent tolerant *Pseudomonas* sp. is novel in this regard (Ogino *et al.,* 1999; Geok *et al.,* 2003 and Gupta and Khare, 2005).

In view of the above facts, study in the present chapter, deals with the effect of hydrophobic and hydrophilic organic solvents on the catalysis of Sj-2 alkaline protease. Susceptibility of the haloalkaliphilic alkaline protease in the presence of solvent with respect to different pH, salt and temperature was also looked into.

5.2 MATERIAL AND METHODS

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5.2.1 Microorganism and culture conditions

Haloalkaliphilic sp. Sj-2 was isolated from saline soil collected from the seashore near Jodiya (Latitude 22.28 N, Longitude 69.4, 60º E) in Gujarat, Western cost of India. The bacterium was isolated as described in Chapter 3.

Due to significantly high level of protease production, Sj-2 was selected for further study. Based on 16S rRNA gene sequencing, the isolate in the present study, was phylogenetically nearest to *Bacillus Pseudofirmus* strain Sj2. The accession number of the 16S rRNA gene sequences for Sj-2 is EU090232.

5.2.2 Enzyme production

For protease production, the Sj-2 was grown at 37ºC in CMB medium (pH 9.0) consisting of (g/l): glucose, 10.0; KH_2PO_4, 10.0; yeast extract, 5.0; peptone, 5.0; casein acid hydrolysate, 5.0 and NaCl, 100.0. The 24 hour grown mother culture (A_{660}; 1.0, 3 ml) was inoculated to 100 ml of production medium, which contained (g/l): gelatin, 30.0; casein acid hydrolysate, 10.0 and NaCl, 100; pH 9. The culture was incubated at 37 ºC at 100 rpm and 96 h grown cells were harvested by centrifugation at 5,500 g for 10 min. The supernatant was used as crude enzyme preparation. The crude enzyme preparation was stored at 4 ºC until further use.

5.2.3 Enzyme purification by ammonium sulphate precipitates

The crude enzyme was concentrated by ammonium sulphate (75% saturation, w/v) and precipitates were suspended in a minimum volume of 20 mM Borax-NaOH buffer (pH 10). The protease activity was measured by Anson–Hagihara's method (Hagihara *et al*., 1950), as described earlier in chapter 3.

5.2.4 Solvents selected for the study

Methanol, ethanol, propanol glycerol, diethyl ether, hexane, octane, and butanol with the corresponding Log Pow values: 1.9, 1.25, 3.9, 2.8, 2.13, 2.69, 2.92 and 0.95 were selected for non-aqueous enzymatic studies of Sj-2 alkaline protease.

5.2.5 Effect of organic solvents on the catalysis of protease

Protease activity was measured in a reaction mixture of 0.5 ml enzyme and Casein Solution (0.6%, w/v) prepared in NaOH-Borax buffer (20mM, pH10) with 10, 20 and 30% (v/v) of methanol, ethanol, Propanol, diethyl ether, hexane, octane and glycerol. Activities were calculated by Anson– Hagihara method as discuss earlier.

5.2.6 Effect of pH on the catalysis of alkaline protease

Effect of pH on protease catalysis with organic solvent was determined by protease assay at different pH in presence of ethanol. The buffers (20mM) used were Sodium Phosphate (pH 5.5 - 8), Tris-HCl (pH 8 – 9.5), NaOH-Borax (pH 9.5 - 10) and Glycine - NaOH (pH 8 - 12). The enzyme was incubated with 10, 20 and 30% (v/v) of organic solvents along with respective buffers.

5.2.7 Effect of NaCl on protease activity in non-aqueous condition

To examine the influence of NaCl and organic solvents on enzyme activity, the reaction mixture was supplemented with 0.5 to 4M NaCl. Protease assay was carried out at 37°C with 5, 10 and 20% (v/v) of ethanol. Percent residual activities were calculated. The protease activity in the absence of additional NaCl and solvent was considered as 100%.

5.2.8 Effect of Temperature on protease catalysis and temperature optima

The temperature profile for Protease activity was examined by incubating the assay reaction mixture at different temperatures in the range of 37-80°C. The Protease activity was determined as mentioned earlier.

5.3 RESULT

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The present study describes the partial purification of an alkaline protease from a Haloalkaliphilic bacterium strain Sj-2 isolated from the saline soil near Jodiya from Coastal Gujarat, followed by the characterization of the enzyme in the presence of organic solvents. The enzyme was partially purified by ammonium sulphate precipitation. The purification results are summarized in Table 5.1. With 30.63 % yield and 1,383.9 U/mg specific activity, 3.96 fold purification was achieved.

Preparation	**Volume (ml)**	**Total protein (mg)**	**Total activity (U)**	**Specific activity (U/mg)**	**Yield (%)**	**Purification fold**
Crude	750	139.6	48,700	348.85	100	-
Ammonium suphate	10	10.78	14,919.5	1,383.9	30.63	3.96

Table 5.1- Purification of Sj-2 protease by ammonium sulphate precipitation

5.3.1 Protease catalysis in the presence of organic solvents

Sj-2 alkaline Protease was quiet active in the presence of the tested solvents. The enzyme was noticeably active up to 20 % (v/v) of glycerol and ethanol while in case of methanol and propanol, it was active up to 10% (v/v). Quiet reduced activity was evident in presence of propanol. Catalysis in presence of 10 % (v/v) glycerol and ethanol was comparable to control. While with 10% (v/v) methanol and propanol, the activities were nearly one third of the control. With methanol and propanol even at 20 % (v/v) total loss of activity were recorded.

Protease in presence of 30% (v/v) ethanol, exhibited 5 % residual activities. However, with 30% (v/v) glycerol, methanol and propanol, the protease activity was totally lost.

With 20% (v/v) ethanol, glycerol, methanol and propanol alkaline protease retained 98.14, 55.42, 7 and 6 % residual activities correspondingly.

The enzyme was noticeably active up to 30% (v/v) of diethyl ether, octane and n-hexane. Relatively reduced activity was evident in presence of butanol. Catalysis in the presence of 10% (v/v) diethyl ether and octane was comparable to control. While with 10% (v/v) n-hexane, the activity was nearly the same as control, while with butanol even at 10% (v/v), there was total loss of activity.

Partially purified enzyme with 10% (v/v) n-hexane, diethyl ether and octane exhibited 95.8, 92.1 and 68.98% residual activities, respectively. However, with 10, 20 and 30% (v/v) butanol, Protease activity was totally lost. Enzyme retained 88.2, 79.3 and 68.21% residual activities with 20% (v/v) n-hexane, diethyl ether and octane, correspondingly. In presence of 30% n-hexane, diethyl ether and octane, the activities further reduced to 77.3, 62.18 and 64.77%, respectively (Figure 5.1).

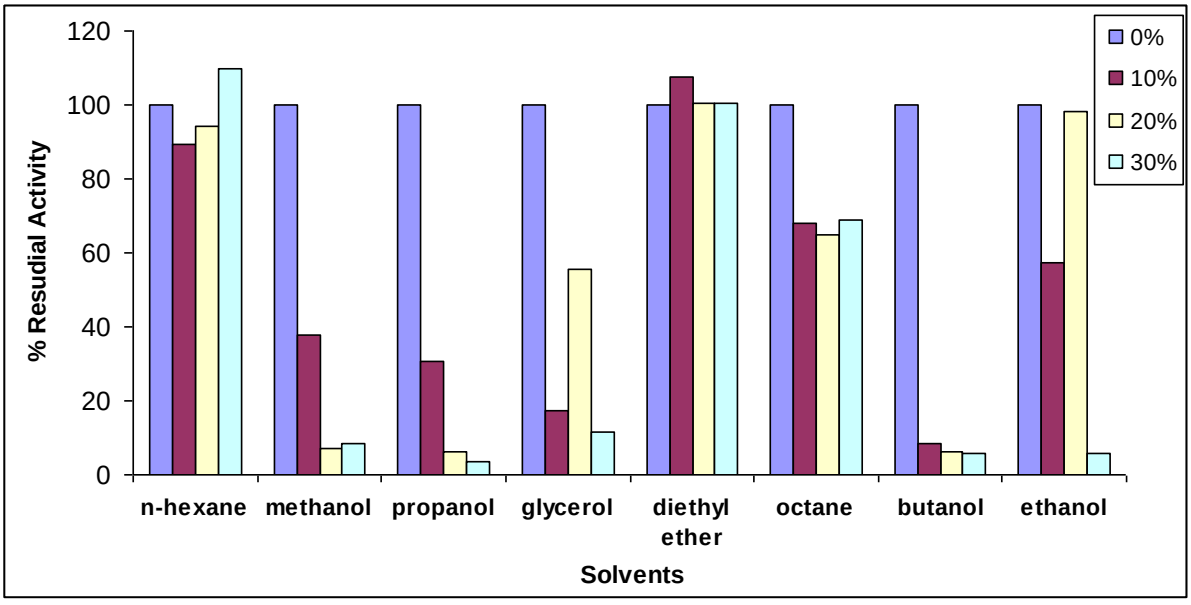

Figure 5.1- Catalysis of Sj-2 Alkaline Protease in presence of various organic solvent

5.3.2 Effect of pH on the catalysis of protease

Effect of pH on Sj-2 alkaline protease was assessed in the presence of ethanol in which enzyme was active in alkaline pH range, 8 to 12. The activity at pH 10, without any solvent, was considered as 100%. At several combinations of pH and solvent concentrations, the residual activities were monitored. Protease retained 73, 65 and 62 % residual activities with 10, 20 and 30% (v/v) ethanol at pH 8. The loss of activities of partially purified enzyme under similar conditions of solvent and pH 9 were quite comparable to those of at pH 8. Enzyme had 90, 76 and 72 % residual activities with ethanol at pH 9.

Protease exhibited 119, 106 and 89 % residual activities at pH 10. Enzyme was exhibiting maximum activity at pH 10. At higher pH, the enzyme had reduced activities. At pH 11, protease retained 82, 65 and 53% residual activities in presence of 10, 20 and 30% (v/v) of ethanol. With the same concentrations of ethanol, Sj-2 Protease had 54, 38 and 24% residual activities, respectively at pH 12 (Figure 5.2).

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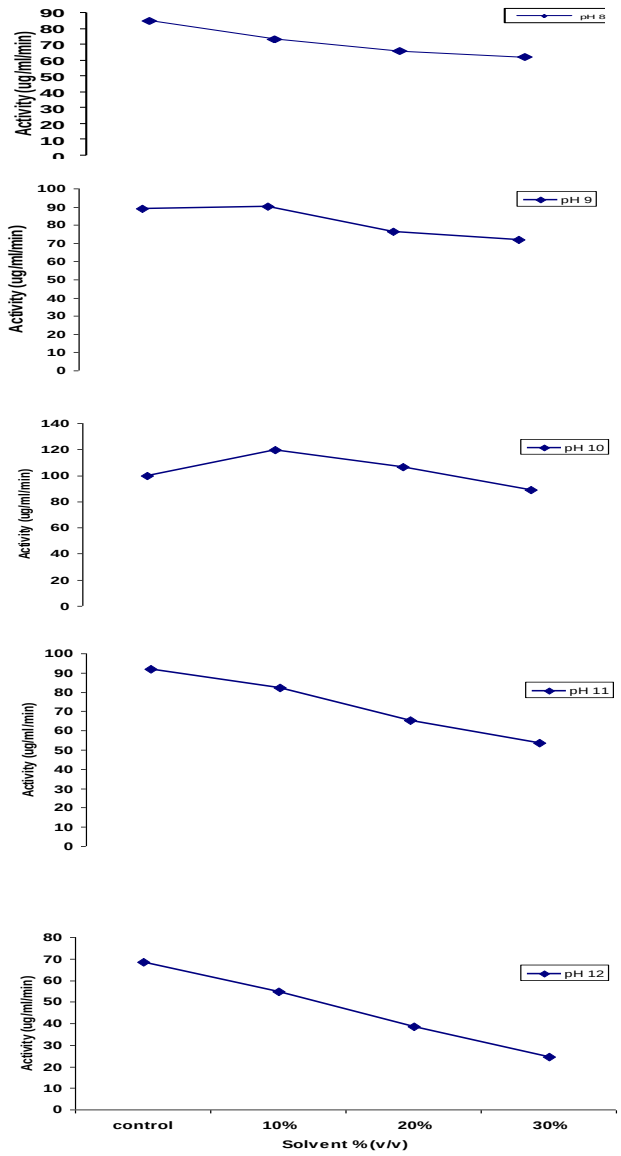

Figure 5.2- Effect of pH on catalysis of Sj-2 Alkaline Protease in the presence of ethanol

5.3.3 Effect of salt on catalysis of Sj-2 protease

Effect of 0.5 - 4M NaCl on Sj-2 protease catalysis was examined in the presence of 10 - 30% (v/v) ethanol. NaCl at 3M was optimum for Sj-2 protease catalysis. While at 2M NaCl, the activity was quiet similar to optimum salt concentration. In presence of 0.5 M NaCl, the protease retained 58, 50 and 43% residual activities with 10, 20, and 30 % (v/v) ethanol.

At 1 M, comparatively better enzyme activity was observed with the tested solvents. As compared to activity at 0.5 M salt, nearly two fold enzyme activities were recorded at 1 M salt. With 1 M salt, the residual activities were 102, 98 and 86% at 10, 20 and 30% (v/v) ethanol, respectively. Activities at 2M salt were similar to optimum level. The Sj-2 alkaline protease with 10, 20 and 30 % (v/v) ethanol, exhibited 139, 124 and 97 % residual activities, correspondingly.

At 3 M NaCl in reaction medium, enzyme exhibited 149, 130 and 104% residual activities with 10, 20 and 30% (v/v) of ethanol, respectively. 3M Salt concentration was optimum for alkaline protease which was unaltered in presence of different concentrations of ethanol. With various salt concentrations, better activity was noted at 3 M. At 4 M NaCl; 80, 65 and 27% residual activities were recorded at tested concentrations of ethanol.

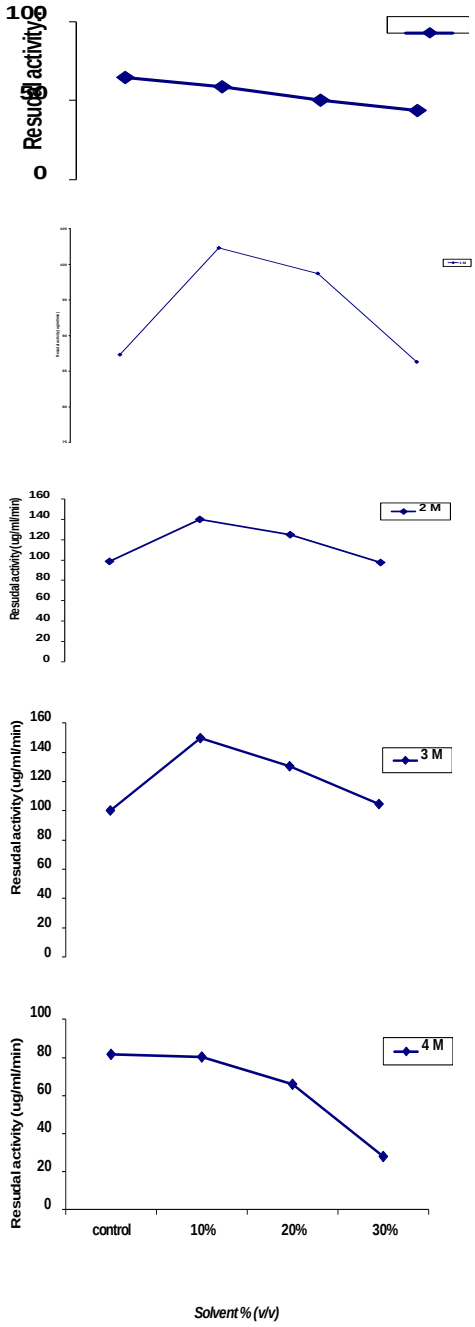

Figure 5.3-Effect of salt on Sj-2 alkaline protease in presence of ethanol

5.3.4 Temperature profile of Sj-2 protease in presence of n-hexane

Figure 5.4 displays the effect of temperature on the catalysis of Sj-2 Protease in the presence of n-hexane. Optimum temperature for alkaline protease was 50°C, which shifted to 60°C in the presence of n-hexane. At 10 % (v/v) n-hexane, protease retained comparable activities as control coupled with enhanced temperature optima. At 30% (v/v) solvent, the enzyme retained nearly 50% of the residual activity.

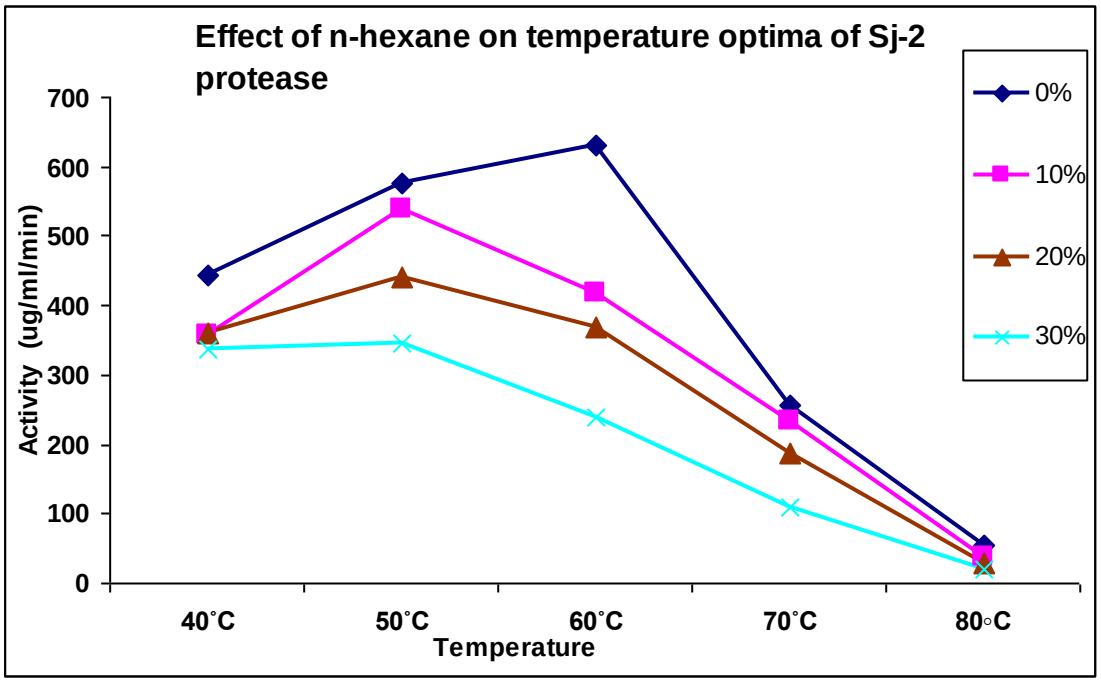

Figure 5.4- Effect of hexane on temperature optima of Sj-2 alkaline protease

DISCUSSION

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As highlighted earlier, due to many limitations only 1-5 % of the microbial word has been explored. The most concentrated and widespread occurrences of organisms are generally observed in moderate environments. However, there are extreme environments thought to prevent the existence of life (Eugster *et al.,* 1978). Organisms which thrive in extreme saline and alkaline environments offer us the opportunity to appreciate the range of adaptive possibilities that evolution can bring to bear on fundamental biological processes and they constitute unique models for investigations on how biomolecules are stabilized when subjected to extreme conditions. Halophilic and haloalkiliphilic micro-organisms offer a multitude of actual or potential applications in various fields of biotechnology (Zeynep *et al.,* 2001).

The biodegradation (transformation or mineralization) of a wide range of hydrocarbons, including aliphatic, aromatic, halogenated and nitrated compounds, has been shown to occur by many halophilic and haloalkaliphilic microorganisms (Ward *et al.,* 1978). Therefore, study of organic solvent tolerance in the presence of high salt concentrations and alkaline pH is of interest for the bioremediation of oil- polluted salt marshes and industrial wastewaters, contaminated with aromatic hydrocarbons or with chlorinated hydrocarbons (Margesin *et al.,* 2002). Over the last twenty years, biocatalysis in organic solvents has emerged as an area of systematic research and industrial development, fueled mainly by chemical and pharmaceutical interest. Attention has been especially focused on enzymes as catalysts for asymmetric synthesis.

Proteases from haloalkaliphilic bacteria represent an interesting resource for both fundamental enzymology and biotechnology, as they are active under high salt and alkaline pH. The haloalkaliphilic bacteria in the present study were able to grow and produce alkaline protease in the presence of solvents. Only limited literature is available on the enzymes from haloalkaliphilic bacteria with respect to non-aqueous biocatalysis

(Herbert *et al*.,1992; Gupta *et al.*, 2005, Madigan *et al*., 1997; Niehaus *et al*.,1999, Karan *et al*. 2010 and Eichler *et al*. 2001). Some intra and extra cellular enzymes from extremely and moderate halophilic and haloalkaliphilic bacteria and actinomyctes have been characterized, which might have potential applications in food, chemical, pharmaceutical, leather, tanning, paper pulp and waste-treatment industries (Costa *et al.*,1998; Mehta *et al*. 2006; Patel *et al*. 2005; Jogi *et al*. 2005; Patel *et al*. 2006, Thumar *et al*. 2007 and Wejse *et al*. 2003). Studies on haloalkaliphilc proteases with respect to their tolerance against organic solvents have not been investigated in great deal. However, some haloalkaliphilic archaea, actinomycetes and their relationship with organic solvents have been investigated during the recent years (Tadamasa *et al*., 2005; Thumar *et al*., 2009 and Saraiva *et al*., 1996). It is well reported that enzymes are inactivated in the presence of organic solvents, and catalytic activities in non-aqueous environment are generally lower than those in aqueous system (Ogino *et al*., 2001 and Ru *et al*., 1999).Therefore, it was quite interesting to study a protease from haloalkaliphilic bacteria in non-aqueous medium. A haloalkaliphilic protease reported in this study was screened against various organic solvents. The protease displayed varying responses against these solvents. Catalysis of Sj-2 protease was in the presence of methanol and propanol was inhibited, with other tested solvent, the enzyme was quiet active. Catalysis with 30% (v/v) water miscible solvents as ethanol and glycerol indicated the robust nature of the enzyme.

Sj-2 alkaline protease was active over wide range of pH, 8-11; the optimum being at 10. These values are marginally higher than those reported for a protease from an alkaliphilic Bacillus sp. (Igrashi *et al*., 1998) and significantly higher than those reported for another Protease from *Halobacterium salinarum* (Good *et al*., 1970). Other organisms, such as thermophilic and halotolerant bacteria, *Halothermothrix orenii* are reported to have proteases with optimal activities in the similar range of pH (Mijts *et al*., 2001). Halophilic enzymes, in general, are not stable in low salt concentrations, because of ionic charges and salt dependent structural stability (Madern *et al*., 2000; damson *et al*., 1997; Bonnete *et al*., 1994 and Martinez *et al*., 2009). Therefore, an increase of activity with salt concentrations is a common feature of the halophilic enzymes (Dym *et al*., 1995). The

protease from Sj-2 in the present study displayed an upward shift in activity with salt from 0.5 to 2 M. Salt affects the binding between the enzyme and substrate. Most of the halophilic and haloalkaliphilic enzymes are inactivated at NaCl or KCl concentrations below 2 M (Camacho *et al*., 1995). As described earlier, the protease activity in Sj-2 increased with increasing salt concentrations, indicating an overall effect of salt on the reaction. The enzyme was quiet active with 1M NaCl even in the presence of solvents. Optimum catalysis at comparatively low salt and its behavior to retain activity with broader range of salt concentrations in the presence of solvents is quite relevant to haloalklaiphilic bacteria. The findings are quite comparable to a moderately halophilic and aerobic bacterium, *Halomonas meridiana* (Coronado *et al*., 2000). At front of solvent tolerance, Sj-2 protease had non differential effects in response to salt as optimum 3M salt concentration was unaltered with varying concentrations of solvents.

The optimal temperature (50^0C) for Sj-2 protease was quite comparable with the enzyme from *Halobacterium salinarum* (Good *et al*., 1970). However, the enzyme was active at higher temperatures, and with 30% v/v n-hexane. It retained significant activity up to 70^0C. Comparable to our studies, the enzyme from an alkaliphilic *Bacillus sp*. also exhibited the optimal temperature at 60^0C (Igrashi *et al*., 1998). Other Halophilic enzymes, such as NAD and NADP glutamate dehydrogenases from Halobacterium salinarum displayed maximal activity at 70^0C, with higher temperature stability (Bonete *et al*., 1987). The temperature profiles and stability were quite comparable to a thermophilic Protease from *Thermus sp.* AMD33, which had temperature optima at 70^0C (Nakamura *et al*., 1993), or with a halophilic and thermophilic bacteria *Halothermothrix orenii*, with an optima at 65 ^{0}C (Mijts *et al*., 2001). The thermophilic nature of our enzyme was also reflected by a shift in temperature optima to higher range. The high optimal temperatures for enzymatic catalysis in halophilic organisms may be considered an adaptive feature as these enzymes have to endure in their natural salt environments, such as, slatterns exposed to intense sunlight. The thermophilic nature has been further reported for several halophilic enzymes (Mijts *et al*., 1995 and Marhuenda *et al*., 2002).

In conclusion, the enzyme described in the present report highlighted several features quite similar to those found in other halophilic enzymes, including salt-dependent

activity. Further, the temperature profile and thermal stability closely resembled to features reflected in thermophilic organisms. The findings on the haloalkaliphilic extracellular protease with respect to its catalysis and enzymatic stability under multitude of extremities; salt, temperature and organic solvents would add to the knowledge on non-aqueous enzymology.

Chapter 6

Purification and Characterization of Alkaline Proteases

6.1 INTRODUCTION

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Organic solvent's tendency to create partition in cytoplasm and disrupt the vital functions of cell, make it extremely toxic for living cells. Their accumulation in cell membrane can change the structural and functional integrity. It accumulates in the bacterial cell membrane and changes in structural and functional integrity may lead to cell lysis (Adams *et al.,*1987, Affleck *et al.,*1992, Adinarayana *et al.,* 2003). With quick and high multiplication rate and shorter life span, organic solvent tolerant bacteria are a relatively suitable approach to overcome toxic effect of solvent polluted environment. Explorations of Solvent tolerant isolate have another great intersect. Due to their natural tolerance for organic solvent, their enzymes have innate stability against organic solvents. Screening of such a solvent tolerant enzyme can avoid the different physical and chemical methods to stabilize it in organic solvents (Bustard *et al.,* 2003).

The enzymatic route of peptide synthesis demands a solvent tolerant protease, which can cope up with harsh and instability causing industrial condition. Biocatalysis in non-aqueous environment have many advantages as increased solubility of hydrophobic substrates, altered enantio-selectivity, reduced microbial contamination and curtailed water-induced side reactions (Bonete *et al.,* 1987).

Several solvent tolerant halophilic isolate have been reported (Beg *et al.,* 2003, Colby *et al.,* 1977, Costa *et al.,* 1998, Dodia *et al.,* 2006, Diego *et al.,* 2006). These microorganisms deal with high osmotic pressure by efficient efflux pump. This is a

positive asset to refrain from structural disorganization. Precipitation in high saline conditions is avoided on account of the increased negative charges on the surface of halophilic proteins due to large number of acidic amino acid residues (Fausnaijgh *et al.*, 1984). With such adaption, most of halophilic proteins perform their functions with high concentrations of NaCl. Optimal activity has been recorded in the NaCl concentration range of 4–5 M (Gimenez *et al.,* 2000). High salt reduces water activity, a feature also generated by the organic solvents. Therefore, halophilic proteins offer valuable tools for non-aqueous enzymology (Gupta *et al.,* 2005).

While number of studies are available on the organic solvent tolerant microorganisms (Gessesse *et al.,* 2003 and Gomes *et al.,* 2004), only limited work is available on haloalkaliphiles in this context (Hagihara *et al.,* 1958, Heidari *et al.,* 2008, Izotova *et al.,* 1983, Isken *et al.,* 1998). Enzymatic characteristics from haloalkaliphilic bacteria under nonaqueous conditions are further restricted (Inoue *et al.,* 1989 and Ikura *et al.,* 1997*).*

In view of the above facts, the present work has focused on the solvent tolerant haloalkaliphilic Bacterium Sj-1 and AH-6 from the saline habitats of Coastal Gujarat in Western India. Haloalkaliphilic Bacterium grew well in presence of high concentrations of solvents. It produced an extracellular protease, active in various organic solvents. In the present work, we also illustrated a single step purification protocol based on hydrophobic interaction chromatography for the efficient purification of the protease. The catalysis and stability of the enzyme under non-aqueous conditions are studied in detail.

6.2 MATERIAL AND METHOD

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6.2.1 Materials

Phenyl sepharose 6 FF was purchased from Sigma (St. Louis, MO, USA). Casein was from Sisco Research Laboratories (Mumbai, India) and other media components were purchased from Hi Media Laboratories (Mumbai, India). All organic solvents (analytical grade) were purchased from Rankem (New Delhi, India).All other chemicals were of analytical grade.

6.2.2 Bacterial strain

Haloalkaliphilic bacteria Sj-1 and AH-6 were isolated from soil sample collected from Jodiya (Latitude 22°43'11"N, Longitude70°16'48"E) Western Coast of Gujarat, India**.** Protease producing isolate was screened by inoculating actively grown culture on gelatin agar plates ((g/l): Gelatin, 30; Peptone, 10; NaCl, 100; pH, 9 and Agar, 30) and incubated at 37°C for 24-48 hour. The Sj-1 strain exhibited considerable protease production and, therefore, was selected for further study.

6.2.3 Growth and protease production with organic solvents

To study the growth kinetics of Sj-1 and AH-6 with various solvents, gelatin Broth media containing (g\l): Gelatin, 30; Peptone, 5; NaCl 100 at pH; 9 was prepared with 10 - 30% (v/v) organic solvents; n-hexane, methanol, propanol, butanol, dodecane, decane, isooctane, heptanes, xylene and cyclohexane. Solvents were added separately. For preparation of inoculums a loop full of pure culture was added into 25 ml sterile gelatin broth medium (NaCl, 10% w/v; pH 9). Medium was incubated at 37ºC for 24 hour.

Further, solvent containing 50 ml gelatin broth medium was inoculated with 5% of the activated culture. Medium was incubated under shake flask conditions (140 rpm) at 37ºC. Control of each set was carried out simultaneously. Up to 120 hours culture aliquots were withdrawn at 24 hour interval and microbial growth was measured at 660nm.The experiments were performed in three independent replicates.

Culture aliquots were centrifuged at 10,000 rpm for 10 minutes at 4^{o}C and cell free supernatant was used as crude enzyme preparation.

6.2.4 Enzyme purification by hydrophobic interaction chromatography

At the first step, 75% saturation (w/v) Ammonium Sulphate was gradually added in the crude enzyme with gentle stirring. The precipitate collected by centrifugation at 12,000g for 20 minutes, was suspended in a minimum volume of 20 mM Borax-NaOH buffer (pH 10). Purification was performed by hydrophobic interaction chromatography. The partially purified enzyme preparation (10.0 ml in 1M Ammonium Sulphate) was loaded on a phenyl sepharose 6 fast flow columns (1 cm × 6.5 cm).

The column was pre-equilibrated with 0.1 M sodium phosphate buffer (pH 8.0) containing 1 M ammonium sulfate. Elution of bound enzyme was done by 0.1 M sodium phosphate buffer, pH 8.0 containing a decreasing step gradient of ammonium sulfate (1.0–0.1 M). Fractions were collected at a flow rate of 0.7 ml min^{-1} and analyzed for protease activity. The active fractions were pooled and used for further characterization.

6.2.5 Enzyme assay and estimation of protein

The proteolytic activity of the enzyme, with casein as the substrate, was determined by Anson–Hagihara's method (Hagihara *et al.,* 1958). An aliquot of protease (0.5 ml) was added to 3.0 ml substrate solution (0.6% casein in 20 mM borax–NaOH buffer, pH 10.0) and the reaction mixture was incubated at 37ºC for 20 minute. The reaction was terminated by the addition of 3.2 ml TCA mixture (containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid). The inactivated reaction mixture was maintained at room temperature for 20 minute, filtered through Whatman filter paper No. 1 and absorbance was measured at 280 nm with tyrosine as a standard.

In control, the enzyme was added after adding TCA mixture. One unit of alkaline protease activity was defined as the amount of enzyme required to produce peptide equivalent to 1.0 g of tyrosine per minute per ml at standard assay conditions. Protein was estimated by dye binding method, using bovine serum albumin as standard protein.

6.2.6 Effect of pH on activity and stability of Protease

The effect of pH in the presence of isooctane on purified protease was examined by assaying the enzyme at pH:8.0-12.0, using different buffers (20mM); Sodium Phosphate (pH 5.5 - 8), Tris-HCl (pH 8 – 9.5), NaOH-Borax (pH 9.5 - 10) and Glycine - NaOH (pH 8 - 12).The enzyme was incubated with 5, 10 and 20% (v/v) of solvent along with respective buffer. To investigate solvent's effect on stability of the enzyme at different pH, the pH adjusted to 5-13 with above buffers in the presence of 30% isooctane. After incubation for 30-180 minute, residual activities were estimated.

6.2.7 Effect of NaCl on activity and stability of Protease

The effect of NaCl and isooctane in conjunction on enzyme activity was assessed by supplementing the reaction mixture with 0.1 to 0.4 M NaCl for AH-6 and 1-4 M NaCl for Sj-1. Protease assay was carried out at 37°C with 5, 10 and 20% (v/v) of isooctane. For the stability of protease in response to salt and solvent, the enzyme was incubated with 2 M NaCl (Sj-1) ,0.2 M NaCl (AH-6) and solvent and the aliquots were withdrawn at regular time intervals for monitoring residual activities. The protease activity in the absence of extra NaCl was considered as a 100%.

6.2.8 Effect of Temperature on activity and stability of enzyme

The effect of temperature on purified protease was examined by incubating the reaction mixture at different temperatures in the range of 37-80°C. The protease activity was determined as mentioned above. The temperature stability was studied by incubating the enzyme at different temperatures (37-80°C). The aliquots were withdrawn at 30, 60, 90, 120 and 180 minute and reaction mixture was incubated at optimum temperature. The residual enzyme activities were measured.

6.2.9 SDS-Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli using 12% crosslinked polyacrylamide gel. The protein bands were visualized on the gel by Coomassie blue staining.

6.3 RESULT

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Microbes from exposed and unpopulated habitats have been explored comprehensively for the study of their sustainability and performance of their enzyme in various organic solvents (Laane *et al.*, 2006, Lama 2005). However, the exploration of Haloalkaliphilic bacteria for similar studies is quite limited. In the present study, organic solvent tolerance of a haloalkaliphilc bacterium and its extra cellular Protease has been undertaken. The haloalkaliphilic bacterium was aerobic and gram-positive having thin long rod. It has simple nutritional requirement having the ability to grow at alkaline and saline conditions.

6.3.1 Sj-1

6.3.1.1 Effect of organic solvents on growth and protease production

Growth of Sj-1 was monitored at various concentrations of different organic solvents and the patterns are highlighted in Figure 6.1 In general, better tolerance was observed with the organic solvents of higher log Pow values. In hexane (log Pow:3.9), dodecane (log Pow: 2.92), decane (log Pow: 5.98), methanol (log Pow: 0.82) , cyclohexane (log Pow: 3.4), isooctane(log Pow: 4.5) and heptane (log Pow: 4.66), the organisms grew well. At 10 % (v/v) of these solvents, the organism started growing after 24 hour .

With xylene (log pow: 3.20) and propanol (log Pow: 0.25), growth was recorded after 48 hour. While in butanol (log Pow:0.9), the growth was further delayed and observed at 72 and 96 hour s, correspondingly.

Growth patterns of Sj-1 with hexane and isooctane were similar to control even at 30% (v/v). It reached to nearly 70% cell density at 120 hour. With 30 % (v/v) heptane, dodecane, decane, methanol and propanol, Sj-1 maintained 50% cell density of control. Butanol retarded the growth most effectively. At 10% (v/v) butanol, significant growth was recorded at 120 hour, while at higher concentrations, it reduced to negligible level.

Considerable protease production was recorded with various solvents (Figure 6.2). In hexane, methanol, dodecane, decane, hepaten and isooctane, considerable protease production was observed at all concentrations. Even at 30% (v/v) solvent, significant enzyme production was apparent. Especially with isooctane, hexane, dodecane and decane, the enzyme production was comparable with control. In case of propanol and xylene, enzyme production was recorded at 10% (v/v) concentration. Protease production was totally inhibited with butanol and cyclohexane.

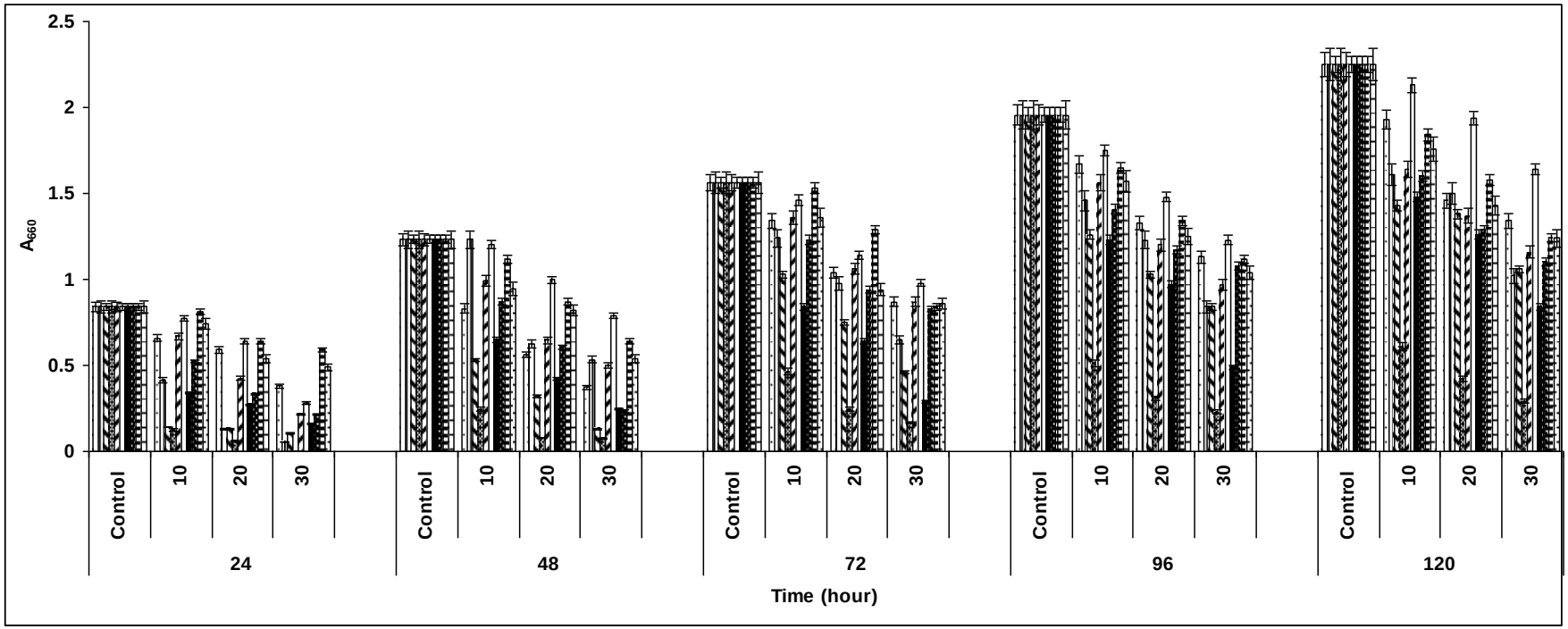

Figure 6.1 1 - Growth of Sj-1 in presences 10,20 and 30% (v/v) Hexane ( ), decane ( ), propanol ( ), butanol ( ), dodecane ( ), methanol ( ), isooctane( ),xylene ( ), cyclohexane ( ), heptane ( ) at different time interval

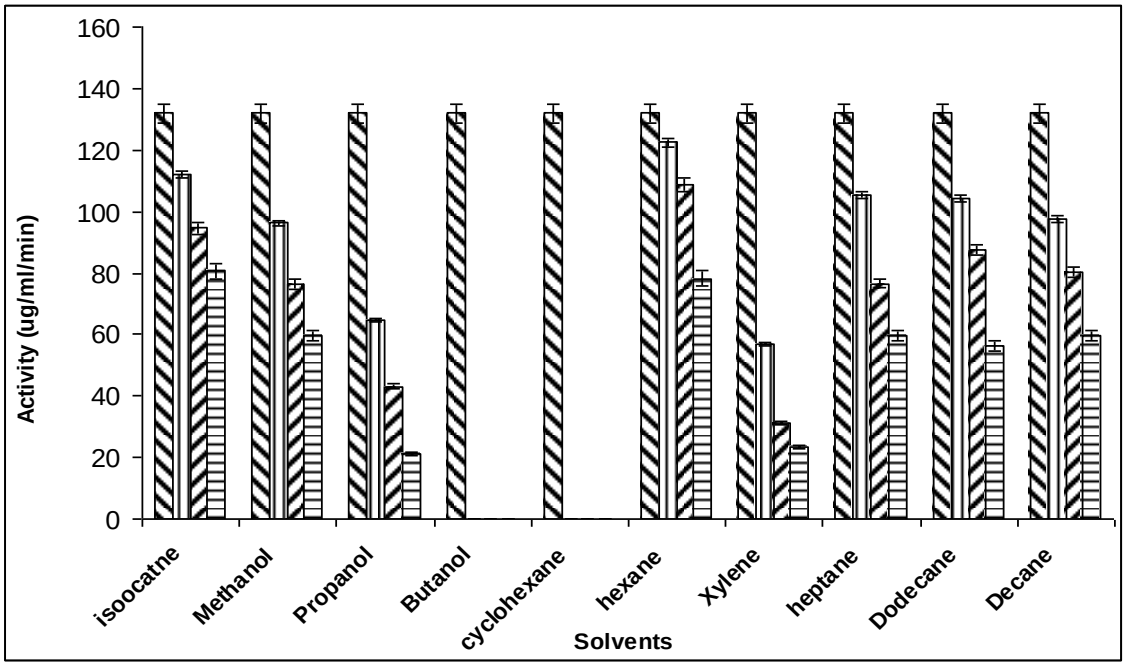

Figure 6.2– Sj-1 Protease production at 120 hour in control (▧), 10(▩), 20(▨) and 30(▤) % (v/v) of various organic solvent

6.3.1.2 Protease purification by hydrophobic interaction chromatography

The enzyme was purified by hydrophobic interaction chromatographic, leading to 27.83 fold purification, specific activity at 9611 U/mg and yield 28% (Table 1). Hydrophobic interaction chromatography has been effectively applied for the purification of alkaline proteases. Purified protease was observed as a single band on SDS-PAGE (Figure -6.3) and its apparent molecular weight was determined as 30 kDa

Preparation	Volume(ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (u/mg)	Yield %	Purifiation Fold
Crude	1000	168.26	58000	344.70		
$(NH_4)_2SO_4$	10	16.28	20365	1250.92	35	3.62
Phenylsepharose 6FF HIC	10	1.725	16580	9611.59	28	27.83

Table 6.1- Purification of Sj-1 protease by hydrophobic interaction chromatography

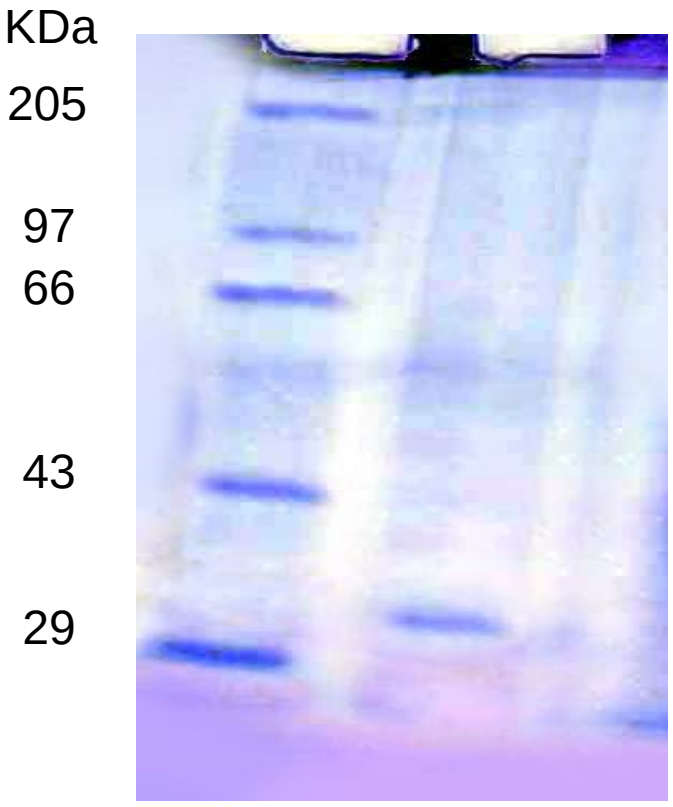

Figure 6.3 - SDS PAGE analysis of Sj-1 protease

6.3.1.3 Effect of pH on the activity and stability of Protease

Effect of pH in combination of organic solvents was studied with 10-30 % (v/v) isooctane and pH 8-12. Optimum catalysis was at pH 10 and it did not change in isooctane. At pH 8 with 10, 20 and 30% (v/v) isooctane, the enzyme had 60, 56, and 48% residual activities, respectively. At pH 9 and 10, for the same set of reactions; 80, 73, 56 and 108, 90, 70 % residual activities were recorded, correspondingly. At higher pH11 and 12; 67, 50, 38 and 43, 30, 24% residual activities were present, correspondingly, with the tested concentration of isooctane. Activity without solvent at pH 10 was considered as 100% (Figure 6.4A).

Stability of Sj-1 protease in the presence of isooctane was evaluated at pH 10. Enzyme was capable to retain its catalysis for 18 hours in 30% (v/v) isooctane (Figure 6.4(b). Enzyme activity without solvent at 0 minute was treated as 100%. After 3 hours, with tested concentration of isooctane, the enzyme retained 85, 80 and 63 % residual activities. After 6 hours incubation with the same concentration of solvent; 77, 62 and 60 % residual activities were recorded. After 6 hours, the enzyme activity started decreasing. The residual activities at 12 and 18 hours in the presence of 10, 20 and 30 %(v/v) isooctane, were 52, 40, 37 and 40, 34, 27 %.

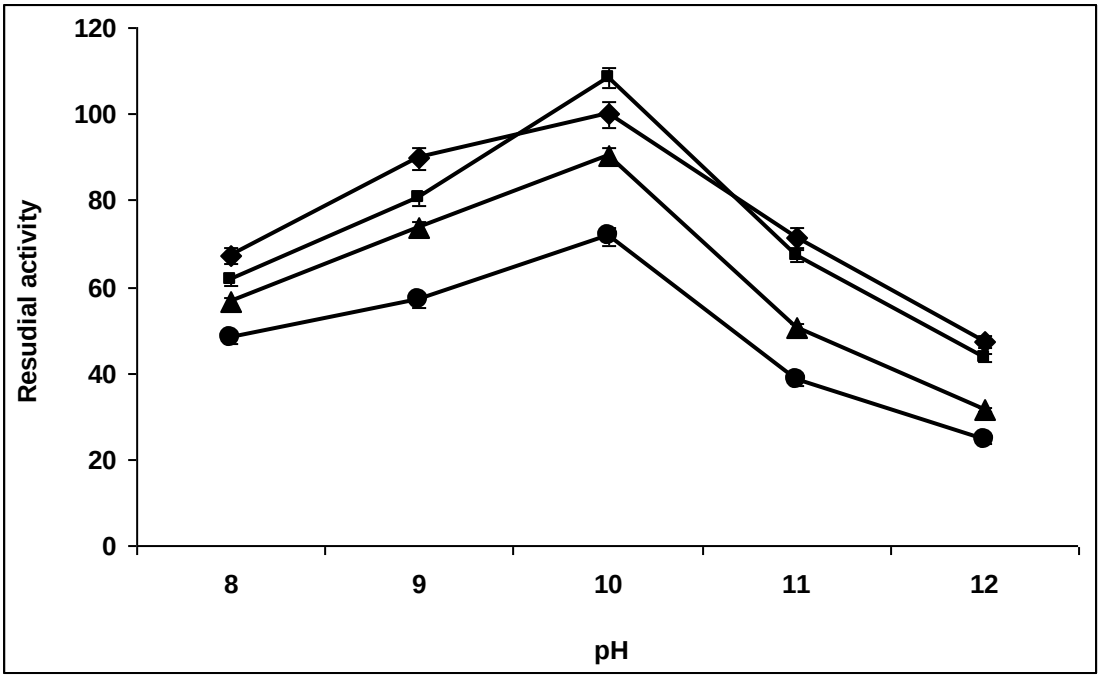

Figure 6.4- A Effect of pH on catalysis of Protease in presence of 0 (♦), 10(■), 20(▲) and 30 (×)% (v/v) isooctane

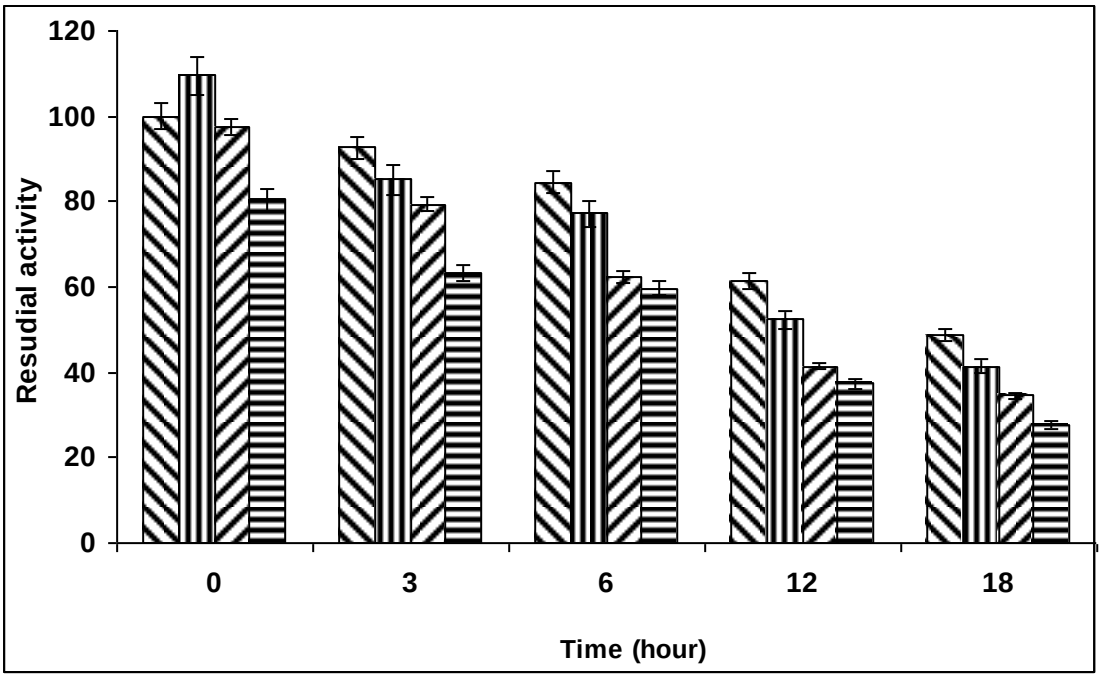

Figure6.4 B- Effect of pH on stability of Protease in presence of 0 (▧), 10(▥), 20(▨) and 30 (▤)% (v/v) isooctane

6.3.1.4 Effect of salt on Protease catalysis

The effect of salt on Sj-1 protease catalysis in non-aqueous condition was assessed with 1 – 4M NaCl and 10, 20 and 30% (v/v) isooctane. At border range of salt, considerable activities were recorded and at 2 M salt (w/v), it was highest. The activity at 1M NaCl without any solvent was considered as 100%. With 10, 20 and 30% (v/v) isooctane and 1 M NaCl, the residual Sj-1 activities were 94, 86 and 78 %, respectively. At 2 M salt, moderately enhanced activities were observed, while at the examined concentrations of solvent; 96, 90 and 83% residual activities were evident. Optimum activity was observed with 3 M (w/v) salt and the enzyme retained 105, 98 and 90% residual activities (Figure 6.5 A).

Sj-1 protease stability with salt was studied in isooctane at optimum salt concentration. With various concentrations of isooctane, stability was significantly retained for 18 hours. Activity at 0 % (v/v) solvent was considered as 100%. After 3 hours of incubation with the tested concentrations of the solvent, the enzyme retained 90, 74 and 56 % residual activities, while after 6 and 12 hours; 77, 70, 42 and 65, 57, 38 % residual activities were observed, respectively. However, at 18 hours, the enzyme with isooctane had 52, 50 and 24 % residual activities (Figure 6.5 B).

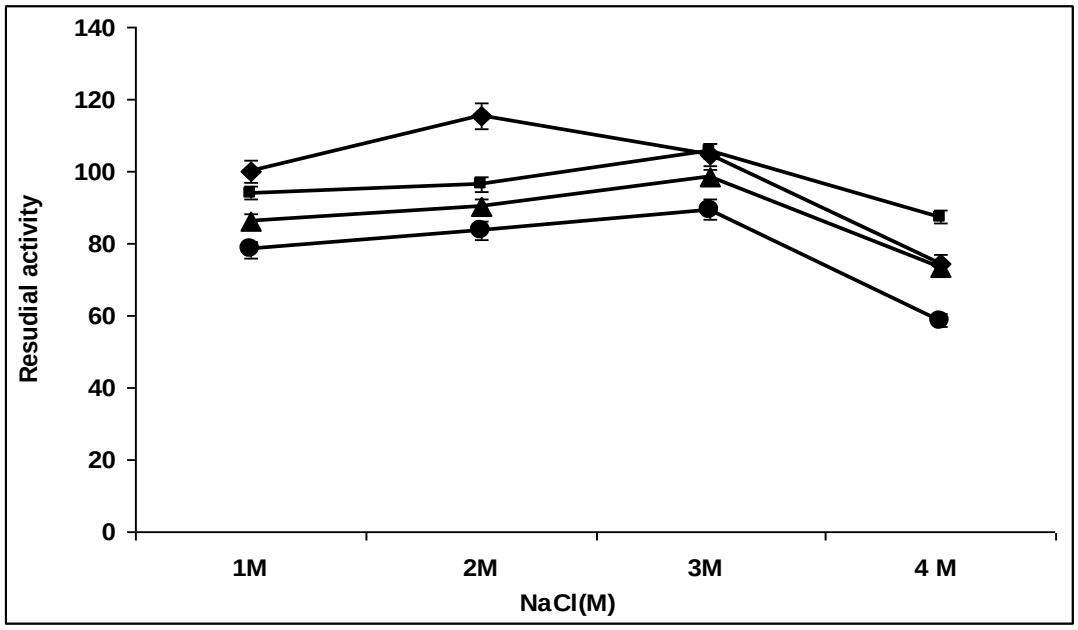

Figure 6.5 A Catalysis of Sj-1Protease in presence of 0 (♦), 10(■), 20(▲) and 30 (●) % (v/v) isooctane and various salt concentration

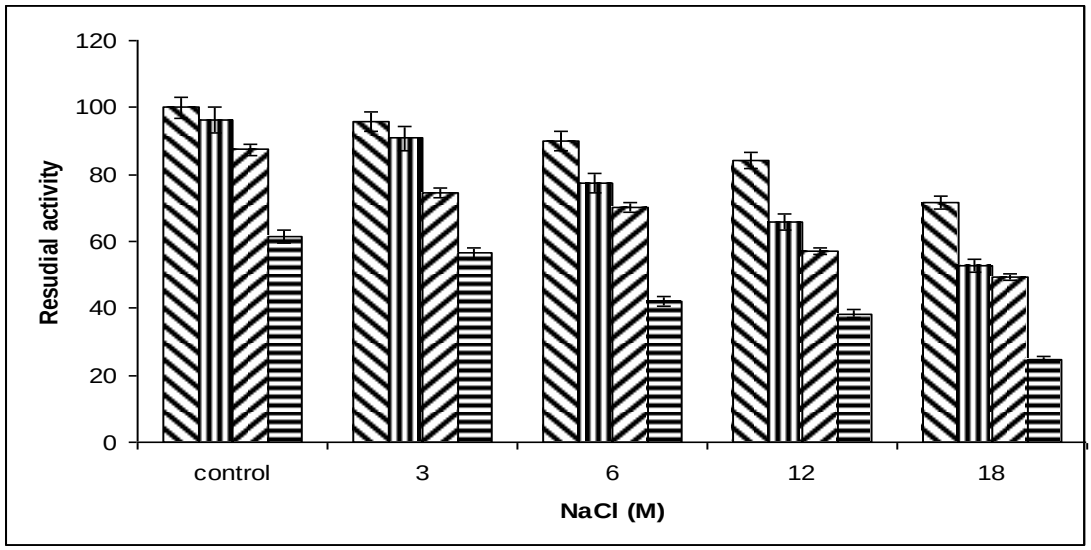

Figure 6.5B Stability of Sj-1Protease in presence of presence of 0 (▧),10(▥), 20(▨) and 30 (▤)% (v/v) isooctane

6.3.1.5 Effect of solvent on temperature profile and stability of protease

Effect of Solvent on temperature optima of Sj-1 protease is summarized in Figure 6.6. The optimum temperature was 50°C, which remained unaltered in isooctane up to 30% (v/v). At various concentrations of isooctane, the enzyme was quite active up to 70°C. It retained around 54% residual activity with 10% v/v isooctane at 70°C. The enzyme exhibited 58, 70, 77, 60 and 38 % residual activities at 40, 50 60, 70 and 80°C, respectively, with 30 %(v/v) isooctane (Figure 6.6 A).

The enzyme was quiet stable at wide range of concentrations of isooctane: 10-30% (v/v) (Figure 6.6B). Stability of protease was investigated at its optimum temperature of 50°C. After 3 hours of incubation; 88, 80 and 64% residual activities were evident. At 6 and 12 hours; 70, 60, 50 and 60, 53, 42% residual activities were recorded. However, after 18 hours incubation with the examined concentrations of isooctane, only 40% of the residual activities as compared to control were observed.

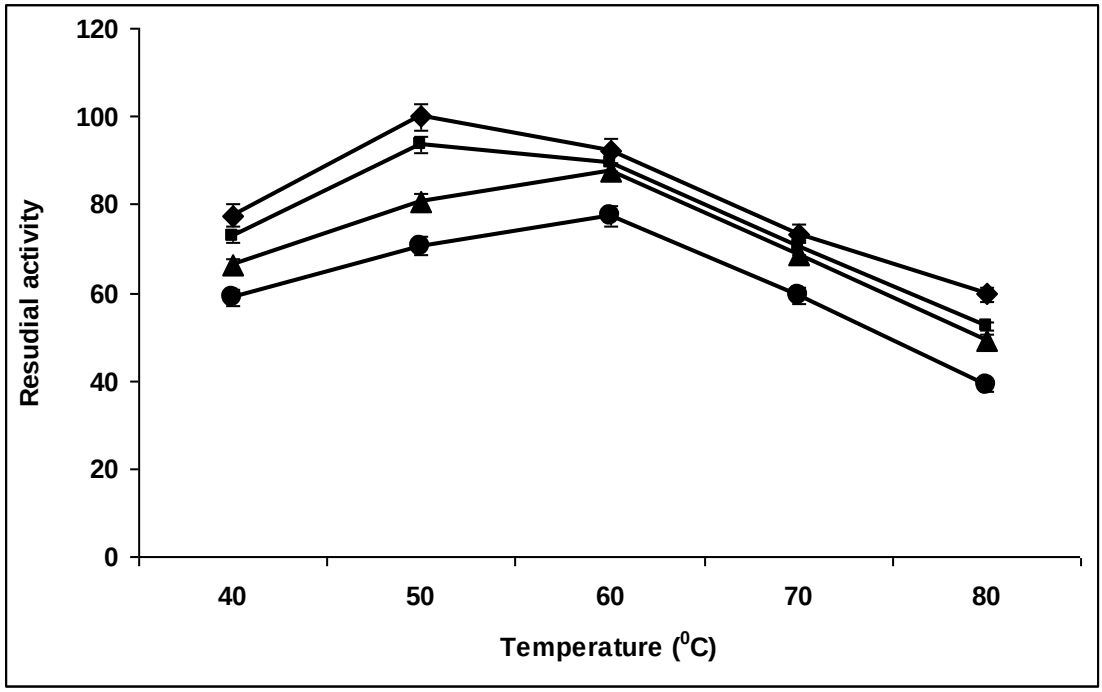

Figure 6.6 (a)- Effect of temperature on protease in presence of 0 (♦), 10(■), 20(▲) and 30 (●) % (v/v) isooctane.

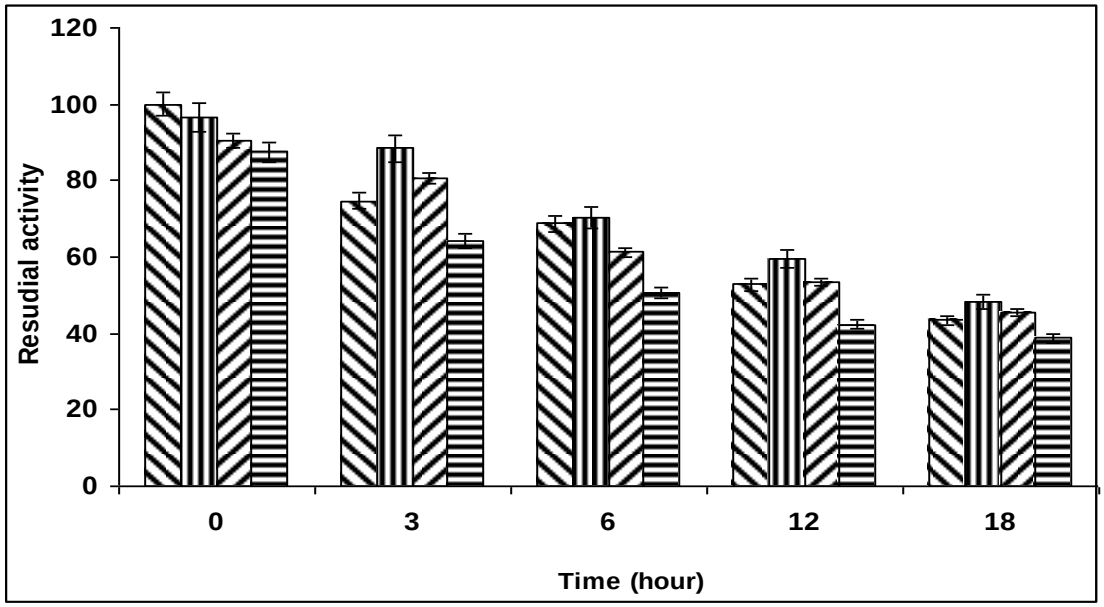

Figure 6.6 B- Effect of temperature on the catalysis of Protease in the presence of 0 (▤),10(▧), 20(▥) and 30 (▨)% (v/v) isooctane

6.3.2 AH-6

6.3.2.1 Effect of organic solvents on growth and protease production

The growth of AH-6 was monitored in the presence of varying concentrations of different organic solvents. Figure 6.7 illustrates the growth patterns of AH-6, where the organism tolerated solvents with higher logPow. AH-6 grew in the presence of hexane (log Pow:3.9), propanol (log Pow: 0.25), dodecane (log Pow: 2.92), decane (log Pow: 5.98), isooctane (log Pow: 4.5) and heptanes(log Pow: 4.66) . With 10 % (v/v) of these solvents, the growth started after 24 hour. In the presence of xylene (log pow: 3.20) and cyclohexane (log Pow: 3.4), growth was visible after 48 hour, while for methanol (log Pow: 0.82) and butanol (log Pow:0.9), it appeared at 72 and 96 hour, respectively. Growth in dodecane, decane, isooctane and heptanes, even at 30% (v/v), was quiet comparable with control. With 30 % (v/v) hexane, methanol and propanol, the growth was nearly 50% of control. Butanol most effectively retarded the growth.

Protease production is represented in Figure 6.8. In the presence of hexane, methanol, dodecane, decane, hepaten and isooctane, considerable protease production was observed as compared to control, at all tested concentrations of solvents. Significant enzyme production, especially with hexane, dodecane and decane was recorded even at 30% (v/v) solvent concentrations. In propanol and xylene , protease production was observed only at 10%(v/v) solvent. However, no enzyme was recorded with butanol and cyclohexane. On the other hand, better enzyme production in comparison to control was apparent with 10 % (v/v) hexane.

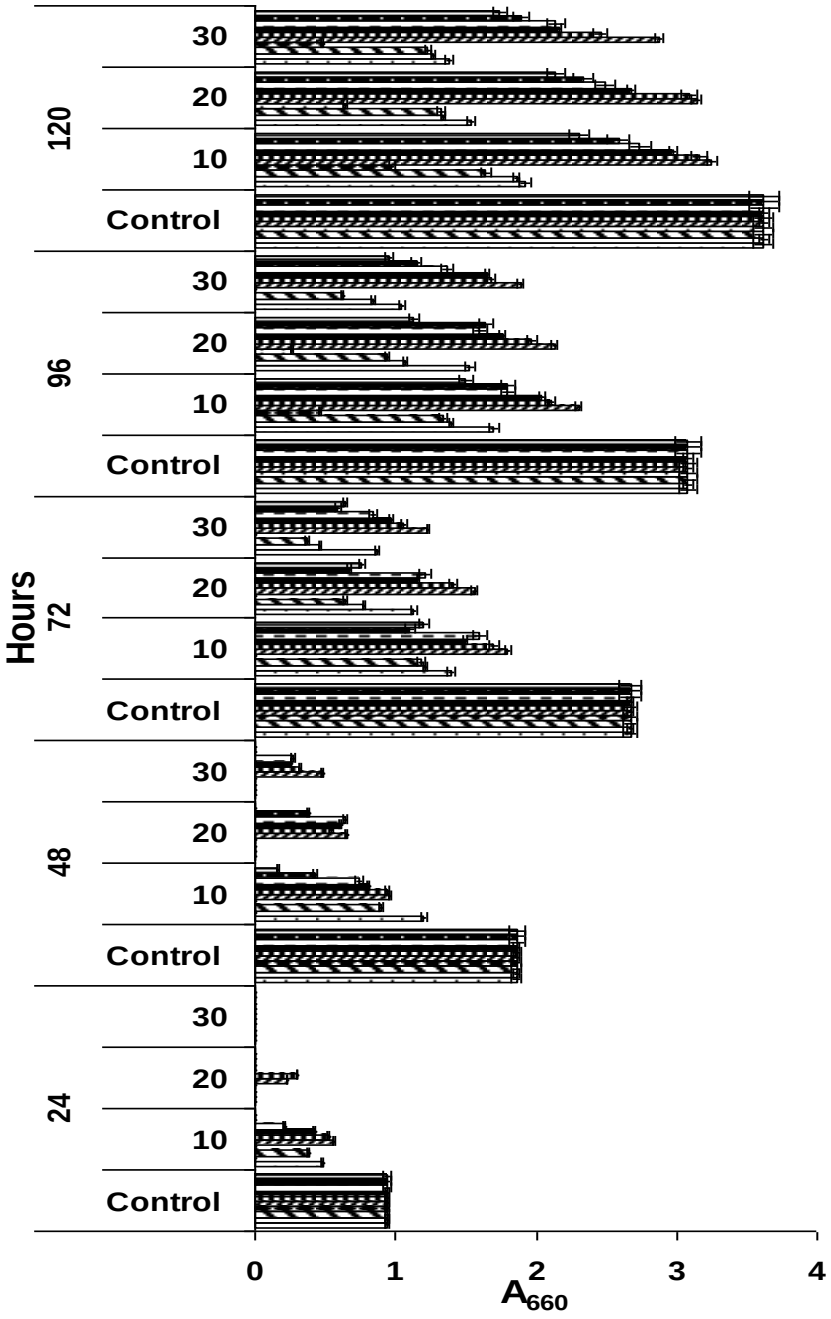

Figure 6.7 - Growth of Ah-6 in presences 10,20 and 30% (v/v) Hexane (▒)**decane** (▥), **propanol** (▧), **butanol** (▩), **dodecane** (▨), **methanol** (□), **isooctane**(■),**xylene** (▓), **cyclohexane** (▤), **heptane** (☷) **at different time interval**

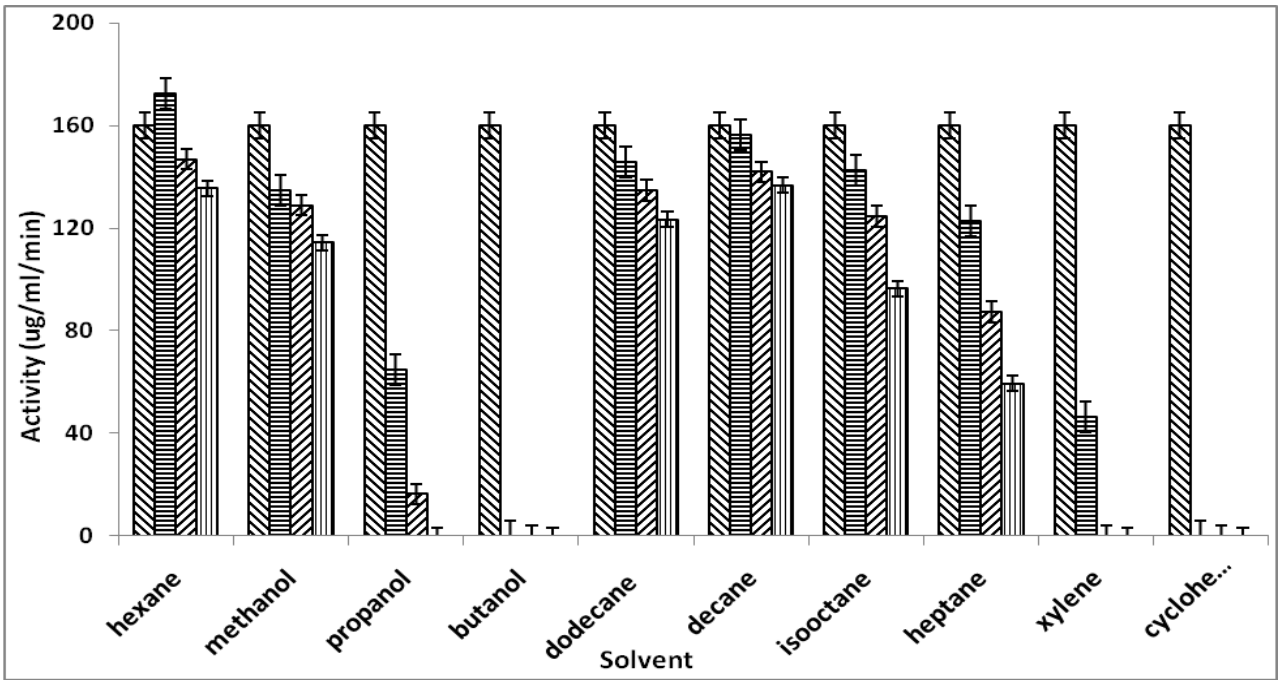

Figure 6.8- AH-6 Protease production at 120 hour in control (▧), 10(▤), 20(▨) and 30(▥) % (v/v) of various organic solvent

6.3.2.2 Protease purification by hydrophobic interaction chromatography

The purification results are summarized in Table 1. 18-fold purification of AH-6 protease was achieved by a single step purification method with phenyl sepharose 6 FF with a specific activity of 7,312 U/mg and 22% yield (Table 1). The purified enzyme migrated as a single band in SDS-PAGE (Fig. 6.9) and apparent molecular mass was determined as 40 kDa.

Preparation	**Volume (ml)**	**Total Protein (mg)**	**Total Activity (U)**	**Specific Activity (U/mg protein)**	**Yield %**	**Purification Fold**
Crude	750	142.68	49000	343.61		
(NH4)2SO4	10	12.46	16832	1350.88	34	3.93
Phenyl sepharose 6FF HIC	10	1.915	14003	7312.27	28	21.30

Table 6.2 Purification of AH-6 protease by hydrophobic interaction chromatography

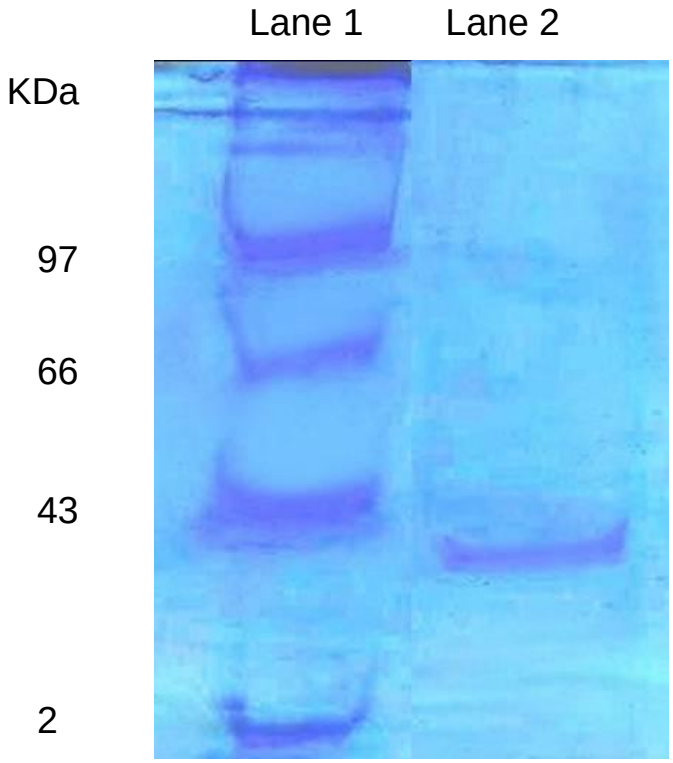

Figure 6.9 – SDS PAGE analysis of AH-6 protease

6.3.2.3 Effect of pH on activity and stability of Protease

Effect of pH: 8-12 on AH-6 protease catalysis was examined in the presence of 10-30% (v/v) hexane. Optimum catalysis was at pH 10, which remained unaltered in hexane. At pH 8, AH-6 Protease with 10, 20, and 30 % (v/v) hexane, had 46, 40 and 38 % residual activities, respectively. At pH 9 and 10, for the same set of reactions, 97, 76 ,`63 and 108, 85, 76 % residual activities were recorded, correspondingly. At higher pH, decreased activities were observed. At pH 11 and 12 with 10, 20 and 30% hexane; 51,43,32 and 34, 21 and 12 % residual activities were observed, respectively. Activity of protease at pH 10, without solvent was considered as 100% (Figure 6.10A).

The enzyme was considerably stable up to 18 hours at pH 10 in the presence of 30% (v/v) hexane (Figure 6.10B). The initial enzyme activity without solvent was considered 100%. After 3 hours, the enzyme retained 84, 78 and 65 % residual activities with 10, 20 and 30% (v/v) hexane. While at 6 hours, it was 61, 54 and 48 % with the same concentrations of the solvent. Fast decline in the activity was observed after 6 hour and at 12 and 18 hours with 10, 20 and 30 % (v/v) hexane, the enzyme retained 35, 30, 19 and 25, 19 and 10 % residual activities, respectively.

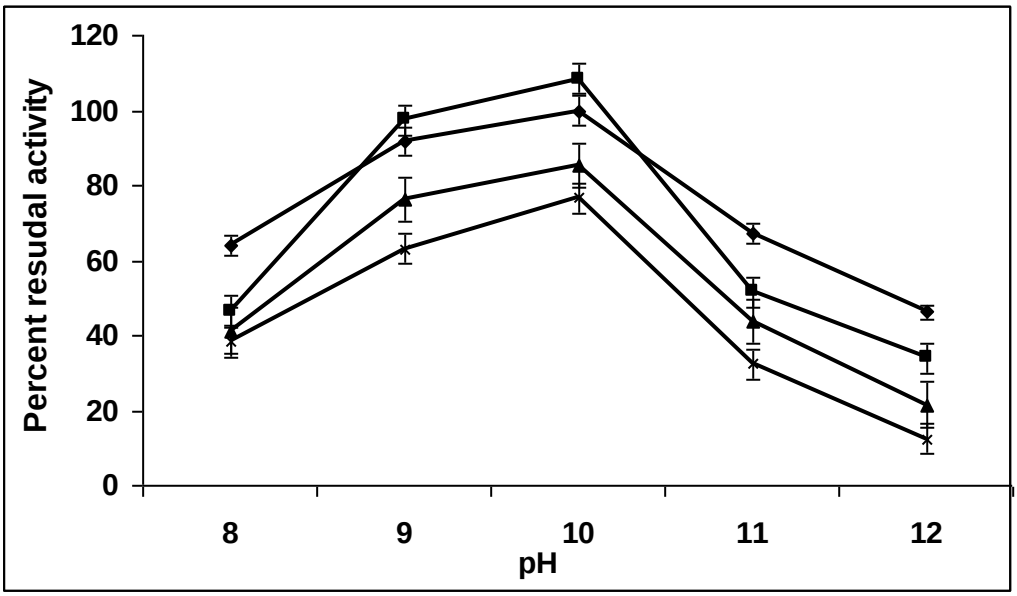

Figure 6.10 A

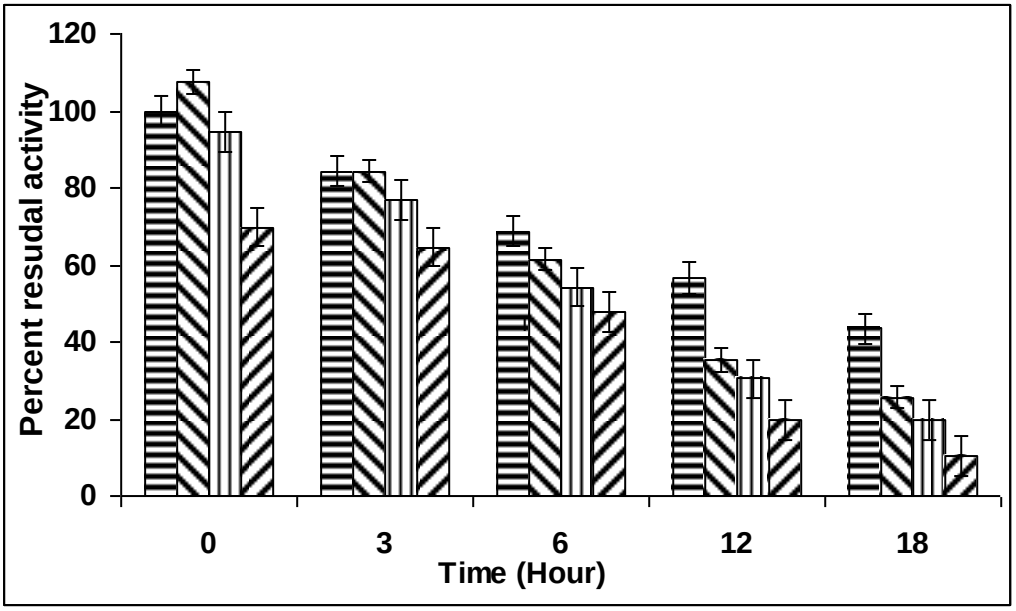

Figure 6.10 B

Figure 6.10 A - Effect of pH on the catalysis of Protease in the presence of 0 (♦), 10(■), 20(▲) and 30 (×)% (v/v) hexane

Figure6.10 B - Effect of pH on the stability of Protease in the presence of 0 (▤),10(▧), 20(▩) an30(▨)%(v/v) hexane

6.3.2.4 Effect of salt on Protease catalysis

Effect of NaCl (0 – 400mM) on enzyme catalysis was investigated in the presence of 10, 20 and 30% (v/v) hexane. The enzyme was active at wide range of salt concentrations, with the optimum at 200 mM. Activity in the absence of salt and solvent was considered as 100%. In presence of 10, 20 and 30% (v/v) hexane, without salt, the residual activities were 95, 84 and 81. With 100 mM salt; 116, 97 and 75% residual activities were recorded with the tested concentration of hexane. At optimum salt concentration in the presence of tested concentrations of hexane, 125, 105 and 87 % residual activities were observed. At higher salt concentrations, the enzyme rapidly lost activity(6.11 A).

Effect of salt on stability of AH-6 protease in the presence of hexane was investigated at optimum salt concentration, 200mM (w/v). Enzyme maintained 77, 86 and 43 % residual activities after 3 hour. While at 6 and 12 hours, the residual activities with 10, 20 and 30% (v/v) hexane were; 58,74, 36 and 56,48 and 23 %, respectively. After 18 hours, the enzyme retained 34, 38 and 112 % residual activities (Figure 6.11B).

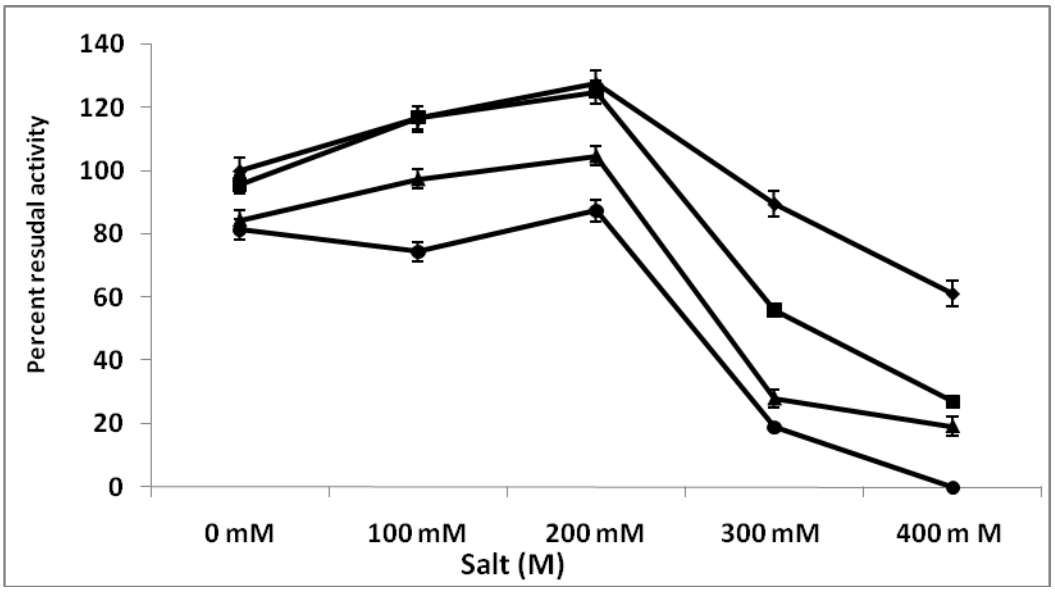

Figure 6.11 A

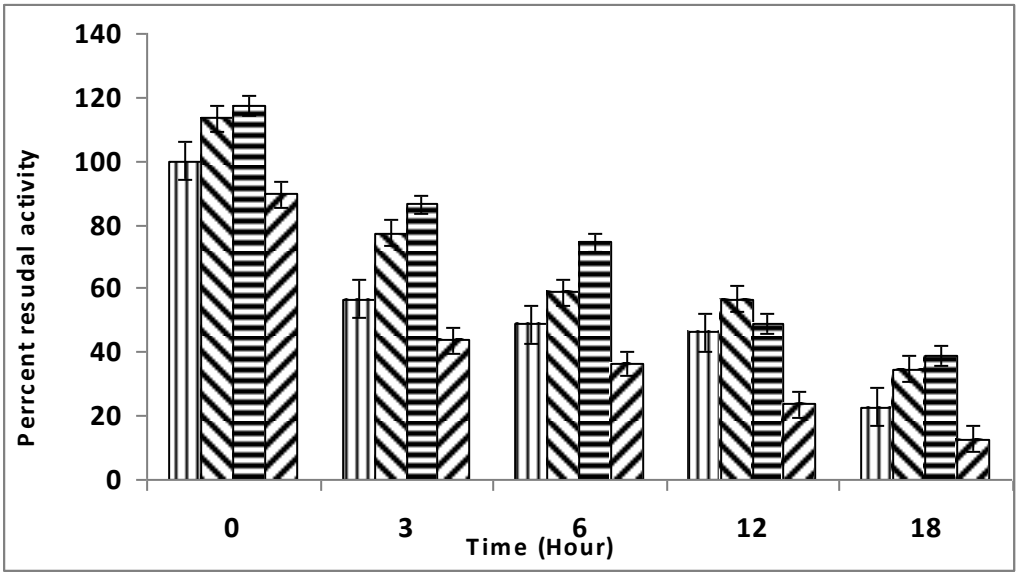

Figure 6.11 B

Figure 6.11 A -Catalysis of AH-6 Protease in the presence of 0 (♦), 10(■), 20(▲) an 30 (●) %(v/v) hexane and various salt concentrations

Figure 6.11B- Stability of Ah-6 Protease in presence of presence of 0 (▤), 10(▧),

20(▥) and 30 (▨)% (v/v) hexane

6.3.2.5 Effect of solvent on temperature profile and stability of protease

Figure 6 (A) reveals the trends of temperature profile of AH-6 alkaline protease in the presence of n-hexane. The optimum temperature at 50°C in the absence of solvent remained unaltered with up to 30% n-hexane. At all tested concentrations of hexane, enzyme was quite active up to 70°C. Enzyme retained around 55% residual activity with 10% (v/v) hexane at 70°C. The enzyme exhibited 84, 90, 80, 40 and 20 % residual activities at 40, 50 60, 70 and 80°C with 30 % (v/v) hexane (6.12A).

AH-6 protease was highly stable over a broad range of hexane from 10 to 30% hexane (v/v) (6.12B). Stability of enzyme was examined at 50°C, which was optimum for it's catalysis. The protease retained nearly 70 and 50 % of the original activity at 6 and 12 hour, respectively, with 10 % (v/v) hexane. With 20 % (v/v) hexane; 53, 40 and 25% residual activities were recorded at 6, 12 and 18 hours. However, in the presence of 30 % (v/v) hexane, the enzyme was stable for shorter duration. At the same concentration of hexane, nearly 50 and 40 % residual activities were observed at 6 and 12 hours, correspondingly.

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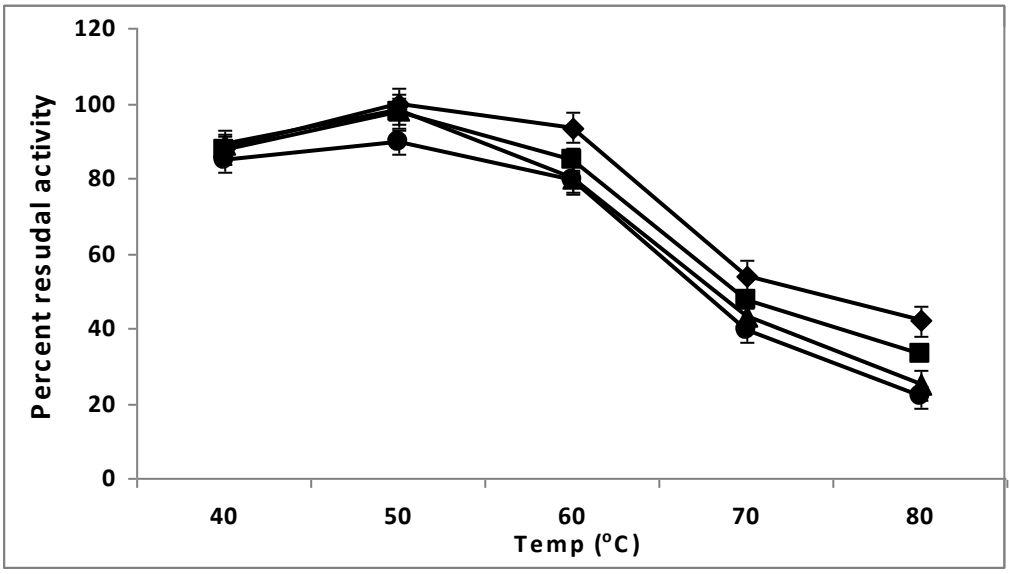

Figure 6.12A

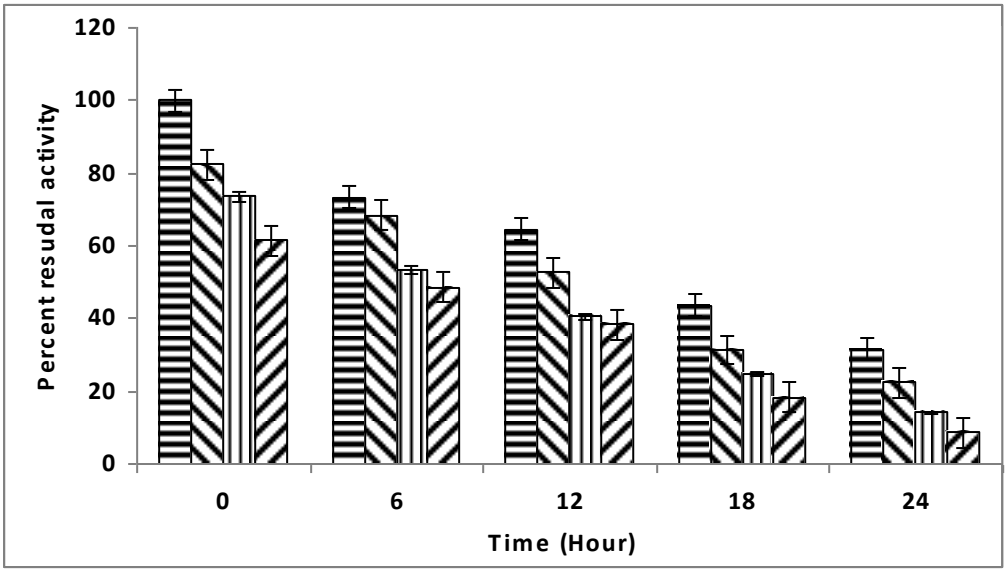

Figure 6.12 B

Figure 6.12 A- Effect of temperature on protease in the presence of 0 (♦), 10(■), 20(▲) and 30 (●) % (v/v) hexane.

Figure 6.12 B- Effect of temperature on the catalysis of Protease in the presence of 0 (▤),10(▧), 20(▥) and 30 (▨)% (v/v) hexane.

DISCUSSION

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Bioremediation of organic solvents and cost-effective source of valuable enzymes for biotechnological industry are the major attractions for exploration of organic solvent tolerant microbes. Various reports have been published on microbes dealing with non-aqueous catalysis. Haloalkaliphilic Bacteria and their enzymes, adapted to sustain in saline and alkaline conditions have not been attended well with respect to their organic solvent tolerance and possible role in bioremediation. Though there are some reports on the haloalkaliphilic archaea and their interactions with organic solvents (Heidari *et al.*, 2007, Tadamasa *et al.*, 2005), only scarce attention has been paid to haloalkaliphilic bacteria in this context (Colby *et al*., 1987).

Haloalkaliphilic bacteria Sj-1 tolerated most of the tested immiscible and miscible organic solvents such as methanol, propanpl, hexane, heptanes, isooctane, dodecanese, decane and cyclohexane. Corresponding to an earlier report, butanol inhibited and delayed the growth of Sj-1. Applicability of Sj-1 gets strength on account of the constant tolerance against organic solvents, even after several sub culturing. Therefore, Sj-1 can be useful in the remediation of organic solvent pollutants from saline and costal areas.

Organic solvents alter the active quarterly structure of enzyme leading to inactivation and therefore, specific catalytic activity in organic solvents are usually less than aqueous conditions (Ogino *et al.,* 2001, Ru *et al.,* 2000). Protease production in 30% organic

solvents: hexane, heptane, dodecane, isooctane and methanol were compared with control. In the presence of butanol and cyclohexane, the enzyme was totally inhibited. The results are relevant with the proposal by lane et al., (Laane *et al.*, 2005) which stated that hydrophobic solvents with lower log Pow do not suit to enzymatic catalysis.

Catalysis of Sj-1 protease in various solvents was related to α-amylase from *Halarcula* sp. Strain S-1 (Manikandan *et al*., 2009). Reduced stability in organic medium is mentioned in many reports (Ogino *et al.*, 1999, Izotova *et al*., 1983). In aqueous medium, water helps to maintain structural flexibility and mobility of protein molecule. Organic solvents may cause deamidation of Asn and Gln residues and hydrolysis of peptide bonds, leading to unfolding of enzyme molecules and loss of enzymatic activity (Affleck *et al.*, 1992).

AH-6 was capable to tolerate various water miscible and immiscible organic solvents, such as, methanol, propanpl, hexane, heptanes, isooctane, dodecanes, decane and cuclohexane. In butanol delayed growth was observed which corresponded with an earlier work (Gimenez *et al*., 2000). The genetic basis and constitutive nature of the solvent tolerance in AH-6 gained strength on account of its constant tolerance even after several sub-culturing in solvent free-medium. With these features in the background, the isolate may emerge as a capable candidate for remediation of organic solvent in saline areas.

It is well established that enzymes are highly inactivated in organic solvents and specific catalytic activities in non-aqueous environment are generally lower than those in aqueous system (Ogino *et al* ., 2001 , Ru *et al.*, 200). AH-6 strain produced protease with up to

30 % solvents; hexane, hepatane, decane, dodecane, isooctane and methanol. Comparing to control, it retained quiet appreciable level of activity. The enzyme activities in propanol and butanol were quite reduced. The results corresponded with the proposal by Lane ., (Laane *et al.,* 2002), according to which, hydrophilic solvents (log P< 2) are not suitable for enzymatic catalysis; however, the ionization state of protein is not taken into account in the log P model. Behavior of AH-6 alkaline protease in organic solvents could be related to α-amylase from *Halarcula sp*. Strain S-1 (Manikandan *et al*., 2009). Some of the earlier reports (Vidyasagar *et al*., 2006, Izotova *et al.,* 1983) documented reduced stability in organic solvents compared to aqueous medium. In water media, water promotes conformational mobility of protein molecule, deamidation of Asn and Gln residues and hydrolysis of peptide bond, causing the unfolding of enzyme molecules and loss of enzymatic activity (Nikolaev *et al*., 2006).

To purify halophilic protease, acetone or ethanol fractionation, ammonium sulphate perception, ultra-filtration, gel filtration, ion exchange or affinity chromatographies have been common techniques (Vidyasagar *et al* ., 2006, Heidari *et al.,* 2008 Xiong *et al.*, 2007). Hydrophobic interaction chromatography is usually preferred method for effectual purification with better yield of 25 to 35 %. Presence of salt around halophilic enzyme assist it in binding of protein and matrix (Gupta *et al.,* 2005 , Namwong *et al.*, 2006, Sánchezp *et al.,* 2003). In the present study, purification of Sj-1 Protease was successfully achieved by hydrophobic affinity chromatography with 27 fold purification and 28% yield. The protease was a monomer protein with the molecular mass of about 30 KDa. Many proteases from halophiles in literature corresponded with the molecular

weight (Vidyasagar *et al* ., 2006 , Xiong *et al.,* 2007, Gessesse *et al*.,2003, Manikandanet *et al.,* 2009).

Our results of AH-6 supported the usefulness of hydrophobic interaction chromatography for purification of haloalkaliphilic protease with 21 fold purification and 28% yield. AH-6 protease was a monomeric protein with a molecular mass of about 40 KDa. The molecular weight of the enzyme was in the range as reported for halophilic archaeal and bacterial proteases (Gessesse *et al*.,2003, Xiong *et al*., 2007).

Activity and stability of Sj-1 Protease at wider pH range 8-11, with optimum at 10, revealed it's alkaliphilic nature, would fall in the category of moderate alkaliphile (Lama *et al.,* 2005, Patel *et al*.,2006, Adams *et a*l.,1998). Our findings are comparable with alkaline proteases from (Salinivibrio Vidyasagar *et al*., 2006, Knubovets *et al*., 1996) and the protease CP1 from *Pseudoalteromonas* sp. strain CP76 (Lama *et al.,* 2005). Stability of Sj-1 protease at various concentrations of isooctane was an attractive feature of this enzyme.

According to studies, optimal activity of halophilic enzymes generally required 1–2 M salt and at lower salt concentrations, these enzymes get inactivated (Affleck *et al*., 1992). Our findings were quiet similar to *Halobacterium halobium* extracellular protease which required 3 M NaCl for optimal activity (Affleck *et al.,* 1992). Sj-1 alkaline protease was not salt dependent, but for its maximal activity, it required it. The enzyme was optimally active at 3 M salt.

AH-6 protease reflected alkaliphilic nature as it was active and stable over wide range of alkaline pH: 8-11, the optimum being at 10. This pH range closely resembled with some earlier reports for protease (Adinarayana *et al*., Gessesse *et* al., 2003). pH profile revealed a moderate alkaliphilic character (maximum activity at pH 9.0). These characteristics are similar to alkaline protease from *Salinivibrio* (Heidari et al., 2007, Lama *et al.,* 2005) and protease CP1 from *Pseudoalteromonas* sp. strain CP76 (Sánchez *et al*., 2003). Stability of protease with various concentration of hexane is attractive part of this study.

It's suggested that the halophilic enzymes generally require 1–2 M salt for their optimal activity and the catalytic activity was lost irreversibly when exposed to lower salt concentrations (Inoue *et al*., 1989). An extracellular protease from *Natrialba magadii* was optimally active with 1–1.5 M NaCl/ KCl (Ruiz *et al.,* 2007) and another extracellular protease from *Halobacteriumhalobium* required 3 M NaCl for optimal activity (Izotova *et al*., 1983). The AH-6 alkaline protease did not require high salt for the optimum catalysis as it was optimally active with 150–200 mM NaCl. In contrast to protease from *Salinivibrio* sp. strain AF-2004 (Heidar *et al.,* 2007) and similar with metalloprotease CP1 from *Pseudoalteromonas* sp. strain CP76 (Sánchez *et al*., 2003), this enzyme needed lower concentration of salt (0.2 M NaCl) for maximum activity.

The Sj-1 Protease was moderately thermophilic in nature, with the optimum activity at 50°C and considerable stability up to 70 °C. Similar findings were also reflected by Khalil Beg and Gupta (Kim *et al.,* 1997) for *Bacillus mojavensis*.

In general, compared to aqueous medium, the enzymes are more thermostable in anhydrous condition. For example, the thermostability of lysozyme with absolute glycerol at 80°C temperature was much greater than in water (Sardessai *et al.*, 2004). Sj-1 protease had significant activity with isooctane at 70°C compared. However, the temperature optimum was unchanged in isooctane. Other halophilic enzymes, such as NAD and NADP glutamate dehydrogenases from *Halobacterium salinarum* displayed maximal activity at 70°C, with higher temperature stability (Tadamasa *et al.*, 2005). The thermophilic nature has been further reported for some other halophilic enzymes (Thumar et al., 2009).

Solvent tolerant proteases are required for effective peptide synthesis while significant activity of most of frequently used commercial protease, such as α-chymotrypsin and A. oryzae protease, drop-down in presence of organic solvents (Gupta *et al*., 2005). Protease from Sj-1 was significantly active and stable in hydrophobic and hydrophilic organic solvents.

AH-6 protease was moderately thermophilic in nature having stability at temperatures up to 70°C, with the optimum at 50°C. Khalil Beg and Gupta (Beg *et al.*, 2003) reported a similar temperature optimum (50°C) for a protease produced by *Bacillus mojavensis.*

The water dependent events cannot proceed in anhydrous organic solvent and therefore, enzymes, in general, are extremely thermostable in such systems. For instance, thermostability of a lysozyme in absolute glycerol at temperatures above 80°C was

greater than that in water (Knubovets, *et al*., 1999). In the present study, AH-6 protease had significant activity with hexane. At 70°C, the protease displayed above 75% activity of control, while that at 80°C was 70%. The temperature optimum at 50°C was unaltered with all tested solvents. Other Halophilic enzymes, such as NAD and NADP glutamate dehydrogenases from *Halobacterium salinarum* displayed maximal activity at 70°C, with higher temperature stability (Bonete *et al.,* 1987). The thermophilic nature has been earlier reported for some halophilic enzymes (Mijts *et al.,* 2001 and Marhuenda *et al*., 2002).

For effective peptide synthesis, proteases stable in organic solvents are highly desirable (Ryu *et al.,* 1994). Interestingly, some of the most widely used commercial proteases, e.g. α-chymotrypsin and *A. oryzae* protease lose significant amounts of their activity in the presence of organic solvents (Gupta *et al*., 2005). In contrast, protease from AH-6 was significantly active and stable in hydrophobic and hydrophilic organic solvents.

Catalysis and stability of protease in organic solvents in combination of salt, pH and temperatures make this study noteworthy. The stability of the protease under alkalinity, high temperature and range of concentrations of NaCl, detergent and organic solvents make the Sj-1 and AH-6 protease a potential enzyme for industrial applications. Besides, the information would add significantly to the knowledge of biocatalysis and be useful for enzymatic applications in remediation.

Concluding Remarks

CONCLUDING REMARKS

==========

The work presented in this thesis addressed the microbial heterogeneity followed by the tolerance of haloalkaliphilic bacterial strains and their extra cellular enzymes. The exploration of enzymatic potential from these bacteria is still in its preliminary stage. In the work presented in this thesis, we have addressed the question of haloalkaliphilic diversity based on organic solvent tolerance from, beyond the boundaries of Soda Lake, Dead Sea and populated sites. In the present work, the natural and manmade saline and hyper saline habitats along the coastal region of Gujarat followed by the exploration of the enzymatic potential of these bacteria for non- aqueous conditions.

Haloalkaliphiles hold many interesting biological secrets, such as the biochemical limits to macromolecular stability and the genetic instructions for constructing macromolecules stable to more than one extremity. Despite the significance of extremophiles, particularly those with dual or multitude extremities, such organisms have been paid only limited attention towards the exploration of biotechnologically relevant products and enzymatic potential. In view of the non-cultivability of the microorganisms, different enrichment conditions were employed with varying pH and salt to isolate maximum population present in the concerned habitat.

A total of 82 bacterial strains belonging to haloalkaliphilic group of bacteria were isolated. The results on the isolation revealed that extremophiles are not restricted to the highly extreme habitats only. Instead, they can also be found in crystallizer pond, saline soil and seawater. Our studies on these saline environments revealed wide diversity among these organisms with respect to their growth in presence of wide range of hydrophobic and hydrophilic organic solvents. One of the facts that emerged from our studies established that with the increasing concentration of solvent during growth kinetics, inferior cell densities were recorded. This clearly indicated that microorganisms dwelling in extreme environments might display poor sustainability.

The isolates under study displayed significant diversity on the basis of the available data on phenotypic and physiological characteristics, these “traditional” taxonomic methods are usually not sufficient to establish their taxonomic positions. Prokaryotic

systematic has seen a large number of changes in the past few decades and increasing attention has been paid towards using genetic data to investigate the evolutionary relationship and to base the taxonomy on that knowledge. Much emphasis has been put on the use of 16S rRNA/DNA sequence data, which has come up as an effective means to ascertain the phylogenetic relatedness of the organisms. Four isolates from our study, identified by 16S rRNA gene homology, resembled to halophilic and alkaliphilic bacteria isolated from various soda lakes. Total 18 haloalkaliphilic isolates were sequenced for 16S rRNA. Their dendogram were revealed 3 major domains and 11 minor domains.

As a recent trend, extremophiles are being looked into for the utilization of organic solvents and production of alternative energy. In realization of the fact that the solvent tolerance of haloalkaliphilic bacteria is rarely studied, we explored the production and catalysis of alkaline protease amylases from isolated bacteria under non-aqueous conditions. Most of tested isolates and their enzyme displayed tolerance towards the solvents having log Pow less than 1, which is highly toxic for any living organisms.

As highlighted earlier, haloalkaliphilic bacteria require not only salt but also alkaline pH for their growth. These two features are favoring for non-aqueous enzymology. Thus, the enzymes from such organisms must be active and stable in presence of organic solvents. Most of the studies related to enzymes have so far concentrated from the halophiles and alkaliphiles; however, the enzymatic potential of haloalkaliphilic bacteria is nearly untouched. In view of this realization, the isolates under study were screened for the most commonly secreted extra cellular enzymes; protease and amylase.

The protease of AH-6, Sj-1 and Sj-2 were highly stable and catalyzed the reaction over the broad range of pH (8-12), optimum being at pH 10-10.5. Our studies revealed the wide occurrence and variation in production and catalysis level of alkaline proteases, only few secreted amylase. The organic solvent range for growth and enzyme secretion varied among the isolates from different samples and enrichment conditions. However, the variation in optimum solvent concentration was more pronounced.

The growth did not necessarily correspond to enzyme secretion as a function of organic solvent levels. In general, protease producers had ability to grow and secret protease over a wider range of organic solvent as compared to amylase producers. However, to enhance production of such enzymes, several research groups have focused their attention by studying the physiology of these unique microorganisms and designing bioreactors that improve growth conditions. Under the larger umbrella of optimization, we looked into the various factors affecting production of proteases and amylases at laboratory scale from certain key strains. Because of low production and unstable nature of the amylases, further studies on production, purification and characterization were focused on the alkaline protease from three potent strain, AH-6, Sj-1 and Sj-2.The protease production was highly influenced by physical conditions and available organic solvent concentrations in media.

The production was enhanced at higher NaCl and alkaline pH and with hydrophobic solvents. The results established that AH-6; Sj-1 and Sj-2 produced alkaline protease substantially in presence of various concentration of different solvent and could prove as potential candidate for process development and various industrial applications especially for peptide synthesis.

Unlike other halophilic proteins, AH-6 protease did not require high salt for their optimum catalysis; however in case of Sj-1 and Sj-2 salt requirement was enhanced and partially purified preparation of Sj-2 and purified preparations of SJ-1 was quite stable in 0-4M NaCl.

It would be further interesting to look into the mechanism of solvent tolerance among these organisms. Recently, the genome sequence of an oil hydrocarbons and solvent degrading marine bacterium, Alcanivorax borkumensis, has been completed (Schneiker et al., 2006). The organism has a streamlined genome with a paucity of mobile genetic elements and energy generation–related genes. This genome sequence may provide the basis for the future design of strategies to mitigate the ecological damage caused by oil spills.

Future perspective

- It would be interesting to locate the phylogenetic positions of other isolates in order to investigate maximum diversity from the coastal region of Gujarat.

- As the genetics organic solvent tolerance by organisms are less attempted and in case of haloalkaliphiles it is not covered at all, it would be interesting to understand genome structure. Development of expression systems for the production of key enzymes and metabolites would be another key point to focus.

- Use of active and stable proteases from potent isolates for peptide synthesis under non- aqueous conditions will add to the applicative part of this study.

- Inducible gene/s responsible for organic solvent tolerance is reported for some microbes, such study for our potent isolates might be an interesting future.

- It would be quite interesting to clone and sequence the alkaline protease genes from haloalkaliphilic bacteria followed by site directed mutagenesis. It will increase the understanding for interaction between enzyme and organic solvents.

Chapter 8

Summary

SUMMARY

==========

Screening and diversity of organic solvent tolerant haloalkaliphilic bacteria

- A total 82 haloalkaliphilic Bacteria from 6 isolation site of Costal Gujarat were screened for organic solvent tolerance, 1 to 5 % (v/v) of 4 solvents: methanol, propanol, n-hexane and butanol were added in growth medium to assess their effect on growth and protease production.

- Majority of isolates were able to grow at the tested concentration of methanol, propanol and n-hexane. Compared to control, in the presence of all solvents, initially cell density was lower.

- Butanol was most growth retarding solvent. Most of the isolates did not tolerate butanol more than 3% (v/v).

- In presence of organic solvents, the isolates exhibited two types of growth patterns. In first type of growth pattern, initially growth rate and cell density were lower while finally cell density was almost similar to control. In second type, difference between control and growth in the presence of solvent remained uneven.

- Isolates Kh-10-9_1 was able to grow in presence of 30% (v/v) acetone. Cell size was enlarged 2 to 2.5 fold. It might be possible that for the storage of entered organic solvents, cell has created vesicles.

- Analysis of the 16S rRNA gene sequence of 18 isolates revealed 3 major and 11 minor clusters.

Effect of organic solvents on catalysis of Haloalkaliphilic Amylase

- Amylases of 9 isolates were subjected for screening of potent amylase for non-aqueous condition. Ve1-10-8_2 and Mi-10-6_2 were selected for further study. Based on 16S rRNA gene sequencing, Ve1-10-8_2 was designated as *Oceanobacillus oncorhynchi* (GQ121034) and Mi-10-6_2 was *Bacillus agaradhaerens* (GQ121032)

- Partial purification of the enzyme was done by ammonium sulphate precipitation with 3.9 fold purification, specific activity of 1246.6 U/mg and 34.87% yield. The crude and partially purified preparations of Mi-10-6_2 amylase were quiet active in the presence of the solvents. The enzyme was noticeably active up to 30 % (v/v) of propanol, hexane, heptane, decane and dodecane.

- Amylase of Mi-10-6_2 was quiet active at alkaline pH range 8 to 12 in presence of solvent propanol and dodecane.

- Mi-10-6_2 amylase catalysis was examined with 0.5 – 4 M NaCl in the presence of 10-30% (v/v) propanol and dodecane. With both solvents, a change in the pattern of salt profile was evident. For crude and partially purified enzyme, the salt optima were 3 and 2 M NaCl, respectively.

- Temperature optimum of Mi-10-6_2 was altered in presence of propanol. Crude amylase was optimally active at 50^{o}C, while in presence of propanol, it was shifted to 60^{o}C.

- In presence of butanol Amylase of Ve1-10-8_2 was active at pH range of 5 to 12, while in presence of methanol, it was active in pH range of 8 to 12.

- Salt profile affected the catalysis of Ve1-10-8_2 amylase. In presence of methanol, it was active with 3M NaCl, while in presence of butanol, it was optimum at 2M

NaCl. Amylase was active up to 70^{o}C, optimally at 50^{o}C. Amylase was active up to 12 hour with 10% solvent.

Effect of organic solvent on catalysis of haloalkaliphilic protease

- Protease of Haloalkaliphilic sp. Sj-2 was subjected for screening such study. The isolate was obtained from Jodiya (Latitude 22.28 N, Longitude 69.4, 60º E).

- With 3.96 fold and 30.63 % yield, Sj-2 protease was purified by ammonium sulphate precipitation. Its specific activity was 1,383.9U/mg. Protease was quiet active with 30% ethanol, glycerol, methanol, propanol, n-hexane, diethyl ether and octane.

- Protease was active with 10-30 %(v/v) ethanol. Optimum activity was at pH 10. Sj-2 protease was optimally active at 2 M salt which was not altered in presence of solvents. Considerable activities were recorded with 3M salt in 20% ethanol.

- In presence of hexane, temperature optima of protease shifted to lower range. In the absence of solvent, temperature optima was 60^{o}C, while in presence of hexane shifted to 50^{o}C.

Purification and Characterization of two haloalkaliphilic alkaline Protease with respect to organic solvents

- Haloalkaliphilic bacteria Sj-1(GQ162111) and AH-6 (EU118361) isolated from Jodiya (Latitude 22°43'11"N, Longitude70°16'48"E) were subjected for purification and characterization.
- In the presence of butanol and cyclohexane, production of protease was inhibited. Sj-1 was able to produce significant amount of protease in the presence of iso-osctan, methanol , propanol, hexane, xylene , heptane, dodoecane and decane.

- With 27 fold and 28 % yield, Sj-1 was purified by hydrophobic interaction chromatography. Its specific activity was 9611 U/mg. Based on SDS-PAGE, it's molecular weight was 30 KDa. Protease was considerably active with 30% ethanol, glycerol, methanol, propanol, n-hexane, dodecane, decane and octane.

- Protease was active at pH range 8 to 12, while it's optimum pH was 10 . Enzyme was considerably stable up to 12 hour with 30 %(v/v) isooctane.

- Salt profile was affected by solvent. In control, the optimum activity of Sj-1 protease was at 2M NaCl, while in isooctane, maximum activity was recorded at 3M salt. At optimum salt concentration in presence of isooctane , protease was stable up to 18 hour .

- Temperature optima were unaltered due to presence of solvent. Protease was optimally active at 50^{o}C and in presence of solvent too it was optimally active at the same temperature.

- AH-6 protease was purified with 18-fold purification with a specific activity of 7,312 U/mg and 22% yield. Based on SDS-PAGE, its apparent molecular mass was determined as 40 kDa.

- Ah-6 protease was active at pH 8-12, maximally at 10. Solvent did not affect pH profile. In the presence of 30 % (v/v) hexane, it was stable up to 12 hour.

- In control and in presence of hexane, protease exhibited maximal activity at 200 mM NaCl. With solvent, it was stable up 12 hour.

- Temperature optimum of AH-6 protease was 50^{o}C and it did not change due to hexane. The enzyme was stable with 20% (v/v) hexane up to 24 hour.

Conclusions

CONCLUSIONS

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- Potent tolerances of the haloalkaliphilic bacteria against range of organic solvents indicated their wide presence beyond the organic solvent polluted land or crud oil extraction sites. A total 82 haloalkaliphilic bacteria were screened against methanol, propanol, hexane and butanol. Decreased sustainability was recorded with increasing concentration of organic solvents, especially hydrophilic solvents.

- Expansion of cell size of isolates can relate with the formation of vesicle as an adaptive modification at cellular level.

- Two different growth patterns in presence of organic solvents is attractive part of this study. These isolates may prove model to understand biochemical and genetic basis of organic solvent tolerance.

- The occurrence of the organic solvent tolerant amylases and Proteases from the isolated strain indicated that this region must be explored for valuable biocatalysts for non-aqueous conditions.

- Amylases had broader range of pH, salt and temperature for the catalysis and stability in presence of organic solvents. Salt and temperature profile was shifted to higher range in the presence of organic solvents.

- Alkaline proteases were purified to the homogeneity by single step purification by affinity chromatography. Catalysis and stability of the Sj-1 and AH-6 proteases at wide range of three extremities of pH, salt and temperature in combination with organic solvent would be quiet useful for harsh conditions of biotechnological industry.

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Appendix

APPENDIX

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APPENDIX I

I. PAPERS COMMUNICATED

Manuscripts Communicated

1. **Pandey, S**. and Singh S.P. Organic solvent tolerance of an amylase from haloalkaliphilic bacteria as a function of pH, temperature and salt concentrations for **Applied Biochemistry and Biotechnology**.

2. **Pandey, S**., Chotaliya, L. and Singh, S. P. Effect of organic solvent on haloalkaliphilic bacteria with respect to growth, protease production and temperature optima of enzyme for **Journal of Industrial Microbiology and Biotechnology.**

3. **Pandey, S**. and Singh S.P. Production and purification of alkaline protease from a solvent tolerant haloalakliphilc Bacillus sp and its catalysis and stability at varying salt concentration, pH and temperature with organic solvents for **Journal of Bioscience and Bioengineering**.

II. BOOK CHAPTERS

PUBLISHED/ACCEPTED/COMMUNICATED

Published

1. Singh, S. P., Purohit, M. K., Thumar, J. T., **Pandey, S**., Rawal, C. M. and Bhimani, H. G. 2008. "Biocatalytic Potential of Haloalkaliphilic Bacteria" in "Biocatalysis Research Progress" NOVA Publisher, New York, USA.

2. Singh, S. P., Purohit, M. K., Bhimani H.G., Rawal, C. M., **Pandey, S**. 2007. Metagenomic: a culture independent approach to study biotechnological

potential of unexplored/uncultivable microorganism. Vak Journal (Saurashtra University).

3. Singh, S.P., Raval, V. H., Purohit, M. K, Thumar J.T., Gohel, S.D., **Pandey, S**., Rawal C.M. and Akbari V.G. 2010. Haloalkaliphilic bacteria and actinobacteria from the saline habitats: new opportunities for biocatalysis and bioremediation. Microbes in Environmental Management and Eiotechnology. Springer Publication.

4. Singh, S.P., Purohit, M. K., Raval, V. H., **Pandey, S**., Rawal C.M., Akbari V.G. 2010. Capturing the potential of Haloalkaliphilic bacteria from the saline habitats through culture dependent and metagenomic approaches.Formatex publication. Spain.

III. SEQUENCES SUBMITTED IN NCBI

16S RIBOSOMAL RNA GENE

1. Singh,S.P., Dodia,M.S., **Pandey,S**. and Joshi,R.H Bacillus pseudofirmus strain SJ2 16S ribosomal RNA gene. GenBank: **EU090232**

2. Singh,S.P., Dodia,M.S., Joshi,R.H. and **Pandey,S.** Haloalkaliphilic bacterium AH-6 16S ribosomal RNA gene. GenBank: **EU118361**

3. Singh,S.P., Joshi,R.H., Dodia,M.S. and **Pandey,S.**Haloalkaliphilic bacterium S-20-9 16S ribosomal RNA gene. GenBank: **EU118360**

4. Singh,S.P., Dodia,M.S., Joshi,R.H. and **Pandey,S**. Halomonas venusta strain Kh-10-91 16S ribosomal RNA gene. GenBank: **EU684463**

5. Singh,S.P., Joshi,R., Dodia,M. and **Pandey,S.** Oceanobacillus oncorhynchi strain VE1-10-82 16S ribosomal RNA gene GenBank: **GQ121034**

6. Singh,S.P., Joshi,R.H., Dodia,M.S. and **Pandey,S**. Halomonas aquamarina strain Ve1-10-83 16S ribosomal RNA gene GenBank:**EU684464**

7. Singh,S.P., Dodia,M.S., Joshi,R.H. and **Pandey,S.** Bacillus agaradhaerens strain Mi10-62 16S ribosomal RNA gene GenBank:**GQ121032**

8. Singh,S.P., Dodiya, M.S., Joshi,R.H. and **Pandey,S**. Oceanobacillus_sp. Sj-1 16S ribosomal RNA gene GenBank **GQ162111**

APPENDIX II

I PAPER/POSTER PRESENTATIONS

PAPERS PRESENTED

1. **Pandey, S**., Meena, A., Thumar, J. T., Joshi, R. H., Dodia, M. S. and Singh, S. P. "Effect of Organic solvent on growth and alkaline protease production in Haloalkaliphilic bacteria and actinomycetes." In DAE BRNS Life Sciences Symposium , BARC , Mumbai (India) 19-21 December 2005.

2. Singh S.P., Purohit, M.K., **Pandey, S.,** Raval,V. and Raval, C "Diversity, Molecular phylogeny and Biocatalytic Potential of Haloalkaliphilic bacteria from Coastal Gujarat"Extremophile-2008, Cape Town, South Africa, 07-11 September 2008.

3. **Pandey, S.** and Singh S.P. " Effect of Organic solvents on haloalkaliphilic bacteria from costal region of western India and their alkaline protease production, stability and thermal stability in presence of organic solvent"Extremophile-2008, Cape Town, South Africa, 07-11 September 2008.

4. Singh S.P., Purohit, M.K., **Pandey, S**., Raval,V, Kikani, B., ,V.G., Akbari "Haloalkaliphilic bacteria from coastal Gujarat: Diversity and Biocatalytic potential under multitude of extremities" in National Conference on " Recent advances in Molecular Biology"at Nirma University of Science & Technology, Ahmedabad (India), 26 March, 2008.

5. **Pandey, S**. and Singh, S.P "Organic Solvent tolerance of Haloalkaliphilic bacteria from costal Gujarat " in national conferences "Recent advances in Molecular Biology" Nirma university, Ahmedabad (India) 26 March, 2008.

6. Singh S.P., Purohit, M.K., **Pandey, S**., Raval,V. and Raval, C "Alkaline proteases among haloalkalipliic bacteria dwelling in saline habitats of coastal Gujarat: distribution, biochemical properties and metagenomics" in National Symposium on Recent trends in Cellular Research at Saurashtra University Rajkot (India), 09 March, 2009.

7. Singh S.P., Purohit, M.K., **Pandey, S**., Raval,V. and Raval, C "Biocatalytic Potential under Multitude of Extremities: Vast Opportunities for Industrial and Environmental Applications" in National Conference on " Microbial Technology on Sustainable Environment" at Gujarat University Ahmedabad (India), 02-03 March, 2009.

8. **Pandey, S**. and Singh, S.P "Organic Solvent tolerance of Haloalkaliphilic bacteria from costal Gujarat " in Science Excellence at Gujarat University, Ahmedabad(India), 10Jan-2010.

9. **Pandey, S**. Singh S.P "Organic solvent tolerance of an amylase from haloalkaliphilic bacteria as a function of pH, temperature and salt" in department of Biochemistry, Saurashtra University, Rajkot (India) 20Feb-2010.

10. **Pandey, S**. and Singh, S.P "Organic solvent tolerant haloalkaliphilic bacteria and their solvent tolerant enzymes from western cost of India" in national conferences " Trends in biological sciences " Department of Biosciences, Saurashtra university, Rajkot (India) 16-17 Sepetember 2010.

II. WORKSHPS/SEMINARS ATTENDED

1. International Seminar on Over expression, system and challenges on 26-28 November 2006 at Center for Cellular Molecular Biology, Hyderabad , India

2. Workshop on Nano-technology at Saurashtra University, Rajkot.Attended National level Workshop on" Current Drug Patent Régime" on 5th March, 2006 held at S.J.Thakkar College, Rajkot

3. National Level Symposium on 'Recent Advances in Molecular Biology & Biotechnology; RAMB-2008.

4. Attended National level Science Symposium organized by Department of Biochemistry on "Recent Trends in Cellular" Research. Rajkot, India 2009.

5. Attended & Presented National level Science Symposium organized by Department of Biochemistry on "A Global Approach from Molecules to Cell". Rajkot, India 2010.

APPENDIX III

I SCHOLARSHIPS/AWARDS

1. Awarded Junior research Fellow (JRF) under UGC- DSA Program from November 2007 to March 2008.

2. Awarded Junior research Fellow (JRF) under UGC- Meritorious fellowship Program from April 2008 to March 2011.

II TRAINING UNDERTAKEN

Organization	Indian Institute of Advanced Research(IIAR), Gandhinagar, Gujarat
Brief Description	Hands on Training Programme in "Bioinformatics"
Duration	3 Days (December 2008).