Screening the organism *Virgibacillus marismortui* for Protease production

A dissertation thesis
submitted to
Nirma University
in Partial fulfillment for the Degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

Submitted by

Belim Sameer (11MBT001)

Under the guidence of

Dr. Mili Das

ACKNOWLEDGEMENT

I take this opportunity to thank **Dr Sarat Dalai**, Director, Institute of Science, Nirma University, for his untiring help and Inspirational words. I thank him for all the facilities he provided me for the completion of my project.

I would also like to express my sincere thanks and gratitude to my Guide **Dr. Mili Das** for her support and encouragement, without whom this work would not have been possible. Her valuable advice, guidance, extensive discussion about my work and constructive criticism was crucial and encouraging. No words can express my sincere and deep sense of reverence for her. I am extremely thankful to her for the scientific attitude she has installed in me which will definitely stand in my future endeavors and it was because of her that I am able to learn so much in this short period of time.

I am thankful to **Dr. Vijay Kothari**, Professor, Institute of science, for providing me the strain Virgibacillus marismortui isolated by his students. I am really thankful to him for his keen interest, valuable guidance, support, and constant encouragement and advice throughout the tenure of this study.

I would also like to express my sincere gratitude for all the faculty members , **Dr. Shalini Rajkumar, Dr. Sriram Seshadri, Dr. Sonal Bakshi, Dr. Amee Nair** and and **Dr. Nasreen Munshi** for their continuous encouragement and support.

I would also like to acknowledge the non teaching staff **Mr. Hasit Trivedi, Mr. Sachin Prajapati, Mr. Bharat Anand** and **Mr.Rajendra Patel** for extending their hands for help, for providing me with all kinds of possible help during my dissertation period.

I enjoyed the opportunity of working with my group members. I convey my special gratitude to all my group members and friends especially for their loving support during difficult times.

It would be unfair not to mention help, guidance and support of **Mr.Palak Patel, PhD scholar, Institute of science, Nirma university** during my dissertation period.

I owe thanks to my Parents, who indeed were a source of inspiration throughout my project and who taught me to stand against all odds of life and face every new challenge bravely whose love and affection strengthened me during my project work. I would also like to thank my brother for taking care of my transportation when I had to stay back to work in college.

Last but not the least, I thank Almighty Who has been very merciful and kind to me and blessed me with His heavenly showers to accomplish my work even in unfavorable conditions without losing faith in Him and myself.

Sameer Belim
11MBT001

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C. List of Abbreviations

BSA Bovine Serum Albumin
TCA Tri chloro Acetic acid
CBB Coomasie Brilliant Blue
APS –Ammonium per Sulfate
SDS Sodium Dodecyl Sulphate
SDS-PAGE Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
DNSA 3, 5 Dinitrosalycylic acid
TEMED- N, N, N′, N′-Tetramethylethylenediamine

Abstract

We have carried out isolation of extracellular industrially important enzymes Protease from haloalkaliphilic soil organism *Virgibacillus marismortui* which was isolated from saline soil of Khambhat. It was observed that *Virgibacillus marismortui* secrete proteases which was confirmed by obtaining zone of hydrolysis on milk agar plates. Further protease activity was confirmed by zymography. We have tried to grow these organisms in different salt concentrations like 15% and 25% NaCl and determined effect of salt concentration on growth and enzyme production. This organism gives higher protease in high salt condition.

Objectives

Screening the organism *Virgibacillus marismortui* for Protease production.

Identification of Protease: SDS-PAGE and Zymography.

Introduction

1. Introduction

1.1 Extremophiles

Extremophiles are organisms that have evolved to exist in a variety of extreme environmental condition. Extremophiles thrive under conditions that would kill most other creatures and many cannot survive in such extreme environments (Gomes and Steiner, 2004; Raven and Johnson, 2012; Burg, 2004). They fall into a number of different classes that include thermophiles, halophiles, acidophiles, alkalophiles, psychrophiles, and barophiles (piezophiles) and others (Fucinos *et al*, 2012). They have adapted to survive in ecological niches such as deep-sea hydrothermal vents, hot springs etc. These microorganisms produce unique biocatalysts that function under conditions in which their mesophilic counterparts could not survive, permitting the development of additional industrial processes (Demirjian *et al*.,2001).The majority of extremophiles have not yet been isolated in pure culture (Hough and Danson.,1999). Extensive studies in recent years have shown that a variety of enzymes isolated from extremophiles are inherently more stable than those from mesophilic sources (Hasegawa *et al*.,1976).

Purification of extremozymes assumes significance in understanding cellular metabolism and regulatory pathways.The halophiles extremozymes exhibit unique structural and biochemical characteristics. The intriguing stabilities of these enzymes under extreme high saline conditions are still unknown. It is speculated that this could be due to the presence of a relatively large number of negatively charged amino acid residues on their surface to prevent precipitation. However, hydrolytic enzymes from halophiles are not only interesting from the basic scientific viewpoint but, they may also be of potential interest in many industrial and biotechnological applications, owing to their stability and activity at low water levels (Sehar and Hameed, 2011).

1.2 Halophile

Halotolerant and halophilic microorganisms, as the name suggests, grow in environments with high salinity concentrations. Halophiles can be categorized as slight, moderate or extreme, by the extent of their halotolerance.. 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).

1.3 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:

1. alkaliphiles and 2.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 %).

1.4 Thermophiles (Life at high temperatures)

Organism grow in high tempereture. Thermophilic organisms produce proteins of extreme stability and they withstand up to the temperature of 120 ºC (Michael and Xavier.,2008). 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).

2. Review of literature

2.1 Micro-organism under study:-

Table 2.1

Organism	Gram charecteristic	Optimal tempreture	Optimal salt concentration
Virgibacillus marismortui.	Gram positive	35 ºC	6 % (can grow upto 25 %)

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 (Ngoc-Phuc *et al*.,2008).

3. Cannot grow in absence of salts .

4. Have optimum salt range-6-10% but can tolerate up to 25% (Tonima *et al*.,2011).

5. Colony characteristics- slightly transparent to opaque, non-pigmented, yellowish white, matt like, smooth, glossy type colonies .

6. Can hydrolyze casein easily.

2.2 Extremozymes in industry:

Microorganisms retrieved from extremely hot, cold 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 *et al.*,2004). *Virgibacilli* secrete large numbers of extracellular proteases (Rajeswari and khare.,2012).

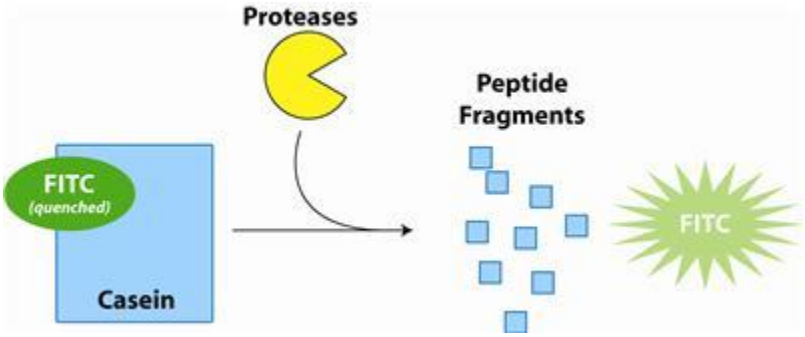
Currently, only 1–2 % of the microorganisms on the earth have been commercially exploited and amongst these there are only a few examples of extremophiles. However, the growing interest that is currently emerging as a result of new developments in the cultivation and production of extremophiles and success in the cloning and expression of their genes in mesophilic hosts is increasing the biocatalytic applications of extremozymes (Cavicchioli and Thomas, 2000*)* Various kinds of thermostable enzymes are required by industries. .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.

2.3 Enzyme under study:-

Proteases:-

Proteolytic enzyme is a class of proteins found in various sources, including animals, plants and microorganisms because of its ubiquitous nature (Aehle.,2004).

Figure 2.1



Alkaline protease is widely used in many industries (verma *et al*.,2011). *Bacillus subtilis* and the related species have been extensively studied for many years (Rao and Bryan.,2000). Recently, alkaline proteases from various strains of *Bacillus* were characterized with unique biochemical characteristics, such as higher thermostability and catalytic efficiency (Jaouadi *et al*.,2008).

Proteases are enzymes, which are able to hydrolyze the peptide bond of proteins (Lima *et al*.,2011). Proteases are naturally produced by microorganisms mainly to degrade large polypeptides in the medium into peptides and amino acids before cellular uptake (Parawira and Zvauya.,2012). They are a highly complex group of enzymes that differ in their substrate specificity, catalytic mechanism and active site (Banerjee and Negi.,2009). Protease also have application in silver recovery from photographic plates and in peptide synthesis which account for about 60% of total industrial enzyme sales (Tambekar and Tambekar.,2012)..The demand of enzymes have considerably increased in different industries such as food, pharmaceutical, leather, paper and silk (Rao *et al*.,2010).Alkaline proteases hold a major share of the enzyme market with twothird share in detergent industry alone (Anwar and saleemuddin.,2000).For effective use in industries, alkaline proteases need to be stable and active at high temperature and pH and in the presence of surfactants, oxidizing agents, and organic solvents (Dutta and chaudhri .,2011). Microbial proteases are utilized extensively in various fields because of their rapid growth and required less space for cultivation (Mahadevan *et al*.,2010).Most of the commercial alkaline proteases are of bacterial origin (Shafee *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 (Visessanguan, 2011; Namwong *et al*, 2005).

Materials & Methods

3.1 Materials:

Casein Peptone and Yeast extract were from Himedia, Ammonium sulfate was from Merck. , Glucose, Agar-Agar powder, Trichloroacetic acid, Tris HCl buffer were from SRL. HCl was from s d fine chemicals.

3.2 Growth medium for *Virgibacillus marismortui*

Table 3.1

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.

3.3 First Approch for screening of proteases.

Table 3.2

Components	**Amount /100 ml**
Casein	1 g
NaCl	25 g
Casein peptone	1 g
Glucose	0.5 g
Yeast extract	0.5 g
Distilled water	Make up 100 ml

The pH was adjusted the to 9.0. Casein in above media had denatured by forming clumps. So we modified media by using second approch.

3.4 Second Approch.

Table 3.3

Components	**Amount /100 ml**
Skimmed milk	5 ml
NaCl	25 g
Casein peptone	1 g
Glucose	0.5 g
Yeast extract	0.5 g
Distilled water	Make up 100 ml

The pH was adjusted the to 9.0.Here skimmed milk was used as sourse of casein. The skimmed Milk was filtered by whatman paper and autoclaved separately. After autoclaving milk is added to media.

Here glucose is used as primary carbon sourse. So it did not easily utilize casein from skimmed milk.

So we modified media by using third approch.

3.5 Third Approch.

Providing specific substrate of enzyme to be screened along with the normal growth media such as (skimmed milk) Casein for protease.

Media composition for protease having skimmed milk as source of casein

Table 3.4

Components	Amount /100 ml
Skimmed milk	5ml
NaCl	25 g
Yeast extract	0.5g

Make up 100 ml by Distilled water. The pH was adjusted the to 9.0 with NaOH .

3.6 Ammonium sulfate precipitation:

Ammonium Sulfate precipitation is a method used to purify proteins by altering their solubility (Kunamneni et al.,2003). It is a specific case of a more general technique known as salting out. The cell-free extracts were fractionated by salting out with solid ammonium sulfate at 100% (w/v) saturation (Guoyu *et al.*,2012).

Ammonium sulfate is commonly used to concenrate protein.

Solid ammonium sulfate was added slowly 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 (Huang *et al.*,2006). The precipitated solution was then centrifuged at 10,000 rpm for 30 minutes (Yan-xia Y *et al*.,2013).

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 pH 9.0. The ammonium sulfate is removed from protein by dialysis.

3.7 Dialysis:

It is necessary to remove the ammonium sulfate before we can proceed to purification process. The simplest way to achieve this is to dialyse the solution. The 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.

Dialyzing buffer should be of same strength and pH in which the protein is resuspended and process must be carried out at 4 ˚C.

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. 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).

3.8 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 20 ml broth from working plate. Inoculated flasks were then kept in shaker incubator at 35˚C and were allowed to grow.

3.9 Zymography (protease activity detection on gel):

15 % Zymograph was run to see protease activity for all samples. Along with the zymograph simultaneously 15% SDS was also run to determine approximate molecular weight of protease. For SDS PAGE, sample was TCA precipitated so as to load more amount of sample.

3.10 Protocol for TCA precipitation

1. Sample was taken in a microfuge tube and 1/10 volume of TCA was added to it and kept on ice for 1 hr.

2 . After 1 hour precipitate formed was centrifuged at 10,000 rpm for 4 minutes.

3. Pellet was resuspended in 10µl gel loading dye and boiled it for 10 minutes.

4. Sample was then loaded into the gel.

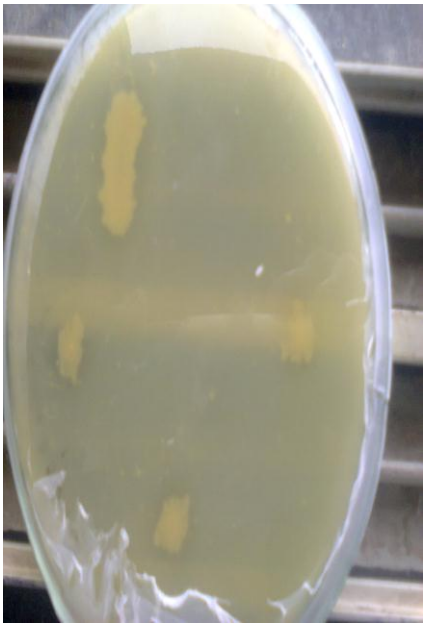
3.11 Protocol for Zymography

For Zymography casein was used as substrate. 15% SDS PAGE containing 0.1% casein were prepared. 0.1% casein was first prepared in resolving gel buffer (1.5mM Tris-HCl pH 8.8). The gel was cast normally as for SDS PAGE. Samples were not boiled but incubated at 37˚C; Electrophoresis was performed as in normal SDS PAGE. After completion of electrophoresis the gel was removed and was soaked in a 2.5% w/v Triton X 100 for one hour. The gel was then washed with distilled water two to three times and incubated with the refolding buffer overnight at 37˚C. Next day gel was washed with distilled water and was finally stained with Coomassie BrilliantBlueand destained to view the bands.

Results & Discussion

4.1 Screening of organism *Virgibacillus marismortui* for protease.

Various media for screening of organism were used in order to know whether the organism produces these enzymes or not. We modified the media by using skimmed milk as a source of casein in place of normal casein. After 48 hours of incubation, clear zone of hydrolysis was obtained.



15 % NaCl concentration

25 % NaCl concentration

Figure 4.1 : Plates showing clear zone of casein hydrolysis on milk agar plates due to protease activity .

4.2 **Gradient gel Elecrtophoresis**

Lane 1 2 3 4 5 6 7 8 9 10

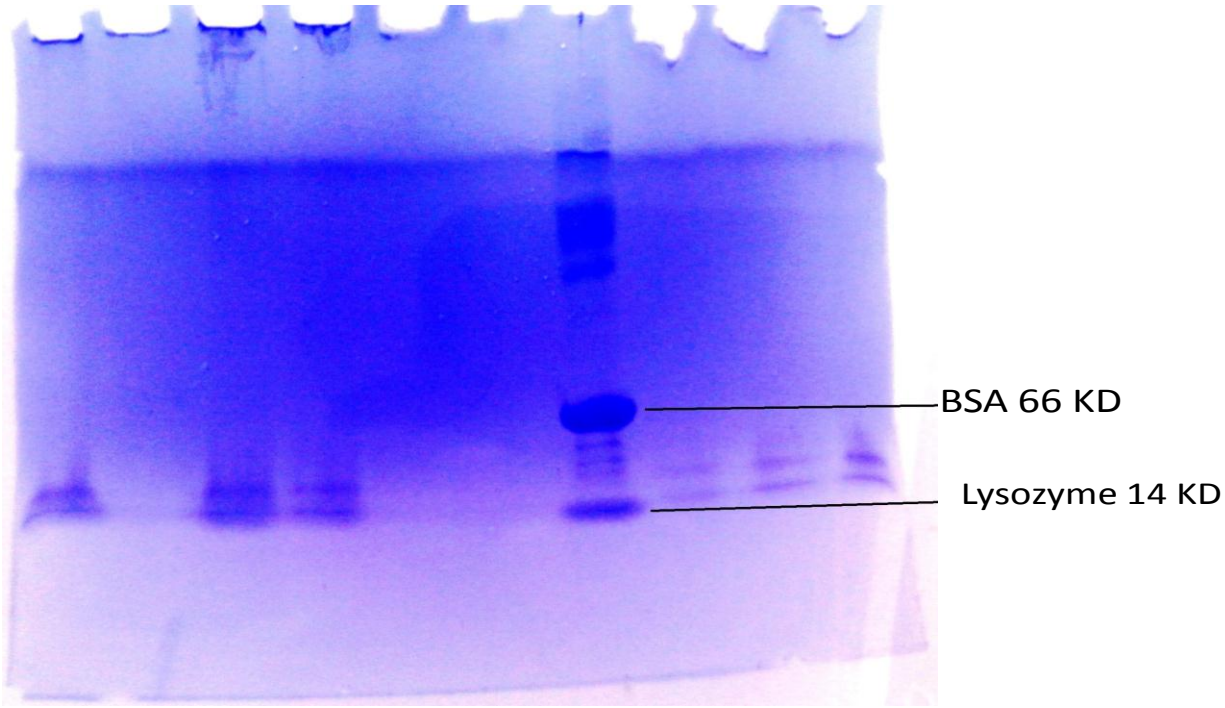


Figure 4.2 :Gradient SDS-PAGE from 5 % to 25 %. TCA precipiteted samples of 25 % NaCl concentration containing sample were loaded in Lane 1 to Lane 6 and in Lane 8 to Lane 10. Lane 7 contains BSA+lysozyme as molecular weight markers.

4.3 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 HCL buffer Ph 9.0. Then in order to confirm that this organism secretes extracellular protease zymogram was done.

At first we conducted zymogram in 15% acrylamide concentration and samples taken were . Ammonium sulfate precipitated 15 % NaCl and 25% NaCl sample. Maximum transparent bands were seen in bottom of the gel.

Lane 1 Lane 2 Lane 3 Lane 4 Lane 5 Lane 6 Lane 7 Lane 8 Lane 9 Lane 10

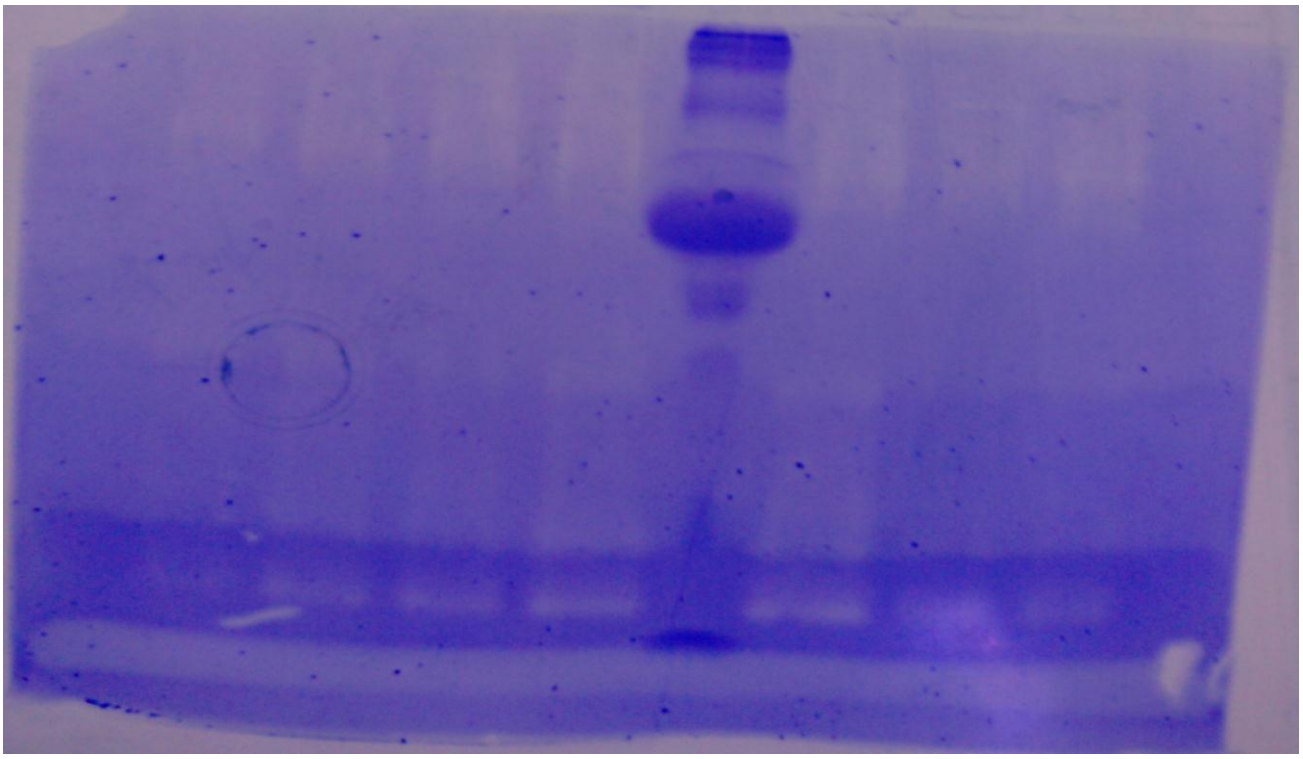


Figure 4.3 : Zymography of dialysed samples of *Virgibacillus marismortui* on 15 % acrylamide gel. Transparent bands seen due to hydrolysis of casein by proteases.

Sample was loaded in the well with loading buffer were

25 % NaCl concentration containing sample (Lane 2 : 5 µL Sample, Lane 3 : 10 µL Sample, Lane 4 : 15 µL Sample, Lane 5 :20 µL Sample, Lane 6 **) BSA** and **Lysozyme** and **15 % NaCl concentration containing sample** (Lane 7 to Lane 8 :20 µL Sample).

4.4 Discussion and Conclusion

Virgibacillus marismortui was grown on media containing different salt concentration i.e. 15% and 25% NaCl. It was observed that when grown in higher salt concentration the time required to achieve full growth is more. Alkaline protease production mainly depends on the availability of both carbon and nitrogen sources in the medium (Shafee *et al*.,2005). The enhancement of protease production by organic nitrogen sources like tryptone, peptone, yeast extract, skim milk and soybean meal was reported (Laxman *et al*.,2005).

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 .

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Future extension

Here we have successfully identified proteases.

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 stability towards heat and chemical denaturation . In future we can work to increase stability of 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 will be interesting to find out the molecular determinants of stability of these extemozymes and to compare them with their mesophillic counterparts.

5. Appendix

- 25 mM Tris-HCL : 3.0285 g Tris-base dissolve in 1 litre of distilled water.

- 2X sample buffer: 0.06 M Tris-Cl, pH 6.8, 2% SDS, 10% Glycerol, 0.025% Bromophenol blue
- 5X Electrode buffer: 0.025 M Tris, 0.192 M glycine, 0.1% w/v SDS, pH 8.3
- 29.2 gm acrylamide and 0.2 gm N, N'–methylene-bisacrylamide add into distilled water and makeup volume to 100 ml.
- Staining reagent: 40% Methanol, 10% Acetic Acid, 0.1% w/v Commasie Blue R-250.
- Destaining reagent: Add 40ml methanol and 37.5 ml acetic acid into 400 ml Distilled water and make up 500 ml.
- Various concentration of resolving gel

Acrylamide Concentration	7 %	10 %	12 %	15 %	20 %
Tris-Hcl pH 8.8	2.50 ml	2.50 ml	2.50 ml	2.50 ml	2.50 ml
Distilled water	5.02 ml	4.02 ml	3.35 ml	2.35 ml	0.69 ml
SDS(10%w/v)	0.10 ml	0.10 ml	0.10 ml	0.10 ml	0.10 ml
Acrylamide(30%w/v)	2.33 ml	3.33 ml	4.0 ml	4.995 ml	6.66 ml
sAPS solution (30 % w/v)	50 µl	50 µl	50 µl	50 µl	50 µl
TEMED	5 µl	5 µl	5 µl	5 µl	5 µl

Procedure for SDS-PAGE.

Procedure:

1. Assemble the gel sandwich plates according to the instruction in manual.

2. Prepare the resolving gel and poured between gel plates which coveredthree 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 and Check the bands visibly after completely destaining.

6. References.

Aehle, W. Enzymes in Industry: Production and Application; Wiley-VCH: New York, NY, USA, 2004.

Anwar A and Saleemuddin M, "Alkaline protease from Spilosoma obliqua: potential applications in bio-formulations," Biotechnology and Applied Biochemistry, vol. 31, no. 2, pp. 85–89, 2000.

Banerjee R, Negi S. Characterization of amylase and protease produced by Aspergillus awamori in single bioreactor. Food Res Int; 42, 443-8.2009.

Bhunia B, Dutta D, and Chaudhuri S, Extracellular alkaline protease from licheniformis NCIM-2042: improving enzyme Activity Assay and characterization,BacillusEngineering in Life Sciences, 11:2,207–215.2011.

Bryan PN. Protein engineering of subtilisin. Biochim. Biophys. Acta 1543:203–222. 2000.

Burg V, Extremophiles as a source for novel enzymes. Curr. Opin. Microbiol. , 6, 213–218.,2003

David C Demirjian, Francisco Morís-Varas and Constance S Cassidy. Enzymes from extremophiles. Current Opinion in Chemical Biology , 5:144–151.2001.

David W Hough and Michael J Danson. Extremozymes. Current Opinion in Chemical Biology , 3:39–46.1999.

Extremophilic Bacteria and Microbial Diversity(ed.), Raven and Johnson's biology, 6th edition, pp:1-12.,2012.

Fucinos P, Gonzalez R, Atanes E, Sestelo AB, Perez-Guerra N, Pastrana L, Rua ML, Lipases and esterases from extremophiles: overview and case example of the production and purification of an esterase from Thermus thermophilus HB27.Methods Mol Bio ;861:239-66.2012.

Ghorbel B, Sellami-Kamoun A, Nasri M. Stability studies of protease from *Bacillus cereus* BG1. *Enzyme Microb Technol* ; 32: 513-8.2003.

Gomes J and Steiner W: Extremophiles and Extremozymes, Food Technol. Biotechnol. 42 (4) 223–235 . Chemical characterization of the recombinant enzyme, Microbiology, 148 2343–2349.2004.

Haki G and Rakshit S, "Developments in industrially important thermostable enzymes: a review," Bioresource Technology, vol. 89, no. 1, pp. 17–34, 2003.

Hasegawa A., Miwa N., Oshima T. and Kazutomo T. Studies on α-Amylase from a Thermophilic Bacterium: Purification and Characterization. J. Biochem., 79:35-42.1976.

Han X-Q, Damodaran S. Isolation, identification, and fermentation of a *Bacillus* species producing a detergent-stable endopeptidase. J. Agric. Food Chem. 45:4191– 4195. 1997.

Huang G, Ying T, Huo P and Jiang J .Purification and characterization of a protease from *Thermophilic bacillus* strain HS08. African Journal of Biotechnology Vol. 5 (24), pp. 2433-2438, 18 December 2006.

Jaouadi B, Ellouz-Chaaboum S, Rhimi M, Bejar S. 2008. Biochemical and molecular characterization of a detergent-stable serine alkaline protease from *Bacillus pumilus* CBS with high catalytic efficiency. Biochimie 90:1291–1305.

Kazumi H,Yasushi N,S Namwong, (2005); Purification and characterization of serine proteinase from halophillic bacterium ilobacillus,sp.RF2,Biosci.Biotechnol.Biochem,69(1),38-44,2005.

Kumar CG. . Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. Lett. Appl. Microbiol.34:13–17.2002.

Kunamneni A,Poluri E, and Davuluri Siva Prasad.Purification and Partial Characterization of Thermostable Serine Alkaline Protease from a Newly Isolated *Bacillus subtilis PE-11* . AAPS PharmSciTech 2003; 4 (4) Article 56. August 19,2003.

Laxman RS, Sonawane AP, More SV, Rao BS, Rele MV, Jogdand VV, *et al*. Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus. Process Biochem* 2005.

Lima AC, Lima Filho JL, Neto BB, Converti A, Carneiro da Cunha MG, Porto ALF. Production and characterization of a collagenolytic serine proteinase by Penicillium aurantiogriseum URM 4622: a factorial study. Biotechnol Bioprocess Eng 2011; 16: 549-60.

Mahadevan S, Dhandapani B, Sivaprakasam S, Mandal AB Batch kinetic studies on growth of salt tolerant Pseudomonas aeruginosa secreting protease in a biocalorimeter. Biotechnol Bioprocess Eng ; 15: 670-5.2010

Michael G and M. Xavier Suresh,.Discrimination of mesophilic and thermophilic proteins using machine learning algorithms. Computational Biology Research Center (CBRC), National Institute of Advanced Industrial Science and Technology (AIST), 2-42 Aomi, Koto-ku, Tokyo 135-0064,2008.

Mona K Gauda. Optimization And Purification of Alkaline Proteases Produced by Marine Bacillus sp.MIG Newly Isolated from Estern Harbour of Alexandria. *Polish J Of Microbiology* Vol.55 No 2,119-126. 2006

Ngoc-Phuc Hua, Amel Hamza-Chaffai, Russell H. Vreeland, Hiroko Isoda and Takeshi Naganuma Virgibacillus salarius sp. nov., a halophilic bacterium isolated from a Saharan salt lake. International Journal of Systematic and Evolutionary Microbiology , 58, 2409–2414.2008.

Patel R, Dodia M, Singh SP. Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: production and optimization. *Process Biochem*; 40: 3569-75. 2005.
Parawira W and Zvauya R.,Production and characterisation of protease enzyme produced by a novel moderate thermophilic bacterium (EP1001) isolated from an alkaline hot spring, Zimbabwe. African Journal of Microbiology Research Vol. 6(27), 5542-5551.2012.

Rahman R, Mahamad S, Salleh AB, Basri M. A new organic solvent tolerant protease from *Bacillus pumilus*. J. Ind. Microbiol. Biotechnol.34:509 –517.2007.

Rajeswari s and khair.Isolation of halophilic *Virgibacillus* sp EMB13; Characterization of its protease for detergent application.Indian journal of biotechnology vol.11.pp 416-426.2012.

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol.
Rev. 62:597– 635. 1998.

Rao CS, Sathish T, Ravichandra P, Prakasham RS. Characterization of thermo- and detergent application of a laundry detergent and organic solvent stable subtilisin-like serine protease (Alzwiprase) from Bacillus subtilis DM-04. Biochem Engg J; 48, 173-80. 2010.

Rehman RN, Geok LP, Basri M, Salleh AB. Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. *Bioresour Technol* 2005; 96: 429-36.

Shafee N, Aris SN, Rehman RN, Basri M, Salleh AB. Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium Bacillus cereus strain 146. J Appl Sci Res; 1: 1-8.2005.

Shama Sehar and Abdul Hameed. Extracellular Alkaline Protease by a Newly Isolated Halophilic Bacillus sp. Global Journal of Biotechnology & Biochemistry 6 (3): 142-148, 2011.

Sumantha, A.; Larroche, C.; Pandey, A. Microbiology and industrial biotechnology of food-grade proteases: A perspective. Food Technol. Biotechnol., 44, 211–220. 2006.

Tambekar S.D. and Tambekar D.H. .Physicochemical properties and production dynamics of alkaline protease of Bacillus odysseyi. Int J Adv Pharm Biol Sci Vol.2, Issue 2, 161-170.2012.

Tanasupawat S, Taprig T, Akaracharanya A,and VisessanguanW , Characterization of Virgibacillus strain TKNR13-3 from fermented shrimp paste (ka-pi) and its protease production , African Journal of Microbiology Research 5(26): pp. 4714-4721.,2011.

Tonima K. Kamat , Shashi K, and Savita K. Antimicrobial potential of *Bacillus marismortui*, A salt pan isolate of cavellosim,Goa,India. International Journal of Advanced Biotechnology and Research ISSN 0976-2612, Vol 2, Issue 3, pp 321-328,2011.

Verma J, Modi DR, Sharma R, Saxena S. 2011. Vital role of alkaline protease in bio-industries: a review. Plant Arch. 11:1083–1092.

Wan M-Y, Wang H-Y, Zhang Y-Z, Feng H.. Substrate specificity and thermostability of the dehairing alkaline protease from *Bacillus pumilus*. Appl. Biochem. Biotechnol. 159:394–403.2009.

Yan-xia Y, Li-zhi N and Shao-nan L.Purification and studies on characteristics of cholinesterases from Daphnia magna. Journal of Zhejiang University. ISSN 1673-1581.mar 2013.