Screening, isolation, purification and characterization of thermophilic and halophilic extremozymes

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Submitted by:

Uzma Zaidi (10MBT020)

Institute of science, Nirma university
S.G.Highway, Ahmedabad

Under the guidance of

Dr. Mili Das

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10MBT020

Dedicated to my parents

Dr.S.S.A.Zaidi

Mrs. Shahana Zaidi

And my brother

Mr.Shahzeb Zaidi

INDEX

A.List of Tables

B. List of Figures

C. List of Abbreviations

BSA Bovine Serum Albumin

TCA Tri chloro Acetic acid

CBB Coomasie Brilliant Blue

SDS Sodium Dodecyl Sulphate

SDS-PAGE Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

DNSA 3, 5 Dinitrosalycylic acid

TEMED- N, N, N', N'-Tetramethylethylenediamine

APS –Ammonium per Sulfate

1. Abstract

We have carried out screening, isolation, and characterization of four different extracellular industrially important enzymes, Amylase, Protease, Lipase and, Pullulanase from haloalkaliphilic soil organism *Virgibacillus marismortui* which was isolated from saline soil of Khambhat and a thermophile *Thermus thermophilus*. It was observed that *Virgibacillus marismortui* secrete proteases which was confirmed by obtaining zone of hydrolysis in milk agar plates. We also found out that maximum protease production occurs in late logarithmic and/or stationary phase. Further protease activity was confirmed by zymographs. It was observed that these organisms do not produce amylase in normal culture however when grown on a minimal media with starch as sole carbon source these organisms were able to grow by producing amylases. The extracellular amylase activity was confirmed by DNSA test and starch iodine test. The crude amylase activity was estimated by DNSA test of ammonium sulphate precipitated sample. When grown on pullulan as sole carbon source, the organism was able to grow which indicates that these organisms are able to hydrolyse pullulan and produce maltotriose which can be detected by DNSA test. After ammonium sulfate precipitation and dialysis of the sample its activity was again checked and crude activity was determined. We also tried to grow these organisms in different salt concentrations like 6%, 10%, 15% and 25% NaCl and determined effect of salt concentration on growth and enzyme production. We also carried out screening of thermophile *Thermus thermophilus* for production of amylase and pullulanase.

2. OBJECTIVES

Organisms that thrive in extreme conditions are called extremophiles and enzymes of these extremophiles are also capable of tolerating extreme environments. Extreme environments such as high salinity, acidity, alkalinity and extreme temperature are also predominant conditions in a variety of industrial processes. As industrial process conditions are harsh, there are high demands for biocatalysts that can withstand the process conditions.

Extremozymes have vast economic potential in many industrial processes (*e.g.* agriculture, food, feed and drinks, detergents, textile, leather, pulp and paper). Keeping in view the enormous demand of enzymes like proteases, amylases in industry etc. the aim of our work is to identify important enzymes which have greater stability with respect to temperature, high salt concentrations, high and low pH etc.

If the organism is able to grow in high salt concentration, it is obvious that enzymes produced by these organism is more salt tolerant which increases it value and application in industries where the enzymes need to be active at high salt concentrations for example, in Detergent industry.

Also we need to find optimum conditions in which these organisms can yield maximum amount of our enzyme of interest. These conditions are to be standardized for a particular organism which then can be used for industrial purposes.

This work was done with the broader objective to study the molecular determinants of protein stability using these extremophilic enzymes as model systems.

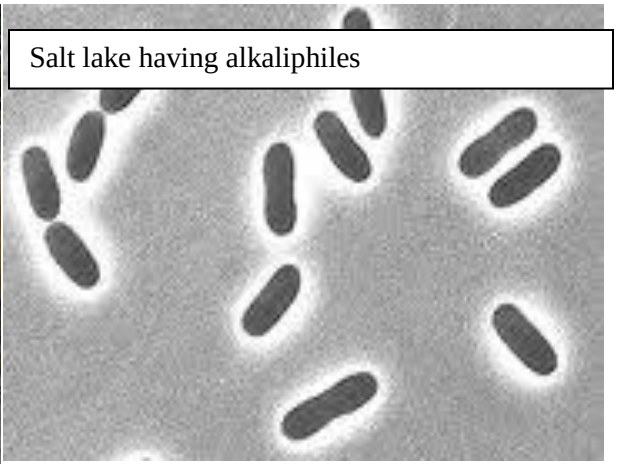
INTRODUCTION

Extremophiles in boiling acid

Halophiles (Pink colour formation)

Thermophiles of yellowstone national park

Salt lake having alkaliphiles



Desulfofrigus oceanense A pshychrophile

3.1 Extremophiles

Many types of micro-organisms have been discovered that are evolved to live in certain types of extreme environments like high or low temperatures, high or low pH levels, high pressures, high salinities and even high radiations

These diverse populations of micro-organisms that can live and reproduce in extreme conditions are called extremophiles. These organisms thrive in such extreme conditions inhabit extreme environments by various mechanisms some of which are still unknown to scientists.

Extremophiles thrive under conditions that would kill most other creatures and many cannot survive in such extreme environments. The main advantage of living in such extreme conditions is that these organisms face a very little competition from other species for food and shelter and can thrive easily as their normal counterparts cannot withstand such extreme conditions. (Gomes and Steiner, 2004; Raven and Johnson, 2012; Burg, 2004)

Extremophiles are of our interest in both basic and applied biology. In a basic sense these organisms hold many interesting biological secrets, such as the genetic instructions for constructing macromolecules stable to one or another extreme and biochemical limits to macromolecular stability and mechanisms by which these organisms thrive in such extreme conditions . It is interesting for scientists to find out as to what drives the mechanisms by which these organisms have developed immunity to such harsh conditions. (Raven and Johnson, 2012)

Some of these extremophiles in particular the hyperthermophiles, lie close to the "universal ancestor" of all forms of life on Earth. Thus, an understanding of the basic biology of these organisms is an opportunity for biologists to get an insight about early life on earth. This exciting fact has propelled much research on these organisms in order to gain insight into nature of propelled primitive life forms, and how early organisms set the stage for the evolution of modern life forms. (Raven and Johnson, 2012)

As far as application aspect is concerned, these organisms have yielded an amazing array of enzymes capable of catalyzing specific biochemical reactions under extreme conditions. Extreme environments such as high salinity, acidity, alkalinity and extreme temperature are also predominant conditions in and variety of industrial processes. As industrial process conditions are

harsh, there are high demands for biocatalysts that can withstand the process conditions. The majority of the enzymes used to date in industries originate from mesophilic organisms and, despite their many advantages; the application of these enzymes in industry is restricted as they have limited stability at the extremes of temperature, pH and ionic strength. Hence extremophiles have an enormous industrial applications and biotechnological potential (Raven and Johnson, 2012).

Several classes of extremophiles are recognized in microbiology and laboratory cultures of representatives of each class are known. This classification of extremophillic organisms is listed in table 1.1

Table 1.1 Classification of extremophiles, their characteristics, products of interest and applications (Adapted from Raven and Johnson, 2012).

Extremophiles	Characteristics	Example	Product	Applications
Thermophile	60-80˚C	Thermus thermophilus	DNA polymerase, amylase, protease etc.	Source of heat stable enzymes in detergents, leather, and food industry
Hyperthermophile	80C -143˚C	*Pyrolobus fumarii*	Thermostable enzymes	Source of PCR enzymes
Psychrophiles	0-4˚C	*Polaromonas Vacuolata*	Protease, lipase	Cold food industry, producers of cold-water laundry detergents
Acidophile	Optimal pH Of 3.0 or below	*Picrophilus oshimae*	Sulphur oxidizers, surface coal mining operations	Waste treatment, desulfurization, animal-feed supplements
Alkaliphile	Optimal pH Of 9.0 or above	*Natrono-bacterium Magadii*	Protease, lipase, amylase	Detergents, paper bleaching
Halophile	15-25% salt	*Halobacterium Salinarum*	Lipase, carotene, protease	Detergent industry, food industry and cosmetics
Barophile	500-700 atm	*Halomonas salaria*	Not yet defined	Yet to be explored
Radiophiles	Can survive 30,000 Grays of ionizing radiation	*Deinococcus radiodurans*	As a cleanup agent for the bioremediation of toxic materials	In repairing soils that are radioactive due to radioactive materials leakage, primarily at nuclear weapons Production sites.

3.2 Extremozymes in industry: Current scenario

Microorganisms retrieved from extremely hot, cold or forbidding environments like those that can grow in both extremes of pH and at high salt concentrations are renewing excitement in the bioprocessing industry for the production of "extremozymes". Examples of such extremozymes include cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases, which have great potential for application in various biotechnological processes (Burg, 2004)

Currently, only 1–2 % of the microorganisms on the earth have been commercially exploited and amongst these there are only a few examples of extremophiles. However, the growing 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 is increasing the biocatalytic applications of extremozymes (Cavicchioli and Thomas, 2000*).*

Current research is expanding rapidly in this field because scientists are discovering that the extremozymes that allow these bacteria to live in such extreme habitats may be utilized for industrial applications. These enzymes expand the range of temperatures and conditions used in manufacturing biotech products, creating opportunity for new, environmentally friendly bioprocesses while saving time and energy (Cavicchioli and Thomas, 2000)

Because extremophiles have such a range of applications, research is expanding rapidly within this field. Currently, about $2.5 billion worldwide is spent on the production of these enzymes each year, "ranging in uses from stone-washed jeans to the genetic identification of criminals and the diagnosis of infectious and genetic diseases" (Madigan et al, 1997).

Microbiologists are even attempting to tailor certain extremozymes to fit certain conditions suitable to industrial usage. One such approach is to clone genes of extremozymes into mesophillic organisms in a way that they can produce high yield of enzymes for industrial purposes under normal conditions itself (Gomes and Steiner, 2004).

Thus, as extremophile research expands, these unique groups of bacteria will prove to be integral in the workings of our society. (Gomes and Steiner, 2004)

Various kinds of thermostable enzymes are required by industries. The detergent, food, feed, starch, textile, leather, pulp and paper and pharmaceutical industries are the major users of enzymes. The starch industry is one of the largest users of thermostable amylolytic enzymes (e.g. -amylases, glucoamylases and isoamylases or pullulanases) for the hydrolysis and modification of starch to produce glucose and various other products.

Amylolytic enzymes are also used in the textile, paper and baking industries. Cellulolytic enzymes are employed in the removal of polyphenolic substances from juices, in detergents for color brightening and softening of textiles, in the bio-stoning of jeans, in the pulp and paper industries and in the pretreatment of plant biomass. (Burg, 2004). Nowadays, biodetergents contain enzymes like amylase; protease, cellulase and lipase, using variants that are resistant to harsh conditions.

Although there are some controversial opinions about the potential of extremophiles, some industrial companies and several research groups are investing money and time searching for these microbes and novel applications of extremozymes and strongly believe that new discoveries will revolutionize biotechnology.

This renewed confidence in enzyme biotechnology may have emerged as a result of the success of genome-based technologies that are currently in use. It is expected that many extremozymes will be discovered in the years to come and the extremozymes will be used in novel biocatalytic processes.

Since the cultivation of extremophiles is associated with many potential difficulties, it is believed that only genetic engineering of the desired extremozymes into mesophilic hosts will allow large-scale production of these extremozymes.

Discoveries of new extremophiles and genetic engineering of the newly isolated as well as of the currently available extreme microbes will offer novel opportunities for biocatalysis and biotransformations.

3.3 Halophiles –Some like it salty

Halotolerant and halophilic microorganisms, as the name suggests, grow in environments with high salinity concentrations. Some halophillic bacteria require salts as a prerequisite component for their growth and cannot grow in absence of salts. Halophilic bacteria are found in different environments such as salt lakes, saline soils and salted foods. Halophiles are found anywhere with a concentration of salt five times greater than the salt concentration of the ocean like in the Dead Sea, and in evaporation ponds.

Halophiles can be categorized as slight, moderate or extreme, by the extent of their halotolerance. High salinity represents an extreme environment that relatively few organisms have been able to adapt to and occupy. Generally high salt concentration in extracellular space results in water moving inside the cell which causes cell to swell and burst resulting in osmolysis and death of bacterial cell. (Raven and Johnson, 2012)

Therefore it is obvious that in order to live in such a high salt concentrations these organisms have some mechanisms to deal with such situations. Hence most halophilic and all halotolerant organisms expend energy to exclude salt from their cytoplasm to avoid protein aggregation ('salting out'). Also in order to survive the high salinities, halophiles prevent desiccation through osmotic movement of water out of their cytoplasm. (Raven and Johnson, 2012; Gomes and Steiner, 2004)

There are two major mechanisms by which halophiles are able to live in salty conditions by preventing dehydration of their cytoplasm.

1) They either produce large amounts of an internal organic solute or

2) By concentrating the solutes from their environment

Proteins from halophilic organisms have a biased amino acid composition in order to remain stable and active at high ionic strength. Halophilic proteins particularly have an excess of acidic amino acids (*i.e.* glutamate and aspartate) on their surface (Danson and Hough,1997), although such a high proportion of acidic amino acids is not present in the amylase from the thermophilic halophile *Halothermothrix orenii* (Mijts and Patel, 2002)

Negative charges on the halophilic proteins can bind significant amounts of hydrated ions, thus reducing their surface hydrophobicity and decreasing the tendency to aggregate at high salt concentration. (Gomes and Steiner, 2004)

The extremely halophilic archaea, the *Halobacteriaceae*, accumulate K^+, while other bacteria accumulate compatible solutes (*e.g.* glycine, betaine, sugars, polyols, amino acids and ectoines), which help them to maintain an environment isotonic with the growth medium. These substances also help to protect cells against stresses like high temperature, desiccation and freezing. (Danson and Hough, 1997; Da Costa and Galinski, 2002)

Also the enzymes of these halophiles, with such salty cytoplasm have evolved to require high doses of compatible solutes like K^+ for their optimal catalytic activity, whereas the membrane or cell wall positioned proteins of these halophiles require Na^+ and are typically stable only in presence of Na+.

Although the halophilic enzymes can perform enzymatic functions similar to those of their normal non-halophilic counterparts, these enzymes have been shown to exhibit substantially different properties, among them the requirement for high salt concentrations (1 – 4 M NaCl) for activity and stability and a high excess of acidic over basic amino residues (Deutch, 2002)

Consequently, in surroundings with lower salt concentrations, the solubility of halophilic proteins is often very low. Halophilic proteins are distinguished from their non-halophilic homologous proteins by exhibiting remarkable instability in solutions with low salt concentrations and by maintaining soluble and active conformations in high concentrations of salt. (Danson and Hough, 1997; Da Costa and Galinski, 2002)

The requirement of high salt concentration for the stabilization of halophilic enzymes is due to a low affinity binding of the salt to specific sites on the surface of the folded polypeptide, thus stabilizing the active conformation of the protein (Deutch, 2002).

The dependence of the stability and the catalytic properties of halobacterial proteins on salt concentration has been the subject of studies for many years. The property of low

solubility of halophilic enzymes has been taken advantage of by applying them in aqueous/organic and non-aqueous media (Klibanov, 2001; Marhuenda and Bonete, 2002).

The mechanisms by which compatible solutes protect enzymes, cell components and cells are still a long way from being thoroughly elucidated, but there is a growing interest in the utilization of these solutes to protect macromolecules and cells from heating, freezing and desiccation. (Burg, 2004)

Halophiles like *Halobacterium, Haloferax, Haloarcula, Halococcus, Natronobacterium* and *Natronococcus* belong to the archaea while *Salinibacter ruber* is a bacterium .Table 1.3 shows some recent reports on halophillic enzymes However, many of these enzymes have not been investigated in detail or applied

Halophiles from the archaeal domain provide the main source of extremely halophilic enzymes. The potentials of halophiles and haloenzymes have been reviewed previously The production of halophilic enzymes, such as xylanases, amylases, proteases and lipases, has been reported for some halophiles belonging to the genera *Acinetobacter, Haloferax, Halobacterium, Halorhabdus, Marinococcus, Micrococcus, Natronococcus, Bacillus, Halobacillus* and *Halothermothrix*.(Kelly et al, 1995; Hough and Danson,1999;Eichler,2001; Sellek and Chaudhuri,1999; Oren, 2002; Danson and Hough,1997; Da Costa and Galinski, 1998; Madern et al,2000) .

Table 1.2 Some recent reports on halophillic enzymes (Taken from Gomes and Steiner, 2004)

Halophiles	Halophilic enzymes	T_{opt}/°C	pH_{opt}	Stability
Halothermothrix orenii	α-Amylase	65	7.5	Tolerates up to 25 % NaCl T_{opt} at 5 % NaCl
Bacillus dipsosauri	α-Amylase	60	6.5	Stable up to 60 °C
Halobacillus sp. strain MA-2	Amylase	50	7.5–8.5	Maximum stable at 5 % NaCl
Haloferax mediterranei	α-Amylase	50–60	7–8	Stable at 2–4 M NaCl Optimum 3 M NaCl
Halophilic bacterium, CL8	Xylanase 1 Xylanase 2	60 65	6.0 6.0	Stable 7 min at 60 °C Stable 192 min at 60 °C (T_{opt} at 4 M NaCl)
Halorhabdus utahensis	β-Xylanase β-Xylosidase	55, 70 65	NA NA	Optimum activity at 5–15 % NaCl Optimum activity at 5 % NaCl
Pseudoalteromonas sp. strain CP76	Protease CPI	55	8.5	Tolerates 0–4 M NaCl Optimum activity at 7.5 % NaCl

NA = not available

The majorities of halophilic microorganisms studied so far produce compounds with great potential in industrial processes and have physiological properties which facilitate its use with commercial aims. Enzymes produced by halophilic microorganisms have developed particular features which confer them stability and solubility at high salt concentrations, thus, low water concentration.

To date a very large number of halophilic bacteria have been grown in culture involving members of all domains of life, including the eukarya. (Burg, 2004). For example, an extracellular protease from Halobacterium halobium has been exploited for efficient peptide synthesis in water/N_0-N_0-dimethylformamide (Kim and Dordick, 1997; Marhuenda and Bonete, 2002)

Recently, a p-nitrophenylphosphate phosphatase (p-NPPase) from Halobacterium salinarum was used in an organic medium at very low salt concentrations after entrapping the enzyme in reversed micelles (Kim and Dordick , 1997).

Under these conditions p-NPPase was active and stable. Similar observations were made with a halophilic malate dehydrogenase .Exploitation of reversed micelles in combination with halophilic enzymes is likely to result in the development of novel applications for these enzymes (Kim and Dordick, 1997)

However, in spite of a growing interest in the use of halophilic enzymes for biotechnological applications, there are relatively few reports in the literature about production and characterization of halophillic enzymes. To date, the use of halophilic extremozymes in organic solvents has been limited to very few enzymes (Sellek and Chaudhuri, 1999).

3.4 Alkaliphiles/haloalkaliphiles

Enzymes from microorganisms that can grow and survive under extremes of pH could be particularly useful for applications under highly acidic or highly alkaline reaction conditions, for example in the production of detergents.

Microorganisms that live at high pH values are simply classified as alkaliphiles which consist of two main physiological groups: alkaliphiles and haloalkaliphiles. Alkaliphiles require an alkaline pH of 8 or more for their growth and have an optimal growth pH of around 10, whereas haloalkaliphiles require both an alkaline pH (pH>8) and high salinity (NaCl up to 33 %).

One of the fascinating properties of acidophilic and alkaliphilic microorganisms is that they maintain a neutral pH internally, and so their intracellular enzymes do not need to be adapted to extreme growth conditions. However, this does not apply for extracellular proteins, which have to function in low or high pH environments in the case of acidophiles and alkaliphiles, respectively. (Burg, 2004; Gomes and Steiner, 2004)

However how these extracellular proteins operate at high or low pH values is yet poorly understood phenomena. Proteases, amylases, lipases and other enzymes that are resistant to and active at high pH and high chelator concentrations of modern detergents are desirable in industry. This has prompted the screening of alkaliphilic and halo-alkaliphilic bacteria and

Archaea for their ability to produce such enzymes. (Gomes and Steiner, 2004; Burg, 2004; Kim and Dordick, 1997)

By these means, several useful enzymes have already been identified and obtained. Combinations of activity screening and homology based PCR have been applied to screen for and detect alkaline proteases in a wide variety of bacterial strains. (Gomes and Steiner, 2004; Burg, 2004)

In an alternative approach, alkaliphilic bacilli that could grow at pH greater than 9.0 were used as a source for oxidation-resistant alkaline proteases. Several enzymes which have been isolated from these organisms are amylases, pullulanases, glucoamylases and glucosidases that work at high pH.

3.5 Thermophiles-Life at high temperatures.

Thermophilic extremophiles have attracted most attention among all other extremophiles. In particular extremophilic proteases, lipases and polymer-degrading enzymes, such as cellulases, chitinases and amylases have found their way into industrial applications. Table 1.3 lists some of the industrially important enzymes of thermophiles

Thermophilic microorganisms can be isolated from virtually any environment that receives intermittent heat, such as soil, decaying plant matter, such as peat bogs and compost, various geothermally heated regions of the Earth, such as hot springs like and deep sea hydrothermal vents. But hyperthermophiles thrive only in very hot environments, including hot springs, both terrestrial and undersea (hydrothermal vents), and active sea mounts. (Raven and Johnson, 2012; Gomes and Steiner, 2004; Burg, 2004)

At elevated temperatures the solubility of many reaction components, in particular polymeric substrates, is significantly improved. Moreover, the risk of contamination, leading to undesired complications, is reduced at higher temperatures. (Gomes and Steiner, 2004)

As a prerequisite for their survival at high temperatures, thermophiles contain enzymes that can function at high temperatures. Thermostability is a feature of most of the enzymes sold for bulk industrial usage and thermophilic organisms are therefore of special

interest as a source of novel thermostable enzymes. Their membranes and proteins are unusually stable at these extremely high temperatures. Thus, many important biotechnological processes use thermophilic enzymes because of their ability to withstand intense heat. . Some of the applications of thermophillic extremozymes are listed in Table 1.2

Table 1.3 Application of some of thermophillic enzymes (Taken from Burg, 2004)

Type	**Growth characteristics**	**Enzymes**	**Applications**
Thermophiles	(Thermophiles Temp >808C (hyperthermophile) and 60–808C (thermophile)	Proteases	Detergents, hydrolysis in food and feed, brewing, baking
		Glycosyl hydrolyses (e.g. amylases, pullulanase, glucoamylases, glucosidases, cellulases, xylanases)	Starch, cellulose, chitin, pectin processing, textiles
		Chitinases	Chitin modification for food and health products
		Xylanases	Paper bleaching
		Lipases, esterases	Detergents, stereo-specific reactions (e.g. trans-esterification, organic biosynthesis

The structural features of thermophilic extremozymes have attracted much attention. Several three-dimensional structures have been solved and compared with those of mesophilic counterparts, with the ultimate goal to identify factors responsible for thermostability. The thermostabilities of enzymes from various hyperthermophiles, referred to as extremozymes, have been documented, and some have been found to remain active up to 140°C. (Gomes and Steiner, 2004)

Extreme thermophiles, growing optimally at 60–80 °C, are widely distributed among the genera *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Rhodothermus*, *Thermotoga* and *Aquifex* .(Kelly et al, 1995; Hough and Danson,1999; Antranikian et al, 1999;Cassidy et al,2001; Irwin et al, 2004; Eichler,2001; Vieille and Zeikus,2001;Haki and Rakshit, 2003; Fujiwara,2002;Sellek and Chaudhuri,1999; Belarbi et al,2000; Antranikian,2002)

On the other hand, most of hyperthermophiles belong to archaea, which consists of four phyla: Crenarchaeota, Euryarchaeota, Korarchaeota and Nanoarchaeota. Some genera belonging to Crenarchaeota are: *Sulfolobus, Acidianus, Pyrodictium, Pyrolobus, Pyrobaculum, Desulfurococcus, Thermoproteus, Thermofilum* and *Staphylothermus.* Euryarchaeota include extreme halophiles (*e.g.Halobacterium, Halobaculum, Halococcus, Haloferax, Halorubrum*), methanogens (*e.g. Methanobacterium, Methanosphaera, Methanococcus, Methanobrevibacter* and *Methanothermus*), extreme acidophiles (*e.g. Picrophilus, Thermoplasma*) and extreme thermophiles (*e.g. Thermococcus, Pyrococcus, Methanopyrus, Archaeoglobus* and *Ferroglobus*).The list of production of extremophillic enzymes by hyperthermophiles is given in table 1.4

Table 1.4 Production of extremophillic enzymes by hyperthermophiles (taken from Gomes and Steiner, 2004)

Hyperthermophiles	Thermophilic enzymes	T_{opt}/°C	pH_{opt}	Stability
Bacteria	Cellulase			
Bacillius, Clostridia, Fervidobacterium pennavorans, Rhodothermus marinus, Rhodothermus obamensis, Thermus caldophilus, Thermoanaerobacter sp.,	Amylase			
	Pullulanase I			
	Pullulanase II			
	α-Glucosidase			
Thermoplasma acidophylum, Thermotoga maritima, Thermotoga neapolitana, Picrophilus oshimae,	β-Glucosidase			
	Glucoamylase			
Picrophilus torridus	Xylanase			
Archaea	Mannanase			
Desulfurococcus mucosus, Pyrococcus furiosus, Pyrococcus woesei, Pyrodictium abyssi, Staphylothermus marinus, Sulfolobus solfataricus, Thermococcus hydrothermalis, Thermococcus litoralis, Thermococcus celer, Thermococcus profundus, Thermococcus aggregans	Pectinase			
	Chitinase			
	Protease			
	Lipase			
	Esterase			
	Phytase			
Alicyclobacillus acidocaldarius	Endoglucanase (CelB)	80	4.0	Stable at pH=1–7, retains 60 % activity after 1 h at 80 °C
Environmental DNA	β-Xylanase	100	6.0	Stable at 90 °C
Methanococcus jannaschii	α-Amylase	120	5.0–8.0	Stable against denaturants
Pyrobaculum calidifontis	Carboxylesterase	90	7.0	½ life: 2 h at 100 °C
Pyrococcus furiosus	Chitinase a and b	90–95	6.0	NA
Pyrodictium abyssi	Xylanase	105	6.0	½ life: 100 min at 105 °C
Rhodothermus marinus	Amylase	85	6.5	½ life: 3 h at 85 °C
	Pullulanase	80	6.5–7.0	30 min at 85 °C
	α-L-Arabinofuranosidase	85	5.5–7.0	8.3 h at 85 °C
	β-Mannanase	85	5.0–6.5	45.3 h at 85 °C
Sulfolobus solfataricus	Xylanase	100	7.0	½ life: 47 min at 90 °C
Sulfolobus solfataricus	α-Glucosidase	120	4.5	Highly thermostable (whole cells used)
Sulfolobus solfataricus	Trehalosyl transglucoylase	75	5.0	Stable at pH=4.5–11.0 after 2 h at 80 °C
Sulfolobus shibatae	α-Glucosidase	98	5.5	Retained 67 % activity after 5 h at 80 °C
Thermococcus litoralis	L-Aminoacylase	85	8.0	½ life: 25 h at 70 °C, 1.7 h at 85 °C
Ralstonia sp. A-471	Chitinase	70	5.0	NA
Thermococcus chitonophagus	Chitinase	70	7.0	½ life: 1 h at 120 °C
Thermoplasma acidophilum	Glucoamylases	90	2.0	½ life: 24 h at 90 °C for *P. torridus* and *T. acidophilum*, 20 h for *P. oshimae*
Picrophilus torridus		90	2.0	
Picrophilus oshimae		90	2.0	

NA = not available

The structural features that are responsible for thermal stability in these proteins are not well understood but a small number of non-covalent features seem characteristic of thermostable proteins. These include a highly non-polar core, which makes the inside of the protein "sticky" and thus more resistant to unfolding, a less surface-to-volume ratio, which confers a compact form on the protein, a reduction in glycine content that tends to remove options for flexibility and thus introduce rigidity to the molecule, and extensive ionic bonding across the protein's surface that helps the compacted protein resist unfolding at high temperature. In addition to these intrinsic stability factors special proteins called *chaperonins* are synthesized by hyperthermophiles. Chaperonins function to bind heat denatured proteins and refolds them into their active form (Raven and Johnson, 2012).

Several factors may combine to prevent DNA from melting in thermophiles. However, the two most important features appear in to be the enzyme reverse DNA gyrase, which catalyses the *positive* supercoiling of closed circular DNA in contrast to , non-hyperthermophiles which contain DNA gyrase, an enzyme that supercoils DNA in a *negative* twisted fashion and possessing various types of DNA binding proteins, including histone-like proteins. It is observed that positively supercoiled DNA is more resistant to thermal denaturation than is negatively supercoiled DNA. Also fact that reverse gyrase seems to be the *only* protein thus far found universally among hyperthermophiles (regardless of their metabolic pattern), points to an important role for it in the heat stability of DNA (Raven and Johnson, 2012)

To prevent their cell membranes from coming apart at high temperatures, hyperthermophiles form their cell membranes as lipid monolayers instead of the lipid bilayers seen in other organisms.(Gomes and Steiner, 2004; Burg, 2004).

3.6 Micro-organisms under study

The micro-organisms selected for our study are listed in Table 1.5

Table 1.5 Organism selected for our study

Organism	**Gram characteristic**	**Optimal Temperature**	**Optimal salt concentration**
Virgibacillus marismortui	Gram positive rod	35 ˚C	6 %(Can grow up to 25% NaCl)
Thermus thermophilus	Gram negative	75˚C	0.2%

3.6.1. Virgibacillus marismortui

Virgibacillus marismortui is a soil oganism isolated by Dr.Vijay Kothari and his students from saline soil of Khambat. This organism has following characteristics:

1. Aerobic and weakly facultative anaerobic
2. Gram positive rods, occur singly/in pairs/in chains
3. Cannot grow in absence of salts
4. Have optimum salt range-6-10% but can tolerate up to 25%
5. Colony characteristics- slightly transparent to opaque, non-pigmented, yellowish white, matt like, smooth, glossy type colonies
6. Can hydrolyze casein easily but cannot hydrolyze starch and Tween 80

3.6.2 Thermus thermophilus (MTCC strain-1494)

Thermus thermophilus was originally isolated from a thermal vent within a hot spring in Izu, Japan by Tairo Oshima and Kazutomo Imahori .*Thermus thermophilus* is a Gram negative eubacterium used in a range of biotechnological applications, including as a model organism for genetic manipulation, structural genomics, and systems biology. The bacterium is extremely thermophilic, with an optimal growth temperature of about65 °C (149 F).

This organism has high G+C content and is sensitive to various antibiotics. Also it has capacity to survive in pH 3.4-9.0 .the outer membrane of *T.themophilus* consists of phospholipids, lipopolysaccharides and peptidoglycan

3.7. Enzymes under study

Table 1.6 shows the list of enzymes that we have selected for our study.

Table 1.6 List of enzymes for screening of organism

Name of the Organism	**Growth characteristics**	**Enzymes to be screened**			
Thermus thermophilus	Thermophile	Amylase		Pullulunase	
Virgibacillus marismortui	Halophile	Protease	Amylase	Lipase	Pullulunase

3.7.1 Protease

Proteases are catabolic enzymes which catalyze the cleavage of peptide bonds in other proteins. Proteases represent class of enzymes which occupy a vital position with respect to their physiological roles in living beings as well as their commercial applications.

They perform both catabolic and synthetic functions. Since they are physiologically necessary for living organisms, proteases occur ubiquitously in a wide diversity of sources such as plants, animals, and micro-organisms. Microbes are an attractive source of proteases owing to the limited space required for their cultivation and their ready susceptibility to genetic manipulation.

Currently, proteases are classified on the basis of three major criteria,

1. Type of reaction catalyzed that is whether they are exopeptidase or endopeptidase,

2. The chemical nature of the functional group at the active site as serine proteases, aspartic proteases, cysteine proteases, and metalloproteases etc.

3. Its evolutionary relationship with a reference structure (Deshpande et al, 1998).

Proteases find extensive applications in the food and dairy industries. Alkaline proteases refer to proteolytic enzymes which work optimally in alkaline pH. As our organisms are haloalkaliphillic in nature, the protease secreted by them is expected to be alkali in nature too (Mabrouk et al, 2008). Alkaline proteases hold a great potential for application in the detergent and leather industries due to the increasing trend of developing environment friendly technologies. The enormous diversity of proteases, in addition to the specificity of their action, has attracted worldwide attention for exploiting their industrial and biotechnological applications e.g. food and feed industry, peptide synthesis, leather industry, management of industrial household waste, photographic industry, medical usage, silk gumming and detergents industry (Deshpande et al,1998)

There is increasing interest in using proteolytic enzymes as targets for developing therapeutic agents. Protease genes from several bacteria, fungi, and viruses have been cloned and sequenced with the prime aims of overproduction of the enzymes by gene amplification, to find

out the role of these enzymes in pathogenecity, and alteration in enzyme properties to suit its commercial application. (Deshpande et al, 1998)

Protein engineering techniques have been exploited to obtain proteases which show unique specificity and/or enhanced stability at high temperature or pH or in the presence of detergents and salts to understand the structure-function relationships of the enzyme. (Deshpande et al, 1998; Visessanguan, 2011; Namwong et al, 2005)

Protein sequences of acidic, alkaline, and neutral proteases from diverse origins have been analyzed with the aim of studying their evolutionary relationships. Despite the extensive research on several aspects of proteases, there is a paucity of knowledge about the roles that govern the diverse specificity of these enzymes. Deciphering these secrets would enable us to exploit proteases for their applications in biotechnology. (Deshpande et al, 1998; Visessanguan, 2011; Namwong et al, 2005)

3.7.2 α –Amylase

Starch is a ubiquitous and is an easily accessible source of energy. It is composed exclusively of α -glucopyranose units that are linked to each other by α -1,4- or α -1,6-glucosidic bonds. The two high-molecular weight components of starch are α -amylose (representing a 15 to 25% weight fraction of starch), which is a linear polymer composed exclusively of α -1,4-linked glucopyranose residues, and amylopectin (representing a 75 to 85% weight fraction of starch), which is also an α -1,4-linked glucopyranose polymer but in addition contains α -1,6-glycosidic linkages representing branch points occurring at every 17 to 26 residues (Rajeshwara et al,2007; Kenji et al,2007).

Several amylolytic enzymes, such as α-amylase (EC 3.2.1.1; glycosyl hydrolase family 13), β-amylase (EC3.2.1.2; glycosyl hydrolase family 14), and glucoamylase (EC 3.2.1.3; glycosyl hydrolase family 15), with different specificities can contribute to starch degradation. (Rajeshwara et al, 2007; Kenji et al, 2007)

Amylases have potential application in a number of industrial processes such as in the food, textiles, paper industries, bread making , glucose and fructose syrups, detergents, fuel ethanol from starches , fruit juices , alcoholic beverages , sweeteners , digestive aid and spot

remover in dry cleaning etc (Suman and Ramesh, 2010) The spectrum of amylase application has widened in many other fields, such as clinical, medical and analytical chemistry, pharmaceutical aid for the treatment of digestive disorders. (Suman and Ramesh, 2010)

3.7.3 Lipase

Lipolytic enzymes represent a hydrolase group, which specifically work over carboxylic ester. Lipases are defined as a carboxylesterases which catalyses the hydrolysis and synthesis of long-chain acylglycerols with trioleoylglycerol being the standard substrate. Lipases are able to catalyse hydrolysis, esterification, perhydrolysis, alcoholysis, intersterification and aminolysis reactions (Svoboda et al, 2008).

Lipases shape a versatile group of enzymes, due to a big amount of catalyzed reactions, therefore, a high potential of applications such as detergent, flavor development, paper recycle, chemical systems, racemic mixtures, and so on.

Lipases and esterases have been recognized as very useful biocatalysts due to its wide-ranging versatility in industrial applications. Biotechnological potential of lipases, due to their estereospecificity, is enormous and they attracted a high interest for food, agricultural, chemical, pharmaceutical, medical and cosmetic industry among other areas (Kouker et al, 1987).

Most of catalyzed reactions by lipases show a high selectivity and efficiency, and they occur under moderate conditions. These reactions occur without added cofactors and with low energy requirements, this properties contribute to reduce industrial conversions cost, and justify the growing interest in lipases. (Svoboda et al, 2008).

3.7.4 Pullulanase

Pullulan is a polysaccharide polymer consisting of maltotriose units, also known as α-1,4- ;α-1,6-glucan'. Three glucose units in maltotriose are connected by an α-1,4glycosidic bond, whereas consecutive maltotriose units are connected to each other by an α-1, 6 glycosidic bond. Pullulan is produced from starch by the fungus *Aureobasidium pullulans*. Four types of pullulan-hydrolyzing enzymes have been reported in literarture which are shown in figure 1.7

Figure 1.7 List of four types of pullulan hydrolyzing enzymes

Enzyme	**Product formed**	**Acts on**
Glucoamylase	Glucose	Non reducing ends
Pullulanase	Maltotriose	α-(1-6)-glucosidic linkages
Isopullulanase	Isopanose	α-(1-4)-glucosidic linkages of pullulan
Neopullulanase	Panose, glucose and maltose	α-(1-4) as well as α-(1-6) activity

Pullulanase is a specific kind of glucanase, an amylolytic exoenzyme that hydrolyses pullulan which is also known as pullulan, 6-glucanohydrolase.When pullulan is used as a substrate; maltotriose is main product that is formed, which can be estimated by DNSA method. (Manama et al, 2006; Delia and Salyers, 1996; Antranikian, 1996)

As an edible, mostly tasteless polymer, the chief commercial use of pollutant is in the manufacture of edible films that are used in various breath freshener or oral hygiene products. Pullulanase is used as a processing aid in grain processing biotechnology (production of ethanol and sweeteners) and serves as a substrate for bio-fuel production (Manama and Kuriki, 1989)

3.8 Reasons for selecting organisms and enzymes

The main reason for selecting these organisms for our study can be summarized as follows:

a. The growth characteristics of these organisms are well known and the organisms can be easily cultured in existing laboratory facilities.

b. The screening assays for enzymes like proteases, amylases, lipases and pullulanases are well known and standardised in various micro-organisms. Hence by these methods we can screen our organisms of interest.

c. There are no reports of screening and identification of these enzymes in these organisms.

d. The whole genome sequence of *Virgibacillus marismorui* is not completely reported. Hence if we identify some enzymes from this organism we can further do some molecular study to gain some insight about molecular determinants of halotolerance as well as for stability of these enzymes.

Materials

&

Methods

4.1 Growth media for organisms

4.1.1 Growth medium for *Virgibacillus marismortui*

Table 2.1 shows the normal growth medium for *Virgibacillus marismortui*. *H*ere the optimum salt concentration is 6% and casein peptone serves as carbon source.

Table 2.1 Growth medium for *Virgibacillus marismortui*

Components	**Amount in grams/100ml**
NaCl	6 g
Casein peptone	1 g
Yeast extract	0.5 g
Glucose	0.5 g

The pH was adjusted the to 9.0 with NaOH

For growing organisms on solid media 2% Agar was added to above media composition

4.1.2 Growth medium for *Thermus thermophilus (*MTCC strain number-1494)

Table 2.1 shows the normal growth medium for *Thermus thermophilus* as prescribed by MTCC.

Table 2.2 Growth medium for ***Thermus thermophilus*** *(*MTCC strain number-1494)

Components	**Amount in grams/100ml**
Polypeptone	0.8 g
Yeast extract	0.9 g
NaCl	0.2 g
Agar	3 g

The pH was adjusted to 9.0 with NaOH

4.2 Screening the organisms *Virgibacillus marismortui* for Protease, Amylase, Lipase and Pullulanase

For the screening of industrial important enzymes two major approaches can be used:

(1) Modification of standard screening assays according to our extremophillic organisms such as high temperature for *Thermus thermophilus* and providing salts i.e. 6% or more NaCl and adjusting pH of growth medium to pH 9.0 for *Virgibacillus marismortui* etc. (Table 2.3, 2.6, 2.8)

(2) Providing specific substrate of enzyme to be screened along with the normal growth media to such as *starch* for Amylase, *Tributyrin* for lipase, *Casein* for protease and *pullulan* for pullulanase. (Table 2.5, 2.7, 2.9)

4.2.1. Screening for Protease

Various different media were used for screening of enzymes in *Virgibacillus marismortui* according to above mentioned approaches. The media composition is given as follows:

Table 2.3 Media composition for protease according to according to approach 1

Media components	**Amount in grams/100ml**
Casein	1 g
Peptone	0.3 g
Yeast Extract	0.5g
NaCl	6 g

The pH was adjusted the to 9.0 with NaOH

NOTE: As the casein in above media had denatured by forming clumps, the above media was modified. In this composition we used skimmed milk as a source of casein for protease screening.

Table 2.4 Modified media composition for protease having skimmed milk as source of casein

Components	Amount /100 ml
Skimmed milk	5ml
NaCl	6g
Casein peptone	1g
Glucose	0.5g
Yeast extract	0.5g
Distilled water	95 ml

The skimmed Milk was filtered by whatman paper and autoclaved separately. After autoclaving milk is added to media, mixed properly and poured on plate.

Table 2.5 Media composition for protease according to according to approach 2

Media components	Amount in grams/100ml
NaCl	6.0 g
Casein Peptone	1 g
Glucose	0.5 g
Yeast extract	0.5 g
Casein	1 g

The pH was adjusted to 9.0 with NaOH volume was made up to 100 ml for all media

4.2.2 Screening for Amylase

Table 2.6 Media composition for amylase according to according to approach 1

Media components	Amount in grams/100ml
Magnesium sulfate	0.05 g
Ammonium sulfate	0.01 g
Starch	2.0 g
NaCl	6 g
Peptone	0.05 g

Table 2.7 Media composition for amylase according to according to approach 2

Media components	Amount in grams/100ml
NaCl	6.0 g
Casein Peptone	1 g
Glucose	0.5 g
Yeast Extract	0.5g
Starch	1.0 g

The pH was adjusted to 9.0 with NaOH volume was made up to 100 ml for all media.

For making plates 2.5 % agar was added (after adjusting pH).

4.2.3 Lipase screening

Table 2.8 Media composition for lipase according to according to approach 1

Media components	Amount in grams/100ml
Peptone	1g
Beef Extract	0.3 g
NaCl	6 g
TBA	1 g

Table 2.9 Media composition for lipase according to approach 2

Media components	Amount in grams/100ml
NaCl	6.0 g
Casein Peptone	1 g
Glucose	0.5 g
Yeast Extract	0.5 g
TBA	0.5 g

The pH was adjusted to 9.0 with NaOH volume was made up to 100 ml for all media.

For making plates 2.5 % agar was added after adjusting pH.

For lipase a pinch of $CaCO_3$ and Nile blue sulfite was added after autoclaving

4.3 Procedure for screening

The organism was grown on solid plates in culture media for their activation. After growth is achieved suitable colony was taken from these plates and organisms were placed in above screening media with the help of nichrome wireloop. Finally the plates were incubated at 35˚C.

4.4 Isolation of protein from cell free supernatant

4.4.1 Ammonium sulfate precipitation:

Ammonium Sulfate precipitation is a method used to purify proteins by altering their solubility. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration.

Two distinct effects are observed: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in.

As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out).

Since proteins differ markedly in their solubilities at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein

Solid ammonium sulfate was added slowly and gradually to the cell free supernatant kept on ice. The ammonium sulfate results in salting out of proteins and the solution becomes turbid. After no more ammonium sulfate dissolves, the solution was kept at 4°C for some time to allow it to precipitate completely. The precipitated solution was then centrifuged at 10,000 rpm for 30 minutes.

The supernatant was discarded and protein was obtained as smear on the walls of centrifuge tube. The protein was further redissolved in minimum volume of 25 mM Tris-Cl buffer. The ammonium sulfate is removed from protein fraction by dialysis

4.4.2 Dialysis:

Having precipitated a protein fraction that contains most of our enzyme, and redissolving it in buffer, it is necessary to remove the ammonium sulfate before we can proceed to subsequent steps in the purification process. The simplest way to achieve this is to dialyse the solution.

As shown in the figure 1.1 the enzyme solution is placed in a bag of selectively permeable membrane (dialysis bag from Sigma Aldrich with molecular weight cut off of 11.28 KDa) immersed in a large volume of buffer that is stirred and maintained at about 4°C.

The membrane has pores that will permit small molecules such as ammonium and sulfate ions to cross, and hence equilibrate in the larger volume of buffer outside, while not permitting large protein molecules to cross. If the buffer is changed several times, allowing several hours each time for the ammonium sulfate to equilibrate, more or less all of it will be removed from the protein solution.

Dialysis will increase the volume of the enzyme solution, because of the initial osmotic effect of the ammonium sulfate. Dialyzing buffer should be of same strength and pH in which the protein is resuspended and process must be carried out at 4˚C.

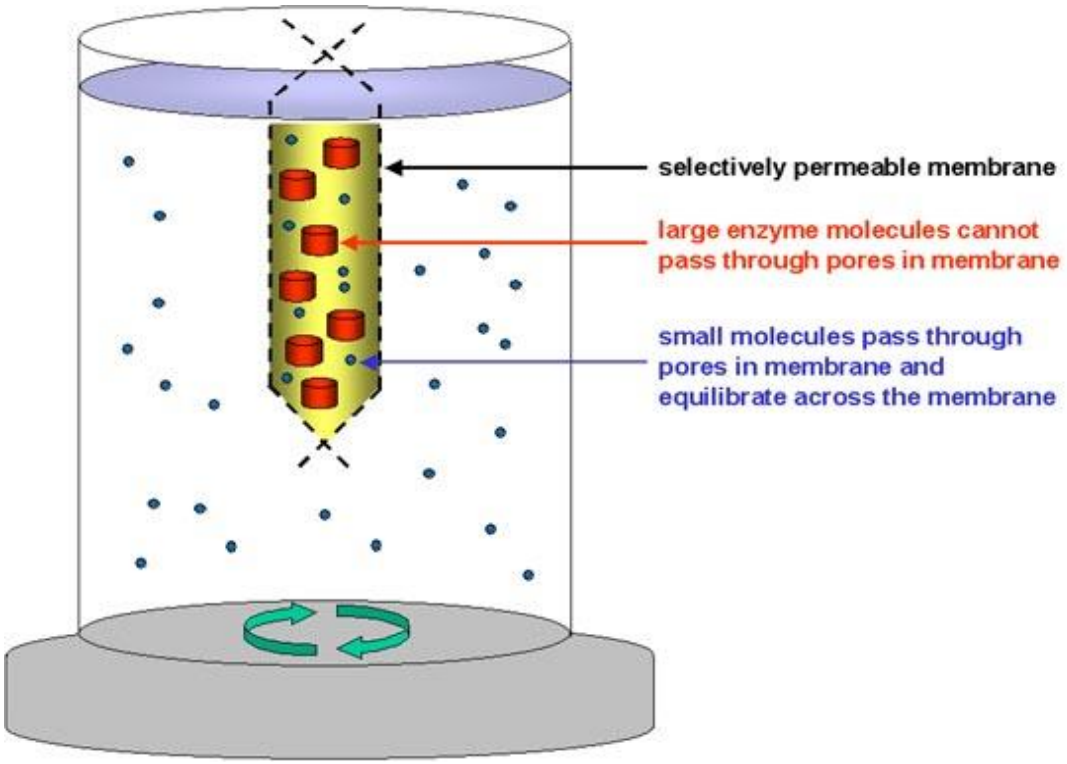


Figure 1.1 Diagrammatic representation of dialysis procedure

Reference: Protein purification protocols, online wiley library.

Dialysis bag was cut according to amount of protein to be dialysed. Then it is washed first with tap water to remove azide solution. Then the dialysis bag is washed with distilled water and boiled for 15 minutes in distilled water. Then it is allowed to cool at room temperature. Once it gets cooled, the bag is sealed at one end and checked whether it is leak proof or not by adding distilled water in it. Finally protein sample to be dialyzed is added in the bag carefully and the bag is sealed at other end. Dialysis bag is tied to the glass rod with rubber band and is dipped in dialysis buffer. Buffer is changed at regular interval (initially after 3hrs and later after 7 to 8 hrs).

4.5 Protease screening

4.5.1 Growth curve for determining the phase of maximum protease activity

The screening procedure carried out for *Virgibacillus marismortui* indicates that this organism produces protease enzyme.

In order to know that maximum protease production occurs in which phase of microbial growth, the growth of organisms was checked at a regular interval and simultaneously assay of protease production was done by centrifuging the culture broth at regular time intervals and loading the cell free supernatant into the bore made on casein agar plates along with measurement of absorbance.

The information about the growth phase in which maximum enzyme production occurs allows us to harvest maximum protein from cultured broth.

4.5.1.1 Preparation for seed culture:

For all assays culture of micro-organisms need to be activated which is called seed culture. The seed culture was prepared by inoculating 50 ml broth from working plate. Inoculated flasks were then kept in shaker incubator at 35˚C and were allowed to grow till O.D reaches to 0.6 to 0.8 (logarithmic phase).

4.5.1.2 Growth curve along with phase of maximum protease production:

5 ml of seed culture were inoculated into the 95 ml broth (5% v/v inoculum).2 ml of culture was taken in an autoclaved microfuge tubes and then culture was incubated in incubator shaker at 35˚C.This is called 0 minute of incubation. The O.D of 0 minute was recorded and broth removed is centrifuged. After that, at regular interval broth is removed and OD was measured at 540 nm and simultaneously culture is withdrawn in a sterile microfuge tube. Microfuge tubes were centrifuged at 10,000 rpm for 10 minutes to remove bacterial cell pellets The pellets were discarded and supernatant were stored at 4˚C. 15 µl of supernatants collected every hour were loaded in a cup made in milk agar plate with the help of cup borer. Then all plates were incubated for 48 hrs at 35˚C. After incubation the zone of protein hydrolysis was observed

4.5.2 Zymography for detection of protease activity

Zymography is an electrophoretic technique, based on SDS-PAGE that includes a substrate co-polymerized with the polyacrylamide gel, for the detection of enzyme activity

The protein needs to be in its native form for zymography. Hence samples are prepared in gel loading dye without beta-mercaptoethanol and are not boiled for the same. Samples were loaded onto SDS-PAGE co-polymerized with 0.1% casein and separated by electrophoresis. After separation, the gel was renaturated in 2.5% Triton X-100 solution at room temperature for 60 min with gentle agitation The acrylamide gel was then equilibrated in developing buffer (50 mM Tris-HCl, pH 9; 200 mM NaCl, 5 mM$CaCl_2$, 0.02% Triton X-100) at room temperature for 30 min with gentle agitation, and incubated overnight at 37°C in fresh developing buffer. Transparent bands of caseinolytic activity were visualized by staining with 0.5% Coomassie Blue R250. Coomassie blue staining of the gel reveals sites of proteolysis as white bands on a dark blue background .In zymography no molecular weight markers are run, so simultaneously an another normal gel of same acrylamide concentration as in zymograph is to be run and to same length. This allows detection of molecular weight of protease to be detected. (Schneider et al, 2008)

NOTE: Initially the sample showed smear of transparent caseinolytic activity as it is more stable, so a pinch of SDS powder was added to the sample-dye mixture before loading it into the gel.

4.5.3 Effect of salt concentration on protease production in *Virgibacillus marismortui*

The organism *Virgibacillus marismortui* is haloalkaliphile which grows on optimal salt concentration 6% NaCl and requires pH 9 for its optimal growth. According to Bergey's manual the genus *Virgibacillus* are halophillic bacteria can grow on salt concentration as high as 16%, and Virgibacillus *marismortui* in particular can tolerate up to 25% NaCl

Hence if organisms can grow on salt concentrations up to 25%, than protease isolated from these cultures can be important in industrial applications.

For this seed culture was prepared at 6% NaCl concentration as earlier. After growth is achieved in seed culture, 5% v/v inoculums were added to broth with different salt concentration while rest of media constituent remained the same salt concentration on which organism was grown were 10%, 16%, 25%. Apart from it, a positive control of flask inoculated with 6% NaCl (inoculated with seed culture) and one negative control of 6% (uninoculated broth) were set up. All flasks was incubated at 37˚C on shaker incubator till the OD reaches to approximate 0.8-0.9

4. 5. 4 Protease activity determination

400µl of 0.5% casein (prepared in 25 mM Tris pH 9.0) was taken in microfuge tube. 100 µl of enzyme sample was added and the tubes were incubated at 37˚C for 20 minutes. After incubation 0.6 ml of 10% w/v TCA were added into it to stop the reaction. It was then centrifuged at 10,000 rpm for 15 minutes at 4˚C. Resultant supernatant was taken into a fresh vial. OD of the supernatant was measured at 280 nm. (Mabrouk et al, 2007)

Note:

Procedure works best only if the protease is an exopeptidase that starts cleaving the peptide from one end and causes the release of amino acids (particularly tyrosine) that are detected at 280 nm, but not for Endopeptidase that cleaves the peptide at internal peptide bonds resulting in polypeptide fragments.

4.6 Amylase screening procedures

Amylase activity was measured by starch iodine assay and by DNSA test.

NOTE: Amylase activity was not found in cell free supernatant of normally cultured broth.as starch is not hydrolyzed by *Virgibacillus marismortui* under normal conditions.

4.6.1 Induction of amylase in *Virgibacillus marismortui* by growing cells in a minimal media with starch as the sole carbon source.

It is well established fact that in presence of easily hydrolysable sugars like glucose and fructose, the organism capitalizes on producing enzymes for these substrates as it saves ATP and energy obtained is faster than that obtained for less utilizable substrates like starch.

Hence for inducing amylase activity we thought to grow these organisms on minimal media which contains all defined components and starch as the sole source of carbon in place of glucose. It is necessary to provide organism with optimal salt concentrations in the minimal media. Hence minimal media was supplemented with 6% NaCl

Table 2.10 Composition of minimal media

Components	g/L
K_2HPO_4	0.7
KH_2PO_4	O.2
$(NH)_2SO_4$	0.1
Glucose	0.1
Sodium citrate	0.05
$MgSO_4.7H_2O$	0.01

4.6.2 DNSA from isolated protein to estimate Amylase activity

This method tests for the presence of free carbonyl group (c=o), the reducing sugar. This involves the oxidation of the aldehyde functional group present; for example; in glucose and the ketone functional group in fructose.

3, 5-Dinitrosalicylic acid (DNS or DNSA, IUPAC name 2-hydroxy-3, 5-dinitrobenzoic acid) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm.

2.5 ml 25 mM Tris buffer pH 9.0 was added in test tubes. Then 2.5 ml of 1% starch and 1ml of 1% NaCl were added. The reaction mixture was incubated at 37˚C for 10 minutes. After incubation different amounts of sample were added and final volume was made up to 1 ml by using 25 mM Tris buffer pH 9.0 .Then again tubes were incubated at 37˚C for 15 minutes. After incubation 0.5 ml of NaOH were added in all tubes to stop the reaction. Finally 0.5 ml of DNSA was added. The tubes were mixed properly and boiled in water bath at 100˚C for 5 minutes then the tubes were allowed to cool down and then OD was measured at 540 nm

NOTE:

For estimating pullulanase activity the DNSA test described above is modified. In this pullulan assay 1% pullulan is used in place of 1% starch.

4.6.3 Estimation of Amylase activity from sample and calculating its crude as well as crude activity per mg:

From the standard curve of maltose we can estimate amount of maltose released in sample. Amount of maltose release per ml of sample protein gives crude amylase activity. The crude amylase activity can be calculated using formula:

$$\textbf{Enzyme activity} = \frac{\text{Net amount of sugar produced}}{\text{Molecular weight}} \times \frac{\text{Dilution factor}}{\text{Incubation time.}}$$

(Lorentz, 1959)

4.6.4 Starch iodine assay

Starch reacts with iodine to give blue color formation. Here we initially incubate the sample with the starch solution so amylase activity in the sample will hydrolyze the starch and hence upon subsequent reaction with iodine color blue colour will develop decreases as compared to blank which has undigested starch.

0.5 ml of buffer was added to all the tubes. Then 0.2 ml of 0.1% starch was added followed by 0.2 ml of 0.01N HCl Incubate all the tubes at 37°C for 15 minutes The 0.1 ml enzyme source was added Incubate all the tubes at 37°C for 30 minutes Add 0.4 ml of 0.01N Iodine and add 8.6 ml of distilled water. The absorbance was measured at 575 nm.

4.7 Pullulanase screening procedures

4.7.1 Minimal media for pullulanase activity

In order to know whether the organism secretes pullulanase and is able to hydrolyse pullulan, we grew the organism on a minimal defined media similar to amylase induction. Here the minimal media (Table 2.10) contained a pullulan as sole carbon source and 6% NaCl .The organism will be able to grow only if it is able to utilize and hydrolyse sole carbon source pullulan.

4.7.2 Effect of salt concentration on pullulanase assay

Once it was confirmed that *Virgibacillus marismortui* is able grow on minimal media containing pullulan as a sole carbon source, we tried to grow it on minimal media with pullulan at different salt concentration i.e. 6%, 10% and 15% and pullulanase activity was checked by DNSA method.

4.7.3 DNSA test for determination of pullulanase activity

The production of maltotriose as a result of pullulanase activity can be detected by DNSA test as described earlier in 4.6.2 (Sinderen et al, 2006). In pullulan assay pullulan is used as a substrate for the assay in place of starch. As the availability of pullulan was less, the total assay was modified as follows

0.25 ml 25 mM Tris buffer pH 9.0 was added in test tubes. Then 0.25 ml of 1% pullulan and 0.1ml of 1% NaCl were added The reaction mixture was incubated at 37˚C for 10 minutes After incubation different amounts of sample were added and final volume was made up to 0.1 ml by using 25 mM Tris buffer pH 9.0 Then again tubes were incubated at 37˚C for 15 minutes After incubation 0.05 ml of NaOH were added in all tubes to stop the reaction. Finally 0.05 ml of DNSA was added.The tubes were mixed properly and boiled in waterbath at 100˚C for 5 minutes then the tubes were allowed to cool down and then OD was measured at 540 nm

4.7.4 Isolation of protein from culture grown at higher salt concentration:

After 48 hrs of incubation, broth of both normal and minimal media from different NaCl concentrations (6%, 10%, 15% for protease and pullulanase and 25% for protease only) were centrifuged to recover cell free supernatant followed by ammonium sulfate precipitation and finally dialysis was performed to remove ammonium sulfate. The proteins recovered by centrifugation after was resuspended in 25mM Tris buffer and was dialysed in the same.

4.8 Screening of Lipase by different assays

Screening of lipase producers on agar plates is frequently done by using tributyrin agar and liquid tributyrin as a substrate. Modifications of these assays use tributyrin in combination with Nile-blue sulfate with calcium carbonate. (Table 2.8, 2.9)

However these substrates are not suitable to detect true lipases because they are hydrolyzed by esterases too. The existence of lipases has to be verified by applying time consuming methods, e.g., a titrimetric test with trioleoylglycerol, olive oil etc which are ideal lipase substrate

Initial screening of lipases on media as described earlier indicates that *Virgibacillus marismortui* are not lipase producers. However to confirm that *Virgibacillus marismortui* are not lipase producers a specific and sensitive assay for lipases were carried out.

4.8.1 Sensitive plate assay for lipases (Kouker et al, 1989)

The halophillic bacteria were cultured in a modified HM MEDIA, with small amount of yeast extract and replacing peptone and glucose with olive oil as sole carbon source Olive oil used here is ideal substrate for lipases in a hydrolysis reaction and Rhodamine B acts as an indicator of the reaction. Rhodamine B reacts with free fatty acids released as a result of lipase activity and results in orange yellow fluorescent halos visible under UV light at 350 nm

Table 2.11 Modified HM media Composition for screening of lipase:

Components	Amount in g/100 ml
NaCl	6 g
Olive oil	3 g
Yeast extract	0.05 g
Agar	3 g

The pH was adjusted to 9.0
The volume was made up to 100 ml by distilled water
A pinch of Rhodamine B indicator and olive oil was added in media after autoclaving
Plates were incubated at 37˚C for around 8 days

All media components except Rhodamine B and olive oil were added to the media and pH was adjusted to 9.0 and autoclaved. A very small amount of olive oil 3% w/v was added in a big test tube and was autoclaved separately. After autoclave add a pinch of Rhodamine B and olive oil and shake vigorously before pouring on plate. The plates were incubated at 37°C for 8 days or more.

4.9 Folin - Lowry's estimation for estimation of total protein from isolated protein.

The principle is similar to that of the biuret method, except that here a second reagent i.e.; the Folin Ciocalteau reagent is added to enhance the color, which depends on the amount of protein present in s sample.

The solutions 2% Na_2CO_3 in 0.1N NaOH, 0.5% $CuSO_4$ and 1% Na- K tartarate solution Were mixed in the ratio of 100:1:1 before use which is called solution C

10µl of sample is taken in a tube and volume is made up to 800 µl. Then 2 ml of solution C is added to it. The tubes are incubated for 10 minutes.120 µl of commercially available Folin Ciocalteau reagent (1N) is then added and tubes are incubated for 30 minute in dark. OD is taken at 660nm

4.9.1 Estimation of total protein for protease:

Estimation of total proteins was carried out for four isolated proteins sample using Folin - Lowry's method.

1) Protein isolated from cultured broth with 6% NaCl concentration
2) Protein isolated from cultured broth with 10% NaCl concentration
3) Protein isolated from cultured broth with 15% NaCl concentration
4) Protein isolated from cultured broth with 25% NaCl concentration

4.9.2 Estimation of total protein for amylase:

The minimal media with starch as sole carbon source was ammmonium sulfate precipitated and Folin Lowry of this sample was carried out in order to estimate total protein.

4.9.3 Estimation of total protein for pullulanase:

Estimation of total protein fractions for cells grown in minimal media with pullulan as sole carbon source at 3 different salt concentrations Folin -Lowry's method:

1) Protein isolated from cultured broth with 6% NaCl concentration
2) Protein isolated from cultured broth with 10% NaCl concentration

3) Protein isolated from cultured broth with 15% NaCl concentration

4.10 SDS PAGE

SDS PAGE was run in order to detect all proteins secreted by the cells into the media. The protein were recovered from cell free supernatant after ammonium sulfate precipitation followed by dialysis. (Refer appendix 3 for preparation of gels)

4.10.1 Protocol for TCA precipitation:

1. 100% TCA was prepared in Milli-Q water
2. Sample was taken in a microfuge tube and 1/10 volume of TCA was added to it and kept on ice for 1 hr
3. After 1 hour precipitate formed was centrifuged at 10,000 rpm for 4 minutes.
4. Finally pellet was resuspended in 10µl gel loading dye and is boiled for 10 minutes.
5. Sample was then loaded in to the SDS PAGE gel.

4.11 Screening of *T.thermophilus* for amylase and pullulanase

The organism themus thermophilus was screened for enzymes amylase and pullulanase by growing them on minimal media with starch and amylase as sole carbon source respectively as mentioned earlier for *Virgibacillus marismortui*

Results

&

Discussion

5.1 Screening of organism *Virgibacillus marismortui* for protease, amylase, lipase and pullulanase

Various media for screening of organism were used in order to know whether the organism produces these enzymes or not. (Refer Table 2.1, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9).For protease screening, initially we faced some problem as clumps were formed in media as casein was denatured due to autoclaving. We then modified the media by using skimmed milk as a source of casein in place of normal casein. (Refer Table 2.2). After 48 hours of incubation, clear zone of hydrolysis was obtained.

Figure 1.2 Plates showing clear zone of casein hydrolysis on milk agar plates due to protease activity

For amylase and lipase, initial screening did not give any significant result; hence other methods were used like DNSA test (4.6.2) and Starch iodine test for amylase (4.6.4) and sensitive plate assay for lipase (4.8.1)

Once it was confirmed that *Virgibacillus marismortui* is able to produce protease, we found out that at which phase of growth the maximum protease production occurs. For this we carried out growth curve along with measurement of protease production (Refer 4.5).

5.1.1 Protease screening

5.1.1.1 Growth curve cum determination of phase of maximum protease production

Table 3.1 Growth curve of Virgibacillus marismortui: Absorbance values of at 540 nm at regular intervals

Time(hours)	O.D at 540 nm
0	0.012
1	0.05
2	0.129
3	0.339
4	0.583
5	0.774
6	0.983
7	1.008
8	1.006
9	0.998
10	0.999

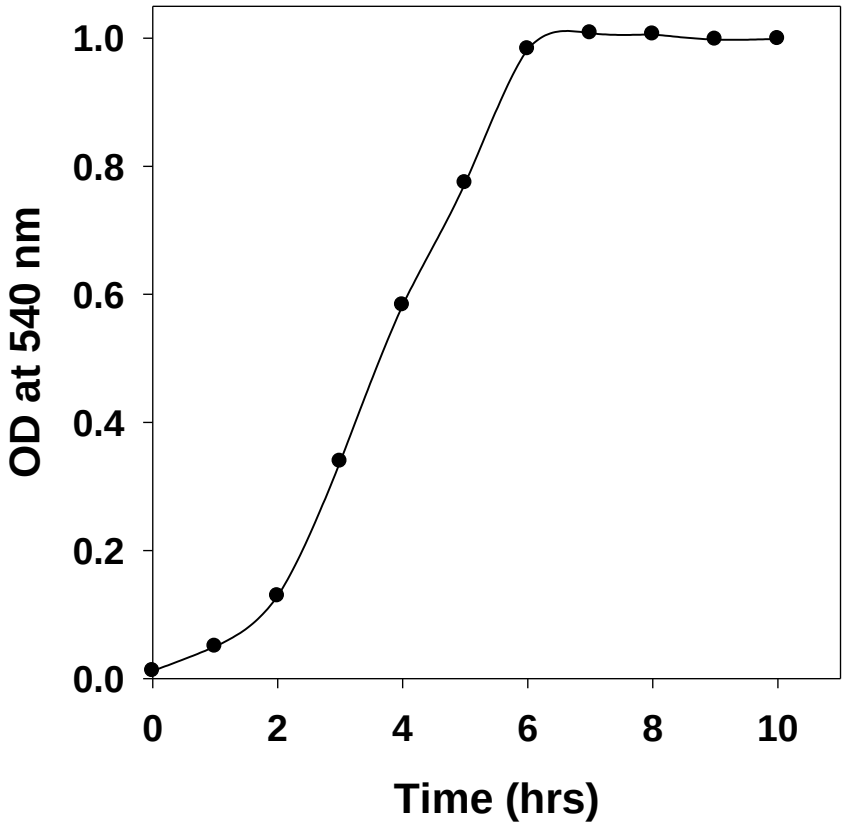
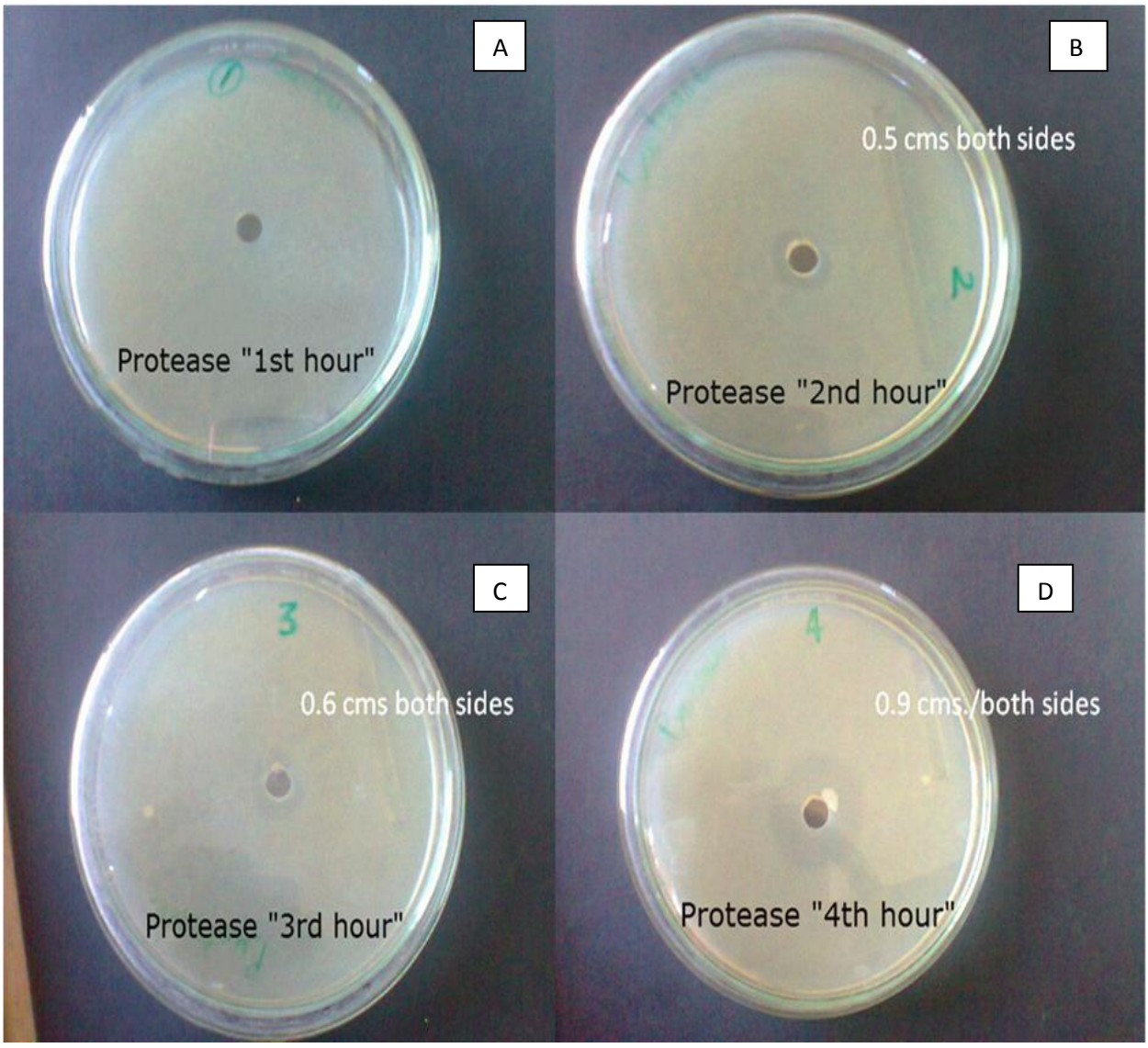


Figure 1.3 Growth curve of *Virgibacillus marismortui*

Result obtained for growth curve indicate that organism enters in to the stationary phase after 8-9 hrs of inoculation of seed culture in the broth while mid logarithmic phase is achieved after 5-6hrs of incubation.

Maximum protease activity occurs in the stationary phase at 9^{th} and 10^{th} hour of inoculation. Images of milk agar plate showing protease activity at all different phases were taken and diameter of zone formed by casein utilization was measured.

Images of milk agar plates in which cell free supernatant removed after each hour of growth was loaded were taken. Zone of hydrolysis increases as time increases and becomes constant afterwards


A
Protease "1st hour"
B
0.5 cms both sides
Protease "2nd hour"
C
0.6 cms both sides
Protease "3rd hour"
D
0.9 cms./both sides
Protease "4th hour"

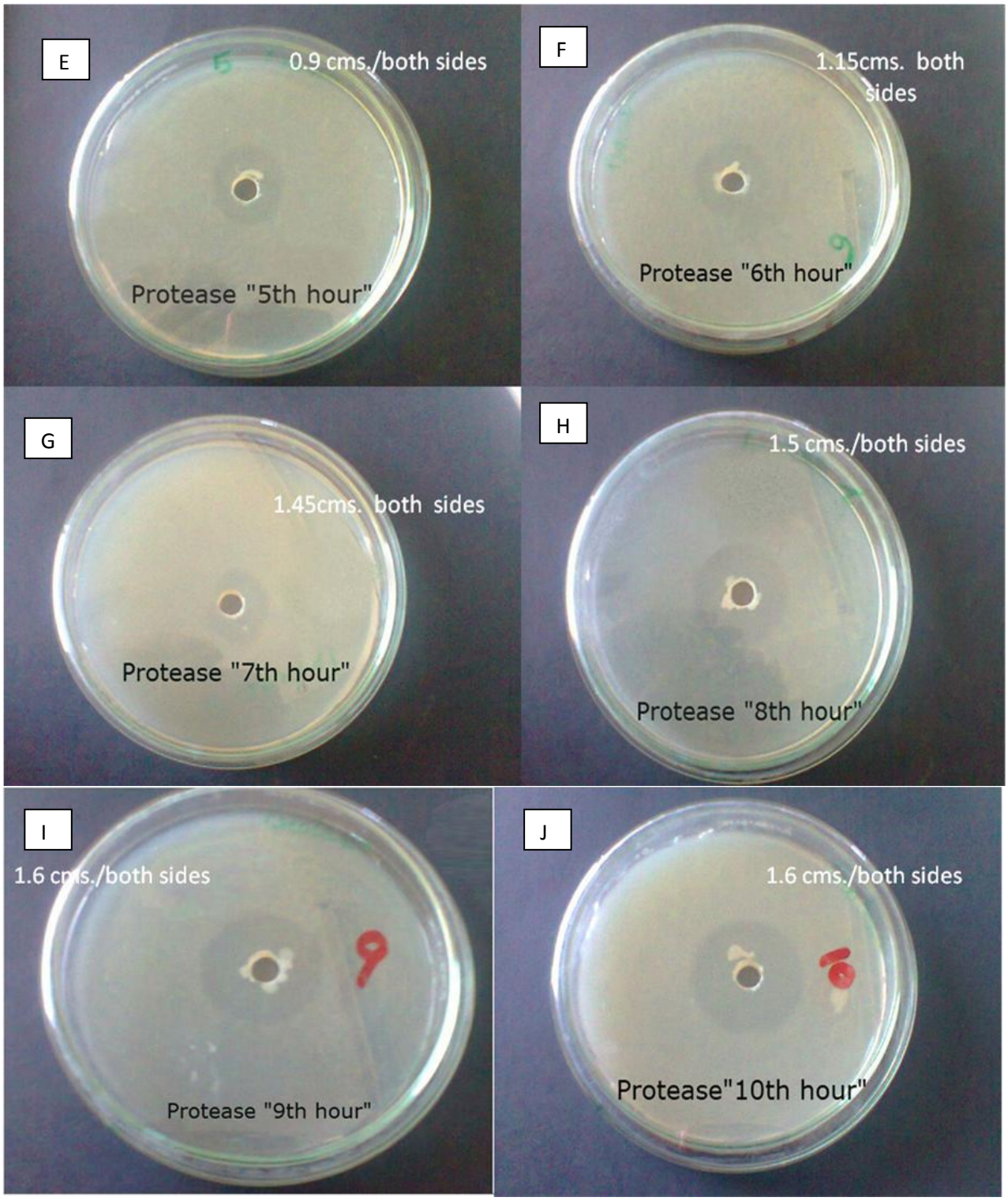


Figure 1.4 Figures A-J showing plates showing clear zone of casein hydrolysis on milk agar plates due to protease activity from cell free supernatant after each hour of growth.

Measurement of protease production

Table 3.2 Estimation of protease activity by measurement of diameter of zone of casein hydrolysis

Time (hours)	Diameter of well (cms.)	Zone diameter (cms)
1	0.8	-
2	0.8	0.50
3	0.8	0.60
4	0.8	0.90
5	0.8	0.90
6	0.8	1.15
7	0.8	1.4
8	0.8	1.45
9	0.8	1.6
10	0.8	1.6

5.1.1.2 Effect of salt on growth conditions of *Virgibacillus marismortui*

Virgibacillus marismortui was grown on media containing different salt concentration i.e. 6%, 10% and 15% NaCl. It was observed that when grown in higher salt concentration the time required to achieve full growth is more than that required for positive control. (Refer 4.5.3)

Table 3.3 Effect of salt concentration in growth of ***Virgibacillus marismortui***

Salt concentration	O.D at 540 nm	Full growth achieved in hours
6%	0.999	9^{th} hour
10%	0.989	12^{th} hour
16%	1.42	24^{th} hour
25%	1.21	27^{th} hour

Figure 1.5 Cells grown on 10% NaCl along with positive control (6% NaCl, inoculated) and negative control (6% NaCl, uninoculated)

Figure 1.6 Cells grown on 16% NaCl along with positive control (6% NaCl, inoculated) and negative control (6% NaCl, uninoculated)

Figure 1.7: Cells grown on 25% NaCl along with positive control (6% NaCl, inoculated) and negative control (6% NaCl, uninoculated)

5.1.1.3 Zymography for confirmation of protease activity

The protein was isolated from above grown cultures by precipitating the cell free supernatant and dialyzing it in 25mM Tris buffer. Then in order to confirm that this organism secretes extracellular protease zymogram was done. (Refer 4.5.2)

At first we conducted zymogram in 12% acrylamide concentration and samples taken were

1. Ammonium sulfate precipitated 25% NaCl sample
2 Cell free supernatant of 15% NaCl sample
3 Cell free supernatant of 10% NaCl sample
4. Cell free supernatant 6% NaCl sample

It was observed that 15% sample did not give any transparent band, while 6% and 10% did give white bands of protease activity. Maximum transparent bands were seen in 25% NaCl sample as it was dialysed and other two were not.

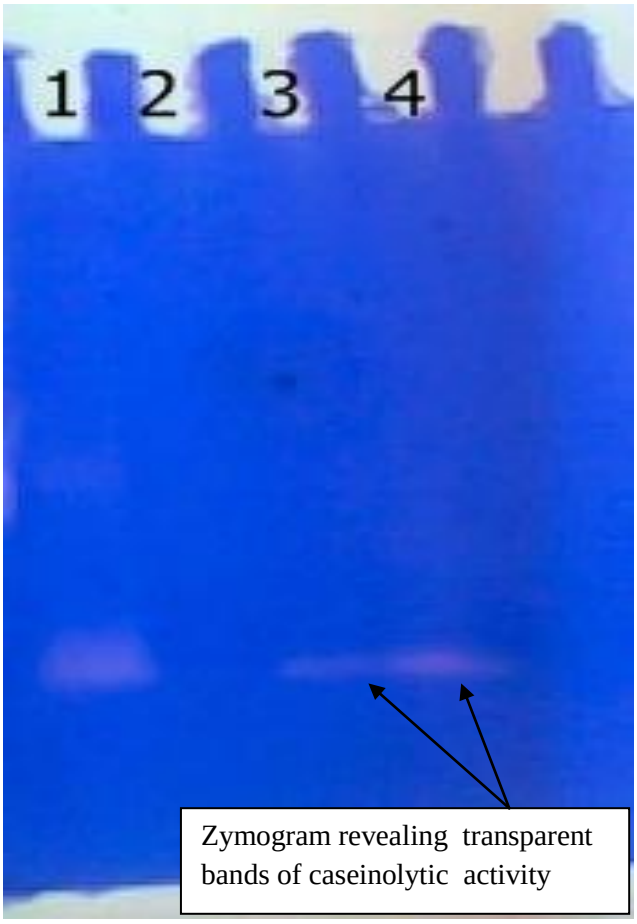


Figure 1.8 Zymography of cell free supernatant of *Virgibacillus marismortui.* Arrows indicate transparent bands due to hydrolysis of casein by proteases

Lane 1 ammonium sulfate precipitated sample of cells grown in 25% NaCl
Lane 2 cell free supernatant of cells grown in 15% NaCl
Lane 3 cell free supernatant of cells grown in 10% NaCl
Lane 4 cell free supernatant of cells grown in 6% NaCl

As we obtained bands of caseinolytic activity at the bottom of the gel, which indicates that the protease produced by organism is very low molecular weight, we decided to go for a 20% acrylamide gel.

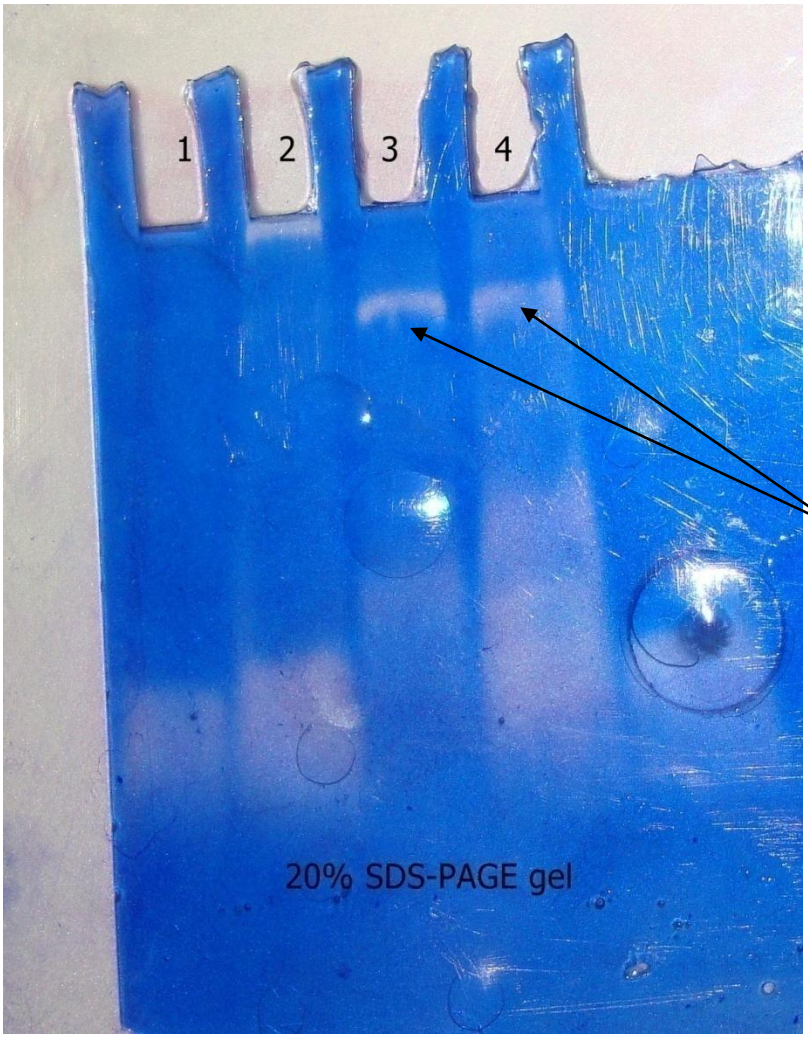


Figure 1.9 Zymogram of 20% acrylamide gel with TCA precipitated sample.

Lane 1 Cell free supernatant of cells grown in 6% NaCl
Lane 2 Cell free supernatant of cells grown in 10% NaCl
Lane 3 Cell free supernatant of cells grown in 15% NaCl
Lane 4 Cell free supernatant of cells grown in 25% NaCl

As we had obtained bands at upper side of gel we can say that 15% and 25 % have a high molecular protease**.** As molecular weight markers are not run in zymogram, a normal zymogram must be run simultaneously at to same length and same voltage. In order to know the molecular weight of protease of interest a normal 20% SDS gel was run simultaneously along with above 20% zymogram.

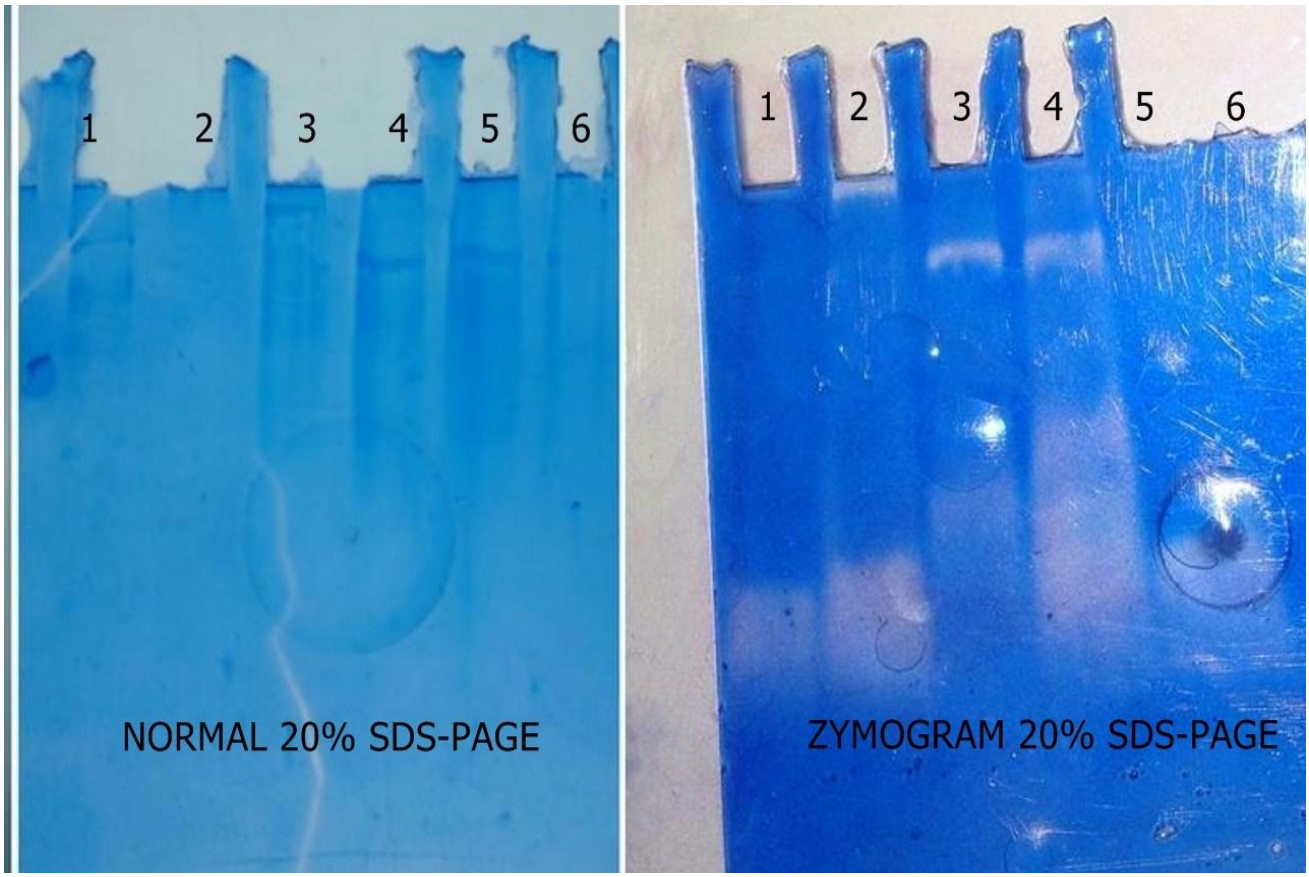


Figure 1.10 Comparative gel pictures showing normal 20% gel (left gel image) and zymogram (right gel image) . Arrows indicate position of proteases in both the gels**.**

Lane 1-Molecular weight marker

Lane 3 (Normal SDS-PAGE) and Lane 2 (Zymogram) Ammonium sulfate precipitated sample of cells grown in 10% NaCl

Lane 4 (Normal SDS-PAGE) and Lane 3 (Zymogram) Ammonium sulfate precipitated sample of cells grown in 15% NaCl

Lane 5 (Normal SDS-PAGE) and Lane 4 (Zymogram) Ammonium sulfate precipitated sample of cells grown in 25% NaCl

5.1.1.4 Protease activity determination:

The protease activity was performed in protease samples of various salt concentrations, did not give any significant absorbance at 280 nm. This may be due to the fact that this assay is based upon detection of free tyrosine that is formed during exopeptidase activity of proteases. As

free tyrosine in not generated by endonucleases, in which tyrosine is still bound to peptide fragments this assay is not applicable to them. As we did not get any significant result in this assay we can conclude that protease of *Virgibacillus marismortui* is an endopeptidase

5.1.1. 5 Folin Lowry estimation of isolated protein

Table 3.4 Folin Lowry estimation of isolated protein

Ammonium sulfate precipitated sample of cells grown in	**Average O.D**	**Concentration of protein in mg/ml**
6% NaCl	0.131	3
10% NaCl	0.124	3.2
15% NaCl	0.358	9.7
25% NaCl	0.278	7.5

Highest amount of protein was observed in 15% NaCl Ammonium sulfate precipitated sample whereas in 6% and 10% NaCl Ammonium sulfate precipitated sample had almost similar protein content.

5.1.2 Amylase screening in *Virgibacillus marismortui*

Initially when we started screening the organism for amylase activity, we were not able to get any amylase activity and it was seen that under normal conditions when the media is supplemented with easily hydrolysable substrates like glucose this organism does not secrete amylase enzyme.

After checking the literature and various reports for *Virgibacillus marismortui* and strain specifications for this organism in Bergey's manual of systematic bacteriology it was confirmed that this organism is not able to hydrolyze starch under normal conditions.

5.1.2.1 Induction of amylase production in *Virgibacillus marismortui*

Although it was confirmed that this organism does not produce amylase under normal growth conditions, we thought to grow it on a minimal media containing starch as a sole source of carbon. In order to survive and grow in these minimal media the organism will be induced to produce amylase

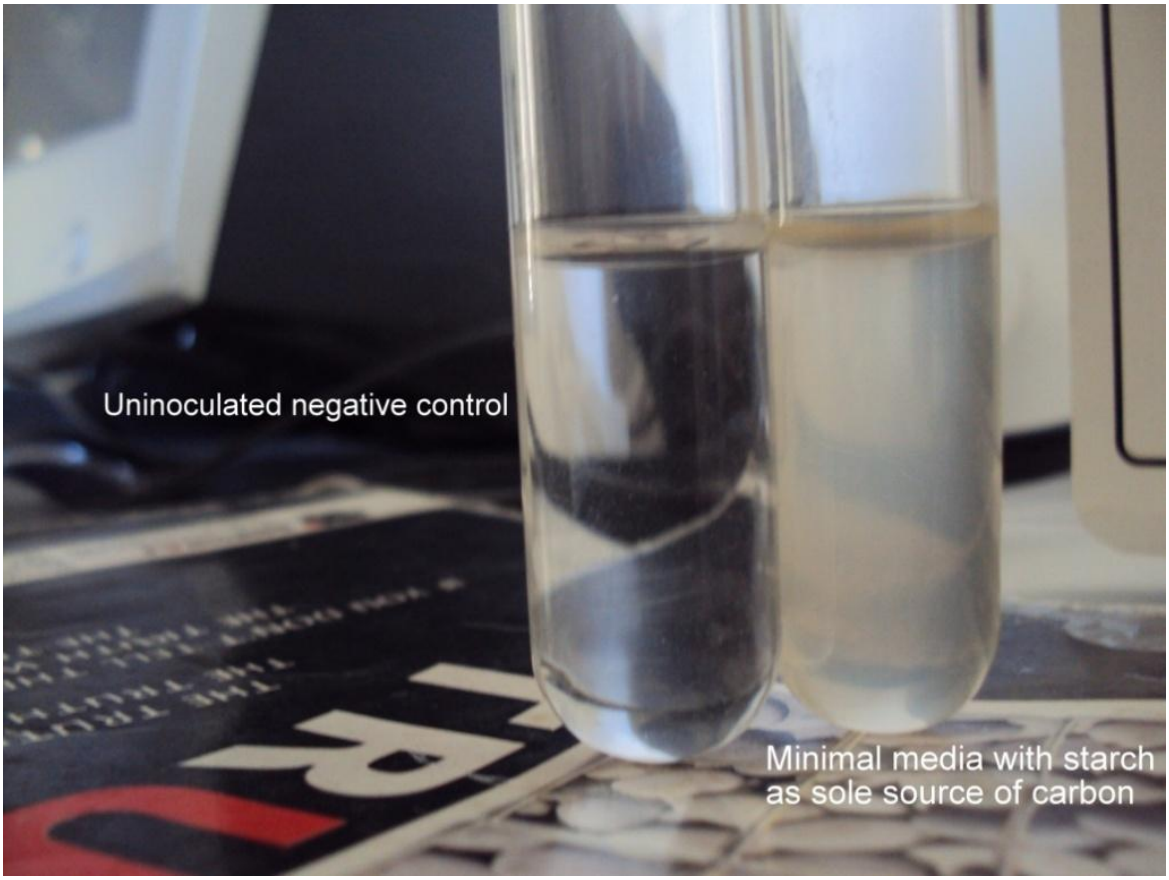


Figure 1.11 uninoculated negative controls of minimal media along with tube containing minimal media with starch as sole carbon source.

When grown on a minimal media containing starch as a sole source of carbon the organisms were able to grow successfully which indicates that this organism can be induced for production of amylase.

5.1.2.2 Isolation of protein from cells grown on minimal media

After the cells were grown to maximum turbidity, the cells were centrifuged and cell free supernatant was precipitated by ammonium sulfate precipitation and finally after redissolving it in minimal volume of Tris-Cl buffer, pH 9.0, was dialysed to obtain the protein sample. The amylase activity was checked for this protein sample by DNSA and Starch-iodine assays.

5.1.2.3 DNSA test for amylase activity.

3 different concentrations i.e. 0.25 ml, 0.5 ml and 0.75 ml of cell free supernatant was used. The total volume of enzyme system was made up to 1 ml by Tris-Cl pH 9.0

DNSA test for cell free supernatant

Table 3.5 DNSA test for cell free supernatant

Volume cell free supernatant(ml)	Amount of Tris buffer added	OD at 540 nm		Average O.D
0.25	0.75	0.143	0.139	0.142
0.50	0.50	0.189	0.196	0.193
0.75	0.25	0.212	0.219	0.214

As the media contains starch already, one cannot do the DNSA with cell free supernatant as it may also contain maltose formed during growth of organism on starch hence for each amount of cell free supernatant taken, a blank is kept in which no starch is added so that only maltose that results from hydrolysis of starch provided in the assay is measured and contribution from maltose originally present in the media is excluded.

After dialysis of the protein sample crude amylase activity was measured.

5.1.2.4 Crude amylase activity determination

On the basis of standard curve of maltose, amount of maltose released in sample and crude activity of sample can be calculated by the formula. 1 Unit of enzyme is defined as amount of maltose released per ml per minute.

Enzyme activity= $\frac{\text{Net amount of sugar produced}}{\text{Molecular weight}} \times \frac{\text{Dilution factor}}{\text{Incubation time.}}$

Table 3.6 DNSA test for isolated protein fraction

Amount of Ammonium sulfate precipitated sample (ml)	**Average O.D**	**Amount of maltose released from graph (µg/ml)**	**Crude amylase activity**
0.1	0.133	1500	0.308

5.1.2.5 Folin Lowry estimation of total protein

Table 3.7 Total protein content of isolated protein sample estimated from Folin Lowry method and determination of crude activity per mg of protein

Crude amylase activity	**Total protein content of isolated protein sample (mg/ml)**	**Crude amylase activity /mg of protein**
0.308	2.8	0.11

5.1.2.6 Starch iodine assay.

In order to confirm that organisms grown on minimal media with starch as a sole carbon source produce amylase, a qualitative assay called the starch iodine assay was performed.

Here for the blank if starch is added than readings of sample are in negative as in them the concentration of starch decrease in comparison to the blank. Hence starch is omitted from the blank. The reading value is obtained by subtracting value of blank for each concentration

Also a blank has to be kept for each amount of cell free supernatant used in which no starch has been added as minimal media may also contain maltose formed by action of amylase during growth of organism on starch as done in DNSA described previously.

The less intense the colour is, smaller the readings are, and more is the amylase activity.

Table 3.8 Starch iodine assay for cell free supernatant of minimal media with starch as carbon source.

Amount of cell free supernatant	**O.D at 575 nm**		**Average O.D**
Blank	0.0		0.0
0.25	0.098	0.092	0.094
0.50	0.062	0.071	0.065
0.75	0.048	0.052	0.051

5.1.3 Pullulanase screening in *Virgibacillus marismortui*

5.1.3.1 Induction of pullulanase production in *Virgibacillus marismortui*

Similar to induction of amylase, pullulanase activity was also checked by growing cells on a minimal media containing pullulan as sole source of carbon. (4.7.1)

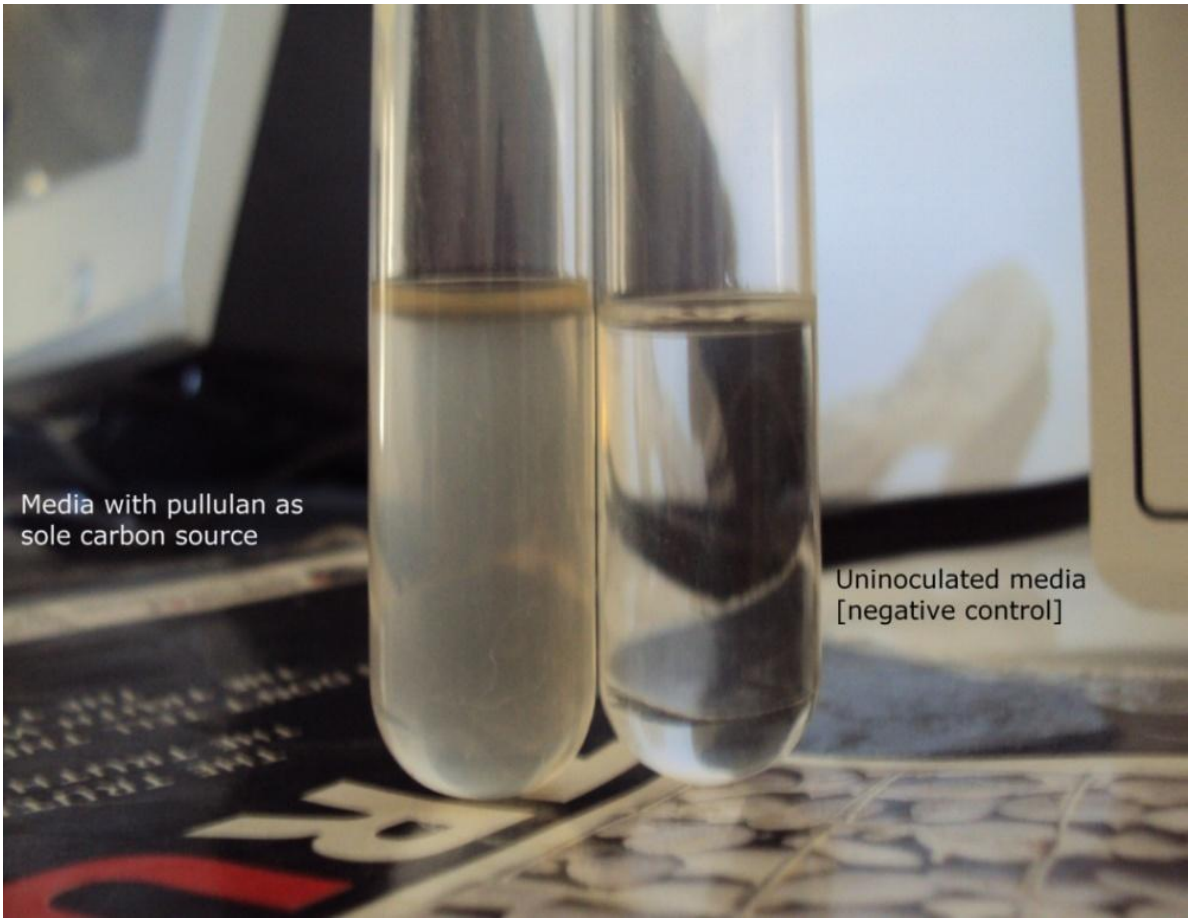


Figure: 1.12 Uninoculated media with minimal media with pullulan as sole carbon source

5.1.3.2 Pullulanase activity at different salt concentration.

Hence we tried to grow this organism in minimal media containing pullulan as sole carbon source and at different salt concentrations 6%, 10% and 15%. (4.7.2). It was observed that this organism was able to grow in minimal media containing pullulan as sole carbon source at different concentrations of salts which indicates its ability to produce pullulanase.

Figure: 1.13 Cells grown in minimal media with pullulan as sole source of carbon and with 10% NaCl concentration along with uninoculated negative control and inoculated positive control with 6% NaCl concentration

Figure: 1.14 Cells grown in minimal media with pullulan as sole source of carbon and with 15% NaCl concentration along with uninoculated negative control and inoculated positive control with 6% NaCl concentration

5.1.3.3 DNSA for pullulanase activity

Similar to DNSA test for amylase, pullulanase assay can be done for amount of pullulan hydrolyzed and maltotriose formed. (4.7.3)

Table 3.9 DNSA test for isolated protein fraction

Sample	**Volume of sample taken (ml)**	**OD at 540 nm**		**Average OD**
6%	0.1	0.093	0.089	0.090
10%	0.1	0.095	0.091	0.092
15%	0.1	0.043	0.039	0.041

5.1.3.4 Crude activity determination from standard maltose curve

Table 3.10 Crude activity determination from standard maltose curve

Ammonium sulfate precipitated sample with salt concentration	**Amount of Ammonium sulfate precipitated sample (ml)**	**Average O.D**	**Amount of maltose released from graph(µg/ml)**	**Crude pullulanase activity**
6%	0.1	0.090	1600	0.362
10%	0.1	0.092	1600	0.362
15%	0.1	0.041	800	0.178

5.1.3.5 Folin Lowry for ammmonium sulfate precipitated protein sample and determination of crude pullulanase activity/mg of protein

Table 3.11 Folin Lowry for ammmonium sulfate precipitated protein sample and determination of crude pullulanase activity/mg of protein

Ammonium sulfate precipitated sample of cells grown in	Average O.D	Concentration of protein in mg/ml	Crude pullulanase activity /mg of total protein
6% NaCl	0.106	2.8	0.129
10% NaCl	0.192	2.6	0.139
15% NaCl	0.026	1.2	0.148

5.1.4 Lipase screening in *Virgibacillus marismortui*

The sensitive lipase assay was carried out in order to determine whether the organism produce lipases and whether they can carry out break down of olive oil as a substrate.

It was observed that this organism cannot grow on modified HM media and hence cannot utilize lipids when present as sole carbon source.

The plates were incubated for about a week and formation of fluorescent halos was checked each day, but no growth was observed.

This confirms previous screening results which were negative for lipase.

5.2 Screening of amylase and pullulanase in *Thermus thermophilus*

The organism was tested for production of enzyme amylase and pullulanase. Similar to amylase and pullulanase screening in *V.marismortui, the* organism was grown in minimal media containing starch and pullulan as sole carbon source respectively

5.2.1 Amylase screening in *Thermus thermophilus*

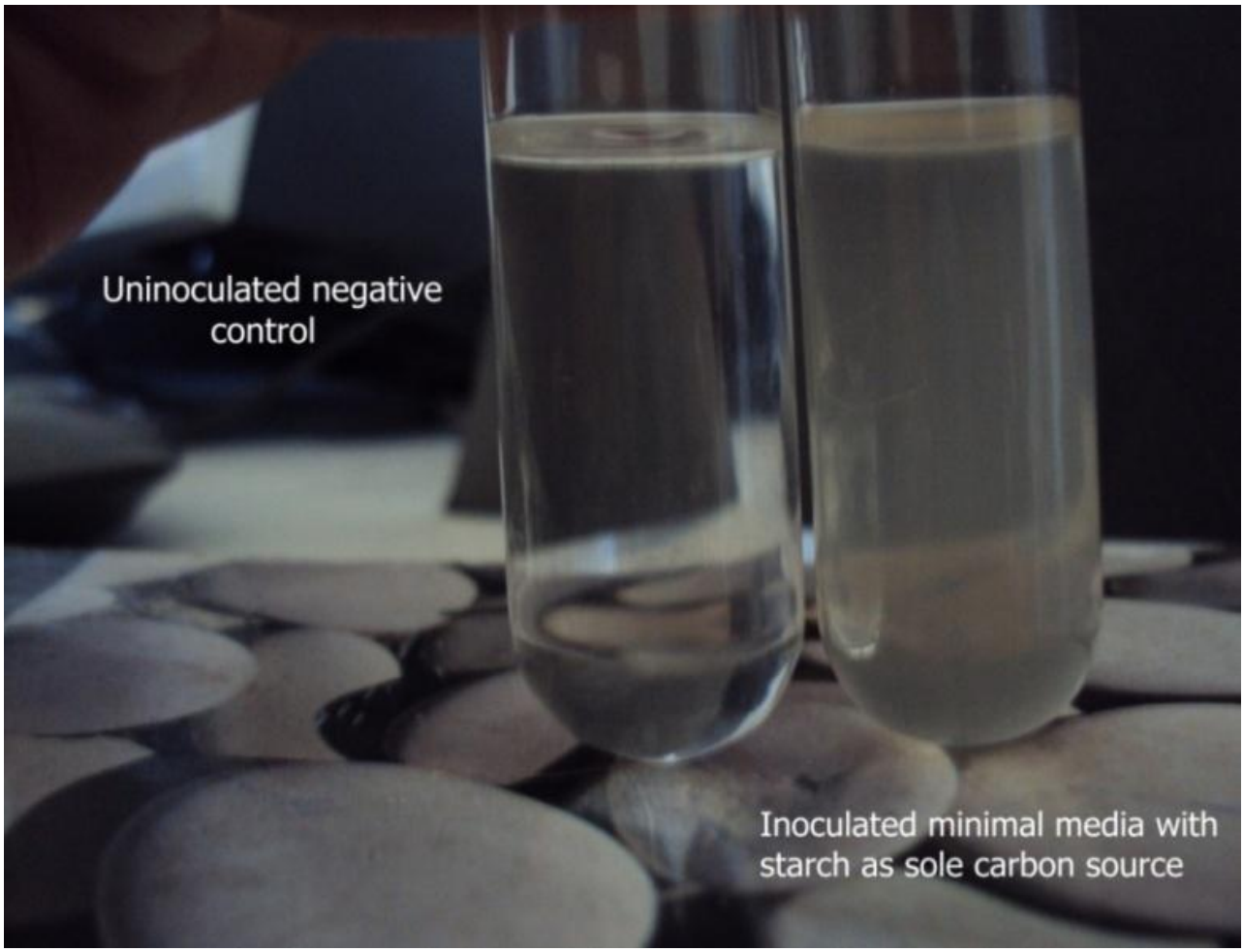


Figure: 1.15 Cells grown in minimal media with starch as sole source of along with uninoculated negative control

5.2.2 Pullulanase screening in *Thermus thermophilus*

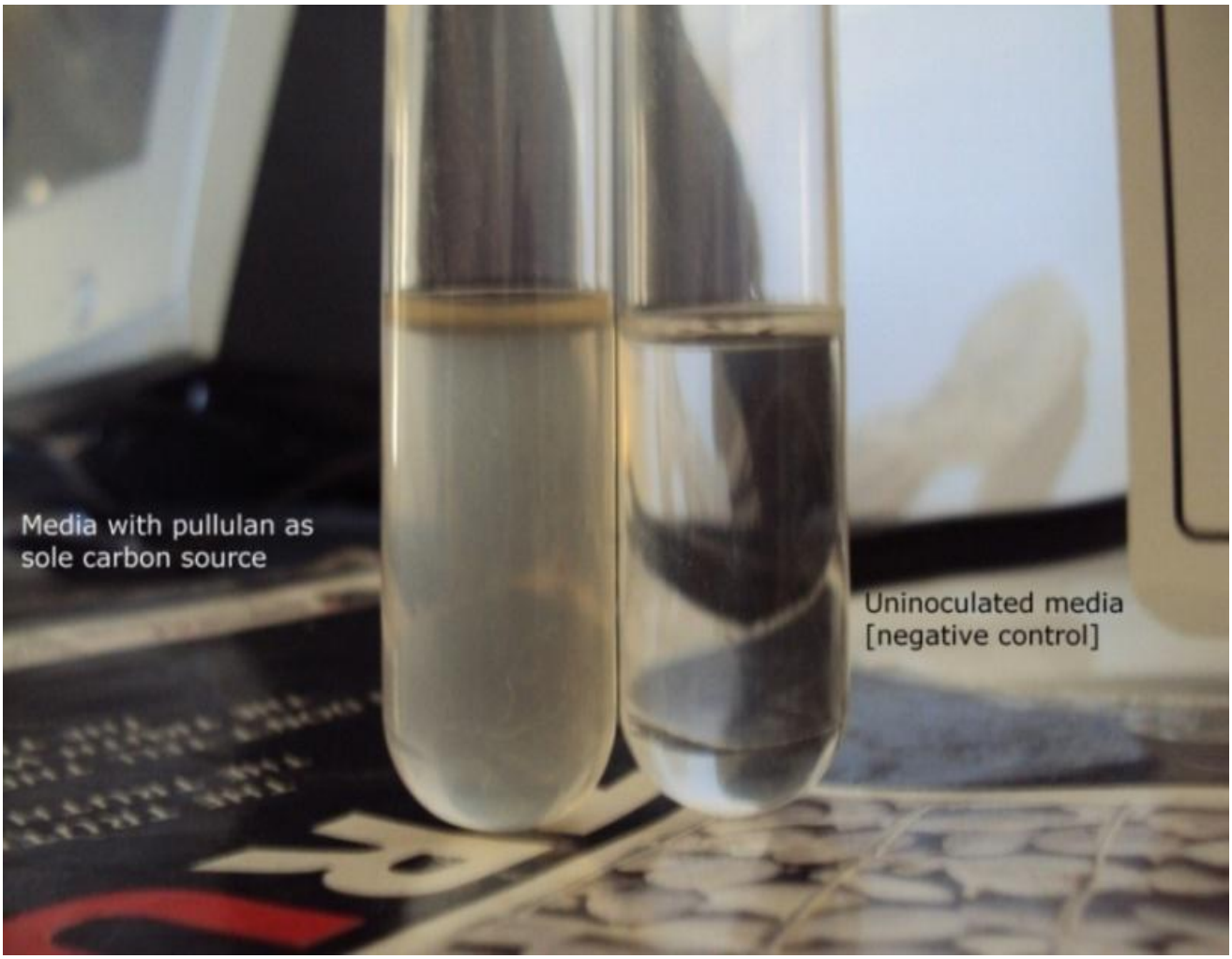


Figure: 1.16 Cells grown in minimal media with pullulan as sole source of carbon along with uninoculated negative control.

6.*Summary*

Extremophiles are of interest in both basic and applied biology. In a basic sense these organisms hold many interesting biological secrets, such as the genetic instructions for constructing macromolecules stable to one or another extreme conditions and biochemical limits to macromolecular stability as well as mechanisms by which these organism strive in such extreme conditions.

As for their application aspect, these organisms have yielded an amazing array of enzymes called *Extremozymes*, capable of catalyzing specific biochemical reactions under extreme conditions. Extreme harsh conditions such as high salinity, acidity, alkalinity and extreme temperature are also predominant conditions in a variety of industrial processes. Hence there are high demands for biocatalysts that can withstand the process conditions. Hence extremophiles have an enormous industrial application and biotechnological potential.

Examples of extremozymes include cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases, which have great potential for application in various biotechnological processes. Cloning and expression of their genes in mesophilic hosts will increase the biocatalytic applications of extremozymes.

We have carried out screening, isolation and characterization of four different extracellular enzymes of industrial importance, Amylase, Protease, Lipase and, Pullulanase from the halophilic soil isolate from Khambhat (Dr.Vijay Kothari) which has later been identified as *Virgibacillus marismortui* by Gujarat State Biotechnology Mission (GSBTM).

This organism secretes proteases which was confirmed by zone of hydrolysis in milk agar plates and maximum protease production occurs in late logarithmic and/or stationary phase. Protease activity was further confirmed by zymographs in which casein was copolymerized in acrylamide gel and clear transparent bands of preotease activity was observed

It was observed that this organism does not produce amylase in normal culture conditions since more easily hydrolysable substrates like glucose are available. However, when these

organisms are made to grow on a minimal media with starch as sole carbon source, it was observed that these organisms are able to grow by producing amylases. The extracellular amylase activity was confirmed by DNSA test and starch iodine test.

To check for pullulanase activity we grew this organism on pullulan, the substrate for pullulanase enzyme. Cells were able to grow with pullulan as the sole carbon source, indicating that they can hydrolyse pullulan and produce maltotriose which can be detected by DNSA test.

Virgibacillus marismortui can grow in salt concentrations upto 25% NaCl. So we tried to grow this organism in different salt concentrations like 6%, 10%, 15% and 25% NaCl. The protease activity in all these concentrations of salt was studied by zymography while the pullanase activty in the presence of different concentrations of salt was also examined.

7.Possible future extension

Here during the course of our work we have successfully screened organisms and identified some important extremozymes. We have also checked its stability in some stress conditions like increasing salt concentration and production of enzymes.

The possible future extension of this project will be purification of these enzymes and characterisation of enzyme activity like optimum pH, optimum temperature, enzyme kinetics (Km, Vmax.) etc

The stability of purified protein will be checked in terms of resistance towards heat and chemical denaturation

Once we have identified enzymes of higher stability in future we can work to increase stability of these enzymes to make them capable to meet the demands of the industry. This can be done by inducing mutations in these genes and checking its effect on thermal and chemical stability of these enzymes.

It is necessary to modify this organism at genetic level so that they can produce functional extremozymes under normal mesophillic conditions. This can be done if we can clone genes of these enzymes into normal bacteria like *E.coli* and make them produce desired enzyme of interest.

Such strain improvement techniques can increase the interest of industry in these enzymes and result in specific high yield of these enzymes for various purposes. It will be interesting to find out the molecular determinants of stability of these extemozymes and to compare them with their mesophillic counterparts

8. *References*

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9. *Appendix*

9.1 Standard curve for Folin Lowry estimation

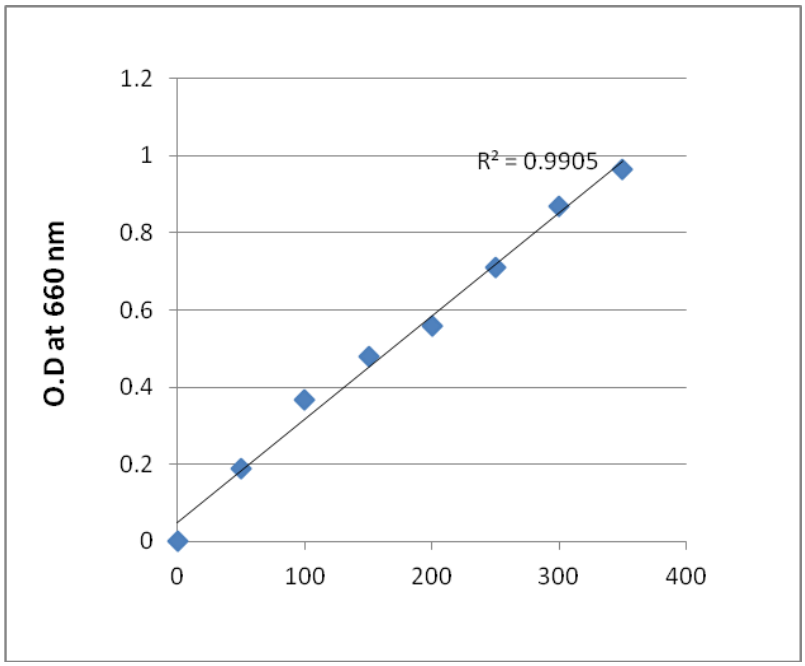

Figure 9.1 Standard curve of Folin Lowry estimation

Table 9.1 Experimental readings for Folin Lowry estimation

Concentration of standard BSA in µg/ml	O.D at 660 nm
0	0
50	0.190
100	0.368
150	0.478
200	0.560
250	0.710
300	0.869
350	0.965

9.1 Standard curve of maltose

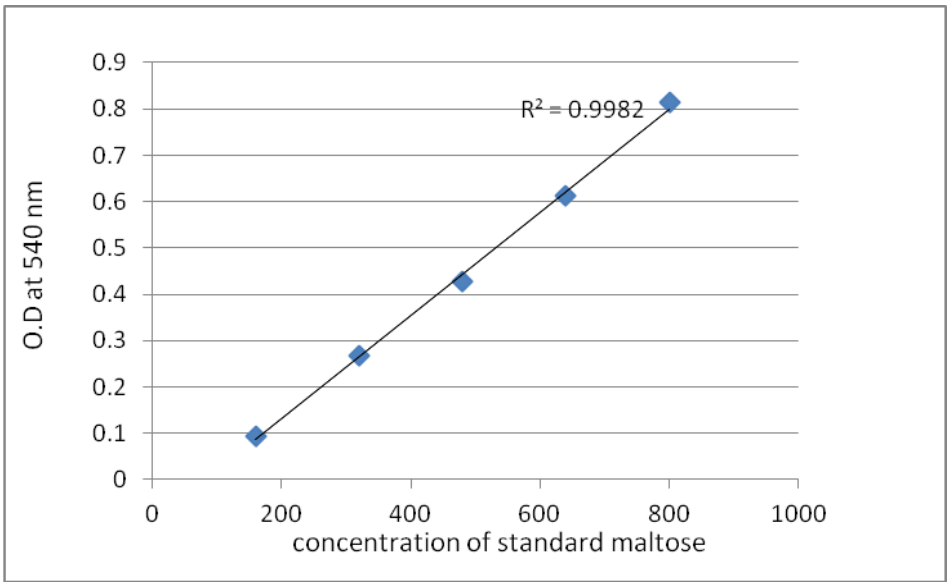

Figure 9.2 Standard curve of maltose

Table 9.1 Experimental readings for maltose

Sr. No	Concentration of standard maltose	O.D at 540 nm (Duplicates)		Average OD
		1	2	
1(blank)	-	0.0	0.0	0.0
2	160	0.096	0.091	0.0935
3	320	0.264	0.269	0.2665
4	480	0.426	0.431	0.4285
5	640	0.61	0.613	0.6115
6	800	0.812	0.816	0.814

9.3 SDS-PAGE:

Table 9.3 Composition of various % of SDS-PAGE gels

Acrylamide concentration	**7%**	**10%**	**12%**	**15%**	**20%**
Distilled water	5.02	4.02	3.35	2.35	0.69
Tris-HCl (1.5 M, pH8.8)	2.50	2.50	2.50	2.50	2.50
SDS solution (10 % w/v)	0.10	0.10	0.10	0.10	0.10
Acrylamide-bis acrylamide solution	2.33	3.33	4.0	4.995	6.66
APS solution (30 % w/v)	50µL	50µL	50µL	50µL	50µL
TEMED	5µL	5µL	5µL	5µL	5µL

2) 2X sample buffer: 0.06 M Tris-Cl, pH 6.8, 2% SDS, 10% Glycerol, 0.025% Bromophenol blue

3) 5X Electrode buffer: 0.025 M Tris, 0.192 M glycine, 0.1% w/v SDS, pH 8.3

4) 29.2 gm acrylamide and 0.2 gm N, N'–methylene-bisacrylamide add into distilled water and makeup volume to 100 ml.

8) Staining reagent: 40% Methanol, 10% Acetic Acid, 0.1% w/v Commasie Blue R-250.

9) Destaining reagent: Add 40ml methanol and 37.5 ml acetic acid into 400

ml distilled water and makeup 500 ml.

Procedure:

1. Assemble the gel sandwich plates according to the instruction in manual.
2. Prepare the resolving gel and poured between gel plates which covered three fourth portion of plate.
3. Layer about 0.5 mL isobutyl alcohol on the top of the resolving gel.
4. Stay remains for half an hour till gel get polymerize.
5. After half an hour make stacking gel and poured over the resolving gel and insert the comb for making wells.
6. Stay remains this for half an hour and after that remove comb.
7. Place plate on the Mini-PROTEAN cell.
8. Add 1X electrode buffer to the lower buffer chamber and upper buffer chamber.
9. Pipette out 15 µl bromophenol blue (tracking dye) into each well to allow visualization of the electrical front.
10. Turn on electric supply for 15 minutes at 100V for running the tracking dye.
11. After 15 minutes add samples with 1X sample buffer in each well.
12. Turn on electric supply. It will take approximately 1 hour for the tracking dye to reach the edge of the gel.
13. Turn off the power supply after complete running of sample & remove plates and separate the gel between plates.
14. Put gel into staining solution for half an hour.

15. Stained gel put into destaining solution for 3 hours on gel rocker.
16. Check the bands visibly after completely destaining.

NOTE:

Only check the pH of electrode buffer. Do not change pH with acid or base.Use gloves when handling acrylamide because it is highly neurotoxic.