CLONING, EXPRESSION, PURIFICATION AND CHARACTERIZATION OF SOME THERMOSTABLE PROTEINS

Prachi Dabhade

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Certificate of Examination

This is to certify that the dissertation titled "**Cloning, Expression, Purification and Characterization of some Thermostable Proteins**" submitted by **Ms. Prachi Dabhade** (Reg. No. MS10009) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr Kausik Chattopadhyay Dr Rachna Chaba Prof. Purnananda Guptasarma

(Supervisor)

Dated: _____

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. Purnananda Guptasarma at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Prachi Dabhade
(Candidate)
Dated: _____

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Purnananda Guptasarma
(Supervisor)

Acknowledgement

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Notation

µ- Micro

APS- Ammonium persulphate

ATP- Adenosine triphosphate

bp- Base pair

DNA - Deoxyribonucleic acid

DW- Distilled water

EDTA- Ethylene diammine tetra acetate

IPTG- Isopropyl-beta-D-thio galactopyranoside

kb- Kilo base pair

kDa- kilo dalton

L- Litre

LB- Luria bertani

M- Molar

mg- Milligram

mL- Millilitre

mM- milli molar

NTP(s)- Nucleotide triphosphate

ºC- Degree Celsius

PAGE- Polyacrylamide gel electrophoresis

PCR- Polymerase Chain Reaction

rpm- Rotations per minute

SDS- Sodium dodecyl sulphate

TAE- Tris-acetate-EDTA

TEMED- Tetramethyl ethylene diamine

x g- times gravity

Contents

ABSTRACT

Proteins from hyperthermophilic organisms are mainly studied for their extraordinary structural stability and applicability in industrial processes carried out at extreme temperatures. Moreover cloning and expressing such proteins in mesophilic organisms such as *E. coli* does not change their properties, making it easy to study, engineer and understand them. In the present study, we have worked upon five such thermostable proteins viz DNA polymerase, Argininosuccinate lyase, and Beta glucosidase from *Pyrococcus furiosus* and Peptidase M50, and Protease Do from *Thermotoga maritima*. Our aim was to clone these genes, express, purify and do a complete biophysical characterization of these proteins. In order to understand the structure and function relationship in the above mentioned proteins, tools like circular dichroism, fluorescence spectroscopy, dynamic light scattering were used as well as denaturation (thermal and chemical) studies were carried out. Since some proteins (DNA polymerase, Beta glucosidase) have industrial importance, such studies are beneficial for achieving scientific insights, as well as exploitation of their commercial potential. The study also provides useful leads for further probing into properties and activities of these proteins.

1. INTRODUCTION

1.1 Proteins

"*The book of life is written in the language of DNA, but the story is made possible only by the activities of myriads of protein molecules.*"

Richard N. Perham

Proteins are macromolecules that consist of one or more chains of amino acid residues. Proteins can be of different lengths and structures depending upon the number of amino acids in the sequence and also on the type of amino acid from 20 different amino acids. We know that the function of the protein is determined by its structure.

Proteins are classified into four different levels of organization viz., primary (sequence of amino acids), secondary (helical or sheet-like structures and beta sheets), tertiary (super secondary structures) and quaternary structure (multiply-folded polypeptide chains).

Proteins are diverse in nature and different proteins behave differently in varied conditions. There are certain proteins that can withstand high temperature; high pressure and are resistant to different kinds of denaturants, etc. Here, we are trying to study some proteins from organisms that thrive in the extremes of nature and can withstand high temperature and pressure, i.e., proteins belonging to hyperthermophilic organisms. Of all kinds of proteins found in nature, some are designed to be optimally active at extremely high temperatures; these proteins are generally derived from hyperthermophilic organisms and are called as hyperthermophilic proteins.

In the present thesis proteins from two thermophilic organisms; *Pyrococcus furiosus* and *Thermotoga maritima* are cloned, expressed, purified and characterized.

1.2 Thermophiles/ Hyperthermophiles under Study

Hyperthermophiles are organisms that survive in extremely hot environments. These display an optimal growth temperature of above 80°C. This thesis describes proteins or enzymes sourced from two main organisms described below:

1.2.1 Pyrococcus furiosus

Pyrococcus furiosus belongs to archaea that is considered as the third domain of life. *P furiosus* has an optimal growth temperature of 100°C. It was originally isolated from geothermally heated marine sediments with temperatures between 90°C and 100°C collected in Italy. The organisms are spherical shaped, 0.8 to 2.5µm in diameter and exhibit monopolar polytrichous flagellation. They are anaerobic and heterotrophic in nature. *Pyrococcus furiosus* has a doubling time of only 37 minutes thus it can easily be used in the laboratory. [1]

It has been shown that *Pyrococcus furiosus* has modified its method of metabolizing sugars. It also has a distinct way to regenerate ATP. The bacterium has enzymes that contain tungsten that is very rarely found in biological molecules. [2]

1.2.2 Thermotoga maritima

Thermotoga maritima(Tm) is a hyperthermophilic bacterium; that has an optimal growth temperature of 80°C. It belongs to the order *Thermotogales.* It was first discovered in the sediment of a marine geothermal area near Vulcano, Italy. The organism is a non-sporulating, rod-shaped, gram-negative bacterium. [3]

Thermotoga maritima catabolizes sugars and polymers and produces carbon dioxide and hydrogen gas as by-products of fermentation. It is also capable of metabolizing cellulose, as well as xylan. [3]

1.3 Hyperthermophilic Enzymes

Enzymes from hyperthermophilic organisms are thermostable i.e. they are resistant to irreversible inactivation at high temperatures. [4] It has been known that hyperthermophilic proteins are highly similar to their mesophilic homologues. [5]

Hyperthermophilic enzymes are best studied for their extraordinary structural stability and applicability in industrial processes carried out at extreme temperatures.

These enzymes have become model systems to study enzyme evolution, enzyme stability and activity mechanisms, protein structure-function relationships, and biocatalysis under extreme conditions. [5]

There are several reasons for such extraordinary stability of hyperthermophilic enzymes, some of which are discussed below:

- Hyperthermophilic enzymes have more number of hydrogen bonds compared to their mesophilic counterparts.
- These have higher packing densities and higher secondary structure content.
- Hydrophobic interactions are improved in them.
- The surface areas in these enzymes are optimized and have tighter packing of the core.
- Stabilization of α-helix dipoles, binding of metal ions and fixation of polypeptide chain termini to the protein core is improved.
- Polypeptide chains are shortened.
- The proportion of proline and β-branched amino acids is increased whereas there is a decrease in the proportion of flexible residues like glycine.
- There is also a decrease in the number of uncharged and non-polar residues accompanied by an increase in the number of charged residues, especially on the surface.
- Electrostatic interactions are improved due to increased number of salt bridges.
- Thermo labile amino acids like asparagine, glutamine, cysteine and methionine are reduced.
- Solvation is reduced due to bulky side chains of amino acids.
- Intra- or inter-subunit contacts are optimized in these enzymes.
- α-helical and β-sheet content are dominated over loops and turns in hyperthermophilic enzymes.

Since the proteins from these organisms exhibit extraordinary stability, studying and understanding their structure, properties and activity is the primary aim of this thesis. We have cloned, expressed, purified and characterized the hyperthermophilic enzymes

mentioned below:

Protein	Organism	Gene ID	Protein ID	PDB ID	Gene Size	Protein Size
DNA Polymerase	*Pyrococcus furiosus*	1468044	NP_577941.1	2JGU	2300bp	90,113 Da
Peptidase M50	*Thermotoga maritima*	898350	NP_228492.1		615bp	23,609 Da
Protease Do	*Thermotoga maritima*	897635	NP_228381.1	1L1J	1380bp	49,924Da
Beta-glucosidase	*Pyrococcus furiosus*	1467902	NP_577802.1	3APG	1500bp	54,665Da
Argininosuccinate lyase	*Pyrococcus furiosus*	1468040	NP_577937.1		1381bp	51,348 Da

1.3.1 DNA polymerase

The DNA polymerase from *Pyrococcus furiosus(Pfu)* is a thermophilic DNA polymerase with an integrated 3'–5' exonuclease activity that corrects errors introduced during the polymerization. *Pfu* DNA Polymerase is a thermostable enzyme having a molecular weight of about 90 kDa. *Pfu* DNA polymerase has superior 'proofreading' properties compared to other thermostable polymerases. [6]

In Polymerase Chain Reaction (PCR), heating at very high temperature is needed to separate the DNA strands and thus many enzymes are not able to withstand such high temperature. Therefore, DNA polymerase from *Pyrococcus furiosus* (*Pfu* DNApol) can be used as it belongs to a hyperthermophile. It can withstand very high temperatures.

Pfu DNA polymerase is known to be monomeric in solution. A single polypeptide chain of 775 amino acids is folded into five distinct structural domains: the N-terminal domain, the 3′–5′ exonuclease domain, the palm domain, the fingers and the thumb domain. [7] We cloned the gene in pET23a vector and expressed in BL21 (DE3)pLysS cells. We looked at the oligomeric state, secondary structure, tryptophan fluorescence, chemical stability, thermal stability and activity of the protein. *Pfu* DNA Polymerase can be further used in the lab for PCR reactions.

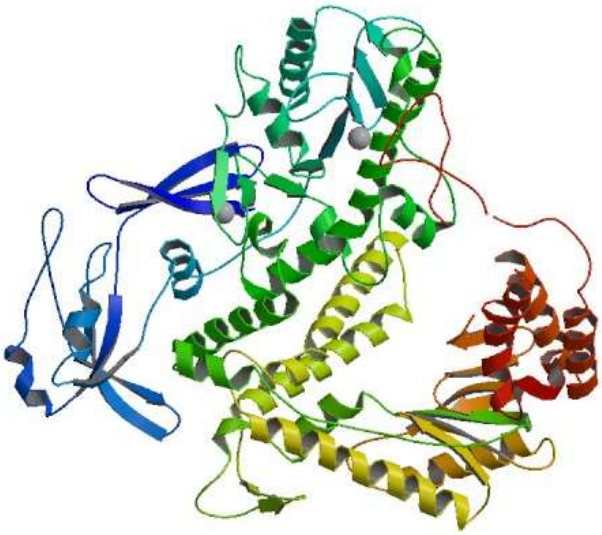


Figure 1.1 Crystal structure of *Pfu* DNA polymerase

1.3.2 Peptidase M50

Peptidase M50 is a metalloendopeptidase from *Thermtoga maritima*. Not much is known in the literature about this protein. We were successful in cloning the gene. We looked at the oligomeric state, secondary structure, tryptophan fluorescence, chemical stability and thermal stability of the protein in order to understand more about this protein. We modeled the structure using HH Pred.

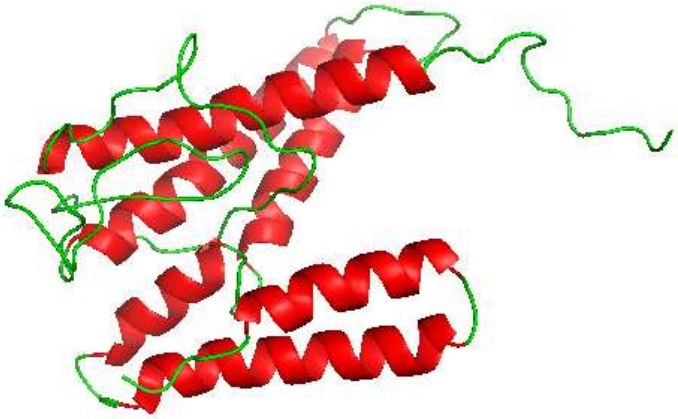


Figure 1.2 Modeled Structure of Peptidase M50 using HHPred

1.3.3 Beta Glucosidase

Beta glucosidase belongs to hyperthermophilic archaeon *Pyrococcus furiosus*. It is involved in the hydrolysis of the β-1, 4-glycosidic bond between the two glucose moieties of a disaccharide. The protein is known to exist in a tetrameric form. Beta glucosidase belongs to the glycoside hydrolase 1 (GH1) family. The enzymes of this family form α/β barrel which hydrolyze the substrate through a retaining mechanism so that the configuration of anomeric C atom remains same. Two glutamate residues serve as a general acid/base or nucleophile in the reaction. It has also been known that the catalytic dyad consisting of Glu207 and Glu372 hydrolyze the β-1, 4 bonds of its substrates. It has been known that it has SDS resistant dimers. [8]

We tried to clone the gene by adding 6 X His tag to N-terminal and also to C-terminal of the gene. The N tagged protein was not stable and was degrading; thus we used C tagged protein in our experiments.

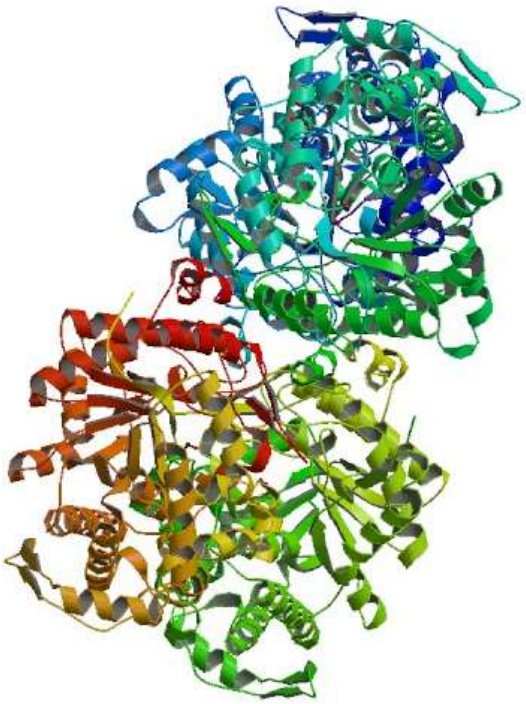


Figure 1.1 Crystal structure of *Pfu* Beta glucosidase

1.3.4 Argininosuccinate Lyase

Argininosuccinate lyase belonging to the hyperthermophilic archaeon *Pyrococcus furiosus* (PfuASL) is an important enzyme of Urea cycle that is involved in the reversible breakdown of argininosuccinate lyase into arginine and fumarate. It is involved in the biosynthesis of arginine. The protein is known to be present in soluble form in the cytosol. We looked at the oligomeric state, secondary structure, tryptophan fluorescence, chemical stability, and thermal stability of the protein.

1.3.5 Protease Do

Protease Do is periplasmic heat shock serine protease belonging to *Thermotoga maritima*. It functions as a molecular chaperone at low temperatures, and its proteolytic activity is turned on at elevated temperatures. It is known that it shows ATP-independent proteolytic activity. It also plays an important role in the degradation of misfolded proteins accumulated by heat shock or other stresses. It has a catalytic triad (Asp^{127}-His^{97}-Ser^{206}) in its active site. It is known to exist as a monomer. [9]

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Table 1: Bacterial Strains and Plasmids

Strains	Genotype
E.coli BL21(DE3)pLysS	*F⁻ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3) pLysS(cm^R)*
E. coli DH5α	*F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–*
E. coli XL1Blue	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[Tn10 proAB$^+$ $lacI^q$ Δ(acZ) M15] hsd17(r_K^- m_K^-)*
pET23a	T7 promoter, pBR322-type ColE1 origin, F1 phage origin for ssDNA production, Multiple Cloning Sites (BamHI to XhoI), ampicillin resistance gene (beta-lactamase), His tag

2.1.2 Media (For Bacterial Cultures)

Luria Broth (LB)

Component	Amount (g/L)
Tryptone	10.00
Yeast extract	5.00
NaCl	5.00

LB agar contains 2% agar in LB medium.

2.1.3 Buffers and Solutions

50X TAE buffer (pH 8)

Tris Cl	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA(pH 8)	100 mL
Deionized water	Up to 1L

Agarose gel (1%)

Agarose	1 g
Deionized Water	100 mL

Acrylamide

Acrylamide	30g
N,N'-Methylene-bisacrylamide	0.8g
Deionized Water	Up to 100mL

Ammonium persulfate (APS, 10%)

APS	100mg
Deionized Water	Up to 1ml

Lower Tris (4X), pH 8.8

Tris	18.17g
10% SDS	4 mL
Deionized Water	Up to 100 mL

Adjust the pH to 8.8 with 6 N HCl.

Upper Tris (4X), pH 6.8

Tris	6.06g
10% SDS	4 mL
Deionized water	Up to 100 mL

Adjust the pH 6.8 with 6 N HCl.

5X Sample loading buffer

Tris Cl(pH 6.8)	0.15M
SDS	5%
Glycerol	25%
β-mercaptoethanol	12.5%
Bromophenol blue	0.06%

Laemmli Buffer

Tris buffer	3g
SDS	1g
Glycine	14.4g
Deionized water	Up to 1L

Gel Staining Solution

Glacial acetic acid	10%
Methanol	40%
Coomassie Brilliant Blue R-250	0.1%
Deionized water	50%

Gel Destaining Solution

Glacial acetic acid	10%
Methanol	40%
Deionized water	50%

Native Lysis Buffer

NaH_2PO_4, pH 8.0	0.05M
NaCl	0.3M
Imidazole	0.01M

Native Wash Buffer

NaH_2PO_4, pH 8.0	0.05M
NaCl	0.3M
Imidazole	0.02M

Native Elution Buffer

NaH_2PO_4, pH 8.0	0.05M
NaCl	0.3M
Imidazole	0.25M

12% SDS Gel Composition

Resolving gel 12%		Stacking gel 5%	
Lower Tris	1.25 mL	Upper Tris	250 µl
Acrylamide	2 mL	Acrylamide	166.5 µl
Water	1.7 mL	Water	583.5 µl
APS	25 µl	APS	6.25 µl
TEMED	5 µl	TEMED	2.5 µl

2.2 METHODS

2.2.1 Preparation of *E.coli* competent cells

The process involves chemically treating the cells with CaCl2. Calcium ions being positively charged form a layer around the bacterial cell wall and help in the uptake of the negatively charged DNA. [10] The competent cells were prepared by following the steps given below:

1. A single colony of *E.coli* was inoculated in LB media and grown to saturation.
2. The culture was re-inoculated into 100 mL fresh LB medium at a dilution of 1:100 and grown to early log phase (A_{600} of 0.3-0.4).
3. The cells were chilled on ice for 15 minutes, centrifuged at 3000 x g for 15 minutes at 4°C in pre-chilled centrifuge tubes. Cells were kept on ice at all subsequent steps.
4. The supernatant was discarded and cells were resuspended in 20 mL of ice cold $CaCl_2$ solution (filtered and autoclaved) after which they were again centrifuged as in step 3.
5. The supernatant was again discarded and step 4 repeated.
6. The cell pellet obtained in step 5 was re-suspended in 2 mL of ice cold $CaCl_2$ solution.
7. Finally aliquots of 80 or 100µl were made from the suspension obtained above and these were used immediately or stored at -80°C till further use.

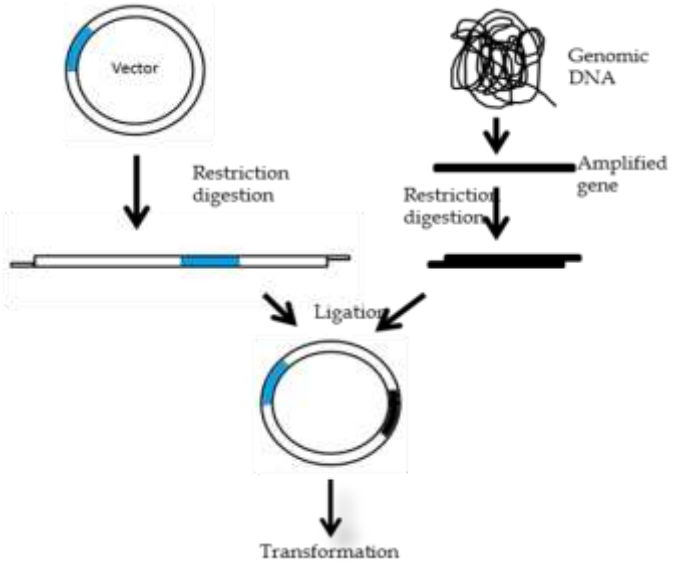


Figure 2.1: Schematic or cartoon representing the steps involved in cloning of a gene into vector.

2.2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is a technique used to amplify a gene. It involves the following steps:

Denaturation – In this step the reaction mixture is heated to 94-98 °C for 30-60seconds. It helps in disrupting the hydrogen bonds between complementary DNA strands and rendering it single stranded.

Annealing – During this step temperature is lowered to 50-65 °C for 20-40 seconds. It allows the binding of primers to the single stranded DNA template.

Annealing temperature in a polymerase chain reaction is decided by the Tm of the primers used for amplification.

Extension – The temperature used in this step depends upon the DNA polymerase used. The extension time depends on both the polymerase and the length of DNA fragment to be amplified. Generally, the temperature for extension step is 72 °C.

For amplification, a mixture of heat stable DNA polymerase, four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP) and forward and reverse primers which are complementary to the template are required. The PCR reactions were performed in vapo.protect from Eppendorf, USA.

Primers used

The following primers were used for amplifying DNA polymerase, Peptidase M50 and Protease Do genes. The forward and reverse primer contains Nde1 and Xho1 site respectively and the nucleotides coding for restriction sites are marked in red.

Table 2 List of primers used in the study

Name of primer	Length	Sequence(5'--------3')
DNA polymerase fwrd	26	5' AGCTATACATATGATTTTAGATGTGG 3'
DNA polymerase rev	33	5' GAATACTCTCGAGGGATTTTTTAATGTTAAGCC 3'
Peptidase M50 fwrd	31	5' AGCTATACATATGGTGAACATGAGGATAATC 3'
Peptidase M50 rev	34	5' GAATACTCTCGAGCCTCACTATCATCCAAACAAA 3'
Protease Do fwrd	35	5' ATTATACATATGAAAAAATTCTTCCTGACCATCGC 3'
Protease Do rev	43	5' ATTACACTCGAGTCTCTGATAGATGAAGCTCACGAAGACTTTC 3'

Optimized conditions for obtaining the amplified PCR products of the genes discussed in the thesis are given below:

Gene	Enzyme	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles
DNA Polymerase	Phusion	98°C 30 seconds	98°C 10 seconds	60°C 30 seconds	72°C 90 seconds	72°C 10 minutes	30
Peptidase M50	Phusion	98°C 5 minutes	98°C 30 seconds	55°C 60 seconds	72°C 90 seconds	72°C 5 minutes	30
Protease Do	Deep Vent	95°C 5 minutes	95°C 30 seconds	56°C 30 seconds	72°C 100 seconds	72°C 5 minutes	30

2.2.3 Restriction Digestion

Restriction digestion is a method to cleave DNA molecules at specific restriction sites using special enzymes called restriction endonucleases. These cleave the sugar-phosphate backbone of DNA. They commonly recognize recognition sequences that are mostly palindromes. Digestion reactions were carried out for 30 minutes (using 1 µl of enzyme/µg plasmid DNA or per 200ng of PCR product) at 37°C. The DNA (plasmid/ insert) to be digested is mixed with appropriate buffer and restriction enzyme/ enzymes of interest (generally 1unit/reaction) and kept at 37 °C. After digestion, DNA samples were separated on agarose gel, gel purified and quantitated. For all the genes mentioned above, Nde1 and Xho1 restriction enzymes were used for digestion. [10]

2.2.4 Ligation

Ligation is a process of joining DNA fragments with T4 DNA ligase or Quick Ligase. It involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. Ligation reactions were incubated at 24°C for two hours in case of T4 DNA ligase while it was set at 25°C for 10-15 minutes in case of Quick Ligase. For all the ligation reactions, insert:vector ratio used was 3:1.

For a particular amount of digested vector (generally 50ng), amount of digested PCR product (insert) required can be calculated using the following formula

$$\text{Amount of insert (ng)} = \frac{\text{Amount of digested vector (ng)} \times \text{molar ratio (insert: vector, 3: 1)} \times \text{size of insert (bp)}}{\text{Plasmid size (bp)}}$$

2.2.5 Transformation

After ligation, the ligation mixture was transformed into the cloning host's cells (XL1-Blue). The protocol for transformation is as follows:

1. The chemically competent cells were thawed on ice for 15 minutes.
2. Further, the ligation mixture was added to the cells and mixed gently. They were then incubated on ice for 15-20 minutes.
3. Heat shock was given to the cells at 42 °C for 90 seconds by incubating the samples in water bath.
4. 1 mL of sterile LB media was added to the cells and was kept in an incubator shaker at 37 °C for 1 hour.
5. The cells were centrifuged at 5000 rpm for 5 minutes and the supernatant was discarded.
6. The pelleted cells were resuspended in 100 µl of fresh media then plated on the LB agar plates with appropriate antibiotics.

The plates were kept inverted for overnight in an incubator set at 37°C and the transformants were observed the next day.

2.2.6 Screening for transformants

The plasmid DNA was isolated from the cultures of transformants/clones and the clones were checked for the integration of insert into the vector. Restriction endonuclease digestion was performed with respective enzymes (Nde1 and Xho1) and was checked by running it on agarose gel. If the insert of expected size is seen on the gel, the clone is confirmed.

Alternative method is by performing a colony PCR, in which colonies were picked up and resuspended in the PCR reaction master mix aliquoted in various tubes. This utilizes vector specific primers which will amplify the gene of interest if it integrates between the restriction sites in the multiple cloning sites.

The plasmid from confirmed clone was first confirmed by sequencing and then transformed into an expression host (BL21(DE3)pLysS) .

2.2.7 Glycerol stock (15%)

For making glycerol stocks1500 µl of an overnight grown culture (37°C) obtained by inoculating a single colony into sterile LB media supplemented with appropriate antibiotics was mixed with 500 µl of 60% glycerol (autoclaved). The stock was stored at -80 °C.

2.2.8 Agarose Gel Electrophoresis

Electrophoresis through agarose is a standard method to separate, identify and purify nucleic acids. An electric field is applied to move the negatively charged DNA through the gel. The percentage of agarose in the solidified gel determines the pore size of the matrix. The molecules travel through the pores in the gel at a speed that is inversely proportional to their size. Thus, a small DNA molecule will travel a greater distance than a large DNA molecule.

Here, 1% agarose gels were used throughout the study.

2.2.9 Purification of DNA fragment(s) from agarose gels

DNA was extracted using a gel extraction kit (Qiagen, Germany). The procedure was as follows:

1. The DNA was visualized using a UV transilluminator and the desired band was excised from the agarose gel and weighed. 3 volumes of solubilization and binding buffer (Buffer QG) was added to the gel slice.
2. It was then incubated at 50°C till complete solubilization of agarose gel is achieved.
3. Further, one gel volume of isopropanol was added and mixed by inverting the tube.
4. This mixture was then poured onto a QIAquick spin column to allow the adsorption of DNA onto the silica gel matrix. For this, centrifugation was done at 13000x g for 1 minute.
5. The flow through was discarded and impurities were washed away with 750µl of an ethanol containing buffer (Buffer PE).
6. DNA was finally eluted in (20-30µL) autoclaved distilled water, and quantitated.

2.2.10 PCR purification

The PCR product obtained from above step was purified using QIAquick PCR purification. The procedure followed is briefly described below:

1. Five volumes of PB buffer was added to one volume of PCR reaction and mixed.

2. To bind DNA, the sample was applied to the column and centrifuged at 13000 rpm for 1 minute. The flow through was discarded and column was placed back in same tube.

3. To wash, 750 µL PE buffer was added to the column. Centrifugation at 13000 rpm for 1 minute was carried out. The flow through was discarded.

4. Centrifuged once more to remove residual wash buffer.

5. To elute DNA, 20 µl deionized water was added to the center of the column and was allowed to stand for 1 minute. Then, centrifugation at 13,000 rpm for 1 minute was carried out.

2.2.11 Plasmid DNA isolation

Isolation of plasmid DNA was carried using QIAprep Spin miniprep kit. The procedure followed is described below:

1. Bacterial cells (3-5 mL) were pelleted by centrifugation at 13,000 rpm. The supernatant was discarded.

2. Pellet was resuspended in 250 µL of P1 buffer and mixed by pipetting.

3. Then 250 µL of P2 buffer was added and mixed gently, by inversion for lysis of the cells.

4. 350 µL of Neutralization buffer (Buffer N3) was then added and mixed by inversion.

5. The mixture was centrifuged at 13,000rpm for 10-15 minutes.

6. Then, the supernatant was loaded onto the columns and it was spun at 13,000 rpm for 1 minute.

7. Flow through was discarded and it was washed with 750 µL of wash buffer (Buffer PE) by centrifuging at 13,000rpm for 1 minute.

8. Centrifuged once more to remove residual wash buffer.

9. The plasmid DNA was eluted in 20µl deionized water in a fresh 1.5 ml microcentrifuge tube.

2.2.12 Purification of 6x His tagged proteins

Cell pellet was resuspended in native lysis buffer at 5 mL per gram wet weight. It was further followed by sonication to break the cells. The lysate was spun at 16,000 x g for 30 minutes at 4°C. The lysis supernatant or the lysate was loaded onto a pre equilibrated (equilibrated with 1X Native lysis buffer) Ni-NTA column. The column's flowthrough was collected after which the column was washed with native wash buffer. The bound protein was eluted with native elution buffer. [11]

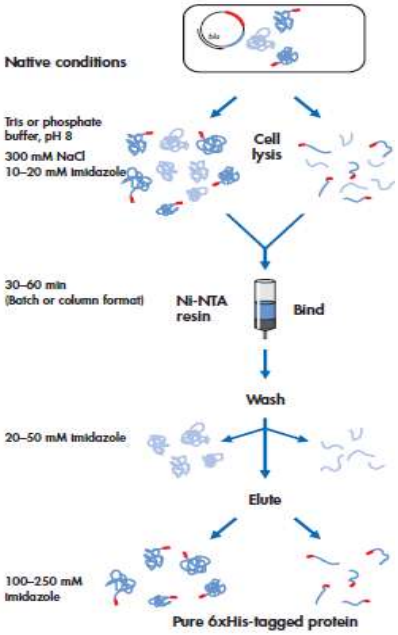


Figure 2.2 Protein Purification (The QIAexpressionist)

2.2.13 Dialysis

The eluted protein was dialysed against 20 mM Tris, pH 8.0 to remove the salts and imidazole.

2.2.14 UV-Vis Absorption spectroscopy

The absorption spectra was collected in the range of 200-600nm using a Cary 50UV-Vis spectrophotometer. The concentrations of the protein were estimated by taking UV absorption readings at 280nm and then calculating the concentration as per the predicted molar extinction coefficient using ProtParam tool (ExPASY Bioinformatics resource tool).

2.2.15 Gel Filtration Chromatography

After purifying the proteins using Ni-NTA column, they were re-purified using size exclusion chromatography. The gel filtration elution profile was collected on GE's Akta Purifier 10 system. The column and pumps were washed with filtered Millipore Elix-3 water and then equilibrated with respective buffer. The column used for the experiments was Superdex 200 10/300 GL (from GE Biosciences). It has a bed volume of 24mL. 500 µl of the

sample was loaded for analysis. The elution volumes were compared with the elution profiles of the standards run on the same column. The elution profile of the proteins depends upon the hydrodynamic volume of the protein. [11]

2.2.16 Fluorescence spectroscopy

The emission spectra was collected on a Cary Eclipse fluorimeter by exciting at 295nm and collecting the spectra in the range of 300-400nm. The slit width used for the experiment was 5nm for both excitation and emission.

2.2.17 Circular Dichroism (CD) Spectroscopy

Far UV-CD spectra for protein were collected on MOS 500 CD spectrometer (Biologic). Protein melting experiments (thermal denaturation) were performed on Chirascan(Applied Photophysics) system and samples were heated from 20 ˚C to 90 ˚C at a ramp rate of 5 degree/min and the signal at 222 nm was monitored. Raw ellipticity obtained was converted into mean residue ellipticity $[\theta]^{+}$ using the following formula

$$[\theta]^{+} = \frac{\theta_{obs}(\text{mdeg}) \times 100 \times \text{MRW}}{1000 \times \text{concentration (mg/ml)} \times \text{pathlength (cm)}}$$

$$\text{MRW} = \text{Mean Residue Weight} = \frac{\text{Total molecular weight of the protein(Da)}}{\text{Total number of amino acids}}$$

θ_{obs} = Raw Ellipticity

2.2.18 Dynamic Light Scattering (DLS)

Hydrodynamic radius of the protein was determined using dynamic light scattering instrument, Zetasizer Nano ZS 90 (Malvern). For this, samples were filtered through 0.2 micron polyvinylidene difluoride filters (Millipore). Hydrodynamic radius distribution versus percent scattering and percent mass data was collected, together with analysis of polydispersity using an isotropic sphere model. DLS measures the changes in scattering of light from molecules with time in solution to determine the translational diffusion coefficient and consequently find the hydrodynamic radius (RH) from the Stokes-Einstein equation. The

R_H depends on the shape of the molecule. It is generally directly proportional to the size and shape of the molecule. A calibration curve can then be constructed to assess the molecular weight of a given protein based on the measured R_H. [12]

3. RESULTS

3.1 DNA POLYMERASE

The DNA polymerase from *Pyrococcus furiosus (Pfu)* is a thermophilic DNA polymerase with an integrated 3'–5' exonuclease activity that corrects the errors introduced during the polymerization. *Pfu* DNA polymerase is a thermostable enzyme having a molecular weight of about 90 kDa. *Pfu* DNA polymerase has superior thermostability and 'proofreading' properties compared to other thermostable polymerases.

3.1.1 CLONING

3.1.1.1 Amplification

DNA polymerase was amplified from *Pfu* genomic DNA. The details for gene amplification or the conditions for PCR are mentioned in the materials and methods section. The desired band (2300bp) was obtained at 60°C using Phusion enzyme.

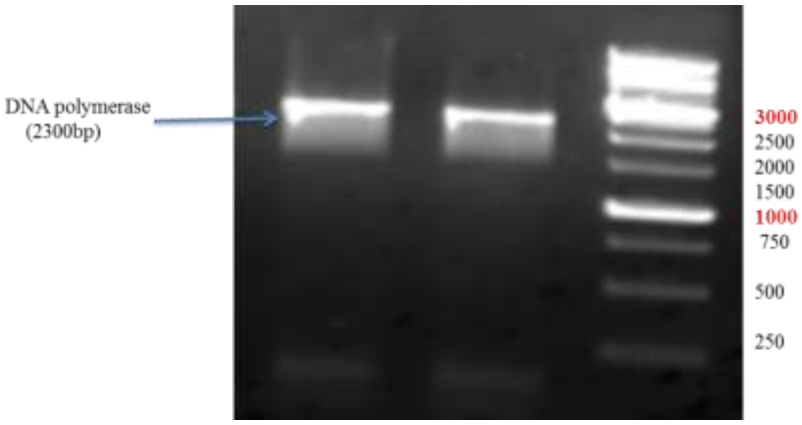


Figure 3.1 Amplification of *Pfu* DNA polymerase

The band of the correct size was excised and gel extraction was performed using kit. The extracted product was used as a template for reamplification of the gene. The PCR reaction was carried out at 44°C, 45°C, 46°C and 48°C using Deep Vent enzyme.

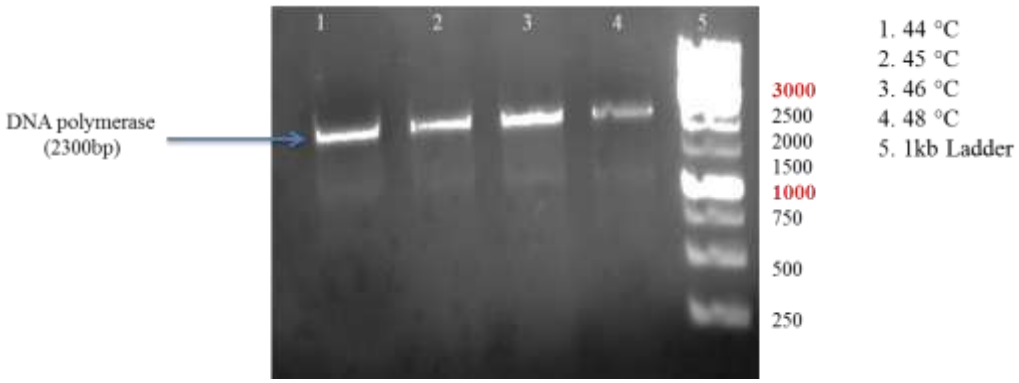


Figure 3.2 Reamplification of *Pfu* DNA polymerase

3.1.1.2 Digestion and Ligation

Further, the bands were excised and gel extraction was performed. For digestion, the clone containing pET23a plasmid was inoculated and grown overnight at 37°C. Plasmid DNA isolation was done the next day. Thus, pET23a and DNA polymerase were digested with Nde1 and Xho1 restriction enzymes by incubating it at 37°C water bath for 1 hour.

After 1 hour, PCR purification of digested DNA polymerase was done and digested pET23a was checked by comparing it with undigested pET23a on 1% agarose gel. Digested pET23a band was excised and gel extraction was done. The concentrations of both digested pET23a and DNA polymerase was checked using NanoDrop (ThermoScientific) instrument.

Ligation reaction was incubated at 25°C for 10-15 minutes with Quick Ligase. For 50ng of digested pET23a, the amount of digested DNA polymerase was calculated using the formula mentioned in the materials and methods section . The insert:vector ratio was 3:1 in this case.

The ligation mixture was then transformed in the cloning host cells, XL1 Blue. Thus, colonies were obtained the next day.

3.1.1.3 Colony PCR

Colony PCR was done to check the positive transformants.

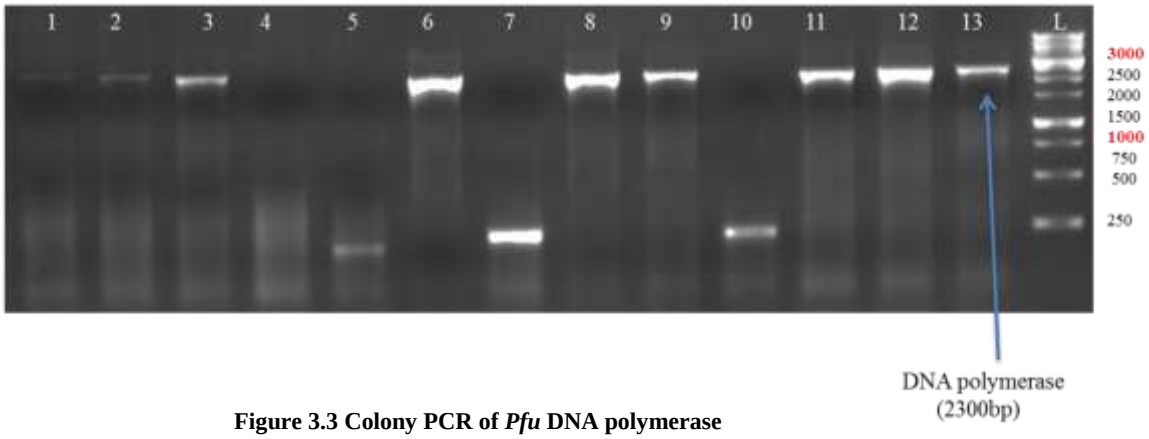


Figure 3.3 Colony PCR of *Pfu* DNA polymerase

The band in the lane 1,2,3,6,8,9,11,12 and 13 show positive colonies. Thus, some of these colonies were inoculated in a fresh LB media and grown at 37°C overnight. Plasmid DNA was isolated and digestion was done to check the integration of the insert in the vector.

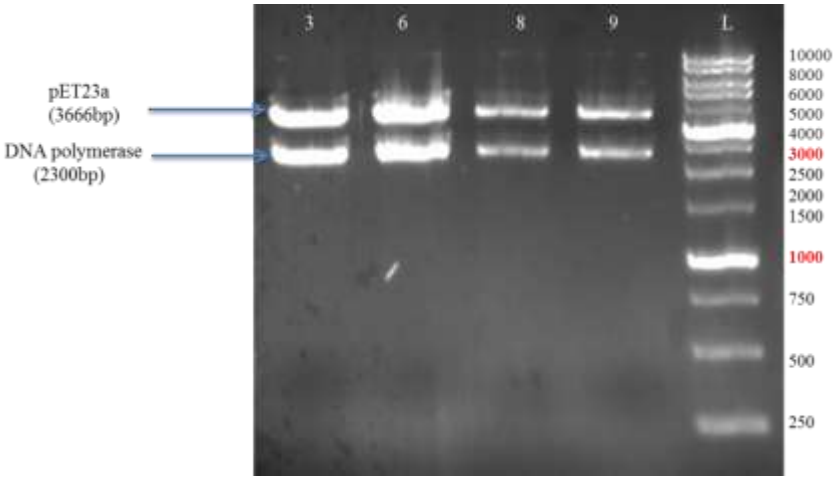


Figure 3.4 Digestion check to see the fall out of insert

Thus, the cloning was confirmed by digestion as we can see the insert (DNA polymerase) and the vector (pET23a) in all the 4 lanes obtained after digestion. This was further confirmed by DNA sequencing.

The clone was transformed in expression host (BL21 (DE3)pLysS) and expression was checked by running the induced and the uninduced cell pellets on SDS-PAGE.

3.1.2 PURIFICATION

Purification was done using Ni-NTA column by native method (Figure 3.5). *Pfu* DNA polymerase was expressed in soluble form and found to be present in the cytosol.

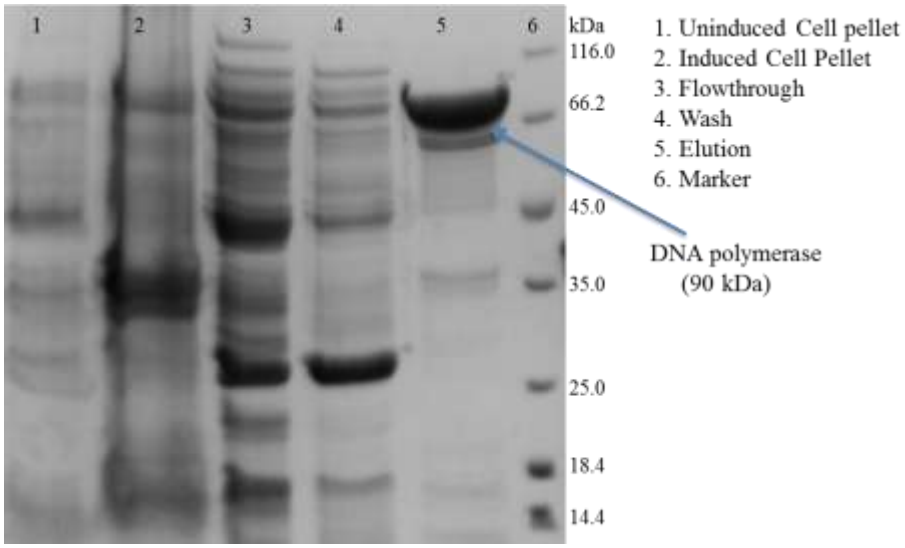


Figure 3.5 Purification of *Pfu* DNA polymerase

3.1.3 BIOPHYSICAL CHARACTERIZATION

3.1.3.1 Oligomeric state of the protein

3.1.3.1.1 Gel filtration

Gel filtration chromatography was done to know the oligomeric status of the protein. The protein was run on Superdex 200 10/300 GL column (24ml). The column was equilibrated with 20mM Tris Buffer and 500µl of the protein was loaded for analysis. The elution volumes were compared with the elution profiles of the standards run on the same column (Figure 3.6).

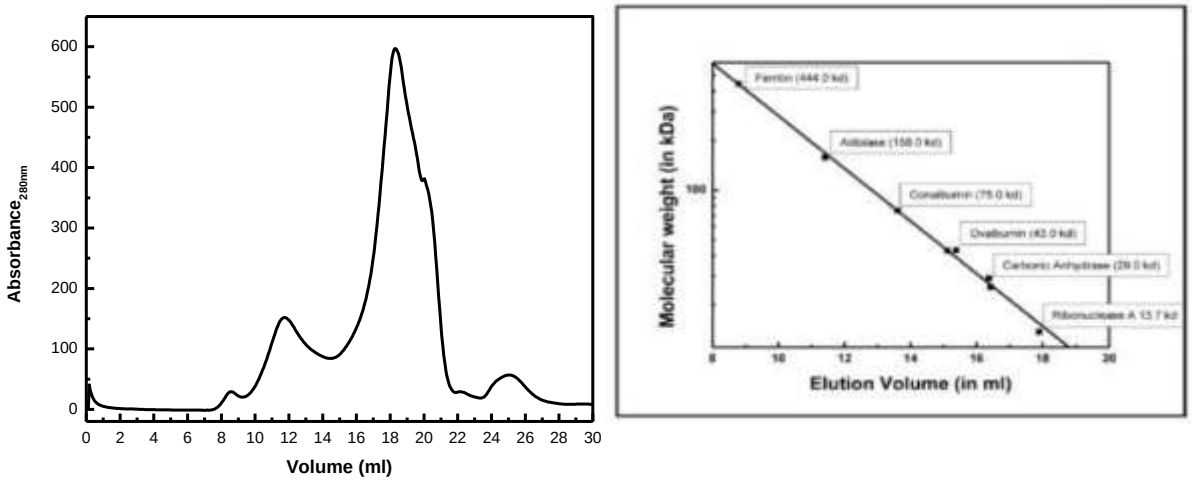


Figure 3.6 Gel filtration chromatogram of *Pfu* DNA polymerase and Elution profile of Standard run

As the protein seems to elute at very high volume, it must be interacting with the column.

Fractions of 1.5 mL were collected and run on 12% SDS gel.

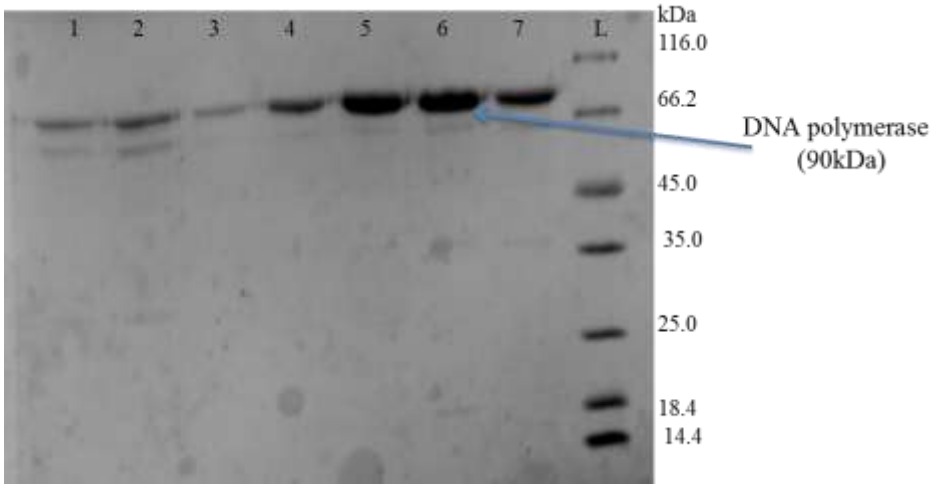


Figure 3.7 Collected fractions of *Pfu* DNA polymerase

3.1.3.1.2 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) was performed to further check the oligomeric state of the protein. DLS tells us about the hydrodynamic radius of the protein.

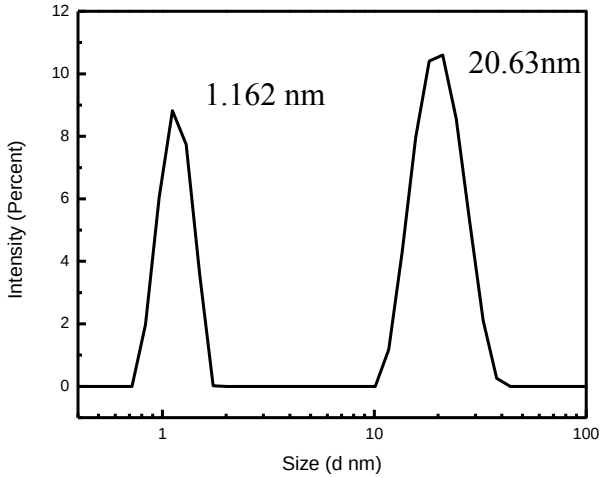


Figure 3.8 DLS of *Pfu* DNA polymerase

DLS data (Figure 3.8) shows two peaks with diameter of around 1.162 nm and 20.63 nm respectively. While the first peak was very small in size and considered insignificant, the second peak suggests that the protein exists in a dimeric form.

This result needs to be further probed by using other techniques such as gluteraldehyde cross linking as the literature reports this protein to be a monomer.

3.1.3.2 Secondary Structure

3.1.3.2.1 Circular dichroism

Far UV CD Spectra (Figure 3.9) was collected to look at the secondary structure of the protein. Concentration of 0.25 mg/ml of the protein was used.

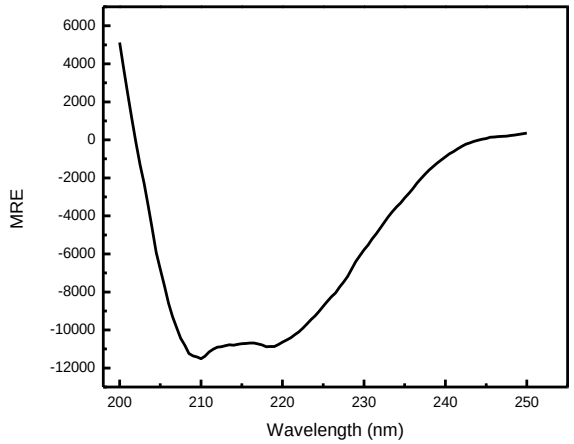


Figure 3.9 CD Spectrum of *Pfu* DNA polymerase

Figure 3.9 represents the far UV CD spectrum of the protein and it shows a characteristic spectra of an α+β protein.

3.1.3.2.2 Tryptophan fluorescence

Fluorescence emission spectra was collected by setting the excitation at 295nm and collecting the spectra in the range of 300-400nm. The slit width used for the experiment was 5nm for both excitation and emission. Since the λ_{max} emission was below 340 nm in the emission spectra, we can say that the protein seems to be well folded and the tryptophan is buried and not exposed.

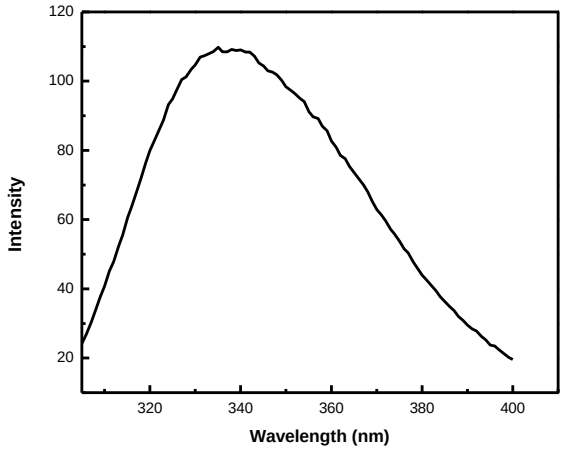


Figure 3.10 Fluorescence emission spectrum of *Pfu* DNA polymerase

3.1.3.3 Stability Studies

3.1.3.3.1 Chemical stability monitored using tryptophan fluorescence

The protein was checked for stability against guanidine hydrochloride (GdmCl) by subjecting the protein to chemical treatment by mixing it with different concentrations of guanidine concentrations viz. 0M, 1M, 2M, 3M, 4M, 4.5M and 5M and incubated overnight. Fluorescence emission spectra were collected for all the concentrations. Fraction folded versus GdmCl concentration was plotted and Cm was calculated. The Cm came out to be 4M in this case. Thus, 50 % of the DNA polymerase molecules have unfolded at a concentration of 4M GdmCl.

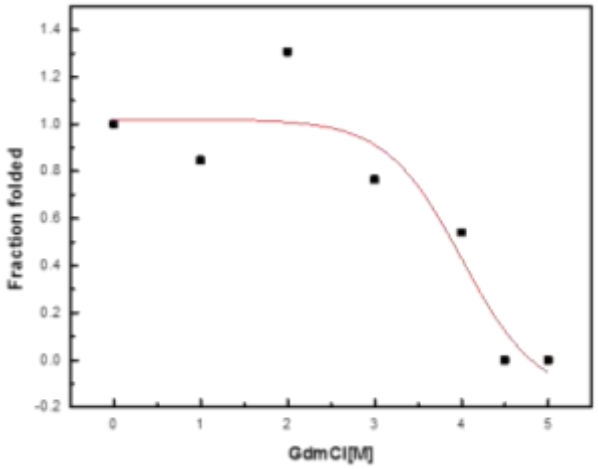


Figure 3.11 Plot of Fraction folded vs GdmCl concentration to monitor chemical stability of *Pfu* DNA polymerase

3.1.3.3.2 Thermal stability monitored using circular dichroism

The thermal stability of the protein was checked by heating the protein from 20 to 90 °C at a constant ramp rate of 5°C/min. 0.25mg/ml of protein was used. After thermal denaturation, the unfolded molecules were allowed to fold back by cooling it down 20°C using the same parameters. Throughout the study the change in the ellipticity value at 222nm was monitored. Figure 3.12 suggests that the protein is quite stable even at high temperatures and regains its structure after cooling.

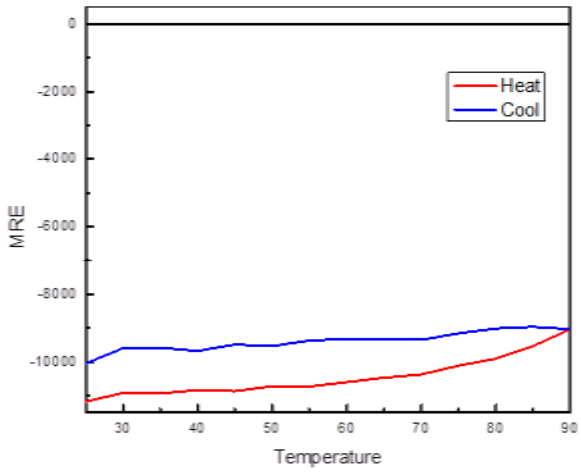


Figure 3.12 Thermal denaturation and renaturation of Pfu DNA polymerase

3.1.4 ACTIVITY ASSAY

The activity of *Pfu* DNA polymerase was checked by amplifying 615bp gene using T7 universal primers. The same gene was amplified using Taq polymerase as a control.

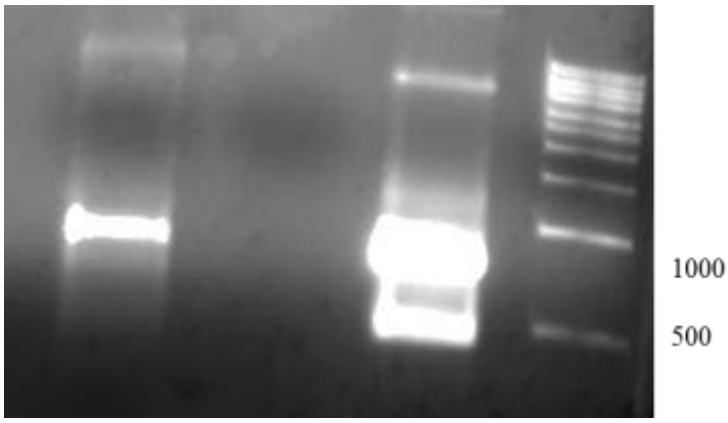


Figure 3.13 Activity assay Lane 1: Amplification using *Pfu* DNA polymerase. Lane 3: Amplification using Taq polymerase

3.2 PEPTIDASE M50

3.2.1 CLONING

3.2.1.1 Amplification

Peptidase M50 is a metalloendopeptidase from *Thermtoga maritima (Tm)*. Peptidase M50 was amplified from *Tm* genomic DNA. The desired band (615bp) was obtained at 55°C using Phusion enzyme.

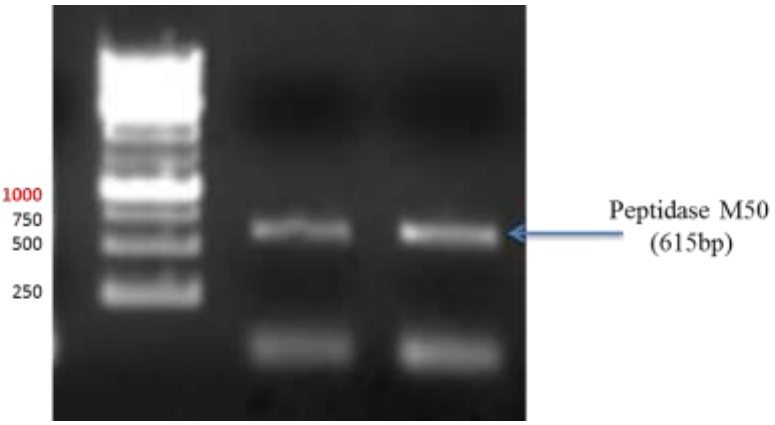


Figure 3.14 Amplification of *Tm* Peptidase M50

The band was further excised and Gel extraction was performed using kit. Thus using it as a template, reamplification was obtained at 55°C from Phusion enzyme.

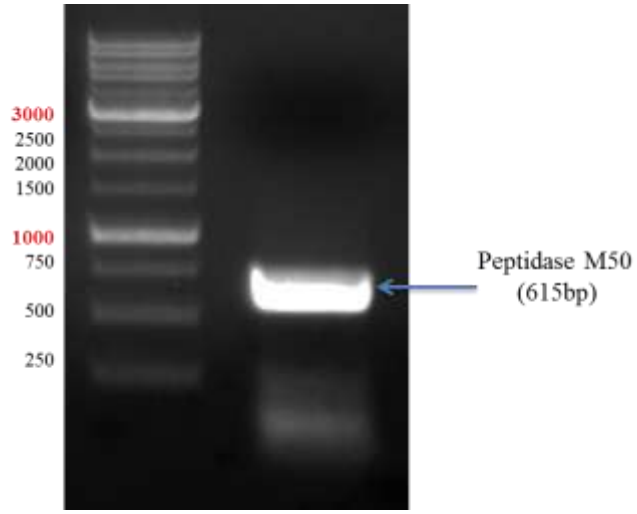


Figure 3.15 Reamplification of *Tm* Peptidase M50

As single band was observed on agarose gel, PCR purification was done for the rest of the amplified product.

3.2.1.2 Digestion and ligation

For Digestion, pET23a plasmid was inoculated and grown overnight at 37°C. Plasmid DNA was isolated the next day. Thus pET23a and Peptidase M50 were digested with Nde1 and Xho1 restriction enzymes by incubating it at 37°C in a water bath for 1 hour.

After 1 hour, PCR purification of digested Peptidase M50 was done and digested pET23a was checked by comparing it with undigested pET23a on 1% agarose gel. Digested pET23a band was excised and gel extraction was done. The concentrations of both digested pET23a and digested Peptidase M50 was checked using NanoDrop instrument.

Ligation reaction was incubated at 24°C for 1 hour with T4 DNA Ligase. For 50ng of digested pET23a, the amount of digested Peptidase M50 was calculated using the formula mentioned in . The insert:vector ratio was 3:1 in this case.

The ligation mixture was then transformed in the cloning host cells, XL1 Blue. Transformation was performed as mentioned in material and methods.

3.2.1.3 Colony PCR

Colonies were obtained the next day. Colony PCR was done to check for positive transformants.

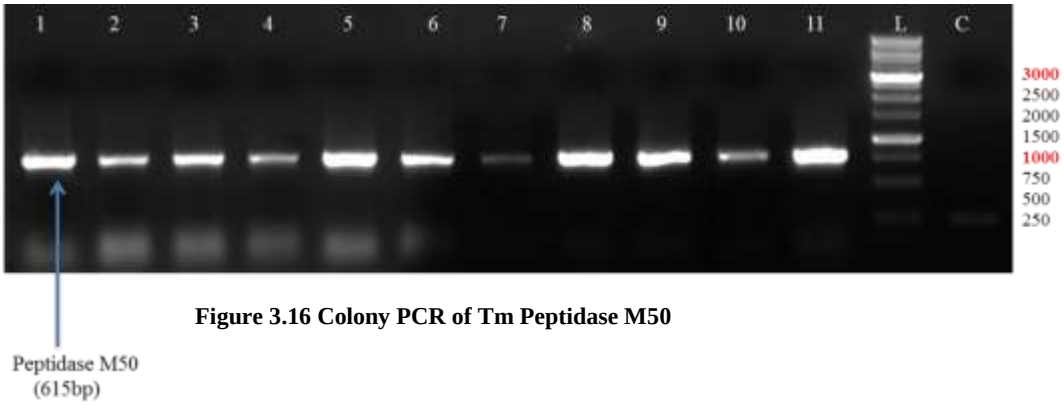


Figure 3.16 Colony PCR of Tm Peptidase M50

All of the selected colonies shown in figure 3.16 were positive. Thus one colony was inoculated in a fresh LB media and grown at 37°C overnight. Plasmid DNA was isolated and digestion was done to check the integration of insert in the vector.

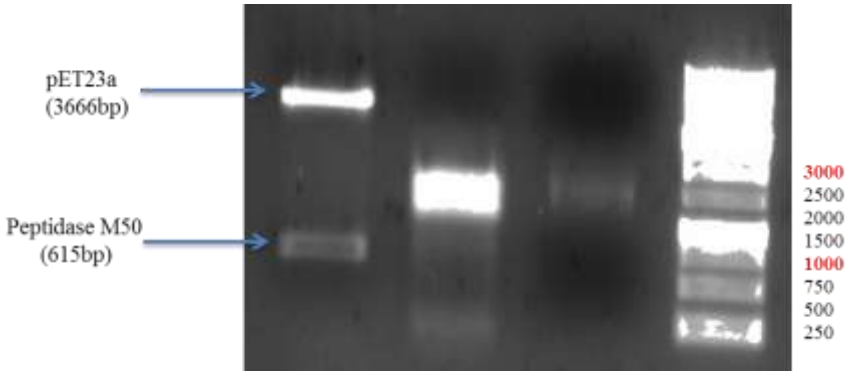


Figure 3.17 Digestion Check to see the fall out of insert

Thus the cloning was confirmed by digestion as we can see the insert (Peptidase M50) from vector (pET23a) in the first lane of the figure 3.17. This was further confirmed by DNA sequencing.

The clone was transformed in expression host (BL21 (DE3)pLysS) and expression was checked by running the uninduced and induced cell pellets on 12% SDS PAGE.

3.2.2 PURIFICATION

Several attempts were made to purify Peptidase M50. Purification was done using Ni-NTA column by Native method. Equilibration of the column was done by 1 X native lysis buffer.

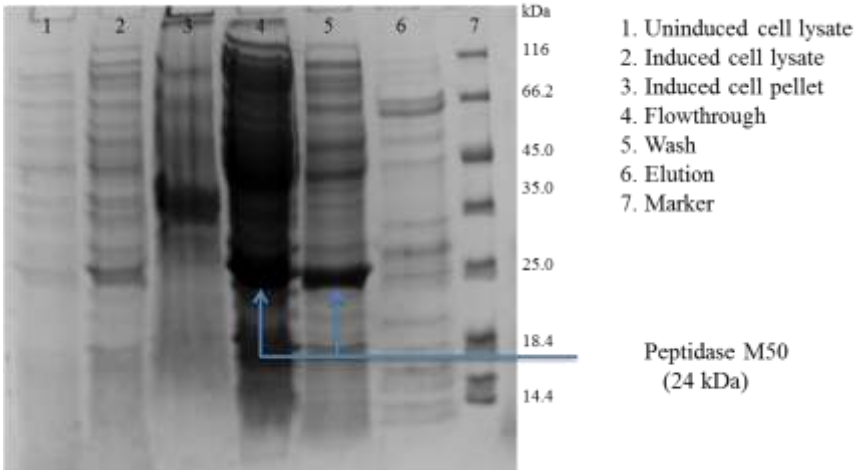


Figure 3.18 Purification of *Tm* Peptidase M50 by native method

In the figure 3.18 it was observed that most of the protein was obtained in flowthrough and wash fractions. Also a lot of nonspecific bands were seen in the elution fraction. Thus the flowthrough was again loaded on the column and washed using 1 X native lysis buffer by adding 700mM NaCl to it. Elution was done using native elution buffer. Also the collected wash fraction (from the previous purification) was loaded on the column by diluting it from 20mM to 5mM imidazole as a large amount of protein was observed in the wash fraction as well. Washing was done with 1 X native lysis buffer containing NaCl to a final concentration of 1M.

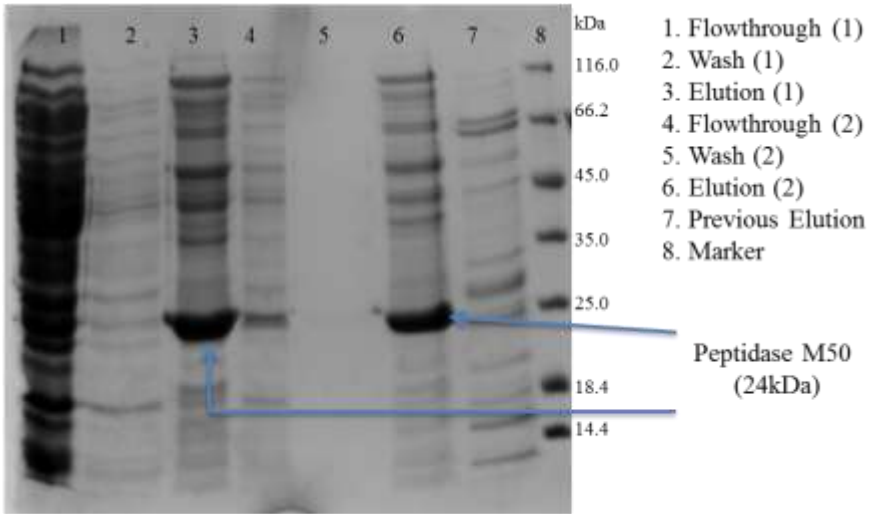


Figure 3.19 Purification of *Tm* Peptidase M50 by increasing salt concentration (1M)

As a lot of impurities (figure 3.19) were still observed in both the elution fractions, fresh purification was done by increasing the NaCl concentration from 1000mM to 1300mM in 1 X native lysis buffer during the resuspension of the induced cell pellet.

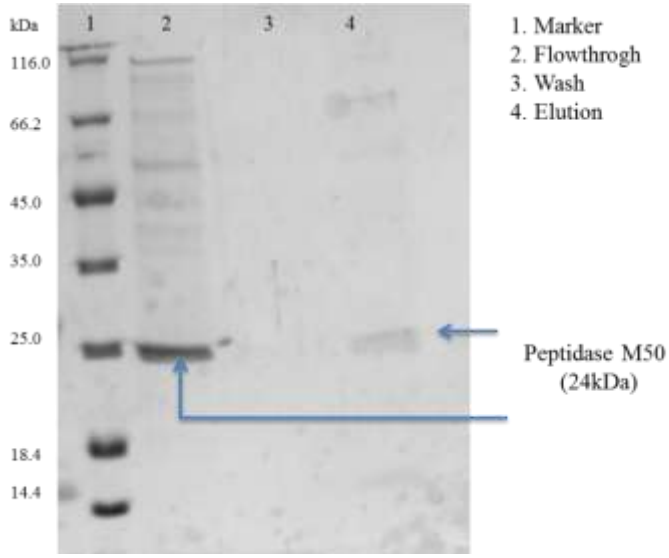


Figure 3.20 Purification of *Tm* Peptidase M50 by increasing salt concentration (1300mM)

From this purification, figure 3.20, a faint band can be seen in the elution lane but still a large amount of protein was observed in the flowthrough fraction. Again fresh purification was done by 1M NaCl to compare with the purification done using 1300mM NaCl in 1 X native lysis buffer.

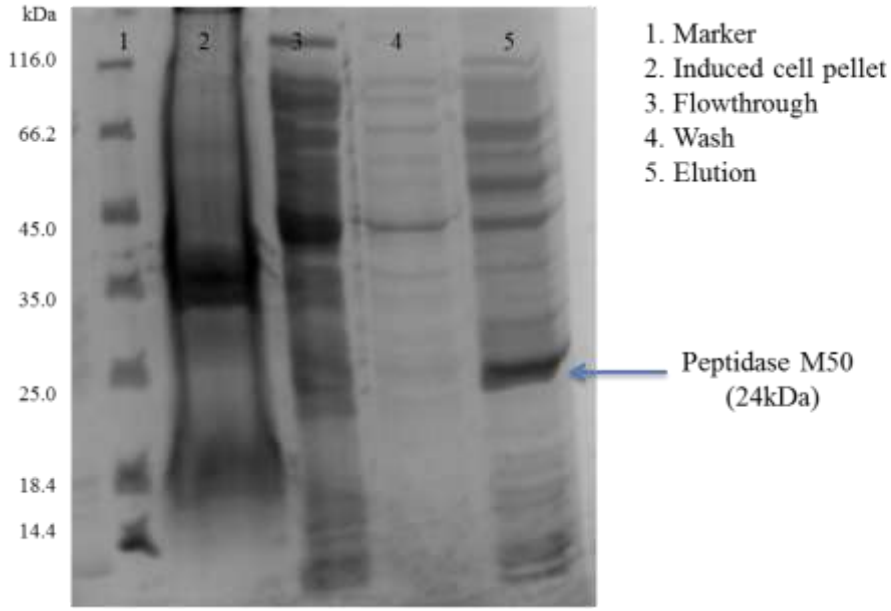


Figure 3.21 Purification of *Tm* Peptidase M50 using 1M NaCl

The desired band of around 24kDa can be seen in the elution lane (figure 3.21) with a lot of impurities. Denaturing purification of the induced cell pellet using 8M urea was also done but still the desired band was not observed.

In order to remove the non specific bands observed in the elution fraction, a fresh purification was done by adding 1M NaCl to 1 X native lysis buffer and heating it at 80°C for 30 minutes. As Peptidase M50 belongs to a thermophile, the rationale behind heating the resuspended cell pellet is to provide the over expressed protein a native environment where it can fold correctly in its native conformation. It also reduces non specific protein load (mainly of the expression host) as the high temperature denatures them.

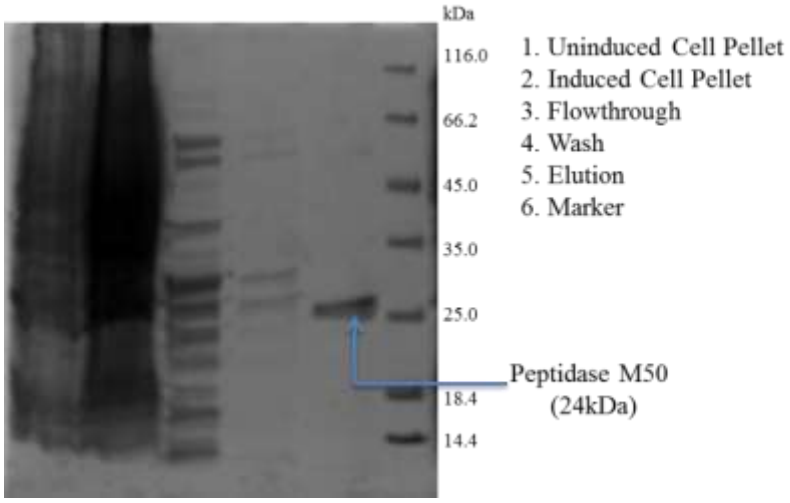


Figure 3.22 Purification of *Tm* Peptidase M50 using 1 M NaCl and Heat

Thus Peptidase M50 was obtained (figure 3.22) as a single band in elution by heating at 80°C and adding 1M NaCl.

3.2.3 BIOPHYSICAL CHARACTERIZATION

3.2.3.1 Oligomeric State

3.2.3.1.1 Gel filtration

Gel filtration chromatography was done to know the oligomeric state of the protein. The protein was run on Superdex 200 column (24ml). The column was equilibrated with 50mM

NaH_2PO_4 and 1M NaCl and 500µl of the protein was loaded for analysis. The elution volumes were compared with the elution profiles of the standards run on the same column.

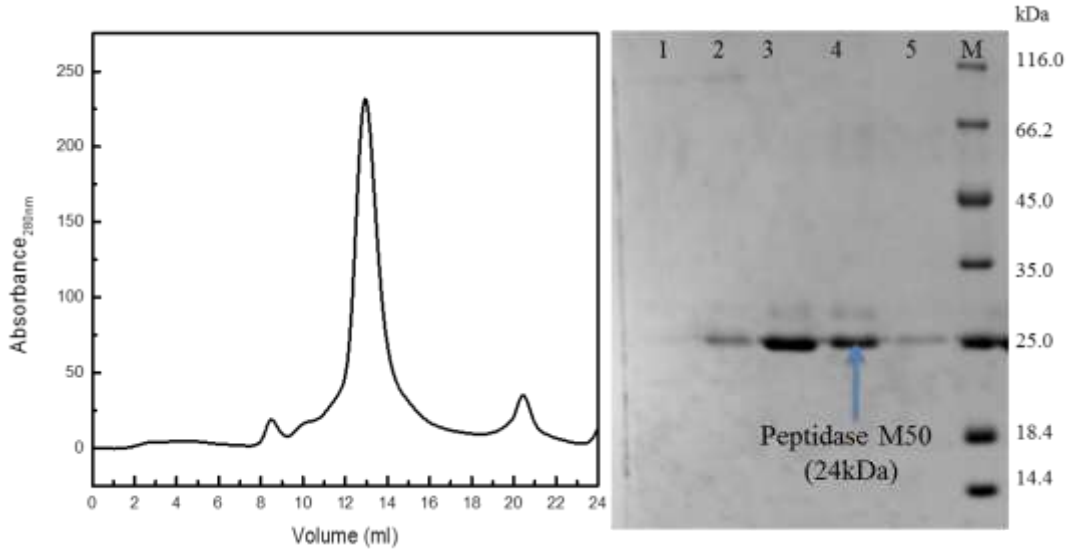
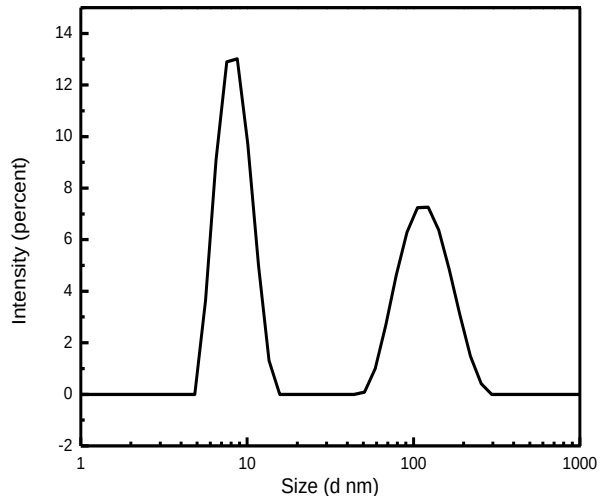


Figure 3.23 Gel filtration chromatogram of *Tm* Peptidase M50 and gel fractions on SDS gel

Fractions collected were run on 12% SDS gel to confirm the protein. Gel filtration data (figure 3.23) suggests that the protein exists in a tetrameric form.

3.2.3.1.2 Dynamic Light Scattering (DLS)

The oligomeric status of the protein was also analyzed using DLS. Figure 3.24 shows the intensity fractions obtained. Peak 1 seems to suggest that Peptidase M50 is a tetramer which is in accordance with the gel filtration chromatogram.



Peak 1- 8.498 nm
Peak 2- 122.4nm

Peak 2 also suggests that the protein exists in tetrameric form.

Figure 3.24 DLS of *Tm* Peptidase M50

3.2.3.2 Secondary Structure

3.2.3.2.1 Circular dichroism

CD Spectra was collected to look at the secondary structure of the protein. 0.75 mg/ml of protein concentration was used. The CD spectrum (figure 3.25) is dominated by β sheet structure.

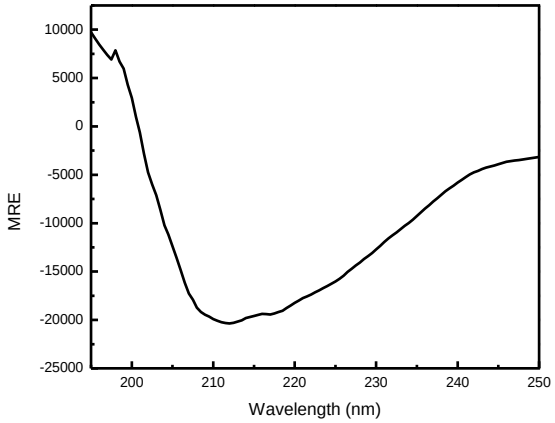


Figure 3.25 CD spectrum of *Tm* Peptidase M50

Since the modeled structure (obtained using HHPred) mainly shows α helical content in the protein so other tools like FTIR must be used to get a better picture of the secondary structure.

3.2.3.2.2 Tryptophan fluorescence

Fluorescence emission spectra was collected by setting the excitation at 295nm and collecting the spectra in the range of 300-400nm. The slit width used for the experiment was 5nm for both excitation and emission. As the peak maxima (figure 3.26) is around 340 nm, the protein seems to be well folded with its tryptophans buried.

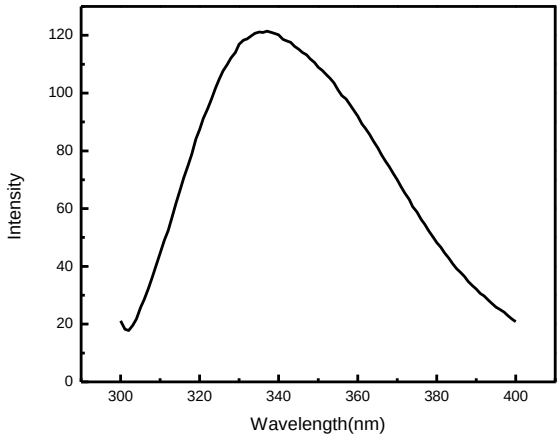


Figure 3.26 Fluorescence emission spectrum of *Tm* Peptidase M50

3.2.3.3 Stability Studies

3.2.3.3.1 Chemical stability monitored using tryptophan fluorescence

Protein samples (0.75mg/ml) were mixed with GdmCl concentrations of 0M, 1M, 2M, 3M, 4M, 4.5M and 5M respectively and incubated overnight. Fluorescence emission spectra were collected for all the concentrations. Thus by plotting fraction folded versus GdmCl concentration (figure 3.27), Cm can be identified which is 2M in this case. Thus half of the Peptidase M50 molecules have unfolded at 2M GdmCl concentration.

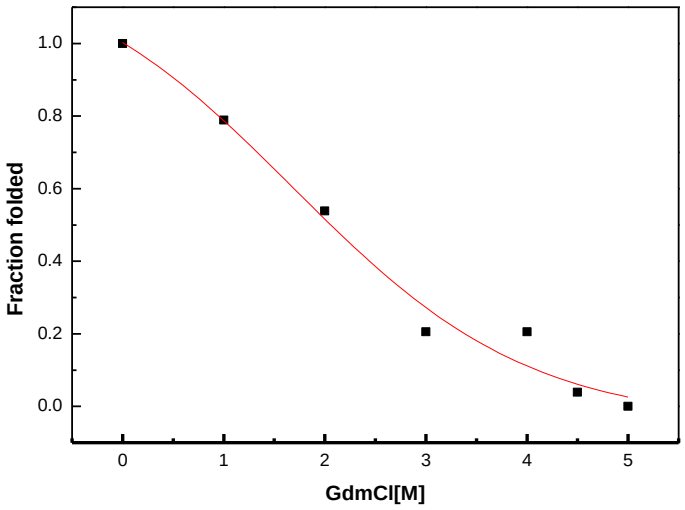


Figure 3.27 Plot of fraction folded vs GdmCl concentration to monitor chemical stability of *Tm* Peptidase M50

3.2.3.3.2 Thermal stability monitored using circular dichroism

0.75mg/ml of protein was heated inside the instrument from 20 to 90°C at a constant ramp rate of 5°C/min and cooled back to 20°C Ellipticity at 222nm was monitored throughout the experiment. Peptidase M50 is quite stable at high temperatures (from 25°C to 75°C) as seen in figure 3.28, after 75°C it starts unfolding rapidly. On cooling it does not regain its structure.

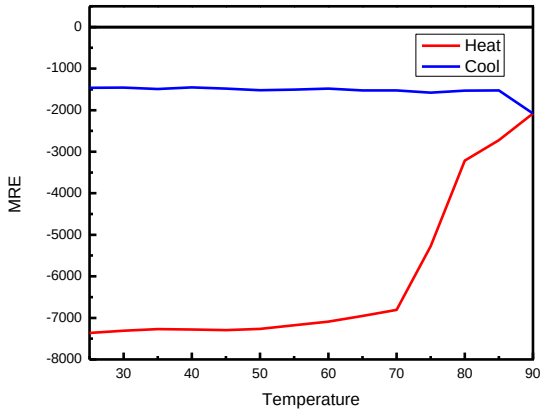


Figure 3.28 Thermal denaturation and renaturation of *Tm* Peptidase M50

3.3 BETA GLUCOSIDASE (Cel B)

Beta glucosidase belongs to hyperthermophilic archaeon *Pyrococcus furiosus*. It is involved in the hydrolysis of the β-1,4-glycosidic bond between the two glucose moieties of the disaccharide.

3.3.1 PURIFICATION

The 1500bp gene was already cloned in pET 28c vector between Nco1 and Xho1 restriction sites. It was expressed in Rosetta cells and purified using Ni-NTA column by native method as seen in figure 3.29.

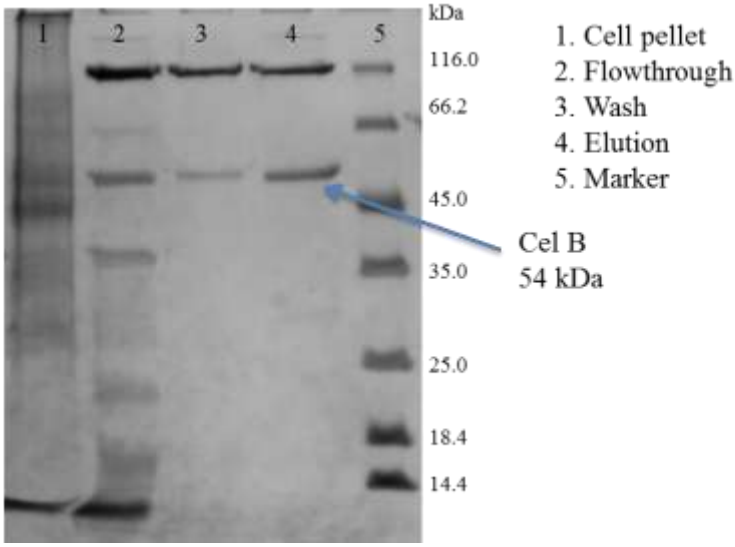


Figure 3.29 Purification of *Pfu* CelB

3.3.2 BIOPHYSICAL CHARACTERIZATION

3.3.2.1 Oligomeric state

3.3.2.1.1 Gel filtration

Gel filtration chromatography was done to know the oligomeric state of the protein. The protein was run on Superdex 200 column (24ml). The column was equilibrated with 20mM Tris Buffer and 500µl of the protein was loaded for analysis. The elution volumes were compared with the elution profiles of the standards run on the same column (figure 3.30).

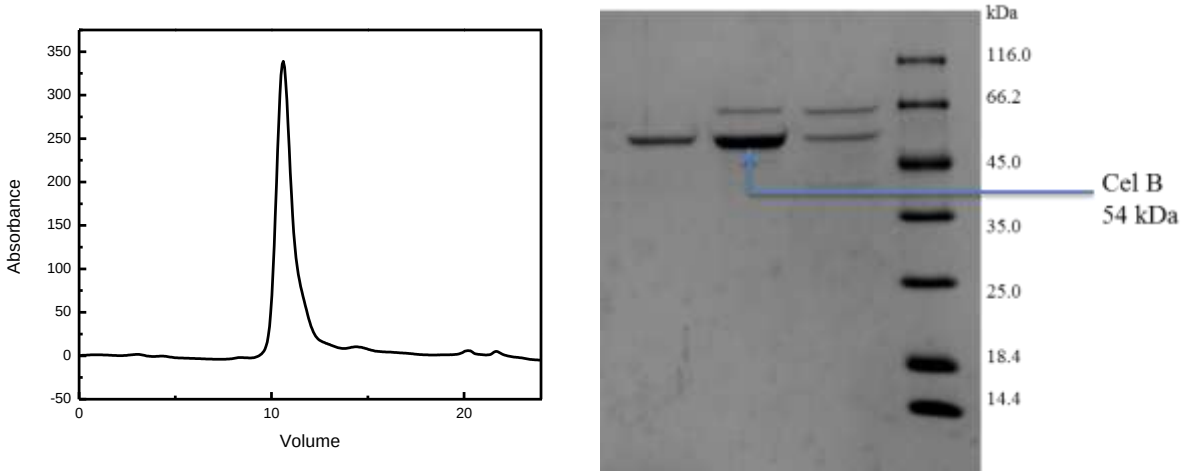


Figure 3.30 Gel filtration chromatogram and fractions collected of *Pfu* CelB

Fractions collected were run on 12% SDS gel to confirm the protein. Gel filtration data suggests that the protein exists in a tetrameric form.

3.4 ARGININOSUCCINATE LYASE (ASL)

Argininosuccinate lyase belongs to hyperthermophilic archaeon *Pyrococcus furiosus*. It is an important enzyme of Urea cycle which is involved in the reversible breakdown of argininosuccinate lyase into arginine and fumarate.

3.4.1 PURIFICATION

The 1381 bp gene was already cloned in pET23a vector between NdeI and XhoI restriction sites. It was expressed in BL21 cells and purified using Ni NTA affinity purification (figure 3.31).

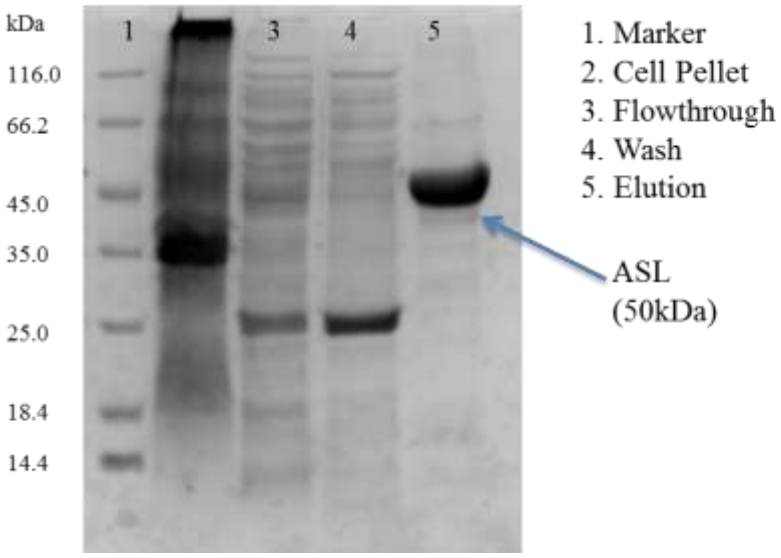


Figure 3.31 Purification of *Pfu* ASL

3.4.2 BIOPHYSICAL CHARACTERIZATION

3.4.2.1 Oligomeric state

Gel filtration chromatography was done to know the oligomeric state of the protein. The protein was run on Superdex 200 column (24ml). The column was equilibrated with 1 X Native Lysis Buffer and 500µl of the protein was loaded for analysis. The elution volumes were compared with the elution profiles of the standards run on the same column. Figure 3.32 shows the gel filtration chromatogram which suggests that the protein exists in tetrameric form.

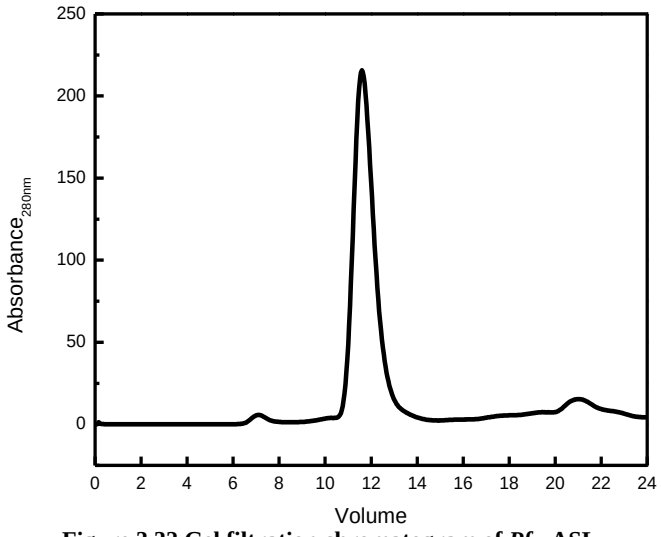


Figure 3.32 Gel filtration chromatogram of *Pfu* ASL

3.4.2.2 Secondary Structure

3.4.2.2.1 Circular dichroism

CD Spectra was collected to look at the secondary structure of the protein. 0.5 mg/ml of the protein was used. The protein is found to be α helical in nature in CD (figure 3.33).

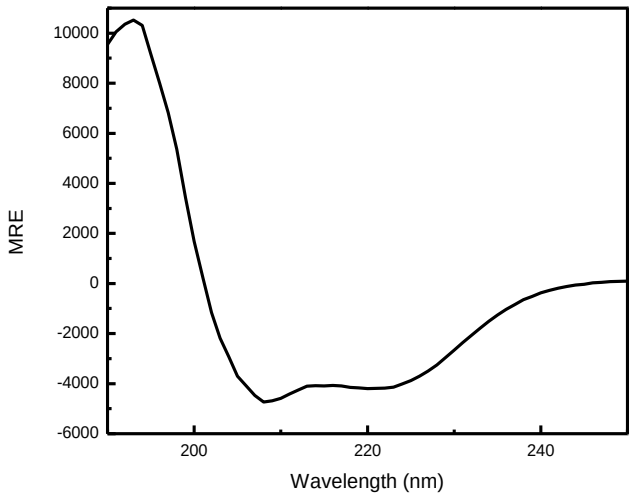


Figure 3.33 CD spectrum of *Pfu* ASL

3.4.2.2.2 Tryptophan fluorescence

Fluorescence emission spectra was collected by setting the excitation at 295nm and collecting the spectra in the range of 300-400nm. The slit width used for the experiment was 5nm for both excitation and emission. By looking at the spectra (figure 3.34), the protein

seems to be well folded. Since the protein contains a single tryptophan residue and many tyrosines (~17) the fluorescence peak is shifted more towards 300 nm suggesting that the tryptophan fluorescence is masked by tyrosine fluorescence.

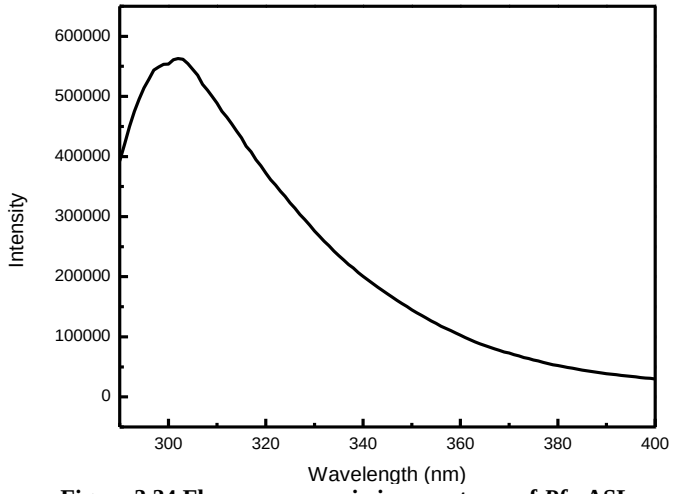


Figure 3.34 Fluorescence emission spectrum of *Pfu* ASL

3.4.2.3 Stability Studies

3.4.2.3.1 Thermal stability monitored using circular dichroism

0.5mg/ml of protein was heated inside the instrument from 20 to 90°C and cooled back to 20°C with a ramp rate of 5°C/minute and intensity at 222nm and 208nm was monitored (figure 3.35). The protein is quite stable at high temperature and traces its path back upon cooling.

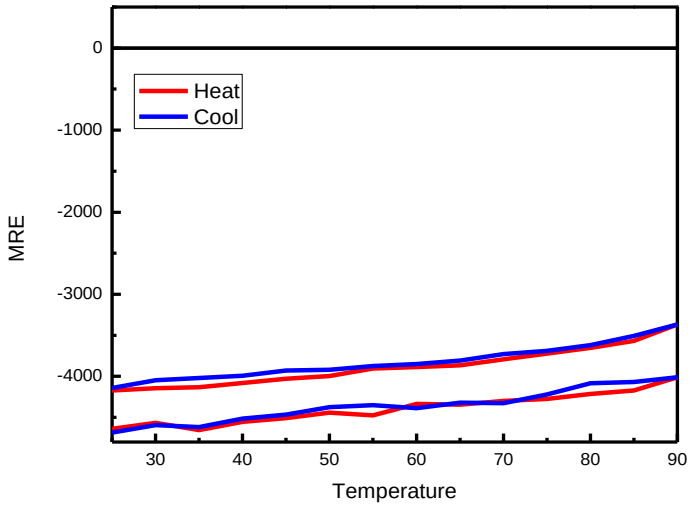


Figure 3.35 Thermal denaturatyion and renaturation of *Pfu* ASL

3.4.2.3.2 Thermal stability monitored using tryptophan fluorescence

The protein was heated inside the instrument from 20 to 90°C and monitored using fluorescence. Denaturation studies using fluorescence (figure 3.36) also co-relate with the CD data showing it to be a highly stable protein since the peak maxima is not shifting. The change in fluorescence intensity is only due to increase in temperature.

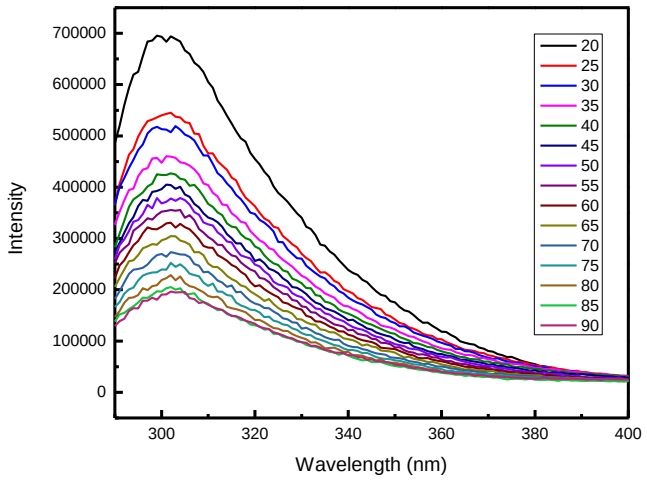


Figure 3.36 Thermal denaturation of *Pfu* ASL

3.5 PROTEASE DO

Protease Do is periplasmic heat shock serine protease belonging to *Thermotoga maritima*. It functions as a molecular chaperone at low temperatures, and its proteolytic activity is turned on at elevated temperatures.

3.5.1 Amplification

Protease Do was amplified from *Thermotoga maritima (Tm)* genomic DNA. The desired band (1380bp) was obtained at 56°C and 57°C using Deep Vent enzyme.

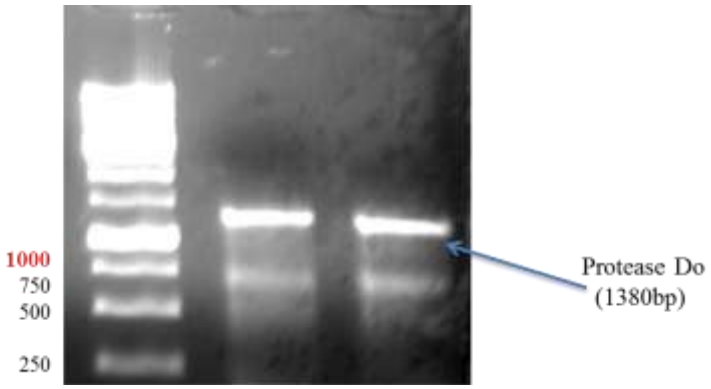


Figure 3.37 Amplification of *Tm* Protease Do

4. SUMMARY

Hyperthermophilic enzymes have drawn the interests of many researchers pertaining to their high thermal and structural stability. They help the organism withstand harsh and extreme conditions. These enzymes have also been put to various industrial uses. It is therefore important to study them and understand their structural features and other aspects in order to understand the thermal stability in proteins. The thesis represent studies on some thermostable proteins from *Pyrococcus fuiosus* and *Thermotoga maritima* namely, DNA polymerase and beta-glucosidase from *P. furiosus* and Peptidase M50 and Protease Do from *T. maritima.* We have cloned, expressed, purified and characterized them. The key findings have been summarized below:

1. DNA Polymerase (Pfu DNAPol)

- DNA polymerase gene (2300bp) was cloned in pET23a vector (Nde1 and Xho1), expressed in BL21 (DE3)pLysS and purified under native conditions.
- The over-expressed protein was found to be present in soluble fraction.
- DLS data suggests that the protein exists in dimeric form but this needs to be probed further using other techniques such as glutaraldehyde crosslinking.
- The protein is well folded and shows a characteristic spectra of an α+β protein.
- The protein starts to unfold at 4M GdmCl.
- The protein is quite stable even at high temperatures and regains its structure after cooling.
- Polymerase activity was also seen in the protein.

2. Peptidase M50 (Tm Peptidase M50)

- Peptidase M50 (615bp) was cloned in pET23a vector (Nde1 and Xho1), expressed in BL21 (DE3)pLysS and purified by adding 700mM NaCl to 1X Native Lysis Buffer and heating it at 80°C for 30 minutes.
- DLS and gel filtration data suggests that the protein exists in tetrameric form.
- The protein is well folded and CD spectrum is dominated by β sheet structure.

- Peptidase M50 is quite stable at high temperatures (from 25°C to 75°C). But, after 75°C it starts unfolding rapidly and on cooling, it does not regain its structure.
- At 2M GdmCl, the protein starts to unfold.

3. Beta-glucosidase (Pfu CelB)

- Gel Filtration data suggests that Beta Glucosidase exists in a tetrameric form.

4. Argininosuccinate Lyase (PfuASL)

- Gel filtration shows that the Argininosuccinate lyase exists in a tetrameric form.
- The protein is well folded and found to be mostly α helical in Far UV CD spectrum.
- The protein is quite stable at high temperature and traces its path back upon cooling.
- Denaturation studies using fluorescence also co-relate with the CD data showing it to be a highly stable protein.

5. Protease Do (Tm Protease Do)

- Protease Do gene (1380bp) was amplified using Deep Vent enzyme.

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