PURIFICATION AND CHARACTERIZATION OF HtrA PROTEIN FROM *Lactobacillus acidophilus*

111567

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May – 2015

Submitted in partial fulfillment of the Degree of

Bachelor of Technology

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY-WAKNAGHAT

(H.P)

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CERTIFICATE

This is to certify that the work titled "**Purification and characterization of HtrA protein from *Lactobacillus acidophilus***" submitted by "**Ms. Poonam Singh**" in partial fulfilment for the award of degree of B. Tech. of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor

Name of Supervisor: Dr. Saurabh Bansal

Designation: Assistant Professor

Date: 25.05.2015

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DECLARATION

I hereby declare that the work reported in the B. Tech thesis entitled "**Purification and characterization of HtrA protein from *Lactobacillus acidophilus***" submitted by "**Ms. Poonam Singh**" at Jaypee University of Information Technology, Waknaghat is an authentic record of our work carried out under the supervision of Mr. Saurabh Bansal. This work has not been submitted partially or wholly to any other university or institution for the award of this or any other degree or diploma.

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ACKNOWLEDGEMENT

I would like to give special thanks to **Dr. R.S Chauhan,** the Dean and Head, Department of Biotechnology and Bioinformatics for providing facilities for carrying out this project work which successfully without any hindrance.

I also like to express my gratitude and sincere thanks to respected research guide **Dr. Saurabh Bansal** Assistant Professor, Department of Biotechnology and Bioinformatics Jaypee University Of Information and Technology, Waknaghat for his constant and valuable guidance, encouragement and support which enabled me to do this project with full confidence.

I would like to express deep gratitude to **Ms.Ritika Verma**, Research scholar, who extended helping hand, valuable encouragement, suggestions and advices throughout the project work. Their timely assistance has made this project a success.

Last but not the least many thanks research scholars for their immense help.

Signature of the student

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ABSTRACT

HtrA is a heat shock-induced serine protease. Homologues of HtrA have been described in a wide range of bacteria and eukaryotes. Its role is to degrade misfolded proteins in the periplasm. As a preliminary step is the understanding of the function of the HtrA protein. Misfolding or unfolding of polypeptides can occur as a consequence of environmental stress and spontaneous mutation. HtrA surface protease in gram-positive bacteria is involved in the processing and maturation of extracellular proteins and degradation of abnormal or misfolded proteins. Inactivation of htrA has been shown to affect the tolerance to thermal and environmental stress and to reduce virulence.The abundance of general chaperones and proteases suggests that cells distinguish between proteins that can be refolded and "hopeless" cases fated to enter the proteolytic pathway. The heat shock protein (HtrA) act as both molecular chaperone and proteolytic activities. The chaperone function dominates at low temperatures, and the proteolytic activity at elevated temperatures. The protease and chaperone activities of HtrA eliminate or refold damaged and unfolded proteins in the bacterial periplasm that are generated upon stress conditions. Function is to protect cells from the deleterious effects of various stress conditions. At temperatures below 28 °C the proteolytic activity of HtrA regarded as negligible and it was believed that the protein mainly plays the role of a chaperone. Substrate recognition probably involves the recently described PDZ domains in the C-terminal half of HtrA. Cells precisely monitor the concentration and functionality of each protein for optimal performance. Protein quality control involves molecular chaperones, folding catalysts, and proteases that are often heat shock proteins. One quality control factor is HtrA, class of oligomeric serine proteases. The defining feature of the HtrA family is the combination of a catalytic domain with at least one C-terminal PDZ domain.

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OBJECTIVE

- Optimization of production of HtrA protein from *Lactobacillus acidophilus.*
- Purification.
- Characterization.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

High temperature requirement A (HtrA) proteins are ubiquitously expressed PDZ-proteases that are critical to maintain protein homeostasis in extracytoplasmic compartments. In contrast to other quality control factors, HtrA proteases are capable to function as chaperone stabilizing specific client proteins. HtrA proteins combine the dual activities of a protease and a chaperone on a single polypeptide, they represent a unique model system to monitor the partitioning of damaged proteins between digestive and refolding pathways. With regards to their structures, HtrA proteins associate via their protease domains to form trimers, the building block of all HtrA oligomers. The projecting PDZ domains either participate in protein degradation presenting substrates to the protease or mediate the binding of allosteric activators that stimulate protease function (Figure 1). The HtrA housekeeping proteases are implicated in important cellular processes such as bacterial virulence, photosynthesis, organization of the extracellular matrix, cell proliferation and ageing. The loss of mammalian HtrA activity is connected with severe diseases, including arthritis, cancer, familial ischemic cerebral small-vessel disease and age-related macular degeneration, as well as Parkinson's disease and Alzheimer's disease.

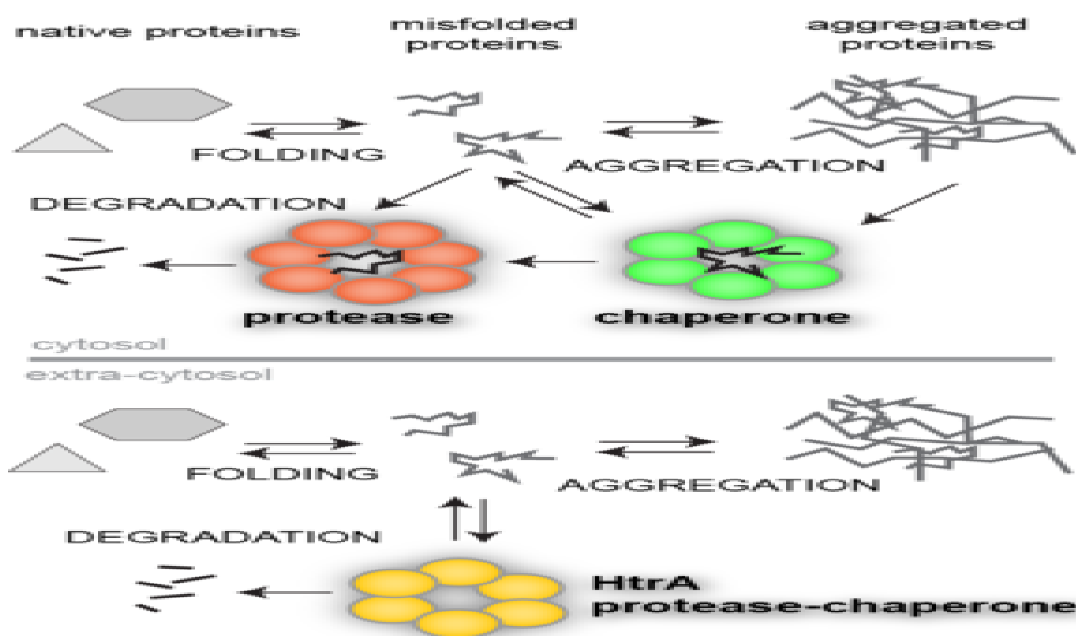

Figure 1: HtrA proteins combine the dual activities of a protease and a chaperone on a single polypeptide.

The High temperature requirement A (HtrA) and its homologues constitute the HtrA familiy proteins, a group of heat shock-induced serine proteases. Bacterial HtrA proteins perform crucial functions, protein quality control in the periplasmic space, functioning as both molecular chaperones and proteases. It exhibits a PDZ domain and a temperature-dependent switch mechanism, which effects the change in its function from molecular chaperone to protease. This mechanism also related to substrate recognition and the fine control of its function. Structural and biochemical analyses of the three HtrA proteins, DegP, DegQ, and DegS, have provided as to the functional regulation of HtrA proteins, as well as their roles in protein quality control at atomic scales.

After proteins synthesized in the cells, these proteins are exposed to a variety of environmental stresses, including heat, chemicals, pathogens, or immune system activities, which can inflict damage to proteins. Damaged proteins, tend to accumulate or aggregate, due to the loss of their proper three-dimensional folding characteristics, as well as their normal activities. In these cases, it is necessary to remove these proteins, or return them to a functional state, as aggregated proteins can vitiate cell viability. This protein quality control is essential for cell survival in all organisms.

1.2 The Role of PDZ Domains

PDZ domains are known to regulate protein-protein interactions, acting via the carboxylate-binding loop. Thus far, however, only three bacterial proteins, HtrA, Tsp (tail-specific protein), and PDZ domains are able to recognize the C-teminal regions of substrates, on the basis of their carboxylate-binding loops. Structural studies of DegP and DegS have determined, their PDZ domains perform different functions. The PDZ domain of DegS operates as a switch for a cellular stress response through the recognition of the C-terminal peptide of OMP, a stress signal . In DegP, two PDZ domains function as proteolytic activity regulators, which restrict substrate access, functioning as gatekeepers of the proteolytic chamber . However, it appears that the second PDZ domain of Tm HtrA is unrelated to the regulation of activity, but involved in dimerization, as the isolated second PDZ domains retain the ability to form stable dimmers . This functional divergence expected, due to the fact that the sequences of the second PDZ domains are not well conserved, unlike those of the protease domain or the first PDZ domain. In the currently-determined crystal structures, the catalytic triads of the HtrA family proteins appear disordered, which is consistent with several previous findings, that the protease domain of E. coli DegP exhibits no protease activity, and that the protease domain of T. maritima HtrA exhibits only a very low level of activity. Therefore, the PDZ domains of the HtrA family may be a prerequisite for proteolyticactivity, due to their

regulation of the rearrangement of the catalytic triad, as well as their substrate access-limiting properties.

1.3 HtrA proteins as targets in therapy of cancer and other diseases

HtrA family proteins are serine proteases that are involved in important physiological processes, including maintenance of mitochondrial homeostasis, apoptosis and cell signaling. These proteins are involved in the development and progression of several pathological processes such as cancer, neurodegenerative disorders and arthritic diseases**.** The HtrA functions/regulations and involvement in diseases (cancer, neurodegenerative disorders, arthritis), and modulation of their proteolytic activity could be used in therapies. HtrA2 is the best target for cancer drug development. Increase in the HtrAs' proteolytic activity could be beneficial in cancer treatment, by stimulation of apoptosis, anoikis or necrosis of cancer cells, or by modulation of the TGF-beta signaling cascade; modulation of HtrA activity could be helpful in therapy of neurodegenerative diseases and arthritis.

1.4 Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response

Human HtrA2 is a novel member of the HtrA serine protease family and it shows extensive homology to the *Escherichia coli* HtrA genes, essential for bacterial survival at high temperatures. HumHtrA2 is also homologous to human HtrA1, also known as L56/HtrA, that is differentially expressed in human osteoarthritic cartilage and after SV40 transformation of human fibroblasts. HumHtrA2 upregulated in mammalian cells in response to stress induced by both tunicamycin treatment and heat shock . Biochemical characterization of humHtrA2 shows predominantly a nuclear protease which undergoes autoproteolysis. This proteolysis is terminate when the predicted active site serine residue is altered to alanine by site-directed mutagenesis. In human cell lines, it is present as two polypeptides of 38 and 40 kDa. HumHtrA2 ,it cleaves β-casein with an inhibitor profile similar to that previously described for *E. coli* HtrA, in addition to an increase in β-casein turnover when the assay temperature is raised from 37 to 45 °C. The biochemical and sequence similarities between humHtrA2 and its bacterial homologues, in conjunction with its nuclear location and upregulation in response to heat shock and tunicamycin suggest that it is involved in mammalian stress response pathways.

1.5 PDZ domains determine the native oligomeric structure of the DegP (HtrA) protease

DegP (HtrA),it is a periplasmic heat shock serine protease of *Escherichia coli* that degrades misfolded proteins at high temperatures. Biochemical and biophysical experiments indicated that the purified DegP exists as a hexamer. To examine whether the PDZ domains of DegP were required for oligomerization, we constructed a DegP variant lacking both PDZ domains. This truncated variant, DegPΔ, exhibited no proteolytic activity but exerted a dominant-negative effect on growth at high temperatures by interfering with the functional assembly of oligomeric DegP. Thus, the PDZ domains contain the information necessary for proper assembly of the functional hexameric structure of DegP.

1.6 The HtrA (DegP) protein, essential for *Escherichia coli* survival at high temperatures, is an endopeptidase

As a initial step in the understanding of the function of the *Escherichia coli* HtrA (DegP) protein, which is indispensable for bacterial survival at elevated temperatures, the protein was purified and partially characterized. The HtrA protein was purified from cells carrying the htrA gene cloned into a multicopy plasmid, resulting in its overproduction. The sequence of the 13 N-terminal amino acids of the purified HtrA protein determined and it was identical to the one predicted for the mature HtrA protein by the DNA sequence of the cloned gene. Moreover, the N-terminal sequence showed that 48-kilodalton HtrA protein is derived by cleavage of the first 26 amino acids of the pre-HtrA precursor polypeptide and that the point of cleavage follows a typical target sequence which is recognized by the leader peptidase enzyme. The HtrA protein has been found to be a specific endopeptidase which has been inhibited by diisopropylfluorophosphate, suggesting that HtrA is a serine protease.

1.7 Proteases and protein degradation in *Escherichia coli*

In *E. coli*, protein degradation plays an important roles in regulation of the levels of specific proteins and in the elimination of damaged or abnormal proteins.*E. coli* possess a very large number of proteolytic enzymes distributed in the cytoplasm, the inner membrane, and the periplasm, but, with few exceptions, physiological functions of these proteases are not known. More than 90% of the protein degradation occurring in the cytoplasm is energy-dependent, but the activities of most*E.*

coli proteases in vitro are not energy-dependent. Two ATP-dependent proteases, Lon and Clp, these are responsible for 70–80% of the energy-dependent degradation of proteins in vivo. In vitro studies withLon and Clp indicate that both proteases directly interact with substrates for degradation. ATP functions as an allosteric effector promoting an active conformation of the proteases, and ATP hydrolysis, required for rapid catalytic turnover of peptide bond cleavage in proteins. Lon and Clp shows virtually no homology at the amino acid level, and thus it appears that at least two families of ATP-dependent proteases have evolved independently.

1.8 Extraction and Partial Characterization of Proteolytic Activities from the Cell Surface of *Lactobacillus helveticus* Zuc2

Proteolytic activities extracted from a dairy *Lactobacillus helveticus* strain and partially characterized. A cell envelope proteinase (CEP) was extracted using a high ionic strength buffer, both in the absence and in the presence of Ca^{2+}. Moreover, cell treatment by 5 *M* LiCl is allowed for the selective removal of the S-layer protein and CEP, suggesting an enzyme ionic linkage to the cell envelope is similar to that observed for the Slayer structure. The enzyme specificity against α_{s1}-CN (f1–23) showed unusual activity on the Lys_3-His_4 bond when compared with other proteinases of the same species. A second proteinase appeared to be linked to the cell membrane because it was extractable after membrane disgregation by detergents. Its specificity against CN fractions and α_{s1}-CN (f1–23) was different from that of the first CEP; moreover, the measured activity was lower than that of CEP.

1.9 HtrA is a key factor in the response to specific stress conditions in *Lactococcus lactis*

The role of *Lactococcus lactis* as housekeeping surface protease HtrA. It involves in the surface properties under regular growth conditions, as *htrA* mutant strain forms longer chains in liquid medium. It also participates in cellular defence against environmental stress conditions: compared to the wild-type strain, the *htrA* mutant strain exhibited increased sensitivity to heat, ethanol, puromycin, and NaCl, but not to pH, H_2O_2, bile salts or to carbon or nitrogen starvation. *htrA* transcription in the wild-type strain showed a transient increase under the stress conditions determined as requiring *htrA*, but not under overexpression of a secreted heterologous protein. Results demonstrate that in *L. lactis*, *htrA* is a key factor in the response to specific stress conditions.

1.10 Environmental stress responses in *Lactobacillus*

Environmental stress responses in *Lactobacillus*, that have been investigated mainly by proteomics approaches, are reviewed. The physiological and molecular mechanisms of responses to heat, cold, acid, osmotic, oxygen, high pressure and starvation stresses are described. Specific examples of the repercussions of these effects in the food processing are given. Molecular mechanisms of stress responses in lactobacilli and other bacteria are compared.

CHAPTER 2
MATERIALS AND METHODS

2.1 Microorganism

The following microbial culture was used:

Lactobacillus acidophilus NCDC-11

Strain was procured from National Dairy Reseach Institute(NDRI), Karnal .

2.2 Medium Composition

Lactic acid bacteria are heterotrophic for many amino acids and they are generally very demanding nutritionally. Traditionally, these bacteria cultivated on the medium which is proposed by the de Man, Rogosa and Sharpe, known as MRS. MRS is a very rich medium in which some nutrients may be supplied in excess.

Organism and inocula preparation

Lactobacillus acidophilus was obtained from Lallemand S.A.(Toulouse , France). The strain grown in De Man,Rogosa and Sharpe (MRS) medium.For preparation of inocula, MRS broth (100ml) was inoculated with 4ml of frozen bacterial suspension and incubated for a period of 7 hours in a 250ml flask at 37°C with magnetic agitation at 250 rpm. The fermentors were inoculated with 3% (v/v) of this culture.

Culture medium

The strain usually grown on a standard medium similar to MRS medium but with different concentrations of some components. Its composition was the following in g/L of water: glucose, 50; peptone E2, (Organotechnie) 12.5, Sodium citrate,2; Sodium- acetate ,3; yeast extract, 12.5; K_2HPO_4, 2; $Mg(SO_4)_2$, 0.2; $Mn(SO_4)$, 0.05; Tween 80, 1.08. According to the different experiments the concentration of the following components was modified: glucose, peptone, yeast extract, sodium-acetate, sodium-citrate.

2.3 Instruments used

- Digital pH meter
- UV –Visible Spectrophotometer
- Incubator
- Weighing balance
- Centrifuge
- Vertical autoclave
- Laminar Air Flow Cabinet
- Orbital shaker
- Hot Plate
- Gradient PCR

2.4 Growth Curve

Requirements for Growth

Physical Requirements

Temperature

The minimum growth temperature, it is the lowest temperature at which the species will grow, and the maximum is the highest. The optimum growth temperature is that at which it grows best.

pH

Most bacteria grow best in a narrow pH range near neutrality, between pH 6.5 and 7.5. Very few grow below pH 4.0. Buffer is sometimes added to media to neutralize acids.

Oxygen

Microbes that are use molecular oxygen are aerobes; if oxygen is an absolute requirement, they are obligate aerobes.

Culture Media

Any nutrient material that is prepared for the growth of bacteria in a laboratory is called a culture medium. Microbes that are growing in a container of culture medium are referred to as a culture. When microbes are added to initiate growth, they are an inoculum. To ensure that the culture will contain only the microorganisms originally added to the medium, the medium must initially be sterile.

Obtaining Pure Cultures

There are the methods for isolating bacteria in pure cultures, which contain only one kind of organism.

Streak Plate Method

The streak plate, probably the most common method of obtaining pure cultures have been shown in (Figure 2). A sterile inoculating needle is dipped into a mixed culture and then streaked in a pattern over the surface of the nutrient medium. The last cells rubbed from the needle are enough apart that they grow into isolated visible masses called colonies.

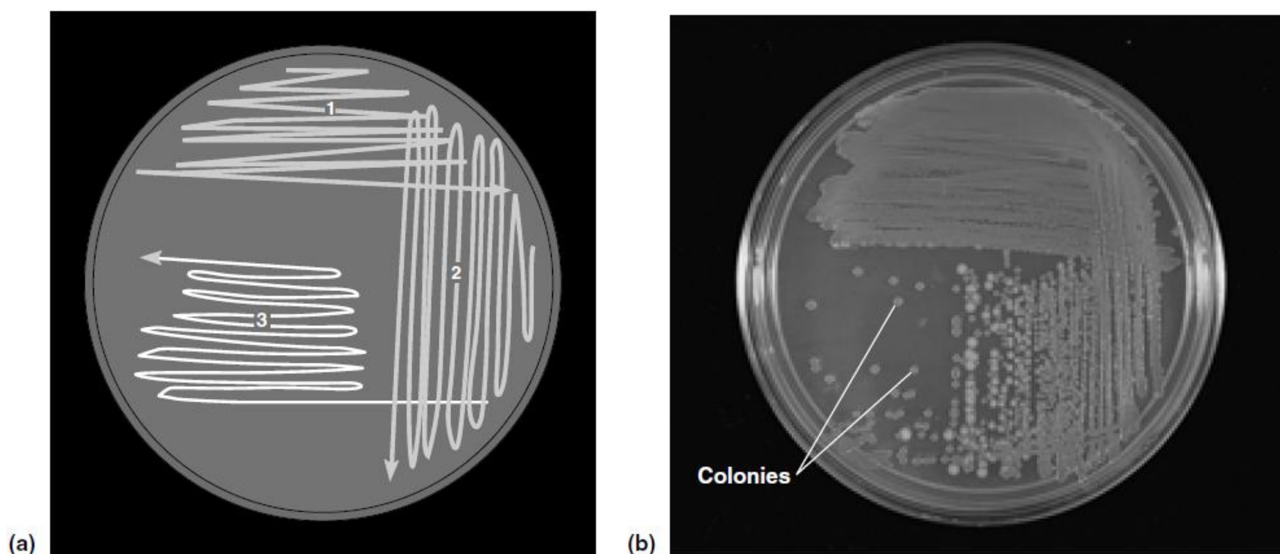

Figure 2: Streak plate method for isolation of pure cultures of bacteria. (a) Direction of streaking is indicated by arrows. Streak series 1 is made from the original bacterial mixture. The sterilized inoculating loop following each streak series. In series 2 and 3, the loop picks up bacteria from the previous series, diluting the number of cells each time. There are many variants of such patterns. **(b)** In series 3 of this example, note that well-isolated colonies of two different types of bacteria have been obtained.

Growth of Bacteria Cultures

Bacterial Division Bacteria normally they are reproduced by binary fission. Genetic material becomes evenly distributed; then a transverse wall is formed across the center of the cell, and it separates into two cells. A few bacterial species reproduce by budding; that is, an initial outgrowth enlarges to cell size and then separates. Some filamentous species produce reproductive spores or simply fragment into viable pieces.

Phases of Growth

Certain phases become apparent, when bacterial population changes are graphed as a bacterial growth curve. The lag phase shows little or no cell division. However, metabolic activity is intense. In the log phase, most actively the cells are reproducing and their generation time reaches a minimum and then remains constant; a logarithmic plot produces an ascending straight line. They are then most active metabolically and most sensitive to adverse conditions. In a chemostat, a population can be kept in such exponential growth indefinitely. Without a chemostat, microbial deaths eventually balance numbers of new cells, and a stationary phase is reached. When the number of deaths exceeds numbers of new cells formed, the death phase, or logarithmic decline, is reached.

Materials required

- Lactobacillus MRS Broth
- Flask
- Tips (1ml) and Eppendorf

Procedure

- Lactobacillus MRS Broth 5.51gm was taken for 100 ml in a flask and raised volume upto 100ml.
- Autoclaved the flask (contains MRS broth), tips, eppendorf.
- 1ml culture was taken and inoculated in the flask (contains Lactobacillus MRS Broth) under the laminar air flow cabinet.
- Then immediately, under the laminar air flow cabinet ,from flask 2ml culture was taken in an eppendorf (marked as 0).
- Eppendorf was taken and put it in the cold room and put flask in shaker at 37 ^{o}C for 30 minutes.
- Then flask was taken from the shaker and put it in the laminar air flow cabinet.
- Under the laminar air flow cabinet, from the flask 2ml culture was taken in an eppendorf (marked as 1).
- Eppendorf was taken and put it in the cold room and put flask in shaker at 37 ^{o}C for 30 minutes.
- Repeated again the above steps till eppendorf (marked as 33).
- After that optical density of each sample was taken by the spectrophotometer.
- Growth curve was obtained.

2.5 Homology modeling of HtrA protein

Homology modeling,it is also known as **comparative modeling** of protein. It refers to constructing an atomic-resolution model of the "*target*" protein from its amino acid sequence and an experimental three-dimensional structure of a related homologous protein (the "*template*"). It relies on the identification of one or more known protein structures that are resemble to the structure of the query sequence, and on the production of an alignment that maps residues in the query sequence to residues in the template sequence. It has been shown that protein structures are more conserved than protein sequences amongst homologues, but sequences falling below a 20% sequence identity can have very different structure.

Naturally occurring homologous proteins have similar protein structure and evolutionarily related proteins have similar sequences. It has been shown that three-dimensional protein structure is evolutionarily more conserved than would be expected on the basis of sequence conservation alone. The sequence alignment and template structure are used to produce a structural model of the target. Because protein structures are more conserved than DNA sequences, detectable levels of sequence similarity usually imply significant structural similarity. The quality of the homology model is dependent on quality of the sequence alignment and template structure.

The method of homology modeling, it is based on the observation that protein tertiary structure is better conserved than amino acid sequence. Thus, even the proteins that have diverged appreciably in sequence but still share detectable similarity will also share common structural properties, particularly the overall fold. Because it is very difficult and time-consuming to obtain experimental structures from the methods such as X-ray crystallography and protein NMR for every protein of interest, homology modeling provides useful structural models for generating hypotheses about a protein's function and directing further experimental work.
Homology modeling created by Discovery Studio 4.1 client.

The **steps in homology modeling** are the following:

- template identification;
- amino acid sequence alignment;
- alignment correction;
- backbone generation;
- generation of loops;
- side chain generation & optimization;

- *ab initio* loop building;
- overall model optimisation;
- model verification. Quality criteria, model quality.

After finding a template it is an absolute requirement that before starting the homology modeling project, make a multiple sequence alignment, which include our sequence, the sequence of the template and some other sequences of proteins are belonging to the same family. This will give an overview of the general features of the protein family, the degree of conservation, the consensus sequence motifs, etc. It would also be desirable to make a secondary structure prediction. Most importantly, the positions of insertions and deletions should be correct, likewise the conservation of important residues, for example active site residues. When the sequence analysis is done and the alignment is corrected then proceed to the modeling. The modeling software will thread the sequence on the template structure, thus creating a preliminary model of protein (backbone generation). After that it will try to build missing parts, generate side chains for replaced residues and optimize side chain conformations, etc. At the last step the overall model needs to be optimized followed by verification of model quality.

2.6 Isolation of genomic DNA from *Lactobacillus acidophilus* by SDS

Materials required

- Flask
- Eppendorf
- 70% ethanol
- Autoclaved distilled water

Reagents

- NaCl- 5M
- EDTA- 0.5M
- Tris HCl- 1M
- Set Buffer (pH 7.5)
- SDS
- TAE Buffer
- Chloroform
- Phenol
- Isopropanol

Ingredients (Extraction Buffer)

	g/100ml
• 75Mm NaCl	0.435
• 25Mm EDTA	0.930
• 20Mm Tris HCl	0.242

Above ingredients were dissolved in distilled water and pH was adjusted to 7.5 before autoclaving.

Procedure

- 2ml culture was taken in an eppendorf
- Then centrifuged at 12000 rpm for 10 minutes
- Bacterial pellet was obtained
- 500 µl of extraction buffer and 50 µl of 10% SDS was added
- Incubated at 65 ^{o}C for 30 minutes
- 5 µl of RNase A (20mg/ml) was added
- Vortex and incubated at room temperature for 5 minutes
- 1/3rd volume of 5M NaCl was added
- Phenol chloroform (1:1) was added
- Centrifuged at 10000 rpm for 5 minutes
- Aqueous layer (upper layer) was taken
- Again phenol chloroform (1:1) was added
- Centrifuged at 10000 rpm for 5 minutes
- Aqueous layer (upper layer) was taken
- Iopropanol (4/2 volume) was added
- incubated overnight (-20 ^{o}C)
- Centrifuged at 15000 rpm for 10 minutes
- Supernatant was removed
- Washed with 70% ethanol (centrifuged at 12000 rpm for 5 minutes)
- Repeated again the above step
- The pellet was air dried(for 15 minutes)
- Suspended DNA pellet in autoclaved distilled water (20 µl)
- Stored at -20 ^{o}C

2.7 Gradient PCR

The sequence and the length of PCR primers generally determine the annealing temperature of the thermal cycling reaction for a specific assay. Although primers are usually supplied with theoretical melting temperatures (Tm), these can be calculated in different ways which may give varying values. Using too low annealing temperature can produce non-specific products whereas if the temperature is too high the PCR yield may be reduced. An annealing temperature optimisation step can avoid these problems and it is especially important when changing a sensitive assay from one thermal cycler to another. The gradient function of the Prime thermal cyclers is a useful feature which can be used to improve the results of a PCR by allowing simple optimisation step that can be performed in a single run. It can also be used to perform the initial optimisation of a number of different assays with various sets of primers, thus saving the user a significant amount.

It is used to amplify a specific DNA (target) sequence lying between known positions (flanks) on a double-stranded (ds) DNA molecule. The PCR can be used to amplify both double and single stranded DNA.

A typical thermal cycle follows:

Heat denaturation at 94 ^{o}C for 20 seconds

Primer annealing at 55 ^{o}C for 20 seconds

Primer extension at 72 ^{o}C for 30 seconds

Materials required

- Eppendorf
- Ice bucket
- PCR vials, Tips

Requirements

- Thermal cycler (thermocycler)
- Distilled water
- Buffer(10x)
- dNTP
- Forward Primer
- Reverse Primer
- Taq Polymerase
- DNA

Master Mix (1×15 µl)

- Distilled water - 88.4 µl
- Buffer (10x) - 12 µl
- dNTP -2.4 µl
- Forward Primer – 4 µl
- Reverse Primer – 4 µl
- Taq Polymerase – 1.2 µl
- DNA – 8 µl

Procedure

- Primer was diluted:

  Working solution – 100 µl

  Forward primer (90 µl distilled water and 10 µl forward primer)

  Reverse primer (90 µl distilled water and 10 µl reverse primer)

- In an eppendorf:

  Distilled water - 88.4 µl

  Buffer (10x) - 12 µl

  dNTP -2.4 µl

  Forward Primer – 4 µl

  Reverse Primer – 4 µl

  DNA – 8 µl were added and at the end taq polymerase was added.

- Take 6 PCR vials and marked as 1,2,3,4,5,6.
- From the eppendorf 15 µl was taken in each PCR vials.
- PCR vials were put in the thermocycler (temperature conditions were: 42 ^{o}C- 47 ^{o}C)
- And then run the PCR.

CHAPTER 3
RESULTS AND DISCUSSION

3.1Growth curve study of *Lactobacillus acidophilus* (shown in Figure 3, Table 1)

Growth can be defined as, the increase in the cell size and cell mass during the development of an organis. Growth is the unique characteristics of all organisms. For their energy generation and cellular biosynthesis, the organism must require certain basic parameters. The growth of the organism is affected by both physical and Nutritional factors. The physical factors, it includes the pH, temperature, Osmotic pressure, Hydrostatic pressure, and Moisture content of the medium in which the organism is growing. The nutritional factors,it includes the amount of Carbon, nitrogen, Sulphur, phosphorous, and other trace elements provided in the growth medium.

Four different phases:

- lag phase
- log phase or exponential phase
- stationary phase
- death phase

During **lag phase**, bacteria adapt themselves to growth conditions. In this the individual bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs.

The **log phase** (called the logarithmic phase or the *exponential phase*) is a period which is characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate so both the number of cells and the rate of population increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time and produces a straight line. The slope of this line is the specific growth rate of the organism, that is a measure of the number of divisions per cell per unit time.

The **stationary phase** is due to a growth-limiting factor such as the depletion of an essential nutrient, and/or the formation of an inhibitory product such as an organic acid. Stationary phase results in which growth rate and death rate are equal. The number of new cells created is limited by

the growth factor and as a result the rate of cell growth matches the rate of cell death.Resulted in a "smooth," horizontal linear part of the curve during the stationary phase.

At **death phase**,(Decline phase) bacteria die. Due to lack of nutrients, a temperature which is too high or low, or the wrong living conditions.

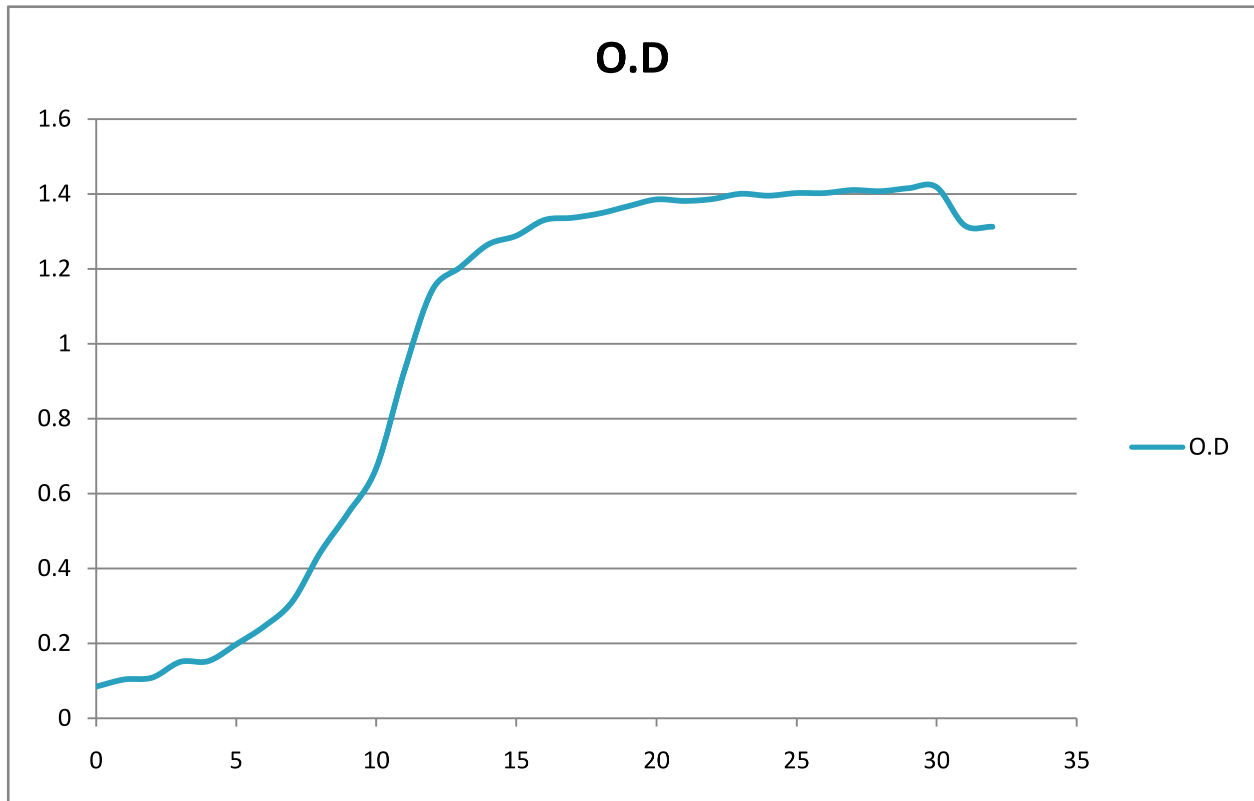

Figure 3: Growth curve of *Lactobacillus acidophilus*.

Table 1: Time interval and absorbance at 600nm.

Time Interval	O.D (600nm)
0	0.084
1	0.103
1.5	0.108
2	0.15
2.5	0.152
3	0.197
3.5	0.245
4	0.311
5	0.442
5.5	0.548

6	0.668
7	0.926
8	1.143
9	1.204
10	1.265
11	1.288
12	1.33
13	1.336
14	1.348
15	1.367
16	1.385
17	1.381
18	1.386
10	1.4
20	1.395
21	1.402
22	1.402
23	1.41
24	1.407
25	1.415
27	1.418
28	1.316
32	1.312

3.2 Homology modeling of HtrA protein

Homology modeling, it relies on the identification of one or more known protein structures likely to resemble the structure of the query sequence, and on the production of an alignment that maps residues in the query sequence to residues in the template sequence.

Figure 4:

- Sequence identity = 19.9%
- Sequence similarity = 33.7%

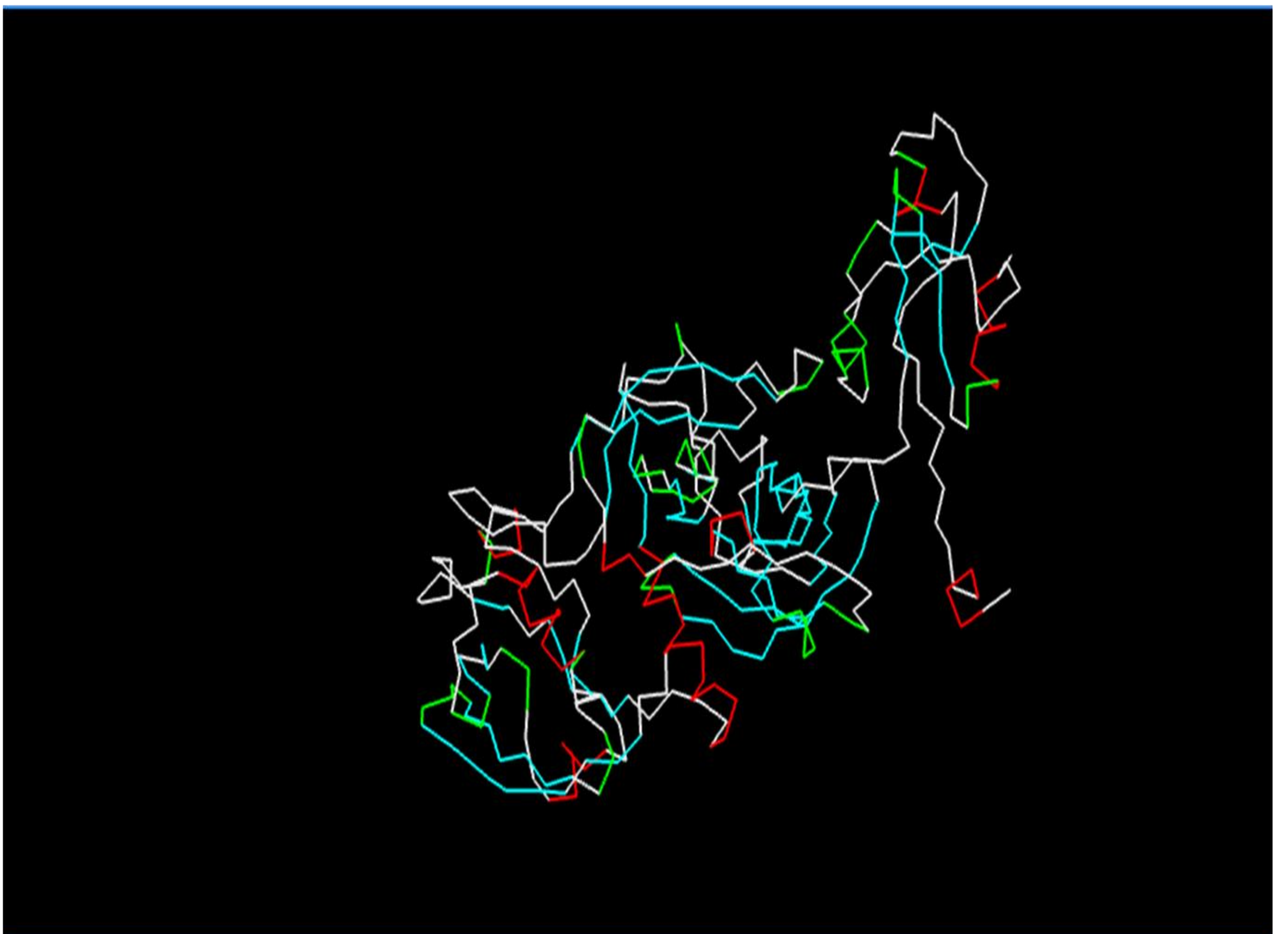

Figure 4: Homology modelling of HtrA protein.

3.3 Isolation of genomic DNA from *Lactobacillus acidophilus* by SDS

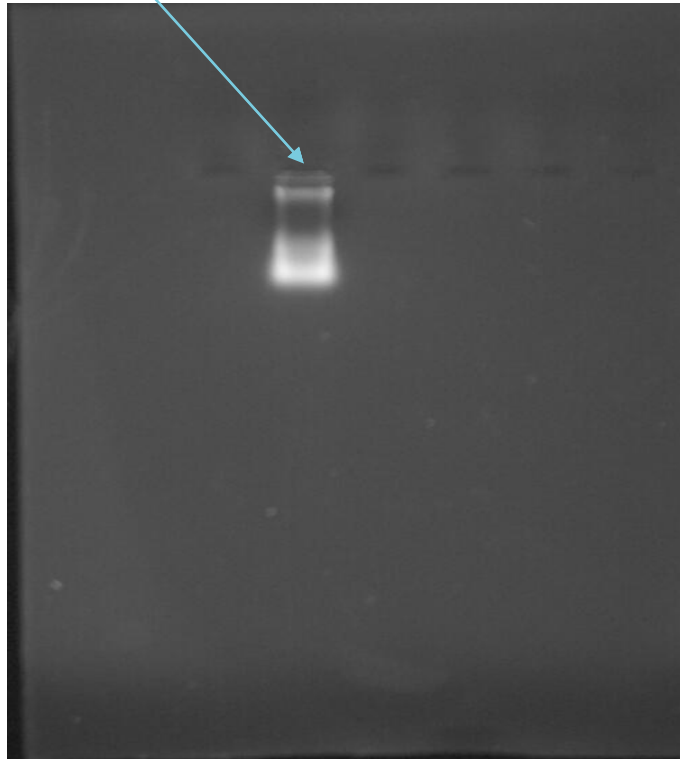

Figure 5: ***Lactobacillus acidophilus*** **NCDC-11**

DNA band can be seen in the strain. Along with band RNA and contaminations are also detected.

CHAPTER 4
CONCLUSION

High temperature requirement A, has been known to be a key player in the protein quality control system in the bacterial periplasmic space. Recent structural and biochemical analyses have suggested that both bacterial responses against variety of stresses, and protein quality control, are subtly controlled by structural and functional changes occurring in the HtrA family proteins. However, the molecular mechanisms underlying protein quality control have yet to be definitively elucidated. In this report, the status of current studies into the structures and functions of the HtrA proteins is briefly summarized. Additionally,structural and biochemical studies may further our understanding of the molecular functions performed by these proteins, as well as the mechanisms underlying their regulation.

REFERENCES

- Catherine Foucaud-Scheunemann, Isabelle Poquet, 2003, HtrA is a key factor in the response to specific stress conditions in Lactococcus lactis, 53-59.

- Carol W. Gray, Sandra Turconi, John B. Davis and Caretha L. Creasy, 2001, Characterization of human HtrA2, a novel serine protease involved in the mammalian cellular stress response,Vol. 267, 1432-1327.

- Dr. Bohr-Gasse, HtrA protease-chaperones, Research Institute of Molecular Pathology (IMP).

- Dong Young Kim and Kyeong Kyu Kim, 2005, Structure and Function of HtrA Family Proteins, the Key Players in Protein Quality Control, Vol. 38, 266-274.

- Lipinska B, Zylicz M, Georgopoulos C,1990, The HtrA (DegP) protein, essential for Escherichia coli survival at high temperatures, is an endopeptidase, Vol. 172(4), 7-1791.

- M. R. Maurizi, 1992, Proteases and protein degradation in *Escherichia coli,* Vol. 48, 178-201.

- Maria De Angelis and Marco Gobbetti, 2004, Environmental stress responses in *Lactobacillus,* Vol. 4, 106-122.

- Nathalie Sassoon, Jean-Philippe Arié and Jean-Michel Betton, 2002, PDZ domains determine the native oligomeric structure of the DegP (HtrA) protease, 1365-2958.

- P.Taillandier, F.Gilis, F.Ramon Portugal, P.Laforce, P.Strehaiano, 1996, Influence of medium composition, pH and temperature on the growth and viability of *Lactobacillus acidophilus*, Vol. 18 No. 7, 775-780.

- Saswati Biswas and Indranil Biswas, 2005, Role of HtrA in Surface Protein, Vol. 73(10), 6923–6934.

- Scolari G, Vescovo M, Zacconi C, Vescovi F, 2006, Extraction and partial characterization of proteolytic activities from the cell surface of Lactobacillus helveticus Zuc2, Vol. 89(10), 9-3800.

- Zurawa-Janicka D, Skorko-Glonek J, Lipinska B, 2010, HtrA proteins as targets in therapy of cancer and other diseases, Vol. 14(7), 79-665.