Cloning and Expression of protease from *Lactobacillus sp.*

Enrollment numbers : 091569 & 091573

Name of Students : Srashti Gopal Goyal & Prakhar Srivastava

Supervisor : Dr. Saurabh Bansal

May 2013

Submitted in partial fulfillment of the Degree of Bachelors of Technology

Department Of Biotechnology and Bioinformatics

Jaypee University of Information Technology

Waknaghat

TABLE OF CONTENTS

CERTIFICATE

This is to certify that the work titled "**Cloning and expression of protease from *Lactobacillus* sp.**" submitted by "**Srashti Gopal Goyal and Prakhar Srivastava**" in partial fulfillment for the award of degree of Bachelor of Technology Biotechnology 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

Date 25/05/2013

ACKNOWLEDGEMENT

The project is an outcome of continuous work and intellectual support from various sources. We take great pleasure in expressing our gratitude and appreciation to those who have contributed to this project.

We are grateful to Dr. R.S. Chauhan, Prof. and Head, Department of Biotechnology and Bioinformatics, JUIT, Waknaghat, for providing us with the opportunity and facilities to carry out the project.

We take this opportunity to thank our mentor and supervisor Dr. Saurabh Bansal, Lecturer, Department of Biotechnology and Bioinformatics, JUIT, for lending us stimulating suggestions, innovative guidance and creative thinking. His practicality, constructive criticism, constant inspiration and advice have helped us in all stages of the project. His scientific views and approach will always be a source of motivation for us. We are grateful to him for the support he provided us in doing things at our pace and for being patient with our mistakes.

We are also thankful to our seniors Mr. Ankit Srivastava, Mr. Swapnil Jain, Ms Shivani Sood, Mr. Aseem Chawla and Mr. J. K. Padhan, who have always been a constant source of encouragement and helped us in numerous ways. We would also like to thank Mr. Baleshwar, Mr. Kamlesh and Mr. Ravikant for their assistance in the lab.

We would also like to thank our friend Ishita Jain for her enormous support and help she provided us with during this project.

Srashti Prakhar

Srashti Gopal Goyal and Prakhar Srivastava

Date 27 May 2013

Summary

Serine protease, HtrA is an enzyme that cleave peptide bonds in proteins and is found ubiquitously in both eukaryotes and prokaryotes, And this particular gene has various role in stress responses like oxidative stress, high temperature, high salt content and also has a pathogenicity factor in various organism as per studied in various other organism like *E.coli or Streptococcus* sp. In the present study we are trying to isolate this particular *htrA* gene from *Lactobacillus* sp. (as this organism is non-pathogenic and user-friendly) through various techniques of rDNA technology and cloning it in suitable expression vector for the purification and for further characterization of enzyme.

List of Figures

List of Table

List of Abbreviations

CTAB :Cetyltrimethyl ammonium Bromide

DNA : Deoxyribose Nucleic Acid

dNTP :Deoxyribonucleoside triphosphate

EDTA : Ethylene Diamine Tetra-acetic Acid

EtBr : Ethidium Bromide

HtrA: High Temperature Resistant Gene A

PCR : Polymerase Chain Reaction

TE : Tris EDTA Buffer `

TAE : Tris Acetic acid EDTA

CHAPTER1

INTRODUCTION

Serine protease HtrA is an enzyme thatis encoded by the *htrA*gene in various prokaryotes and eukaryotes cells.It is commonly known as heat shock protein and its level is basically increased during stress condition.Various studies have been performed in order to check the role of *htrA* gene during various stress condition.*htrA* gene homologue has been found in various microorganisms in which structural studies reveal the stability of this gene at elevated temperature or at various oxidative stress conditions.The presence of different domains like PDZ binding domain, chymotrypsin and trypsin domain have both chaperone activity and proteolytic activity. This regulates the proper functioning of the proteins at various stress condition. The newly formed polypeptide is usually under the condition of degradation by various other proteosome or ubiquitin factors, so the heat shock proteins basically assist these proteins to have a proper folded structure. During diseased condition or in prokaryotes under high stress condition the regulation or activity of these heat shock proteins increases. These heat shock proteins are also present in normal condition and have role in cell differentiation and proliferation.

Recent studies show that they have a major diagnostic role in cancer tumorogenicity, elevation in the level of these proteins act as a biomarker for detection of cancer and various other diseases.

CHAPTER 2

REVIEW LITERATURE

Proteases or peptidases are enzymes that catalyze the cleavage of peptide bonds and are widely distributed in nature, where they perform a variety of different tasks. They serve essential housecleaning functions by degrading damaged proteins and signal peptides and play important regulatory roles by inactivating signaling proteins. In higher organisms, they are involved in such diverse functions as cell growth, apoptosis, allergic reactions, fertilization, control of blood pressure, as well as blood clotting. The serine peptidases are subdivided into six classes on the basis of sequence similarity, tertiary structures, and the sequential order of catalytic residues.

Serine Protease HtrA belongs to the trypsin family where the order of the catalytic triad is His-Asp-Ser. Proteases have a two-domain structure with each domain forming a six-stranded β barrel. The active site cleft is located at the interface of the two perpendicularly arranged barrel domains. All enzymes of this class are endoproteases, and they occur widely in RNA viruses, prokaryotes, and eukaryotes. Many of proteases are expressed as an inactive pro-form with an N-terminal inhibitory peptide and require proteolytic maturation for activation.The active site is constructed by several loops located at the C-terminal side of both barrel domains. The participating loops of the N-terminal β-barrel are termed LA (residues 34–41 using the chymotrypsin nomenclature), LB (59–64), LC (91–102), those of the C-terminal barrel L1 (184–189), L2 (214–228), and L3 (164–181)*(Tim Clausen et al.,2002)*. There are over 180 members of this family (corresponding to 10% of trypsin-like proteases) combine a proteolytic domain with at least one C-terminal PDZ domain.

The stability of proteins depends on proper folding and environmental or cellular factors interfering with the folded state. Mis- or unfolding events are monitored by specialized proteins, chaperons and proteases.These quality control elements can either repair or remove unfolded polypeptides.Some of the most prominent chaperons and proteases are widely conserved in various living system. For example, heat shock proteins(hsp) are usually over expressed at high temperature. Various reports suggested that the activity and expression of various chaperones and proteases become predominant

under the stressed conditions (*Keiichiro Hiratsuet al.,* 1995).Stressed conditionsinclude various kinds of abnormal conditions like high salt content,elevated temperature(*Lipinskaet al.,*1989) low or high pH, high oxidative stress in bacteria.Glonekand his group studied the effect ofoxidative stress on the expression level of htrA proteinin *E. coli*in the presence offerrous sulphate and hydrogen peroxide (*J.Shorko-Gloneket al.,1999).*

In humans, stress can be characterized by various disease conditions. Molecular chaperons recognize hydrophobic domains that are surface exposed in non-native state. These proteins allow single or, if necessary,multiple steps of non-covalent binding and release of polypeptides.Their main task is to prevent aggregation and help in either proper folding or targeting of substrates to one of the many proteases.Heat shock proteins perform these functions with the help of various co- chaperons and helps in maintaining native polypeptide structure.In some cases, chaperons are also involved in targeting to secretion. Proteases remove polypeptides that have not remained in the folded state.Even though chaperons and proteases carry out antagonistic reactions, they are expected to share common features because their substrates are similar. It is thought that this chaperone activity contributes to the efficiency of proteolysis.If prior to proteolysis, misfolded substrate is released from the chaperone domain, refolding into a protease-resistant, and possibly native, conformation can occur(*J.Shorko-Gloneket al.,*1997). Irreversibly damaged substrates will be bound by the protease subunit for degradation. The protease(clP) and chaperone(clpA or clpX) activities reside in separate subunits that constitute a hetero-oligomeric complex.The newly synthesized proteins in the cells are exposed to a variety of environmental stresses, including heat,high temperature(*Lipinskaet al.,* 1990), chemicals, pathogens, or immune system activities, all of which can cause 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 such cases, it is necessary to remove these proteins, or return them to a functional state, as aggregated proteins can cause cell viability. This protein quality control is essential for cell survival in all organisms.

In the bacterial cytoplasm, molecular machines such as ClpXP (AP), where AP is Alkaline Phosphatase which work as a substrate or act as a cleaving site for protease play

important roles in this quality control process, by degrading damaged proteins in an ATP-dependent manner purification and characterization of alkaline phosphatase has been performed by biochemical assay (*Lianna Munson et al.*,1978) in order to see the effect of AP substrate during proteolytic activity. These ATP-dependent molecular machines have two major components, the ATPases and the proteolytic compartments. The ATPase component consists of multimericATPases, and affects the capture, unfolding, and translocation of its substrates to the proteolytic compartments. An ATP-independent chaperone-protease, htrA/DegP, has been implicated as a key player in the control of protein quality in the periplasmic space of Gram-negative bacteria, consists of 474 residues, while the mature protein has 448 residues,lacking the N-terminal signal peptide. This signal peptide is followed by a domain called Q-linker. The next domain is the catalytic domain with the postulated catalytic triad His105, Asp135 and Ser210. At its C-terminus, DegP has two PDZ domains.

HtrA homologues have been isolated in a variety of species, including Gram-negative and -positive bacteria, plants, and mammals. Although these homologues normally exhibit proteolytic activities suggestive of serine protease activity,their physiological roles and structural architecture are rather diverse.

2.1 Bacterial HtrA

In the last few years, various HtrA homologues have been isolated from a variety of bacteria. Bacterial HtrA (DegP) has been implicated in thermal, osmotic, and pH tolerance and peroxide resistance.In addition, DegP seems to be involved in bacterial virulence(*William R.Lyonet al.*,2004), since DegP null mutants of several pathogenic bacteria such as *Salmonella typhimurium*, *Brucellaabortus*, and *Yersinia spp.* are attenuated(*Kerstin Williamset al.*,2009).These attenuated species have major role in vaccines and some species have been studied recently in order to know the pathogenicity of particular microbe. Huston and his research group showed that HtrA protein from *Chlamydia trachomatis* act as both chaperone and protease at 37°C(*Wilhelmina M. Hustonet al.*, 2007),(*Guadalupe Corteset al.*,2002).

The exposure of cells to stressful conditions causes damage to the various cellular components,including proteins. Proteins due to stress, may lose their native conformation

which result into aggregate formation or interaction with inappropriate partners. To prevent these detrimental effects, cells induce sophisticated defense systems, whose role is to suppress the aggregation and refold the denatured proteins, or to degrade the damaged proteins. These functions are carried out by molecular chaperones and proteases, respectively. The HtrA protein has been shown to combine both activities: that of a chaperone and that of a protease. In *E. coli*, HtrA is located in periplasmic space which is indispensable for its survival during the elevated temperatures and under certain conditions of oxidative stress. The proteolytic activity of HtrA was shown to be temperature dependent. In in vitro assay,HtrA cleaved its substrate (β-casein for *E.coli*) efficiently at temperatures above 30^0 C. Maximum activity was observed at temperatures around 45^0 C, which corresponded well with the important role of this protease under heat-shock conditions. At temperatures below 28^0 C, the proteolytic activity was regarded as negligible and it was believed that the protein then plays the function of a chaperone.

HtraA, also known as DegP and probably identical to the protease Do,is a heat shock-induced envelop- associated serine protease that was first described in *E coli* but is now known to have homologous in bacteria as diverse as cyanobacteriaand mycobacteria as well as in some eukaryotes(humans and yeast). There are two homologues of HtrA in *E.coli*: DegS and DegQ.

DegP (HtrA) is a heat shock protein, localized in the periplasmic compartment of *E. coli*. DegP is a serine protease and the intact protein is indispensable for bacterial growth attemperatures above 42^0C. Moreover DegP has been implicated in pH tolerance, H_2O_2resistance and bacterial virulence. Beside the well-studied protease activity, recent experiments demonstrate that DegP can also act as a chaperone in a temperature-dependent way. At low temperatures, DegP has general molecular chaperone activity, but at elevated temperatures, the protein behaves like a protease. Structural studied had been done on the switch in activity on DegP protease*(Tobias Krojeret al., 2008*). DegP belongs to a large family of related serine proteases,whose members are found in most organisms, including humans. The unprocessed precursor of DegP, also called protease Do, consists of 474 residues, while the mature protein has 448 residues,lacking the N-terminal signal peptide. This signal peptide is followed by a domain of unknown function and a Q-linker. The next domain is the catalytic domain with the postulated catalytic triadHis105,

Asp135 and Ser210. At its C-terminus, DegP has two PDZ domains. DegP forms oligomeric complexes in solution but there are still contradictory results whether it is a hexamer, dodecamer or if there is equilibrium between these two states. Misfolding and unfolding of polypeptides can occur as a consequence of environmental stress or spontaneous mutation. Comparative studies have been performed in wild HtrA and null mutant by Fourier transformed infrared spectroscopy to check the role ofHtrA gene during various stress condition(*J. Shorko-Gloneket al.,1995*).

2.2 Role Of Q Linkers

HtrA family members possess Q-linkers of various sizes. The Q-linker of DegP, corresponds to loop LA in the structure, consists of a long loop which is involved in the formation of the hexamer.Hexameric structure of HtrA is responsible for switch in activity and for differentiation between the substrates. Loop LA protrudes into the active site of the opposite subunit, and interacts with loops L1 and L2 in the opposite subunit. Loop LA of DegP thereby forms the side-wall of the proteolytic chamber, which is required for differentiation between substrates. However, the Q-linker of *Thermotogamaritima* (Tm)HtrA forms a short helical lid (HL) within the crystal structure. This helical lid covers the active site, thereby effectively blocking substrate access. However, this lid is expected to operate as a temperature sensor, which is able to activate proteolytic functions which control the exposure of the active site to solvents at high temperatures(*Dong Young Kim et al.,2005)*. Comparisonof Q-linker of *T.maritima* with Q-linker of Gram positive or Gram negative bacteria shows evidence that why *T.maritima* is able to survive at temperature above 80^0 C*(Tracy L.Rivioet al.,2005)*. HtrA tells about the stability of particular gene as *T. maritima* survives at temperature above 80^0C. Sequence alignment has revealed that many of the HtrA family proteins found in Gram-negative and -positive bacteria, including the HtrA from *T. maritima*, constitute *E. coli* DegQ homologues, as the Q-linkers of these proteins are similar with regard both to sizes and sequences. In addition, the secondary structures of the Q-linkers in HtrA and DegQ have both been predicted to be short α-helices. The presence of the Q-linker in DegS is almost negligible, however, due to its short length.Although sequence identities are generally high among the protease domains of *E. coli* DegP, DegQ, DegS, and many

other HtrAs from different species,the length and structure of the Q-linkers may be considered a major criterion with regard to the classification of DegP, DegQ, and DegS subfamilies, as well as in the determination of their functions. In this regard, it has been suggested that the Q-linkers of HtrAs from Gram-positive and -negative bacteria may also function as temperature sensors, as has been observed in Tm HtrA, and that they are not involved in the dimerization of trimers. Similarly, it can be proposed that many of the HtrA homologues which have classically been considered examples of HtrA in Gram-positive and Gram negative bacteria should more appropriately be designated as DegQ variants.

DegP is a member of the widely conserved HtrA family ofserine proteases that are crucial to maintain protein homeostasis in extracytoplasmic compartments. The bacterial representatives DegP and DegS have key functions in the unfolded protein response of the cell envelope, whereas the four human HtrA proteins are implicated in many severe disorders including Parkinson's and Alzheimer's diseases. HtrA proteins encompass a catalytic domain with a chymotrypsin-like fold and one or two carboxy-terminal PDZ domains,which are well-characterized protein–protein interaction modules.The protease domains of three protomers interact closely to form atrimer that is the basic building block of HtrA oligomers. The projecting PDZ domains either participate in protein degradation by presenting substrates to the protease or offer a binding site for anallosteric activator that stimulates protease function. The reversible activation mechanism ensures that the digestive mode of HtrA proteins can be precisely switched on and off depending on the needs of the cell. The available crystal structures suggest that HtrA proteins differ in their molecular architecture, ranging from trimers with surface-accessible active sites to hexamers that belong to the class of self-compartmentalizing proteases. For these proteases, trimer association positions a regulatory loop in the active site of a neighbouring molecule, thereby blocking substrate access and deforming the proteolytic site.

2.3Human htrA

Human HtrA1 belongs to the widely conserved high-temperature requirement A (HtrA) family of homo-oligomeric and ATP-independent serine proteases. HtrAs of pro- and eukaryotes are implicated in protein quality control. They can act as key stress sensors and regulators of unfolded protein response signaling pathways and can mediate the repair and assembly or the removal of damaged, fragmented and mislocalized proteins. Defining features of HtrA proteases are their homo-oligomeric architecture and the presence of C-terminal PDZ domains that can be involved in substrate processing, sensing of misfolded proteins, mediating allosteric and cooperative regulation of the proteolytic activity and in the switch between various oligomeric states.

Among the four human HtrAs, HtrA 1, HtrA 2, HtrA 3 and HtrA 4, the ubiquitously expressed HtrA1 consists of a signal sequence for secretion, partial insulin like growth factor binding protein (IGFBP) domain, serine protease domain resembling classic serine proteases such as trypsin and one C-terminal PDZ domain. Like *E.coli*, DegP, a prototypic HtrA protease involved in protein quality control.HtrA1 is activated by oligomerization. Substrate binding triggers the switch between the resting and the active conformations and between various oligomeric states. HtrA1 has at least three cellular localizations and a multitude of functions. Extracytoplasmic HtrA1 is involved in the homeostasis of the extracellular matrix.Elastin,fibulin, nidogen, fibronectin, fibromodulin, aggrecan and decorin have been identified as substrates for the extracytoplasmic HtrA1. Intracellular HtrA1 was localized to microtubules and to the nucleus. Cytoplasmic HtrA1 has been implicated in the degradation of tuberin thereby modulating cell growth and proliferation*(Mara Campioniet al.,2010)*. Furthermore, microtubule-associated HtrA1 degrades tubulins thereby inhibiting cellmigration.Consequently, HtrA1 has been implicated in several severe pathologies including cancer, age-related macular degeneration, Alzheimer's disease, arthritis and familial ischemic cerebral small vessel disease.Human HtrA degrade Tau protein aggregates which are critically involved in neuron disorders*(Annette Tennstaedtet al.,2012)*.

2.4PDZ 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 YaeL, have been determined to actually harbor PDZ. It has suggested that the *E.coli* PDZ domains are also able to recognize the C-terminal regions of substrates, on the basis of their carboxylate-binding loops. Structural studies of DegP and DegS have determined that their PDZ domains perform different functions. In DegP, however, 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 is involved in dimerization, as the isolated second PDZ domains retain the ability to form stable dimers. This functional divergence was expected, due to the fact that the sequences of the second PDZdomains are not very 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, namely, 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 well be a prerequisite for proteolytic activity, due to their regulation of the rearrangement of the catalytic triad, as well as their substrate access-limiting properties. Basically DegP protease domain and PDZ domain have combined role in stress condition they have a role in elimination of misfolding proteins*(TobiasKrojeret al.,2008).*

2.5 Role ofDegP/HtrA in plants:

Deg proteases are located inchloroplasts,one in peroxisomes, one in mitochondria, and one in the nucleus. The chloroplast-located Deg/HtrA proteases were reported to be involved in the degradation of damaged photosynthetic proteins, especially the photosystem II (PSII) reaction under light stress conditions. Bacteria and Eukarya cluster into four distinct classes, whereby plants are the only organisms containing proteases from all four classes. However Deg/HtrA proteases are found in relatively high number in

plants with a great diversity.Same localizedproteases have a similar domain arrangements, and comparable sizes, carries a high risk of confusion. This is potentiated by the fact that during genome annotation of vascular plants (ė.g.*A. thaliana* and *O. sativa*). DegP/HtrA proteases were numbered according to the order of their discovery, thus giving orthologous proteins different numbers and names depending on the organism.

2.6 Folded And Misfolded Proteins:

The majority of HtrA proteins act on misfolded proteins. From the crystal structure, different mechanism for the sterically controlled access to the active site can be distinguished.In DegP,the dimension of the inner cavity achieves substrate discrimination.The height of cavity($15A^0$) excludes folded protein from entering the proteolytic sites.In the HtrA 2 crystal structure, the PDZ domain is rotated onto the body of protease domain such that an arrow active site canyon is formed on the inner side of trimeric funnel. Structural alignment with related proteases suggests that this canyon might be too small to accommodate the protein substrate and that the PDZ domain has to reorient prior to substrate binding. However besides different strategies in substrate discrimination, both the HtrA2 and DegP binding sites are hydrophobic which facilitate distinction between misfolded and native proteins.

2.7 Regulation Of Protease Activity And Function Of PDZ Domain:

In DegP, a complex network of interactions is established between the two trimeric rings. The enlarged protease loop LA of one ring protrudes into the active sites of the opposite ring.Here loop LA intimately interacts with loop L1, L2. The resulting loop trio not only prevent formation of S1-pocket but also completely block the entrance to the active site, Further the L1 loop containing the active site is stretched into an extended conformation such that the turn structure to form the NH cradle oxyanion cannot be formed(Fig 2.1).Thus,at low temperature the protease domain of DegP is present in completely inactive conformation, in which substrate binding as well as catalysis is prevented, cleavage site and substrate specificity were studied in DegP and DegQ periplasmicendoprotease*(Herald Kolmaret al., 1996)*. Activation requires large conformational changes, in particular disruption of loop trio L1-L2-LA. Both substrate

binding and temperature-induced conformational changes might be involved in this process. The L1 loop is strongly kinked into a beta-turn structure,as a result the NH donors of the oxyanion hole are flipped onto the opposite site of the protein backbone precluding stabilization of the oxyanion intermediate(Fig 2.1). Furthermore, access to the proteolytic site is restricted. Instead of loop LA and L2 the PDZ domain is the steric block of HtrA2. Consequently, the PDZ domain was proposed to be the main regulator activity and thus to have a different function as in DegP. According to the model of *(Liet al.2002)*,the HtrA2 protease activity can be fine-tuned by the position of the PDZ domain. Interaction with a ligand could lead to reorientation of the PDZ domain, thereby making the active site accessible for substrates.According to the hypothesis of Shi and coworkers*(Li et al., 2002)*,this specificity might be directed against other trimeric apoptotic proteins,such as the cell death receptor, the interaction with which could directly affect the activity of HtrA2. On the other hand, the flexible peptide binding site of DegP may be required to act on a broad spectrum of misfolded proteins that may be formed, for example,during heat shock.A residue common to both binding pocket is Arg262. This arginine forms part of the carboxylate binding groove and also contribute to the protease-PDZ hinge. Thus interaction of substrate with Arg262 might trigger reorientation of PDZ domain.

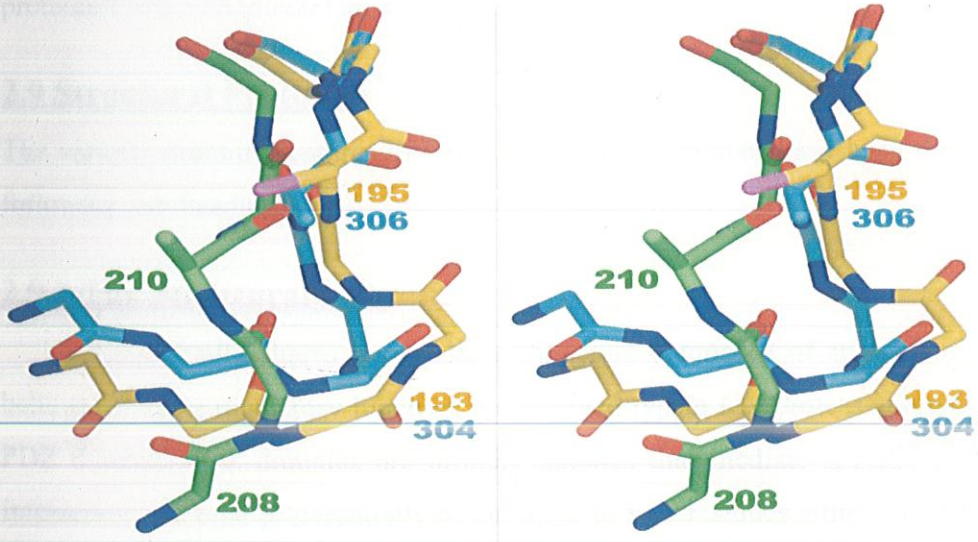


Fig 2.1 Oxyanion hole, a pocket in the structure of an enzyme formed during proteolysis by chymotrypsin.

Another remarkable feature is that HtrA protease activity can switch reversibly.This is mediated at least in part by geometric arrangement of catalytic triad.This novel strategy may have evolved to guarantee the reversible switch from chaperone to protease which may be necessary to rapidly respond to environmental changes that cause folding problem or affect protein composition.

2.8 Switch In Activity:

The proteolytic activity of DegP is negligible below 20°C, but then rapidly increases with temperature. Refolding by DegP is most productive at lower temperature.Thus at low temperature DegP dominantly acts as molecular chaperones by stimulating refolding of non-native proteins. As temperature increases, DegP switches from protein folding to protein digesting machine.While the mechanism of this switch remains elusive, the molecular players can be discerned from the crystal structure. The switch in activity mainly depends on the interplay of loops LA and L2. Especially,an extended L2 loop seems to be required for protease inhibition. Accordingly this loop may indicate which members of the HtrA family are able to act as a chaperone one. Loop LA and L2 not only fulfill a regulatory role but also carry several hydrophobic residues that line the inner cavity and should form the binding platform for misfolded proteins*(ChristophSpiesset al.,1999)*.

2.9 Structural Features:

The various structure features of HtrA from various sources have been discussed in the following sub-headings:

2.9.1 The Structural Unit:

HtrA family shares a modular architecture composed of an N-terminal segment believed to have regulatory function, a conserved typsin like protease domain and 1 or 2 PDZ domains.PDZ domains are protein modules that mediate specific protein-protein interaction and bind preferentially to the three to four residues atthe C-terminal of target protein.PDZ domains of HtrA contains a 20 residue following the first Beta strand that is important for inter and intra molecular contacts within the oligomer.The functional unit

of HtrA appears to be a trimer which is stabilized exclusively by residues of protease domain. The basic trimer has funnel like shape with the protease domains located at its top and PDZ domains protruding to the outside. Once substrate has been bound, they have to be delivered into the interior of the funnel and the proteolytic sites.

2.9.2 Sequential Comparison Of DegP, DegQ And DegS:

After the initial identification of HtrA (High temperature requirement A, DegP) as an essential protein for cell survival at high temperatures *(Lipinska et al., 1988; Strauch andBeckwith, 1988),* the hosts of HtrA homologues were isolated from a variety of species. These HtrA family proteins normally contain two conserved core domains, a chymotrypsin-like protease domain, and at least one C-terminal PDZ domain.In *E. coli*, DegP, DegQ, and DegS comprise the HtrA family of proteins. These three proteins exhibit a high degree of sequence homology in their protease domains. However, DegP and DegQ harbor two PDZ domains, whereas DegS contains only one. Many ofthe other HtrA homologues isolated from Gram-positive bacteria, cyanobacteria, and mammals also harbor only onePDZ domain. In contrast to the high sequence homology found in htrA homologues of various species, one specific region in the protease domain exhibits only minimal sequence homology. This region of DegP is called the Q linker, as many Gln residues have been discovered in the DegP of *E. coli(Woottonet al., 1989).* Structurally, the Q-linker is positioned between the β1 and β2of the protease domain. The Q-linker of DegP encompasses approximately 40 amino acids, whereas in *E. coli* DegQ and many HtrA homologues, the Q linker isonly about 20 amino acids long. In the DegS found in *E. coli* or in human HtrA2, only a few, if any, amino acid residues are located within this region. Upon comparison of the sequences of the HtrA family proteins found near the Q linker, it was determined that many HtrA homologues exhibit sequence homology with the Q-linker of *E. coli* DegQ, which is of a similar length. Therefore, we propose that the HtrA homologues found in many bacteria should be redesignated as DegQ homologues.

2.10 Function of Deg P, DegQ, and DegS

Bacterial proteolysis is a vital component of a variety of essential cellular functions, including protein turn-over, recycling, activation, and the degradation of

abnormal proteins. Bacterial HtrA proteins perform several functions, which include non-destructive protein processing *(Carvardet al., 1989),* modulation of signaling pathways mediated by the elimination of regulatory proteins *(Alba et al., 2001),*resistance to heat stress*(Lipinskaet al., 1988; Li et al., 1996; Poquet et al., 2000; Wonderlinget al., 2004)* and virulence. *E. coli*HtrA/DegP was initially identified as a required protein for bacterial survival at high temperatures, or for the degradation of abnormalperiplasmic proteins. It has been proposed that the primary role of DegP is the digestion of abnormal proteins within the periplasmic space. *E. coli* DegP is a serine protease which forms a catalytic triad (His105, Asp135 and Ser210)and its proteolytic activities are inhibited only by diisopropylfluorophosphate (DFP). A number of proteins have been described as natural substrates for *E.coli* DegP. These proteins include colicinAlysis protein, K88 and K99 pilin subunits, MalSand PapApilin. DegP does not cleave folded proteins, such as native MalS, α-lactalbumin, or insulin, but does appear to degrade substrates which are at least partially unfolded. Therefore, it has been suggested that the access of substrates to active sites might be restricted. In addition to proteolytic activity, DegP has also been reported to exhibit a chaperone activity. Interestingly, DegP exhibits this molecular chaperone activity only at low temperatures, and its proteolytic activity increases rapidly between temperatures of32°C and 42°C. Therefore, DegP is believed to possess a temperature dependent functional switch, which enables a change in its function, from chaperone to protease.

The DegQ and DegS genes were identified on the basis of their sequence homology to DegP.DegQ was initially identified as a multicopy*E.coli* suppressor with a prc null mutation, and it has also been observed that DegQ is a functional substitute for DegP when overexpressed in *E. coli*. However, the DegQ gene is not heat-inducible, nor is it relevant to cell growth under normal conditions. DegQ, a serine protease, exhibits a substrate specificity profile very similar to that of DegP, and recognizes both the Val/Xaa and the Ile/Xaa sequences at the P1 site. However, many HtrA homologues, which have the sequence homology with *E. coli* DegQ in Q-liner region, are assumed to play vital roles in bacterial protein quality control, because many bacteria lack DegP and DegS. Differential studies have been performed by characterization of *E.coli* DegQ and DegS genes encoding homologues of DegP(*Patrick R.H Waller et al.,*1996). For instance,*T.*

maritima, a hyperthermophilic bacterium, possesses only a DegQ homologue, HtrA. This protein also exhibits dual function, acting as either a protease or a chaperone, depending on the conditions. Various studies have been conducted regarding crystal structure of protease domain of HtrA*T.maritima* in order to have its structural feature and to see orientation of various amino acids in it(*Dong Young Kim et al.,*2002). Taken together, it is assumed that DegQs might have an essential role in protein quality control and their activities are also modulated from molecular chaperone to protease in temperature-dependent manner(*Xiao-chenBaiet al.,*2011). Genetically, *E.coli* strains with mutated *DegS* genes are characterized by the small colony phenotype under all growth conditions. However, it is now known that DegS, a membrane-anchored periplasmic protease, performs a critical function in the σE-stress response pathway. This pathway is triggered by the binding of C-terminal peptides of OMPs, upon exposure to heat stress, to the PDZ domain of DegS. The structural rearrangement of DegS results in the activation of the catalytic site of the protease domain, thereby affecting the cleavage of the periplasmic domain of RseA, which is an anti-σ factor.

All living organisms employ dedicated chaperones and proteases to monitor and control the state of cellular proteins. Failure of this quality control can lead to protein aggregation, a malfunction that is correlated with fatal protein-folding diseases. DegP is a member of the widely conserved HtrA family of serine proteases that are crucial to maintain protein homeostasis in extra cytoplasmic compartments. The bacterial representatives DegP and DegS have key functions in the unfolded protein response of the cell envelope, whereas the four human HtrA proteins are implicated in many severe disorders including Parkinson's and Alzheimer's diseases. HtrA proteins encompass a catalytic domain with a chymotrypsin-like fold and one or two carboxy-terminal PDZ domains, which are well-characterized protein–protein interaction modules.The protease domains of three protomers interact closely to form a trimer that is the basic building block of htrA oligomers*(Jiansen Jiang et al., 2008)*. The projecting PDZ domains either participate in protein degradation by presenting substrates to the protease or offer a binding site for an allosteric activator that stimulates protease function. The reversible activation mechanism ensures that the digestive mode of HtrA proteins can be precisely switched on and off depending on the needs of the cell.

2.11 Regulation of HtrA:

A complex network of signal transduction pathways regulates expression of HtrA.

2.11.1 The RpoEregulon:-

The *htrA* gene resides in a single gene operon at 3.9 centisomes on the *E.coli* chromosomes.Transcription of *htrA* depends on alternative sigma factor,RpoE(sigma-E).RpoE is activated in response to signal generated by misfolded proteins in periplasm.The activity of RpoE is modulated by the product of three genes,*RseA, RseB, RseC*,which lie downstream of the *rpoE* gene.RseA is an inner membrane protein with terminal cyoplasmic domain and large coiled-coil periplasmic domain.Under basal condition the cytoplasmic domain binds RpoE,preventing transcription of RpoEregulon,i.e. it act as anti-sigma factor .The periplasmic domain of RseA, interacting with periplasmic protein RseB senses when misfolded proteins are present in periplasm, an conformational signal causes RpoE to be released from the cytoplasmic domain.

2.11.2 Cpx pathway:-

It is a classical two component regulatory system comprised of a sensor transmembrane protein CpxA and a response regulator CpxR.CpxR is stimulating transcription of the degP promoter only in the phosphorylated state. Phosphorylation of CpxR is controlled by CpxA which can act both as protein phosphatase and as a kinase. The switch from phosphatase to kinase activity is again controlled by concentration of misfolded proteins.This control is mediated by another protein,CpxP which upon interaction with unfolded proteins no longer binds to CpxA and thus allow CpxA to activate CpxR(Fig 2.2).

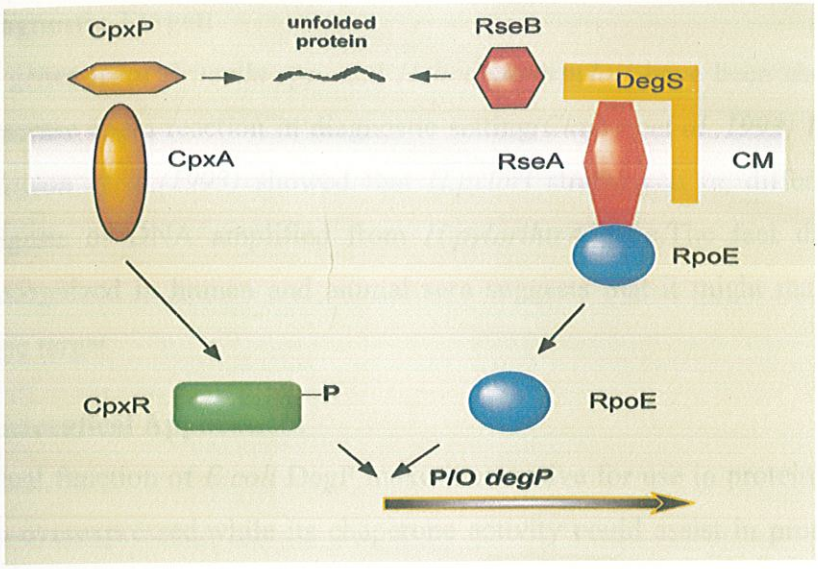


Fig 2.2 Regulation of HtrA by Cpx pathway

2.12HtrAas a tool:

2.12.1 As Vaccines:

HtrA mutants of *S.tymphirium* and *Y.entrocolitica* are not just attenuated in mice but also act as vaccines, protecting mice against subsequent challenge with virulent wild-type strains.An aroC, aroD,htrA mutant of *S.tymphirium* has recently shown promise as atyphoid vaccine in clinical trials in human.Recent study has highlighted the potential of a multivalent vaccine consisting of an HtrA*Salmonella* mutantexpressing epitopes from Herpes simplex virus and tetanus toxin*(Chabalgoityet al., 1996). Tothet al.(1995)* have shown that recombinant vaccinia virus expressing HtrA is able to elicit an antibody response against HtrA in mice,raising the possibility of using HtrA protein as a vaccine component although no protective effect has been demonstrated but these findings and research can lead to various other possibilities in production of various vaccines.

2.12.2 As Cloning Hosts:

Expression of several recombinant proteins has been shown to be improved in HtrAnegative hosts-examples include truncated derivatives of diphtheria toxin*(Zdanovskyet al.,1992)*, heat-labile enterotoxin mutant proteins*(Wulfing and Rappuoli, 1997)* and the recombinant S1subunit of pertussis toxin*(Barbieriet al.,1990)*.

2.12.3 As Diagnostic Target:

HtrA genes from *Brucella spp.* and *Helicobactorpylori* have been used as target for the polymerase chain reaction in diagnostic setting*(Clayton et al.,1993; Da Costa et al.,1996). Clayton et al.(1993)* showed that *H.pylori* strains can be differentiated by restriction digests of DNA amplified from *H.pylorihtrA* gene.The fact that HtrA is commonly recognized in human and animal sera suggests that it might make a useful sero-diagnostic target.

2.12.4 Pharmaceutical Application:

The dual function of *E.coli* DegP make it attractive for use in protein production when it is co-overexpressed,while its chaperone activity could assist in protein folding and could thus increase the amount of recombinant protein, its protease activity should remove misfolded product. Potential applications include the use of DegP and DegS as drug targets for antimicrobials.DegP is an essential virulence factor and has been studied in various other organisms*(Marco Cassoneet al.,2012)*and DegS is an essential protein even under laboratory conditions. Furthermore, 15% of known human proteases are under investigation as potential drug target. Based on current reports,HtrA1 and HtrA2 could be explored as potential drug target for osteoarthritis, cancer, and aging. Anti-cancer drugs could be envisaged that selectively activate the HtrA 1 promoter. In cases where the protease activity of HtrA would be involved in causing disease, for example, via degradation of extracellular matrix proteins during arthritis, inhibitors could be generated, which would selectively inactivate proteolysis but not substrate binding. These would not only prevent substrate degradation but may even lead to an increased life span of substrates because binding of HtrA could protect against or degradation by other proteases.

2.13 *Lactobacillus* Protease:

Lactobacillus acidophilus is a homofermentativeGram positive bacterial species, fermenting sugars into lactic acid, and grows readily at pH values 6.2 to 6.6 and has an optimum growth temperature of around 35°C. *L. acidophilus* occurs naturally in the human and animal gastrointestinal tract, mouth, and vagina.

Protease is found in both prokaryotes and eukaryotes. Very few studies have been conducted on *Lactobacillus* species regarding this particular gene. Studies on *L. helveticus* has been conducted in order to characterize the stress inducible gene i.e. *htrA*.The deduced amino acid sequence of this gene exhibited 30% identity with the HtrA protein from *Escherichia coli*.The putative catalytic triad and a PDZ domain that characterize the HtrA family of known bacterial serine proteases were also found in the sequence. Expression of the *L. helveticus htrA* gene was analyzed under the various stress conditions at the transcriptional level.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials: All the chemicals prepared were listed in APPENDIX A.

3.1.1 *Microbial culture*:-

Lyophilized *L. acidophilus*culture (MTCC No. *10708*) was obtained from *IMTECH CHANDIGARH.*

3.1.2 Chemicals:

The chemicals used in the present study were obtained from Merck Limited and Qualigens fine chemicals Limited. The various media used in the study namely Agerose gel, MRS Agar, MRS Broth, were manufactured by Merck Ltd. (Appendix-A)

3.1.3 Primers:

Forward primerwas designed using an initial stretch of 20 nucleotides from gene of interest and NheI restriction site was added with few additional bases (ATTT) at the 5' end.

5'-ATT TGC TAG CAT GAT ATT AGG TAA TAT GAG-3'

Tm	53.4°C
GC content	30%

Reverse primer was designed using reverse compliment of last 20 nucleotides from gene of interest XhoI restriction site was added with few additional bases (ATTAA) at the 5' end.

5'-ATT AAC TCG AGT TAA TTA CCT TCA AGT TTT AC-3'

Tm	54.2°C
GC content	28.1%

3.2 Methods:

3.2.1 Media preparation:

MRS medium were used as solid or liquid media for growth of *L. acidophilus* which was prepared by mixing a desired concentration of media in distilled water and then followed by autoclaving at 121°C and 15 lb/in² pressure for 15-20 minutes.

3.2.2 Inoculation:

After media sterilization, transferred Lyophilized *L. acidophilus* to the media in Laminar Airflow Chamber. Then the media was incubated at 37°C in incubator shaker for 48 hours.

3.2.3 Streaking on MRS AGAR Plate-

L. acidophilus was streaked on the MRS agar plate from the culture flask. Plates were incubated at 37°C in incubator for 48 hours.

3.2.4 Gram's Staining

1) A primary stain (crystal violet) was applied to a heat fixed smear of a *Lactobacillus* culture, stain was left on the smear for 1 minute. Stain was washed with water so that excess stain was washed off.
2) Gram's Iodine was added, which binds to crystal violet and traps it in the cell. Washing was done so that excess stain was removed.
3) Rapid decolorizationwas done with ethanol.
4) Counterstaining was done with Safranin.
5) Slide was observed under microscope at 100X magnification.

3.2.5Competent Cells Preparation

A primary culture was prepared from LB plate on which *E. coli* culture was grown. Primary culture: A colony was inoculated from LB plate into 10 ml LB liquid medium and incubated at 37 °C for overnight.

Secondary culture : 1ml primary culture was taken and inoculated into 100 ml LB medium and keep it in at shaker at 37°Ctill it attained the OD of 0.25-0.3 at 600nm (usually it takes about 1.5-2 hours).

Cells are transferred into 2 ml eppendorftubes and kept it at ice for 15 min and then were centrifuged for 10 min at 3000rpm at 4°C.

Medium was discarded and the cell pellet was resuspended in 1 ml cold 0.1M $CaCl_2$. Cells were kept on ice for 30 min andcentrifuged again. Supernatant was discarded and cell pallet was resuspended in 1 ml 0.1 M $CaCl_2$ solution plus 15% glycerol.

The tubes were freezedin liquid nitrogen and then transferred to -80°C freezer.

3.2.6Plasmid Vector pET28aIsolation

5 ml LB medium containing proper antibiotics was inoculated with a single bacterial colony. The tube was incubated at 37°C overnight with vigorous shaking at 200 rpm. Bacteria palletwas recovered from the culture by centrifuging it at 10,000rpm for 5 minutes at room temperature. Supernatant was discarded. Bacterial pellet was resuspened in a total of 100 µl ice-cooled solution I (25mM).200µl of solution II was added at room temperature to the suspension and mixed thoroughly by repeated gentle inversions.150 µl ice-cold Solution III was added to the lysate and mixed thoroughly by repeated gentle inversion. The mixture was centrifuged at 12,000g for 5 minutes at 4°Cresulting supernatant was recovered. Equal volume of PCI was added to precipitate the plasmid DNA and mixed thoroughly by repeated gentle inversions. Upper layer was taken and washed with isopropanol. Then again washing was done with 70% ethanol. dH_2O or TE was used to dissolve the pellet. 2µl RNAseA (10mg/ml) was added to the mixture and was incubated for 30 minutes at 37°C temperature to remove RNA.

3.2.7 DNA Extraction Using CTAB

Cells were centrifuged at 10000rpm for 5minutes.700µl of CTAB solution was added. The mixture of pallet and CTAB was incubated for 1hr at 60°C in water bath. To each tube 700µl of Chloroform: Isoamyl alcohol (24:1) was added and mixed by gentle inversion. After proper mixing, solution was centrifuged at 10000 rpm for 10 min was done. The upper aqueous phase was transferred to a clean microfuge tube. To each tube

500µl of ice cold isopropanol was added.Tubes were inverted slowly several times and were placedin -20°C for 1 hr after the addition of isopropanol to precipitate the DNA. To wash the DNA, the precipitated form was transferred into a microfuge tube containing 200µl of ice cold 70 % ethanol and centrifuged at 10000 rpm for 2 minutessupernatant was discarded and the DNA pellet was left to dry. In the DNA pallet was resuspened in sterile DNAse free water. RNAse A (10µg/ml) was added to the water prior to dissolving the DNA to remove any RNA in the preparation (10µl RNAse A in 10ml H2O).

3.2.8 DNA quality confirmation

- 1 % solution of agarosewas prepared by melting 1 g of agarose in 100 ml of 0.5x TAE buffer in a microwave for approximately 2 min.
- 2.5µl of ethidium bromide was added.
- Gel was castedusing a supplied tray and comb and was left to solidify.
- The DNA sample was loaded into the following separate wells

  3µL 1kb ladder

  5µL sample + 2µL 6x Loading Buffer
- Gel wasrun for 30 min at 100 V.
- The gel was observed under UV in GelDoc.

3.2.9Gene Amplification through PCR

S. NO.	Stage	Step	Temperature	Time
1.	STAGE 1 *1 CYCLE		94° C	5 mins
2.	STAGE 2 *30 CYCLES	STEP 1	94° C	1 min
		STEP 2	45°C	90 sec
		STEP 3	72° C	1min
3.	STAGE 3 *1 CYCLE		72° C	12mins

PCR Master Mix:

Components	Volume
Template DNA (50ng)	5µl
Primers (10µmole)	1µl (each)
10X PCR Buffer(with Mgcl2)	5µl
10mM DNTPs	1µl
Autoclaved Water	36µl
Taq DNA Polymerase	1µl
Total	50µl

To perform several parallel reactions, master mix was prepared containing autoclaved distilled water, buffer, dNTPs, primers and Taq DNA polymerase and template DNA solutions was added at lastin a single tube, and aliquoted into individual tubes. Samples were placed in a thermocycler PCR.

3.2.10Agarose gel electrophoresis of PCR Products

The amplified PCR product 8µl mixed with 2µl of gel loading dye was loaded on 1.2 % agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding 5µl ethidium bromide working solution (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad)

3.2.11Restriction Digestion (plasmid vector)

Restriction digestion is basically done by restriction endonuclease in order to cleave the restriction sites in the vector plasmid and the gene of interest with the same enzyme in order to proper ligation and cloning within a suitable expression host.

The plasmid vectors were digested using following restriction enzymes:

1) Forward Primer:-Nhe 1 (5' G/CTAGC 3')

2) Reverse Primer:-Xho 1 (5' C/TCGAG 3')

Single Digestion

Vector Plasmid	8µl
Forward NheI Primer	1µl
10X Assay Buffer(2.1)	1µl
Total	10µl

Double Digestion

Vector Plasmid	7µl
Forward NheI Primer	1µl
Reverse XhoI Primers	1 µl
10X Assay Buffer(2.1)	1µl
Total	10µl

Procedure:-

1) The reaction mixture was made upto 10µl for each reaction as described above given table.

2) The sample was incubated for 4 hours at 37^0C.

3) The digested mixture was loaded on 1 % Agarose Gel (2µl product+ 1µl Dye) with a ladder of 1Kb.

4) The gel was exposed to UV light and photograph was taken.

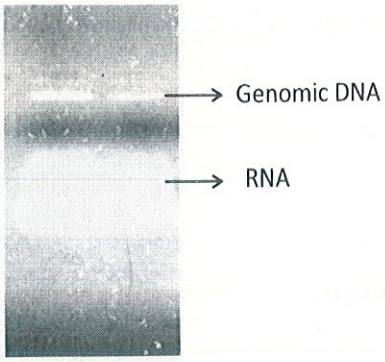


Fig 4.2 Genomic DNA isolated from *L. acidophilus.*

4.3pET 28aPlasmid Vector Isolation

The Plasmid pET28a was isolated from transformed *E.coli* DH5α cells usingstandard protocol.All the samples were loaded on to the gel showing the presence of plasmid in all the wells. 1Kb ladder was also used in order to check the definite size of the plasmid,Three separate bands of plasmid were observed almost in all wells. Linear plasmid band was found corresponded to ~5.2 kb.

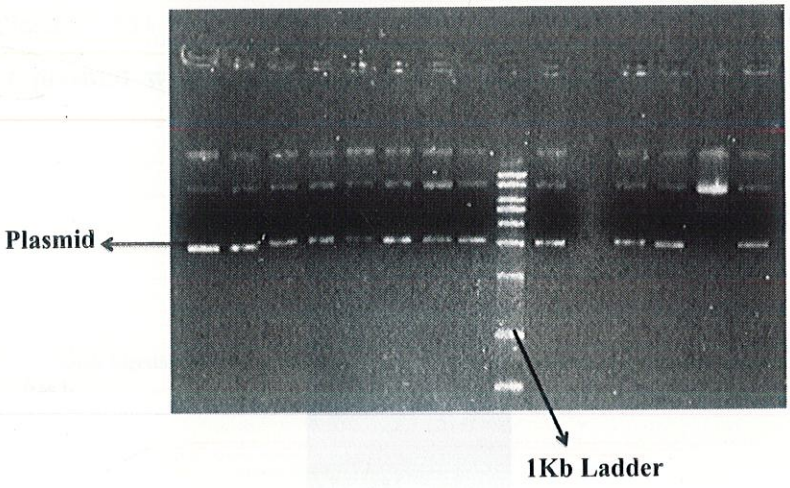


Fig 4.3 pET 28a plasmid vector on 1 % Agarose gel

4.4 PCR amplifications of HtrAby using specific primers.

A band of ~1.2 kb was observed on 1 % agarose gel. This size was found to be equivalent to HtrA gene of *L. acidophilus*.

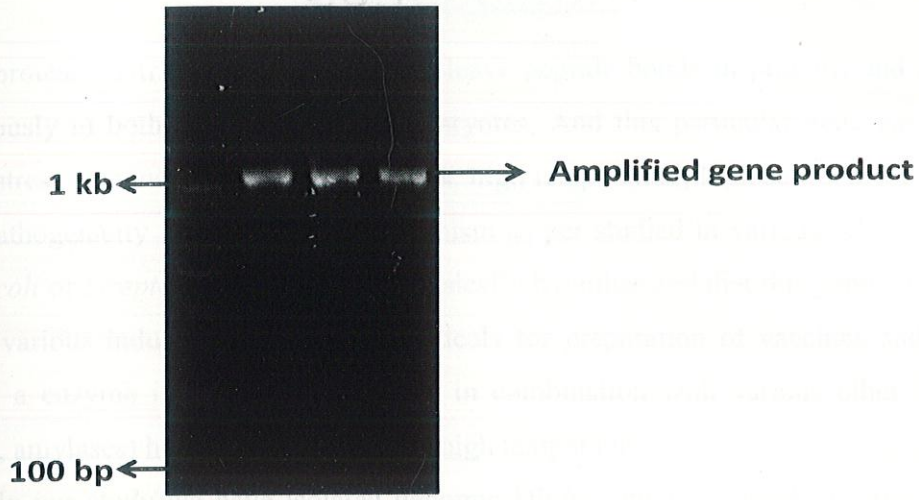


Fig 4.4 PCR amplified HtrA gene of *L. acidophilus*

4.5 Restriction Digestion of plasmid vector

The isolated Plasmid was treated with two restriction enzymes i.e. Nhe 1 and Xho 1. A single band of plasmid with a size of ~ 5 kb was observed after single and double digestion.

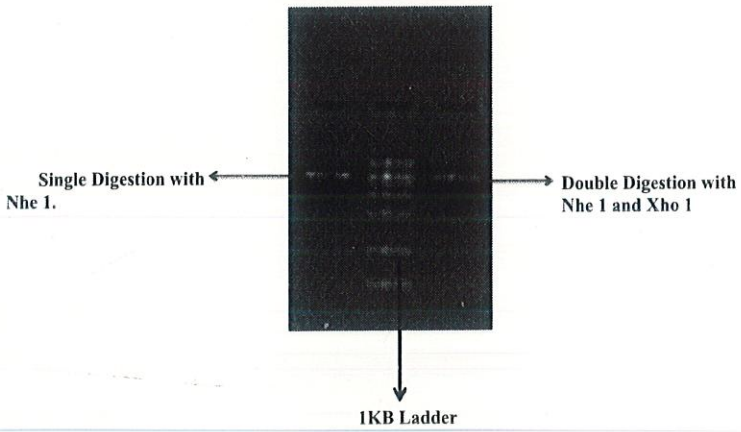


Fig 4.5 Single and double digestion of pET 28a plasmid vector (Lane 1 and lane 3 respectively)

CHAPTER - 5

CONCLUSION

Serine protease, HtrA is an enzyme that cleave peptide bonds in proteins and is found ubiquitously in both eukaryotes and prokaryotes, And this particular gene has various role in stress responses like oxidative stress, high temperature, high salt content and also has a pathogenicity factor in various organism as per studied in various other organism like *E. coli* or *Streptococcus spp.* So we basically hypothesized that this gene might have role in various industries like pharmaceuticals for preparation of vaccines and can be used as a enzyme in detergent industries in combination with various other enzymes (lipases, amylases) having high stability at high temperature.

In our study we have isolated genomic DNA from *L. acidophilus*, and pET28a plasmid vector from *E. coli* DH5α. We were able to optimize PCR conditions for the amplification of HtrA gene from *L. acidophilus* using specific Primers and obtained an amplified ~1.2 kb gene fragment.

APPENDIX-A

List of chemicals:

Competent cells chemicals:-

- Single colony of E. coli cells to be transformed
- LB medium
- M CaCl2, ice cold
- LB amp plates
- 42 °C water bath
- 1M CaCl2 +15% glycerol, sterile

Plasmid Isolation chemicals:-

Lysis Buffer(solution 1):-

50mM Glucose	10ml
0.5M EDTA	1ml(pH-8.0)
1M Tris	12.5ml(pH-8.0)
Autoclaved water	85.5ml(store at 4^0C)

Solution II (Lysis buffer II): Freshly prepared 0.2 N NaOH, 1% SDS. Store at room temperature (RT)

Isopropanol: Stored at -20 °C

Solution III(Lysis buffer II):

5M Potassium Acetate	30 ml
Glacial acetic acid	5.75 ml
Autoclaved water	14.25 ml

1M Tris:

30.28 g of Tris in 250 ml of dH2O and adjust the final pH to 8.

0.5M EDTA:

46.53 g of EDTA in 250 ml of dH_2O and adjust the final pH to 8.

TE Buffer(1M)

Composition of TE Buffer (100ml)

0.2 M Tris base	50ml
0.1M EDTA	50ml

Chloroform – Isoamyl alcohol (24:1) – 5ml

Chloroform	4.8ml
Isoamyl alcohol	0.2ml

Extraction Buffer (100ml)

1% CTAB	1gm
50 mMTrisHcl	5ml of 1M
50mM EDTA	10ml of 0.5M
700Mm Nacl	70ml of 1 M
Water	Raise volume to 99ml and autoclave
1% β-Mercaptoethanol (added after autoclaving)	1ml

TAE Buffer

50X Stock solution of TAE was prepared by adding the following:

Composition of TAE Buffer

Tris base	24.2gm
Glacial acetic acid	5.71ml
EDTA(0.5M, pH 8.0)	10ml
Distilled water	Make up the vol. to 100ml

Gel loading dye(6X)

Composition of Gel loading dye

Bromophenol Blue	0.25%(w/v)
Xylene Cyanol	0.25%(w/v)
Glycerol in DW	30%(v/v)

APPENDIX B

DH5-α *E.coli*

This strain of E. Coli is not a pathogen, and was developed for laboratory cloning use.

lacZ Delta M15 mutation: Allows for blue-white screening for recombinant cells.

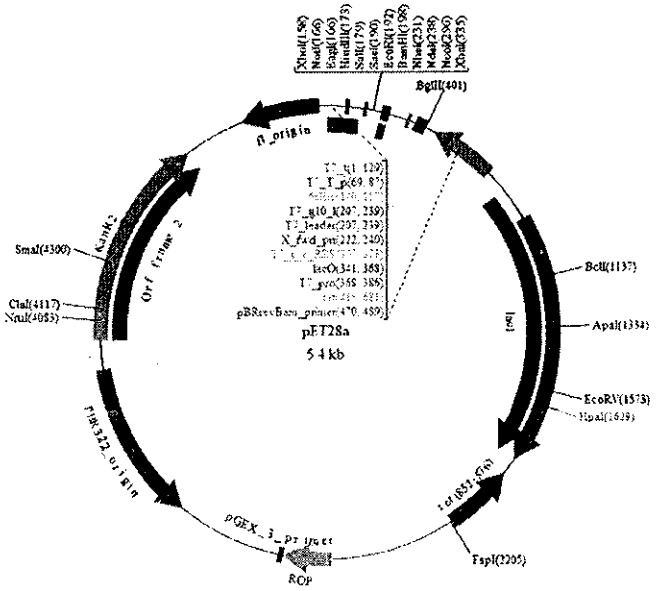
Genome structure

The genomic structure of this strain is a singular circular chromosome consisting of 4,686,137 nucleotides, 4359 genes, and 4128 protein encoding genes.

This strain also contains plasmids, and has the ability to accept plasmid insertion exceptionally well.

pET28a Vector

The pET28a-LIC vector was derived from expression plasmid pET28a. It is used for T7 promoter driven expression of recombinant proteins with the addition of a 19 amino acid N-terminal fusion tag containing a 6X His-tag followed by a thrombin protease cleavage site. Two stop codons areincluded in the vector at the C-terminal cloning site.



Information related to *L. acidophilus* HtrA

PID	58336431
Start	85570
Stop	86841
Strand	Direct
Synonym	LBA0083
Gene	htrA
Product	putative heat shock related serine protease
Upstream 100 bases	>100_bases TTTAAAAATCATTGATACAGAGCCGAATCACCCAACAAAATTAATTGAAATTTAGC AGTTTTAGCACCTTTTGTTCATATAATTTTCAAATTTTATCTGT
Gene Sequence	>1272_bases ATGATATTAGGTAATATGAGGGGAGAGTTAAGTATGGTAGAAAATCAAAATAATA ATCAACGACCAAGAAAAAATAGTAATGCAAAGATCATCACTACTGCAGCTATTGT AGGTGTAGTTGGTGGTCTGATCGGCGGTGGCGTTTCATATTATGCAGCTGATCAAA TGAATAACGCTACTGATACTACTACGGCACAAACTAGTGTATCTTCAAATAGTAGT AAGGTATCCGAAAAAAGTGCTAAAACCAGTGGTACGATGACTACTGCTTATAATG ATGTAAAAGGGGCTGTAGTGTCCGTTATTAACTTAAAGAGACAATCATCCTCAAGT AGCGCTAACTCTCTTTACAGCAGCTTATTTGGGGATGATAGCGATAGTTCTTCAGG TAAGAGCGGCAAGCTTGAGACTTACAGTGAAGGTTCCAGTGTAGTTTATATGAAGT CAAATGGTAAAGGCTATATTGTAACTAATAATCACGTTATTTCAGGCAGTGATGCA GTTCAAGTGCAACTTGCTAATGGCAAGACTGTTAGTGCAAAGGTTGTTGGGAAAG ATAGTACTACTGACTTAGCTGTTTTATCAATTGACGCTAAGTACGTAACACAAACA GCCGAATTTGGCGATTCTAAGAGTCTTCAAGCTGGTCAAACTGTAATTGCTGTAGG TTCACCACTTGGTAGTGAATATGCTTCTACGGTAACGCAAGGTATTATATCAGCAC CGGCTAGAACTATCTCAACTTCATCTGGTAATCAGCAAACAGTTATTCAAACAGAT GCAGCCATTAACCCAGGTAACTCAGGTGGTGCATTGGTTAACTCAGCTGGTCAAGT TATCGGTATTAATTCTATGAAGCTTGCTCAATCAAGTGATGGTACTTCTGTAGAAG GTATGGGATTTGCTATTCCTTCGAATGAAGTTGTAACTATCGTAAATGAATTGGTT AAGAAGGGTAAGATTACTCGTCCACAACTTGGTGTAAGAGTAGTTGCTCTTGAAG GTATTCCTGAAGCATACAGAAGTCGCTTAAAGATTAAGTCAAACCTTAAGAGTGGT

	ATCTATGTTGCTTCAATTAATAAGAATAGTTCAGCTGCAAATGCAGGCATGAAGAG CGGTGATGTCATTACTAAGGTAGATGGCAAGAAGGTTGATGATGTAGCATCATTAC ACAGTATCCTTTACAGTCACAAGGTTGGTGACACTGTGAACATAACTATTAATAGA AATGGTAGAGATGTCAACTTAAAGGTAAAACTTGAAGGTAATTAA
Downstream 100 bases	>100_bases TAAGTTTATTAGATAAAGAAAAAGGCTAGGTTACGAAATATAAACGTAATTTAGC CTTTTTTACTAACAATTGCTGTGTGTAACAAAAGATTATTCGTAT
Protein Sequence	>423_residues MILGNMRGELSMVENQNNNQRPRKNSNAKIITTAAIVGVVGGLIGGGVSYYAADQMN NATDTTTAQTSVSSNSSKVSEKSAKTSGTMTTAYNDVKGAVVSVINLKRQSSSSSANS LYSSLFGDDSDSSSGKSGKLETYSEGSSVVYMKSNGKGYIVTNNHVISGSDAVQVQLA NGKTVSAKVVGKDSTTDLAVLSIDAKYVTQTAEFGDSKSLQAGQTVIAVGSPLGSEYA STVTQGIISAPARTISTSSGNQQTVIQTDAAINPGNSGGALVNSAGQVIGINSMKLAQSS DGTSVEGMGFAIPSNEVVTIVNELVKKGKITRPQLGVRVVALEGIPEAYRSRLKIKSNL KSGIYVASINKNSSAANAGMKSGDVITKVDGKKVDDVASLHSILYSHKVGDTVNITIN RNGRDVNLKVKLEGN

BIBLIOGRAPHY

Ahmad Jomaa, Jack Iwanczyk, Julie Tran, And Joaquin Ortega.
Characterization of the Autocleavage Process of the *Escherichia coli*HtrA Protein: Implications for its Physiological Role, JOURNAL OF BACTERIOLOGY, Mar. 2009, p. 1924–1932.

Andre`AsSmeds, PekkaVarmanenAndAiriPalva,
Molecular Characterization of a Stress-Inducible Gene from *Lactobacillus helveticus,* JOURNAL OF BACTERIOLOGY, Dec. 1998, p. 6148–615 Vol. 180, No. 23.

Annette Tennstaedt, Simon Pöpsel, Linda Truebestein, Patrick Hauske, AnkeBrockmann, Nina Schmidt, Inga Irle,Human high temperature requirement serine protease a1 (htra1) degrades tau protein aggregates, JBC Papers in Press. Published on April 25, 2012 as Manuscript M111.316232Copyright 2012 by The American Society for Biochemistry and Molecular Biology, Inc.

Barbara Lipinska, MaciejZylicz, And Costa Georgopoulos
Department of Cellular, Viral and Molecular Biology, University of Utah Medical Center,Salt Lake City, Utah 84132, The htrA (DegP) Protein, Essential for Escherichia coli Survival atHigh Temperatures, Is an Endopeptidase, JOURNAL OF BACTERIOLOGY, Apr. 1990, p. 1791-1797

Barbara Lipinska, Olivier Fayet, Lisa Baird, And Costa Georgopoulos,
Identification, Characterization, and Mapping of the Escherichia coli
htrA Gene, Whose Product Is Essential for Bacterial Growth Only at Elevated Temperatures, JOURNAL OF BACTERIOLOGY, Mar. 1989, p. 1574-1584 Vol. 171, No.3.

Carol W. Gray, Robin V. Ward, Eric Karran, Sandra Turconi, Alison Rowles, Daniela Viglienghi, Christopher Southan, Amanda Barton, Kenneth G. Fantom, Andrew West, John Savopoulos, Namir J. Hassan, Helen Clinkenbeard, Charles Hanning, Bernard Amegadzie, John B. Davis, Colin Dingwall, George P. Livi and Caretha L. Creasy.
Characterization of human htrA2, a novel serine protease involved in themammalian cellular stress response, European.Journal.of Biochemistry.267, 5699±5710 (2000) q FEBS 2000.

C. Hal Jones, Tove' C. Bolken, Kevin F. Jones, Gloria O. Zeller And Dennis E. H. Ruby.
Conserved DegP Protease in Gram-Positive Bacteria Is Essential for Thermal and Oxidative Tolerance and Full Virulence in *Streptococcus pyogenes,* INFECTION AND IMMUNITY,Sept.2001,p.5538-5545.

ChristophSpiess, Alexandra Beil and Michael Ehrmann
Fakultat fur Biologie,Universitat Konstanz, A Temperature-Dependent Switch from Chaperone to Protease in a Widely Conserved Heat Shock Protein,Cell, Vol. 97, 339–347, April 30, 1999.

Dong Young Kim and KyeongKyu Kim
Structure and Function of htrA Family Proteins, the Key Players in Protein Quality Control,Journal of Biochemistry and Molecular Biology, Vol. 38, No. 3, May 2005, pp. 266-274.

Enrico Bertoli, and Fabio Tanfani,
htrA Heat Shock Protease Interacts with Phospholipid Membranes and Undergoes Conformational Changes, THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 14, Issue of April 4, pp. 8974–8982, 1997.

G. Hershkovits,Z. Dubinsky , D. J. Katcoff,
A novel homologue of the prokaryotic htrA gene is differentially expressed in the alga Haematococcuspluvialis following stress, Mol Gen Genet (1997) 254:345 ± 350

Harald Kolmar, Patrick R. H. Waller, And Robert T. Sauer
Department of Biology, Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139, The DegP and DegQ PeriplasmicEndoproteases of *Escherichia coli*:Specificity for Cleavage Sites and Substrate Conformation, JOURNAL OF BACTERIOLOGY, Oct. 1996, p. 5925–5929 Vol. 178, No. 20

Jack Iwanczyk, Daniela Damjanovic, Joel Kooistra, Vivian Leong, Ahmad Jomaa, Rodolfo Ghirlando, and Joaquin Ortega1,
Role of the PDZ Domains in *Escherichia coli* DegP Protein, JOURNAL OF BACTERIOLOGY, Apr. 2007, p. 3176–3186.

Jiansen Jiang, Xuefeng Zhang, Yong Chen, Yi Wu, Z. Hong Zhou, Zengyi Chang, Sen-Fang Sui
Activation of DegP chaperone-protease via formation of large cage-like oligomers upon binding to substrate proteins, Edited by Robert T. Sauer, Massachusetts Institute of Technology, Cambridge, MA, and approved June 19, 2008

Joanna Skorko-Glonek, Anna Sobiecka-Szkatula, Joanna Narkiewicz and Barbara Lipinska, University of Gdansk, Department of Biochemistry, Kładki 24, 80-822 Gdansk, Poland, The proteolytic activity of the htrA (DegP) proteinfrom Escherichia coli at low temperatures, Microbiology (2008), 154, 3649–3658.

Joanna Sko′ rko-Glonek, Barbara Lipin′ ska, KonradKrzewski, Giovanna Zolese, Marco Cassone, Alyssa L. Gagne, Lynn A. Spruce, Steven H. Seeholzer, and Michael E. Sebert The htrA protease from *Streptococcus pneumoniae*digests both denatured proteins and the competence stimulating peptide, JBC Papers in Press. Published on September 25, 2012 as Manuscript M112.391482

J. SkoÂrko-Glonek á D. Zurawa á E. Kuczwara
M. Wozniak á Z. Wypych á B. Lipinska, The Escherichia coli heat shock protease htrA participates in defense against oxidative stress, Mol Gen Genet (1999) 262: 342±350

Joanna Skorko-Glonek, KonradKrzewski, Barbara Lipinska,EnricoBertoli,
Comparison of the Structure of Wild type htrA Heat Shock Protease and Mutant htrA Proteins, Journal of Biological Chemistry,Vol270,No.19,Issue of May 12,pp.11140-11146,1995.

Kathryn L. Strauch, Kit Johnson, And Jon Beckwith,
Characterization of degP, a Gene Required for Proteolysis in the Cell Envelope and Essential for Growth of Escherichia coli at High Temperature, JOURNAL OF BACTERIOLOGY, May 1989, p. 2689-2696 Vol. 171, No. 5

Keiichiro Hiratsu, MitsukoAmemura, Hiroko Nashimoto, Hideo Shinagawa, Kozo Makino
The *rpoE*Gene of *Escherichia coli*, Which Encodes sigma E, Is Essential for Bacterial Growth at High Temperature, JOURNAL OF BACTERIOLOGY, May 1995, p. 2918–2922 Vol. 177, No. 10.

Lianna Munson &R.Ray Fall
Department of Chemistry,University of Colorado,Boulder, Colorado,U.S.A, Purification and characterization of *E.coli* Alkaline Phosphatase.A Biochemical Experiment,july 1978, VOL.6,No.3.

Mara Campioni, Anna Severino, LucreziaManente
The Serine Protease htrA1 Specifically Interacts and Degrades the Tuberous Sclerosis Complex 2 Protein,*Mol Cancer Res* 2010;8:1248-1260. July 29, 2010

Marie Westerlund, HomiraBehbahani, Sandra Gellhaar, Charlotte Forsell, Andrea Carmine Belin, Anna Anvret, Anna Zettergren, Hans Nissbrandt, Charlotta Lind,OlofSydow,Caroline Graff, Lars Olson, Maria Ankarcrona, and Dagmar Galter,
Altered enzymatic activity and allele frequency of OMI/htrA2 in Alzheimer's disease.

Patrick R. H. Waller And Robert T. Sauer
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, Characterization of *degQ*and *degS*, *Escherichia coli* Genes Encoding Homologs of the DegP Protease, JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 1146–1153 Vol. 178, No.4.

Tobias Krojer, Justyna Sawa1, Eva Scha¨fer, Helen R. Saibil, Michael Ehrmann& Tim Clausen, Structural basis for the regulated protease and chaperone function of DegP, Vol 453| 12 June 2008| doi:10.1038/nature07004

Tracy L.Raivio
Department of Biological Sciences, University of Alberta, Edmonton, Canada, Envelope stress responses and gram negative bacterial pathogenesis, Molecular Microbiology(2005).

Wilhelmina M. Huston, Joaquim E. Swedberg, Jonathan M. Harris, Terence P. Walsh,Sarah A. Mathews, Peter Timms,
The temperature activated htrA protease from pathogen Chlamydia trachomatis acts as both a chaperone and protease at 37^0C,VFEBS Letters 581 (2007) 3382–3386.

William R. Lyon and Michael G. Caparon,
Role for Serine Protease htrA (DegP) of *Streptococcus pyogenes*in the Biogenesis of Virulence Factors SpeB and the HemolysinStreptolysin S *Infect. Immun.* 2004, 72(3):1618. DOI: 10.1128/IAI.72.3.1618-1625.2004.

Zeller and Dennis E. Hruby C. Hal Jones, Tove' C. Bolken, Kevin F. Jones, Gloria O
Conserved DegP Protease in Gram-Positive Bacteria Is Essential for Thermal and Oxidative Tolerance and Full Virulence in *Streptococcus pyogenes* 10.1128/IAI.69.9.5538-5545.2001.

BRIEF RESUME:

Srashti Gopal Goyal, 091569

C.G.P.A: 5.8

B.Tech Biotechnology, JUIT

Interests: Microbiology, Animal Tisuue Culture and Molecular Biology.

Prakhar Srivastava, 091573

C.G.P.A: 6.2

B.Tech Biotechnology, JUIT

Interests: Microbiology, Molecular Biology and Genetic Engineering.