# Studying an Enzyme Chimera through Swapping of β/α Loops between Homologous β/α Barrel Xylanases

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# Certificate of Examination

This is to certify that the dissertation titled **"Studying an Chimera through Swapping of β/α Loops between Homologous β/α Barrel Xylanases"** submitted by **Mr. Manmeet Singh** (Reg. No. MS08032) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by theInstitute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Rajesh Ramachandran Dr. Rachna Chaba Prof. Purnananda Guptasarma
(Supervisor)

# DECLARATION

The work presented in the dissertation has been carried out by me under the guidance of Dr. Purnananda Guptasarma at the Indian Institute of Science Education and Research, Mohali. The 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.

Manmeet Singh
(Candidate)

Dated:

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

Prof. Purnananda Guptasarma
(Supervisor)

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Having spent more than a year working under with him, I would like to express with utmost sincerity, a heartfelt gratitude to my guide Dr. Purnananda Guptasarma. More than a guide, he has been an inspiring and an exceptionally kind and patient teacher. His unique approach to science and life in general has influenced me deeply. Moreover his logical thinking and enthusiasm as a scientist greatly helped me in understanding the basic concepts of this work.

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# Abstract

Xylanases are proteins that degrade xylan, which is a component of plant cell walls. Thermostable xylanases are useful for the paper and pulp industry because they can be used as substitutes for chlorine in certain methods. The prerequisite of a useful industrial xylanase is that it should be thermostable, but also active at lower temperatures (40ºC-60ºC) than most thermostable xylanases (which tend to be thermo-active, as well as thermostable). Further, in cold climes, a xylanase needs to be able to act at even lower temperatures, to degrade plant waste. In order to try and create a xylanase with unusual characteristics normally absent in naturally-occurring xylanases, we attempted to make a 'chimeric' xylanase by transplanting catalytic and other loops from a beta-alpha barrel xylanase derived from *Cryptococcus adeliae*, an Antarctic yeast adapted to cold environments, onto the structural scaffold of a mesophile xylanase of known high kinetic thermostability (the NG-27 Xylanase, also known as BSX, or Bacillus sp. xylanase). The chimeric xylanase was constructed on the basis of principles of protein engineering. NG-27 Xylanase shows optimal activity at 70 ºC while *C. adeliae* xylanase is expected to show optimal activity in the range of 0-10 ºC. So, a encoding the novel protein-engineered xylanase was constructed by one of my seniors, by transplanting the latter's loops onto the former's scaffold. However, there was a frame shift mutation in the gene which introduced a stop codon. The first step of this project was, therefore, to correct this mutation. After correction of the mutation, the idea was to produce the xylanase, and study various characteristics including whether it folds and whether it is active. Since it is a novel protein, there was a need of optimizing the conditions under which it can be expressed and purified. This was achieved by exploring various different conditions at each and every step during purification and expression. After optimizing the conditions for expression and purification, we proceeded to check whether the chimera formed was active or not. Standard xylanase degradation assay (Miller 1962) was made use of for checking the

activity in which the reducing sugars react with DNSA and gives colour which is monitored at 540nm. Also, plate-based assays were tried, to check for activity through visual evidence of degradation of substrate (xylan) in the form of zones of clearance of suspended (turbid) xylan around cavities in which enzyme was placed. To characterize the protein and have a better understanding of the system, we also used different techniques like CD (Circular Dichroism) and gel filtration chromatography. While performing these experiments, the main findings were: a) the protein was expressed and present in inclusion bodies, so it was extracted out with the help of denaturants, b) it can be refolded by removing denaturant using dialysis, or through on column refolding, or rapid removal of denaturant using a desalting column, c) the refolded protein displays signature features of alpha helix and beta sheet in the CD spectrum, suggesting that the protein folds into mixed beta alpha structures, d) refolding through dialysis leads to formation of soluble aggregates containing polypeptides in structured beta-alpha form, but refolding through desalting-based removal of denaturant leads to formation of some dimer population too, and e) the soluble aggregate does not show activity under any of the conditions or temperatures tested, indicating that such refolding and activity assays need to be optimized, while the dimers obtained through desalting have not yet been fully tested for activity (although initial tests at one pair of chosen high, and low, temperatures suggests that there is no activity even in the dimer). Further studies are needed.

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# CHAPTER 1

# INTRODUCTION

# 1.1 Xylanase

Xylanases are enzymes that degrade xylan. Xylan is a linear polysaccharide. It is a component of the hemicelluloses in plant cell walls. Xylanases are mainly produced by fungi and bacteria that feed on plant matter. The substrate of this enzyme, xylan, is commonly referred to as being the 'second most abundant saccharide in nature'. Therefore, this enzyme evokes great interest from the paper and pulp industry. Xylanases are beginning to be used in bleaching of paper pulp, and appear to improve paper quality, while reducing use of chlorine (which produces toxic organic compounds). The most important sub-class of enzymes used for bleaching are the endoxylanases (i.e. β-1,4-xylanases) as these hydrolyse the β-1-4 glycosidic bond in the backbone of xylan. This study uses one such endoxylanase, derived from a mesophile, obligate alkaliphile *Bacillus* sp. strain NG-27. The NG-27 xylanase produced by this organism (also known as BSX, or *Bacillus sp.* Xylanase), shows optimal activity at 70°C and pH 8.4. While the intrinsic thermostability of this enzyme is highly desirable in the pulp industry, a more proficient enzyme for applications at colder temperatures would be one that is thermostable, but shows optimal activity at room temperature, or below room temperature. An enzyme with these (desired) characteristics was constructed using principles of protein engineering, by transplanting all beta-alpha loop residues from a low-temperature xylanase from *Cryptococcus adeliae*, an Antarctica yeast adapted to cold environments. The aim of this thesis was to correct a frame shift mutation in the gene encoding the protein-engineered xylanase, express it, purify it and investigate further characteristics of the enzyme. In the first part, the aim was to correct the frame shift mutation and investigate conditions under which this novel protein can be expressed efficiently. Second aim was to study biophysical characteristics and check whether it shows any activity or not.

## 1.2 An Introduction to Protein Engineering

Protein engineering is a discipline which focuses on design and construction of novel proteins using recombinant DNA technology. One of the aims of protein engineering is to optimize native proteins for industrial application or medicinal purposes. Often, protein are

engineered to effect a change in their substrate specificity, or their thermal tolerance, or resistance to proteases. Protein engineering requires comprehensive understanding of protein sequence-structure relationships because the structure and sequence of a protein are intricately linked, and it is not feasible to make changes to either one without disrupting the other, if changes are made in a blind fashion. However, even till today it is not possible to accurately predict the effect of alterations made in the sequence of a protein upon its structure. Thus, all designs have to be made and tested.

## 1.3 An Introduction to the NG-27 Xylanase

NG-27 xylanase (GenBank accession no. XYNAF015445) is an extracellular single domain glycosidase which catalyzes the endohydrolysis of 1,4-β-D -glycosidic linkages in xylan, breaking down the polysaccharide into xylose. It is a highly thermostable enzyme that is surprisingly secreted naturally by a mesophilic organism, Bacillus sp. NG-27, which is observed to grow optimally in the laboratory at a temperature of ~ 27°C (Ghosh A. *et al*, 2000). The enzyme is 358 residues long and has a molecular mass of 41.3kDa. The NG-27 xylanase contains no cysteines, and has neither free sulfhydryl groups nor disulfide bonds stabilizing its native structure. Under natural conditions, the protein is monomeric. It displays facultative dimerization or tetramerization when exposed to low salt concentrations (Ghosh A. *et al*, 2000). Chaotrope induced unfolding of most globular proteins generally require overnight incubation (with urea or guanidinium chloride) at room temperature. NG-27 xylanase on the other hand requires more than a week to attain to a completely unfolded state. Such slow unfolding by chaotropes has been reported for proteins of thermophilic origin, but not for proteins of mesophilic origin, by and large. Interestingly, the thermal unfolding of this protein is found to occur at rates much slower than those seen with most mesophilic globular proteins. In many cases, the postulated cause for slow unfolding is not high thermodynamic stability (i.e. a large difference in the free energy of native and unfolded enzyme). To rationalize this behaviour it has been proposed that the native state of the enzyme is kinetically trapped. This might be a mechanism that evolved to allow the NG-27 xylanase to function at elevated temperatures for long periods of time. The NG-27 Xylanase also shows considerable thermal stability in terms of its ability to retain activity at high temperatures. Microstructures of enzymes (including catalytic loops) tend to be labile and

susceptible to denaturation; more so than protein cores. Thus loss of enzyme activity precedes changes in 3-D structures. The NG-27 xylanase deviates from this trend. Structurally, NG-27 xylanase is homologous to family 10 endoglycanases. Xylanases of family-10 show considerable homology, both at amino acid sequence and 3-D structural levels, with members adopting an α/β barrel structure (Ramakumar S. *et al*, 2006). The $(\alpha/\beta)_8$ barrel or TIM barrel fold of the NG-27 Xylanse is the most common of all domain structures seen in enzymes (Hocker B. *et al*, 2005). Almost all glycolytic enzymes have the $(\alpha/\beta)_8$ structure, and many other enzymes and proteins which bind and transport metabolites adopt this structure. The α/β barrel folding motif consists of 8 parallel β- strands, arranged like staves in a barrel, surrounded by 8 α-helices. The active site is present at the carboxy-terminal ends of the β-strands. In other words, catalytic and binding residues frequently occur in loop regions that connect the carboxy-terminal ends of β-strands with amino-terminal ends of adjacent (succeeding) α-helices (Hocker B. *et al*, 2005). We refer to these loops as β-α loops.

## 1.4 Xylanase from the psychrophilic yeast *Cryptococcus adeliae*

In the current study, catalytic β-α loop units were transplanted from the psychrophilic yeast *Cryptococcus adeliae* onto a core protein scaffold (which constitutes the barrel and the β-α loop units) of the NG-27 xylanase. A psychrophile is an organism that grows optimally at low temperatures, ranging from -15°C to 10°C. In such organisms, temperature is the factor leading to biochemical adaptation to environments (Steiner W. *et al*, 2000). Therefore, biochemical properties of psychrophile organism-derived proteins are different from those of proteins from mesophile or thermophile environments. Studies of enzymes from thermophile microorganisms have demonstrated, for example, that strengthening of their non-covalent intra-molecular interactions leads to thermostable structures, but such changes can be detrimental to the specific activities of enzymes. With psychrophile microorganisms, in order to maintain appropriate metabolic rates, these have to adapt to reduction of reaction rates at subzero temperatures. Therefore, enzymes from such microorganisms are thought to have evolved to more flexible structures, as compared to mesophilic and thermophilic counterparts (Gerday C. *et al*, 2000), through weakening of intra-molecular interactions and this is

assumed to be responsible for increased catalytic efficiency and low thermal stability of psychrophile enzymes (Steiner W. *et al*, 2000). Cold-adapted enzymes are less rigid than their mesophilic homologues. A xylanase belonging to glycoside hydrolase family 10 (GH10) is produced by *Cryptococcus adeliae*. The mature glycosylated xylanase secreted by *C. adeliae* is composed of 338 amino acid residues and shares 84% identity with a mesophilic counterpart from *C. albidus*. The xylanase from *C. adeliae* is less thermostable than its mesophilic homologue when the residual activities are compared. In the range 0°–20°C, the cold-adapted xylanase displays a lower activation energy and a higher catalytic efficiency (Gerday C. *et al*, 2000).

## 1.5 Chimeric Xylanase

A gene encoding the chimeric protein was created by transplanting DNA segments encoding β/α loops of *C. adeliae* xylanase onto segments encoding the NG-27 xylanase beta barrel and alpha helical barrel scaffolds. Loops were selected by aligning amino acid sequences of NG-27 xylanase and *C. adeliae* xylanase. Aligned sequences were used to determine sequence boundaries of loops from *C. adeliae* xylanase for insertion into the NG-27 xylanase. The resultant chimera designed contains 322 amino acids and has a molecular weight of 36.6 kDa. The pI of this protein is 4.44. We expect that this novel enzyme will be thermostable and show activity in the range in which a mesophile-derived xylanase shows activity.

# CHAPTER2

# MATERIALS AND METHODS

## 2.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a biochemical technique for amplifying selected fragments of DNA with the help of specific primers. This highly useful technique was developed by Kary Mullis in 1983. It consists of following steps :

Initial denaturation step: This step consists of heating of reaction mixture at a temperature of 94-96ºC. This is required for the activation of DNA polymerase as well as unwinding of the template DNA.

Denaturation step: This step consists of complete melting of DNA strands by heating them at 95ºC.It causes DNA melting by disruption of hydrogen bonds between complementary bases of parent DNA strand.

Annealing step: This step allows annealing of primers to the single stranded DNA. Typically the annealing temperature is about 3-5ºC below the Tm of the primers used.

Elongation or extension step: In this step the thermostable DNA polymerase synthesis a new DNA strand complementary to template strand by additon of dNTPs in 5'to 3' direction. The extension step is generally carried out at a temperature of 72ºC.
The repeated cycles of denaturation, annealing and elongation steps was performed 30-35 times to ensure that desired gene gets sufficiently amplified.

Final elongation: This step is performed at a 72ºC for 10 minutes after the last PCR cycle to ensure that all remaining single stranded DNA is fully extended.

Final hold: This step allows for an indefinite storage of reaction mixture at 4ºC

### Components:

**Template DNA:** The amount of template DNA used in a 25µl reaction is in the range of 0.01-1ng for plasmid and 0.1-1 µg for genomic DNA. For all reactions performed for this

study the template concentration was 2ng/ reaction.

**Primers**: PCR primers are generally, 18-28 nucleotides in length. The GC content of a primer directly determines its melting temperature and should be maintained between 40-60%. It can be calculated using the formula:

$$Tm = 4 (G + C) + 2 (A + T) \{When\ primer < 25\ nucleotides\}$$

The concentration of primers in the final reaction was 0.5µM.

**dNTPs:** The concentration of each dNTP in a 25µL reaction mixture is usually 200 µM. These are sensitive to freeze-thaw cycles and tend to get used up towards the end of a typical reaction.

**Phusion DNA polymerase**: The DNA polymerase used in this study was Phusion DNA polymerase (*New England Biolabs*). It is a high fidelity enzyme with proof reading activity.

**Method:** Gently vortex and briefly centrifuge all solutions after thawing. Add all the reagents in a thin-walled PCR tube, on ice. Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube. Place samples in a thermo cycler and start PCR.

PCR conditions used for amplification of our templates were the following:

1) Initialization step: 98ºC- 30sec
2) Denaturation step: 98ºC- 10sec
3) Annealing step : 60ºC 30sec
4) Extension step : 72ºC 30sec
5) Final elongation :72ºC 10 min
6) Final extension :4ºC

Steps 2, 3 and 4 were repeated 25-30 times to ensure that we get sufficient amount of product of our gene of interest.

| S no. | Name of Primer | Sequence(5'-3') | Length |
|---|---|---|---|
| 1 | Xylanase mutation reverse | 5'-GTGAATCCCAACAAATATTATG-3' | 22 |
| 2 | Xylanase mutation forward | 5'-ATAATATTTGTTGGGATTCAC-3' | 22 |
| 3 | 10 Reverse Xho1 | 5'-ATTGAATAGCTGGAGTCGATGATACGCC-3' | 28 |
| 4 | 1 Forward Nde1 | 5'-AGATTCTACATATGGTTCAGCCGTTCG-3' | 27 |

In the first step we used primers 4 and 1 mentioned in table above to amplify the template. We obtained Product A(700bp) shown in lane 4,5,6 and 7 of figure given below.

In the second step we used primers 2 and 3 mentioned in above table to amplify the template. We obtained product B (300bp) shown in lane 8,9,10 and 11 of figure given below.

## 2.2 Restriction digestion of plasmid DNA and PCR product

Restriction digestion is a process in which phosphodiester bonds between adjacent nucleotides are broken by restriction enzymes at specific sequences. The restriction sites are most often palindromes and enzymes make either a blunt (straight) or staggered (stair-like) cuts in the double helix. One unit of enzyme cuts 1µg of vector and 200ng of an average sized gene. Generally in a standard digestion reaction, the template is mixed with buffer and enzyme/enzymes of interest (generally 1unit/reaction) and incubated for ½ hour at 37ºC

## Ligation reaction

Ligation involves insertion of the desired gene into a vector with help of DNA ligase. This is the last step in the formation of a genetically modified DNA vector. A conventional ligation reaction, employing the use of T4 DNA ligase is carried out at 16°C overnight. This vector can then be transformed into a host organism and its expression can be checked. However, we have used Quick ligase (*New England Biolabs*) for the ligation reaction in which the components are incubated at 25°C for 15-30 minutes, before being transformed into the cloning host. The insert to vector kept for the ligation reaction was 3:1.

**Components:** The components of a typical ligation reaction are the following-digested vector, digested insert, DNA Quick Ligase, Quick Ligase Buffer. The amount of insert to be added is calculated using the following formula: Amount of insert= Amount of vector× (size of insert/size of vector)×3 {since 3/1 is the molar ratio of vector & insert}

## 2.3 Agarose Gel Electrophoresis

This technique is used to separate DNA fragments based on sizes by running them through an agarose matrix, using an electric field to move negatively charged DNA through the gel. The percentage of agarose in the solidified gel decides the pore size of the matrix. Smaller DNA fragments require higher concentrations of agarose for optimum resolution. Components: Typically, DNA gel electrophoresis uses agarose (a linear polysaccharide obtained from red algae), TAE Buffer (a buffer solution composed of Tris base, acetic acid and EDTA), ethidium bromide (fluorescent nucleic acid stain) and gel loading buffer (containing bromophenol blue dye and glycerol- to ensure complete settlement of DNA containing solution into the wells of agarose gel) Method: DNA fragments were fractionated on 0.8, 1.0, 1.4% agarose gels depending on their sizes. 6X gel loading buffer was added to DNA samples at a final concentration of 1X prior to loading onto the gel. Electrophoresis was carried out in 1X TAE buffer at ~ 8V/cm. Ethidium bromide (0.5 μg/ml) was supplemented in the agarose gel for visualizing DNA on an UV transilluminator. DNA ladders resolved at 100 bp, 500bp and 1 kb were used as markers for calculating the size of DNA fragments from their relative mobility.

## 2.4 Purification of DNA fragment(s) from agarose gels

After electrophoresis, DNA was visualized using a UV transilluminator. Agarose blocks containing the desired DNA fragment(s) were cut and weighed. DNA was extracted using a gel extraction kit (Qiagen, Germany) as described below: (1) Solubilization and binding buffer (Buffer QG,) was added at 3 volumes per volume of the agarose gel slice. (2) Incubation was done at 50°C till complete dissolution of the agarose gel was achieved. (3) The dissolved agarose solution containing the DNA was then poured onto a QIAquick spin column (provided by the manufacturer) to allow the adsorption of DNA onto the silica gel matrix. (4)The impurities were washed away with an ethanol-containing buffer (PE, supplied

by the manufacturer) (5) DNA was finally eluted in autoclaved distilled water, and quantitated.

## 2.5 Transformation

Genetic alteration of a cell resulting from the uptake, genomic incorporation, and expression of foreign DNA is called transformation. Following ligation, the ligation product plasmid is transformed into bacteria for propagation. An untreated bacterial cell cannot - on its own - efficiently take up foreign DNA. For this, cells are given specific treatment to make them competent to readily take up DNA present in their environment. The nature and preparation of competent cells has been discussed in the next section. Components: A typical transformation requires competent cells, plasmid {or ligation mixture} and LB Agar. Method: Add the entire ligation mix or 40-100ng of plasmid to 200µL of competent cells. Incubate on ice for thirty minutes followed by a heat shock of 42˚C for thirty seconds. Dispense this mix in 1mL LB broth and incubate at 37°C for one hour, along with vigorous shaking. This mixture can now be plated on an agar plate containing the antibiotic corresponding to the antibiotic selection marker of the transformed DNA.

## 2.6 Preparation of Competent Cells

Competence is the ability of a cell to take up foreign DNA from its environment. Competence is distinguished into *natural competence*, which is a genetically specified ability of bacteria that is thought to occur under natural conditions as well as in the laboratory, and *induced* or *artificial competence*, arising when cells in laboratory cultures are treated to make them transiently permeable to DNA. The process involves chemically treating the cells with $CaCl_2$. Calcium ions being positively charged form a layer around the bacterial cell wall and help in the uptake of the negatively charged DNA. Components: Transformation requires $CaCl_2$ solution {60mM $CaCl_2$, 15 % glycerol and 10mM PIPES}, Luria Bertani broth, and bacterial secondary culture that has been grown up to O.D. of 0.4. Method: Pre-chill the tips, eppendorfs and CaCl2 {100mM} at -20˚C. Aliquot the broth into the pre-chilled tubes and leave on ice for ten minutes. Centrifuge the cells for seven minutes, 1600g at 4°C. Pour off supernatant, resuspend pellet in 40µL ice cold $CaCl_2$ solution. Centrifuge cells for five minutes, 1100g at 4°C. Discard the supernatant, resuspend each pellet in 20µL ice cold

CaCl2 solution gently. Keep the resuspended cells on ice for thirty minutes. Centrifuge cells for five minutes, 1100g at 4°C. Discard the supernatant, resuspend each pellet in 2.0mL ice cold CaCl2 solution. Resuspend final pellet well. Aliquot 100µL cells into pre-chilled, sterile eppendorf tubes. Freeze immediately at -80°C.

## 2.7 Screening for positive transformants

The presence of the insert was confirmed by electrophoresing the plasmids on 0.8-1.2% agarose gels alongside a control plasmid lacking the insert. This was further confirmed by restriction digestion using the endonucleases used for cloning, or by PCR using the genespecific/vector-specific primers, followed by electrophoresis on gels of appropriate percentage.

## 2.8 SDS-PAGE

SDS-PAGE:Sodium dodecyl sulfate polyacrylamide gel electrophoresis is used to separate the protein according to size and electrophoretic mobility. SDS is an anionic detergent applied to a protein sample to linearize proteins and impart a negative charge to such linearized proteins. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Sample Preparation : Sample can be any material containing proteins. The sample is mixed with SDS an anioinic detergent which denatures the secondary and tertiary structures and applies a negative charge to protein in proportion to its mass. Heating the protein further at 90ºC further promotes protein denaturation and help SDS binding. A dye is also added to the solution, which helps to track the movement of protein solution through the gel in eletrophoretic run. Preparation of Acrylamide gels : The gel consists of acrylamide (30%), bisacrylamide (0.8%),SDS and a buffer adjusted to pH 6.8 in stacking gel and pH 8.8 in resolving gel. Ammonium persulfate (APS) and TEMED are added to initiate polymerization. The polymerization occurs due to bisacrylamide which cross links the two acrylamide chains. In the present work 13% acrylamide was used is resolving gel and 5% acrylamide was used in stacking gel. The amount in which various components have been put is given in below.

Stacking gel composition:

| S no. | Ingredients | Volume |
|---|---|---|
| 1 | Water | 3.067 ml |
| 2 | Acrylamide(30%, 0.8%) | 4 ml |
| 3 | Lower tris (1.5 M, pH 8.8, 10% SDS) | 2.5ml |
| 4 | TEMED | 50µl |
| 5 | APS(10%) | 10µl |

Resolving gel components

| S no. | Ingredients | Volume |
|---|---|---|
| 1 | Water | 1 ml |
| 2 | Acrylamide(30%, 0.8%) | 0.333 ml |
| 3 | Upper tris (1.5 M, pH 8.8, 10% SDS) | 0.5ml |
| 4 | TEMED | 12.5µl |
| 5 | APS(10%) | 5µl |

Running Buffer:

This is the reservoir buffer in which gel was run. The various components used were:

1) Tris buffer.....3gm
2) Glycine.....14.4 gm
3) SDS.....1 gm

After adding all the components, the pH was adjusted to 8.3 and the volume was made upto 1000 ml.

Sample Loading buffer(5x)**:** It consists of glycerol, bromophenol blue, beta mercaptoetanol and 1M tris buffer pH 6.8.It is mixed with protein samples and the samples were boiled for 5min prior to loading them in the gel.

## 2.9 Ni-NTA affinity purification

Immobilized-metal affinity chromatography (IMAC) was first used to purify proteins (Porath et al. 1975) using the chelating ligand iminodiacetic acid (IDA, IDA was charged with metal ions such as Zn 2+, Cu 2+ , or Ni 2+ , and then used to purify a variety of different proteins

and peptides (Sulkowski 1985). IDA has only 3 metal-chelating sites and cannot tightly bind metal ions. Weak binding leads to ion leaching upon loading with strongly chelating proteins and peptides or during wash steps. This results in low yields, impure products, and metal-ion contamination of isolated proteins. Nitrilotriacetic acid (NTA) is a tetradentate chelating adsorbent that overcomes these problems. NTA occupies four of the six ligand binding sites in the coordination sphere of the nickel ion, leaving two sites free to interact with the 6xHis tag. NTA binds metal ions far more stable than other available chelating resins and retains ions under a wide variety of conditions, especially under stringent wash conditions. The unique NTA matrices can therefore, bind 6x His-tagged proteins more tightly than IDA matrices, allowing the purification of proteins from less than 1% of the total protein preparation to more than 95% homogeneity in just one step . The 6xHis tag: The 6xHis affinity tag facilitates binding of polypeptides to Ni-NTA. The peT23a vector which is being used in the present study contains a C-terminal hexahistidine tag. It is poorly immunogenic, and at pH 8.0 the tag is small, uncharged, and therefore does not generally affect secretion, compartmentalization, or folding of the fusion protein within the cell. In most cases, the 6xHis tag does not interfere with the structure or function of the purified protein as demonstrated for a wide variety of proteins, including enzymes, transcription factors, and vaccines. Purification under native or denaturing conditions: The decision whether to purify 6xHis-tagged proteins under native or denaturing conditions depends on protein location and solubility.In native purification we did not denature the cell and protein is purified with buffers that does not contain any denaturing agent. However in denaturing purification some denaturing agent is used like urea or GuHCl.

Buffers for purification under denaturing conditions

Lysis buffers

**Buffer A (1 liter):**

| | |
|---|---|
| 100 mM $NaH_2PO_4$ | 13.8 g $NaH_2PO_4 \cdot H_2O$ (MW 137.99 g/mol) |
| 10 mM Tris·Cl | 1.2 g Tris base (MW 121.1 g/mol) |
| 6 M GuHCl | 573 g guanidine hydrochloride |

Adjust pH to 8.0 using NaOH

**Buffer B (1 liter):**

| | |
|---|---|
| 100 mM NaH2PO4 | 13.8 g NaH2PO4·H2O (MW 137.99 g/mol) |
| 10 mM Tris·Cl | 1.2 g Tris base (MW 121.1 g/mol) |
| 8 M urea | 480.5 g (MW 60.06 g/mol) |

Adjust pH to 8.0 using NaOH

Wash buffer

**Buffer C (1 liter):**

| | |
|---|---|
| 100 mM NaH2PO4 | 13.8 g NaH2PO4·H2O (MW 137.99 g/mol) |
| 10 mM Tris·Cl | 1.2 g Tris base (MW 121.1 g/mol) |
| 8 M urea | 480.5 g (MW 60.06 g/mol) |

Adjust pH to 6.3 using Hcl.

**Buffer E (1 liter):**

| | |
|---|---|
| 100 mM NaH2PO4 | 13.8 g NaH2PO4·H2O (MW 137.99 g/mol) |
| 10 mM Tris·Cl | 1.2 g Tris base (MW 121.1 g/mol) |
| 8 M urea | 480.5 g (MW 60.06 g/mol) |

Adjust pH to 4.5 using HCl.

Buffers for purification under native conditions

**Lysis buffer (1 liter):**

| | |
|---|---|
| 50 mM NaH2PO | 46.90 g NaH2PO4·H2O (MW 137.99 g/mol) |
| 300 mM NaCl | 17.54 g NaCl (MW 58.44 g/mol) |
| 10 mM imidazole | 0.68 g imidazole (MW 68.08 g/mol) |

Adjust pH to 8.0 using NaOH.

**Wash buffer (1 liter):**

| | |
|---|---|
| 50 mM NaH2PO | 46.90 g NaH2PO4·H2O (MW 137.99 g/mol) |
| 300 mM NaCl | 17.54 g NaCl (MW 58.44 g/mol) |
| 20 mM imidazole | 1.36 g imidazole (MW 68.08 g/mol) |

Adjust pH to 8.0 using NaOH.

**Elution buffer (1 liter):**

| | |
|---|---|
| 50 mM NaH2PO4 | 46.90 g NaH2PO4·H2O (MW 137.99 g/mol) |
| 300 mM NaCl | 17.54 g NaCl (MW 58.44 g/mol) |
| 250 mM imidazole | 17.00 g imidazole (MW 68.08 g/mol) |

Adjust pH to 8.0 using NaOH.

We employed both the methods for purification. However the denaturing purification using 8M urea or 6M GuHCl proved to be best suited for the protein under study.

## 2.10 Desalting-column refolding

To obtain protein in soluble form and free from denaturant, we tried refolding by removing denaturant through desalting chromatography using Sephadex G-25 resin (PD-10 column). For this, protein containing denaturant was loaded onto the column of volume 10 ml. The first 2 ml of flow through were discarded and the next 2.5 ml of flow through containing protein were collected. The remaining flow through (containing the denaturant) was discarded. The collected protein fraction was then studied by gel filtration chromatography and spectroscopic methods.

## 2.11 Flash Dialysis:

Dialysis based refolding was attempted for protein purified under denatured conditions (6M GuHCl/ 8M Urea). Dialysis was done using a microdialyser (1ml) against 200 ml of buffer, and using membranes of 10 kDa cut-off, with 4-5 rapid changes, followed by two rounds of extended dialysis. The protein was allowed to refold by the rapid decrease in the concentration of the denaturant. During the process no visible precipitation of protein was observed.

## 2.12 Gel Filtration Chromatography:

The gel filtration elution profiles were obtained on a GE AKTA purifier system. The pumps and the columns were washed with filtered millipore Elix water and then equilibrated with 50 mM tris,150 mM NaCl and pH 8.0 Tris buffer. Superdex 200 column with bed volume of 24

ml was used for gel filtration studies. 500 µl of protein sample was loaded onto the column. Gel filtration was done both with samples refolded through flash dialysis and with samples refolded through desalting-based removal of denaturant.

## 2.13 Circular Dichroism (CD) Spetroscopy:

CD spectra were collected on an Applied Photophysics ChiraScan instrument (for the dialysed sample) and on a Jasco 810 spectropolarimeter (for the desalted sample) in quartz cuvettes of 0.1cm path length. Before switching on the instrument's lamp nitrogen was flushed for 15 minutes. The far UV-CD spectrum (which gives us information about the secondary structure elements in a protein) of the protein was collected in the range of 200-260 nm. Five spectra were collected for the experiment and then data was averaged and smoothed. Raw ellipticity was then converted into mean residue ellipticity using the following formula: Mean residue ellipticity = Raw ellipticity *100* MRW/1000*concentration (mg/ml)*path length (cm), where MRW= Mean residue weight/Total no. of amino acids

## 2.14 DNSA-based Xylanase activity assay:

DNSA(3,5-Dinitrosalicyclic acid) is an aromatic compound that reacts with reducing sugars to form 3-amino 5-nitrosalicyclic acid which absorbs light strongly at 540nm. Preparation of Xylan substrate: 1g of Xylan was mixed into 100 ml of water. The suspension was autoclaved. After that the suspension was sonicated for 20 minute with 5 seconds on and 5 seconds off pulse rate and the amplitude was being set at 20. Activity Reaction: We checked its reactivity at six different temperatures 10ºC ,20ºC ,30ºC ,40ºC ,50ºC and 60ºC. 150µl of substrate was filled in each eppendorf tube and 10µl of enzyme was added to each eppendorf tube and then the total volume was made upto 200µl by addition of 40µl of buffer. For each reaction there was a control in which no enzyme was added. All eppendorf tubes were incubated for one hour at different temperatures described above. After one hour of incubation, 300µl of DNSA was added to all eppendorf tubes and all the tubes were put in boiling water for half an hour to check whether this enzyme chimera is active or not.

In addition, we also did plate assays in which suspended xylan mixed with agar was allowed to react with protein placed in a cavity in the agar, to examine whether there was formation of a zone of clearance around the cavity, showing the protein to be active enzymatically.

# CHAPTER 3

# RESULTS AND DISCUSSIONS

# 3. Results and Discussion

## 3.1 Correction of Frame shift position

It was mentioned in the introduction that there was a frame shift mutation in the cloned gene that we came to know about only after getting the previous clone sequenced (prepared by Kirandeep Kaur). The frame shift mutation was corrected using splicing by overlap extension polymerase chain reaction (SOE-PCR). After the getting the correct sized gene (in which the mutation was also corrected), we inserted our gene into a pet23a vector. The gene was cloned between Nde 1 and Xho1 sites. Various sets of primers used during the PCR reactions are given in the table in the materials and methods section. In the first step, we used primers 4 and 1 mentioned in above-mentioned table to amplify the template. We obtained Product A (700bp) shown in lane 4,5,6 and 7 of figure given below. In the second step we used primers 2 and 3 mentioned in above table to amplify the template. We obtained product B (300bp) shown in lane 8,9,10 and 11 of figure given below.

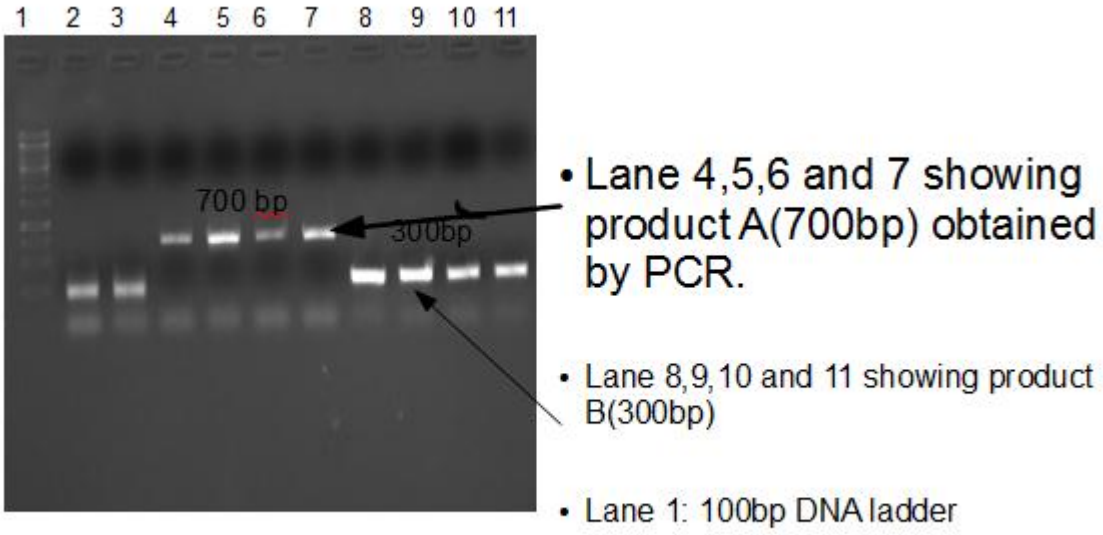



Figure 3.1: Agarose gel showing amplified products

## SOE-PCR

Next step was to amplify our gene using to PCR products obtained above. PCR products obtained above (Product A and Product B) were used as template in SOE PCR and 1F Nde1 and 10 R Xho1 primers were used to amplify the product.

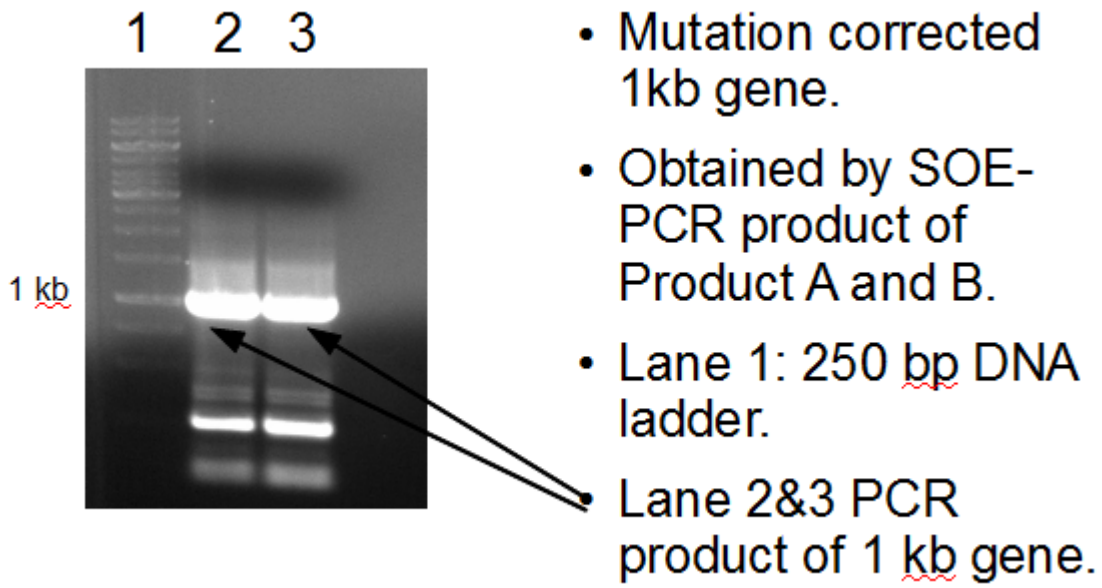
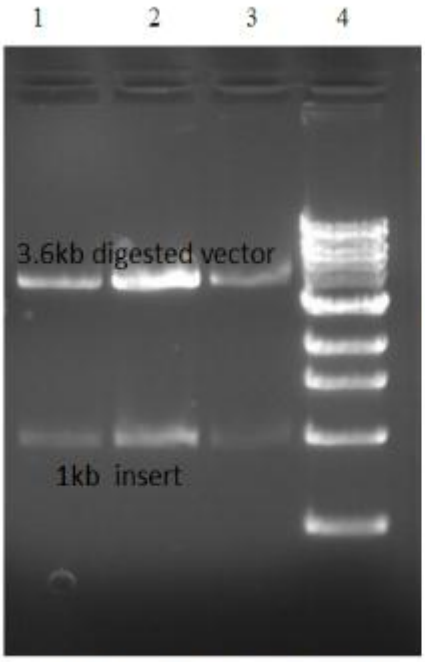



Figure 3.2: Gel electrophoresis picture showing SOE PCR.

## Confirmation of positive clones

Positive clones were confirmed using restriction digestion reaction. Restriction enzymes (Nde1 and Xho1) cut the plasmid containing the insert at specific sites leading to the release of the insert and this reaction when run on agarose gel shows digested plasmid and insert in the positive clones. All the three clones examined were positive showing the band of vector (3600 bp) and the insert (1000bp) [Fig.3.3]




Agarose gel showing seprated vector 3.2kb and insert 1 kb

Lane 1,2 and 3: Separated vector of size 3.6 kb and insert of 1kb size.

Lane 4: 500bp DNA ladder.

Figure 3.3 Agarose gel showing separated vector 3.2kb and insert 1 kb

Alternatively the clones were also confirmed using colony PCR in which 8 colonies were screened for the positive clones using the 1F and 10R primers.Out which we got only two positive clones.

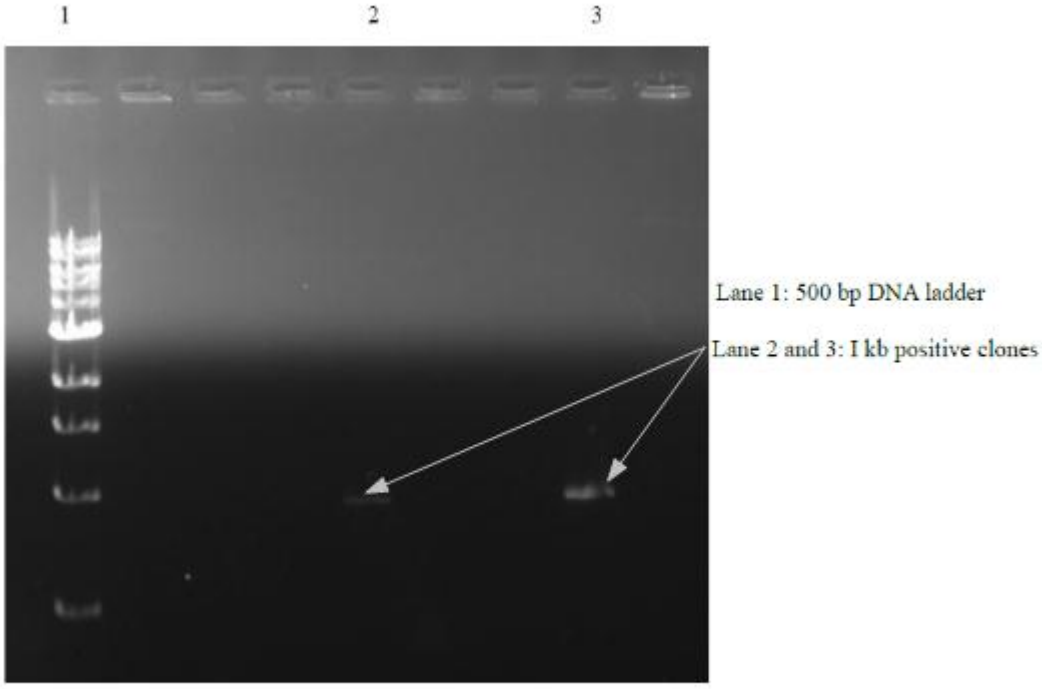



Figure 3.4: Agarose gel showing positive clones using colony PCR

# 3.2 Protein expression and purification

**Protein expression**: After getting DNA sequencing done for our gene of interest, we expressed the gene. The gene was transformed into two variants of BL21 (DE3) viz, BL21 Star (DE3) pLysS and BL21 (DE3)pLysS. BL21 (DE3) pLysS and BL21 Star (DE3) pLysS have antibiotic resistance for chloramphinicol. We observed a better expression in the first variant. So, we continued our further studies using BL21star (DE3) pLysS. Till this point we purified protein using native purification methods, but to our disappointment, the protein was observed only in cell pellet, showing that the protein was retained in inclusion bodies. So, we moved on to denaturing purification. We used 8M urea to extract the protein from the cell pellet. After denaturing purification protein came out of the inclusion bodies and it was observed in elution fractions after purification through Ni-NTA coloumn.

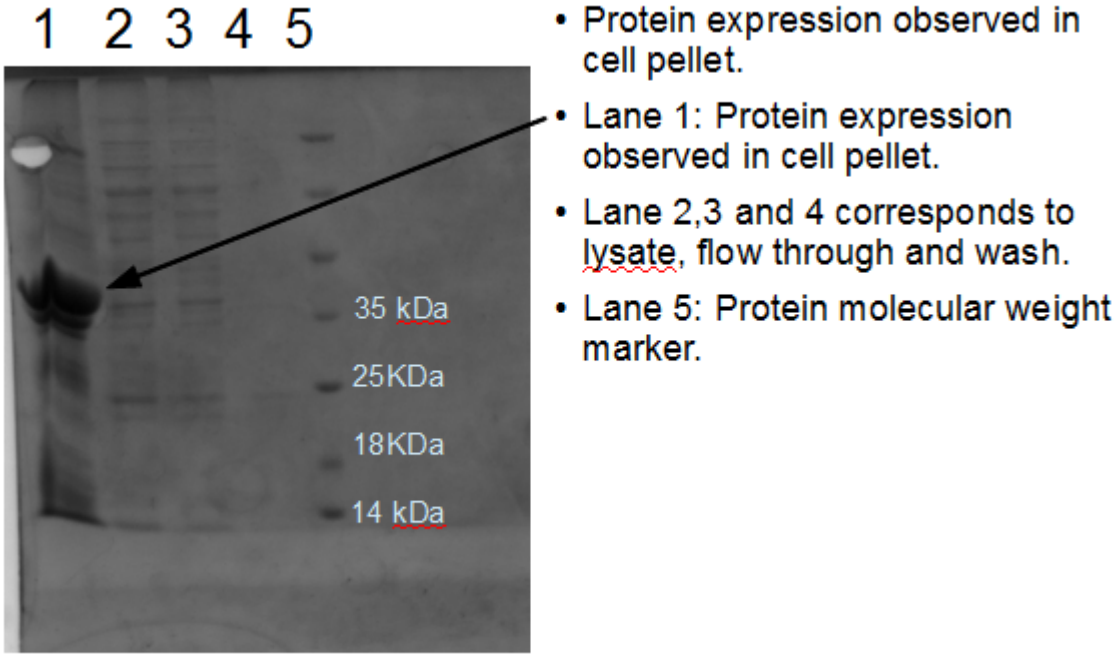



Figure 3.5 SDS Page Gel showing protein in cell pellet

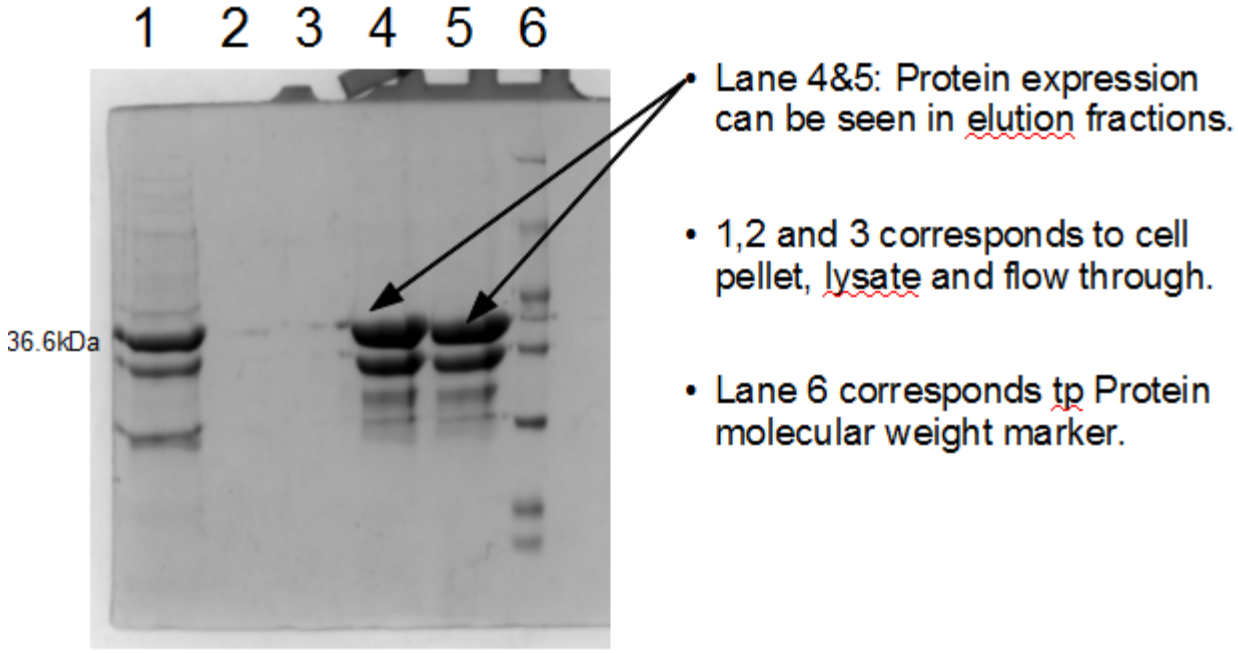



Figure 3.6 SDS Page gel showing Protein in elution fractions

## 3.3 Optimization

Since the cloned protein was a novel one, there was a need to optimize the conditions under which it can be best expressed and purified so that we can get a good yield of protein. We tried different conditions on each and every step of purification protocol to optimize conditions.

a) **Isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration**: IPTG is a compound that triggers the transcription of lac operon. IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon and in promoters designed using portions of the lac operon which are under the control of lac repressor. We tried four different concentration of IPTG to check at what bare minimum concentration of IPTG protein can be expressed. When the secondary culture grown at 37 deg C reaches an OD at 600 nm of 0.6, it was induced with different concentrations of IPTG viz. 1.0 mM, 0.5 mM, 0.25 mM and 0.1mM. We got same amount of expression in all four concentrations [Fig.3.7]. So we moved further taking 0.1 mM as standard IPTG concentration.

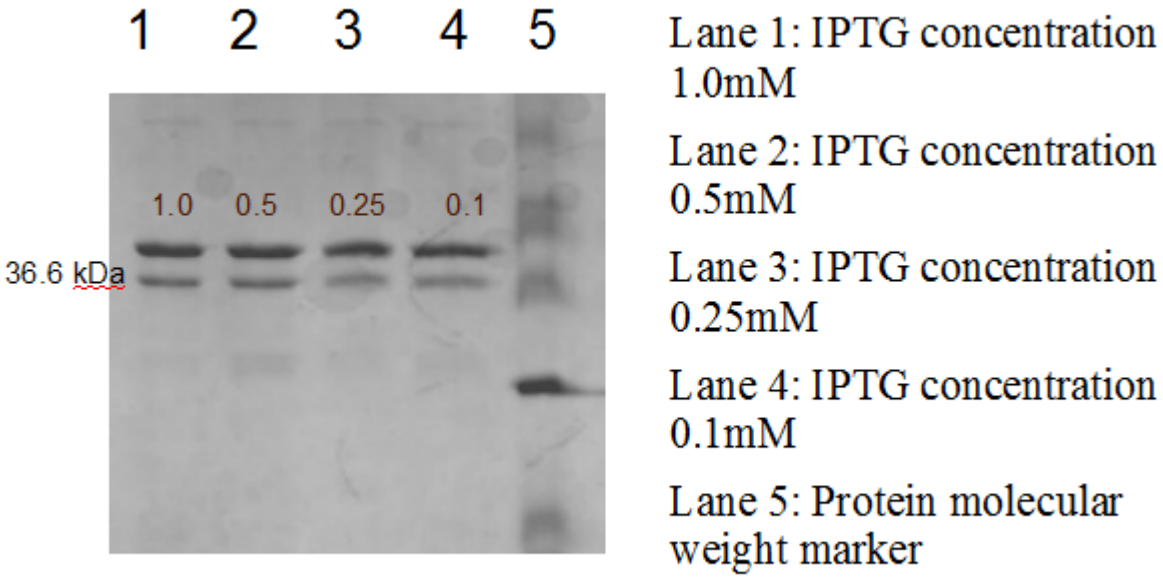



Figure 3.7: SDS Page gel showing protein expression in different concentrations of IPTG

b) **Incubation temperature**: After optimizing IPTG concentration next step was to optimize the incubation temperature at which protein is expressed. Incubation temperature is an important step because if cultures are not provided optimum temperature for their growth, we cannot expect good expression. We tried three different temperatures, 37 ºC, 25 ºC and 18 ºC. The cultures incubated at 25 ºC and 18 ºC after induction were subjected to overnight incubation while the culture incubated at 37ºC was given 5 hours incubation. The amount of expression we observed cultures incubated at 25ºC and 18ºC was very less as compared to expression observed in cultures incubated at 37ºC. Furthermore, there was no soluble protein obtained even at low temperature (where lower hydrophobic interactions can result in some soluble protein), suggesting that there was no point in compromising total protein yield by incubating cultures after induction at lower temperatures. Hence, we used 37 ºC as the standard incubation temperature for further studies [data not shown, as a computer crash destroyed these gel pictures].

c) **Denaturant concentration**: As observed previously, protein remains in cell pellet if purified using native purification protocol since protein enters inclusion bodies. While using denaturing purification protocol, although clearly protein could be extracted using 8.0 M urea, we wanted to optimize concentration of urea required to get protein out of the cell pellet, to see whether protein could be purified under less harsh denaturing conditions. We used five different concentrations of urea to find out what is the bare minimum concentration at which protein comes out of cell pellet. Different concentrations of urea used were 0 M, 1 M, 2 M, 4 M, 6 M and 8 M. We started from 0 M in increasing order towards 8 M. Interestingly, the protein did not come out of the cell pellet till 6 M Urea and it came out at 8M urea. May be protein can come out of cell pellet at any concentration greater than 6 M but we didn't try any concentrations between 6 M and 8 M, So it can be investigated further.

d) **Amount of urea required to keep protein in soluble fraction:** We also tried to investigate the minimum amount urea required to stop protein from aggregating. We got our protein eluted in the buffer which contains 8M urea. In order to refold, urea has to be dialyzed out, but we can't do it suddenly because there is a chance of the protein getting

precipitated if urea is removed too suddenly. So, we have to reduce the concentration of urea gradually. We did it in two different ways listed below:

1) Dialysis
2) Desalting chromatography-based refolding

Using both of the methods listed above we removed urea. Even at 0 M urea concentration, the protein didn't get precipitated so we can say that this protein does not require urea to stay in a folded state.

## 3.4 Biophysical Characterization

## Gel Filtration Chromatography

It is based upon the size exclusion principle. Large molecules tend to elute earlier as compared to molecules which are small in size. This is because molecules with small size enter into gel beads but large molecules are not able to enter in gel beads so they elute out early. We can use this technique to analyze our protein that whether it is oligomerizing or not. If it is oligomerizing then it will elute out earlier than expected.

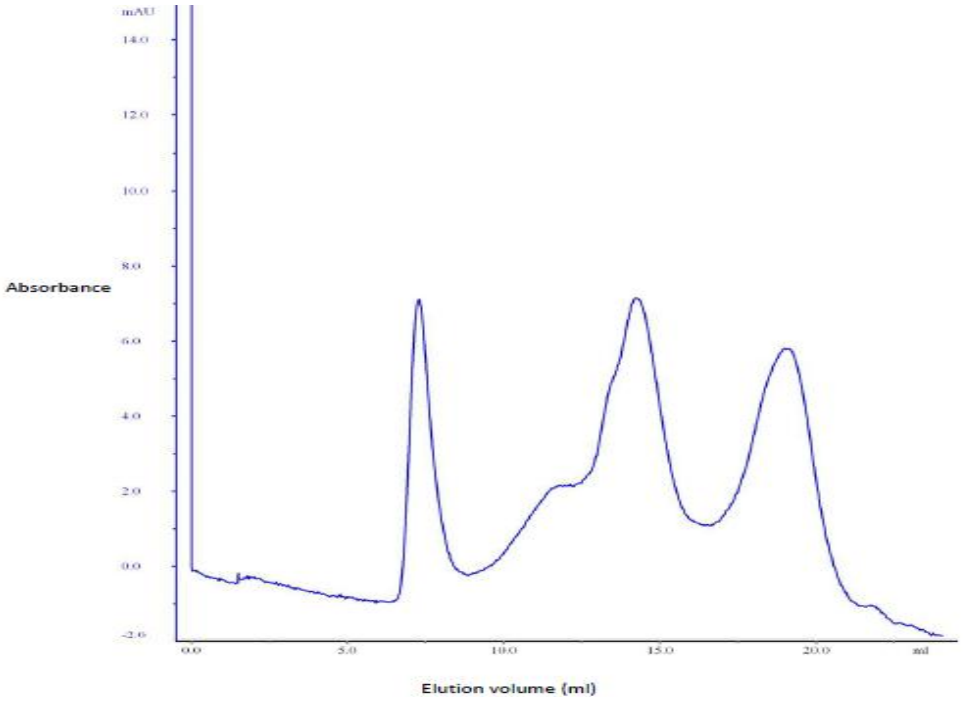



Figure3.8: Gel filtration profile after PD 10 Column refolding

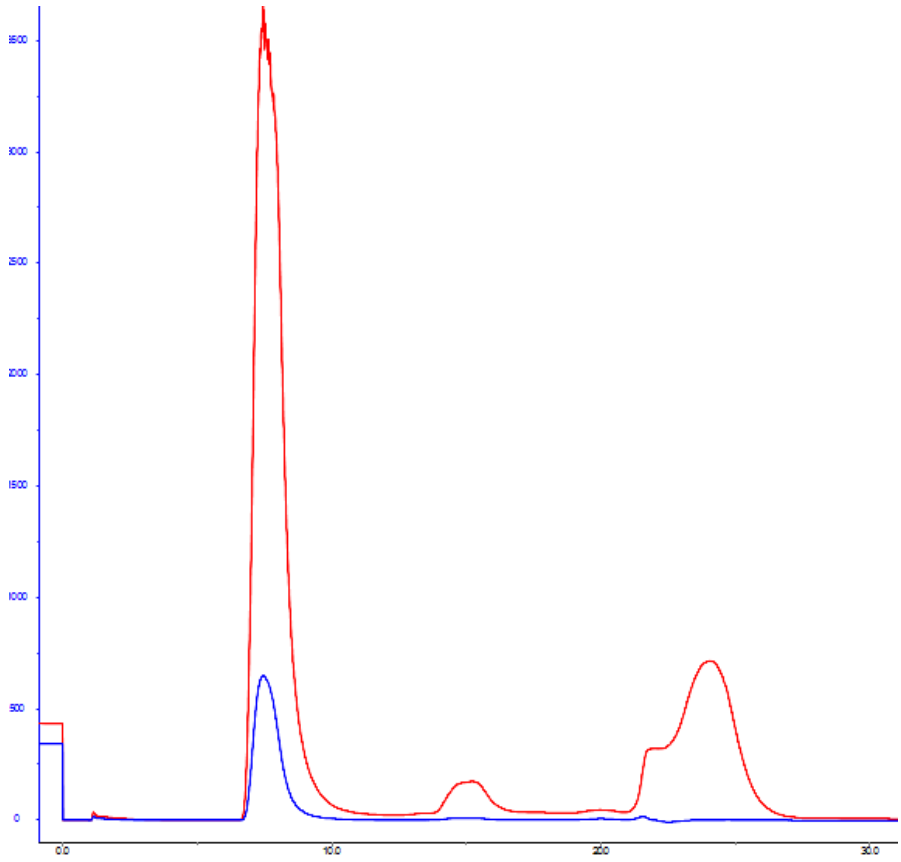


Figure 3.9: Gel filtration profile after dialysis showing absorption at two different wavelengths of 220 nm (red) and 280 nm (blue).

As evident through the (Figures 3.8 and 3.9) the gel filtration profile in both the cases i.e., dialysis and desalting was somewhat similar, in that in both the cases protein was seen to elute out at void volume ~ 8 ml, due to the fact of majority of the population being present as soluble aggregates. However, there is another population which peaks around 15 ml corresponding to the dimeric state of the protein, and this peak is populated significantly in the desalting column-derived sample.

**CD (Circular Dichroism)**: CD is based on differential absorption of left and right circularly polarized light. It is exhibited by optically active chiral chromophores moieties. The far-UV CD spectra of proteins can give us important insights about their secondary structures. CD spectra can be readily used to estimate the fraction of a molecule that is in the alpha helix conformation, the beta sheet conformation, the beta turn conformation, or some other (e.g. Random coil) conformation. It can be used to study how the secondary structure of a molecule changes as a function of temperature or of the concentration of denaturing agents, e.g. GuHCl and urea. We used CD to check whether our refolded protein is structured or not. If refolding was good enough then it will show signature peaks of alpha helix and beta sheet. We also did thermal denaturation to check at what temperature it is getting unfolded.

Two kinds of samples were studied, namely those derived from dialysis and those derived from desalting. As already mentioned, there was some dimer obtained after desalting-based removal of denaturant, but only soluble aggregate obtained through dialysis. The two samples both showed evidence of the formation of mixed beta and alpha structures in their CD spectra. Both spectra had the characteristic 222 nm and 208 nm bands indicative of alpha helical structure and the sample obtained through desalting had a somewhat more intense 222 nm band, perhaps because of a greater content of beta sheet structure (contributing to the signal in this region, due to its negative 218 nm band).

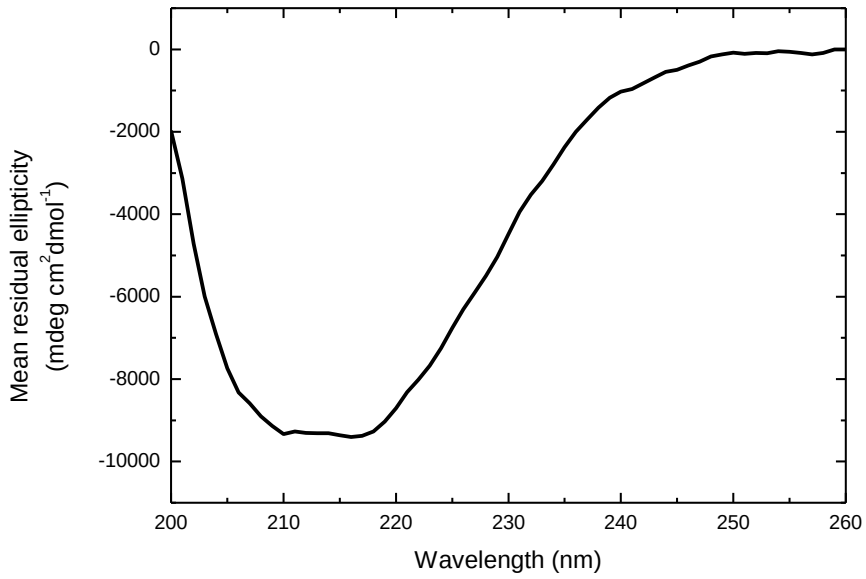



Fig. 3.10: Far UV CD-spectrum of chimeric xylanase refolded after dialysis

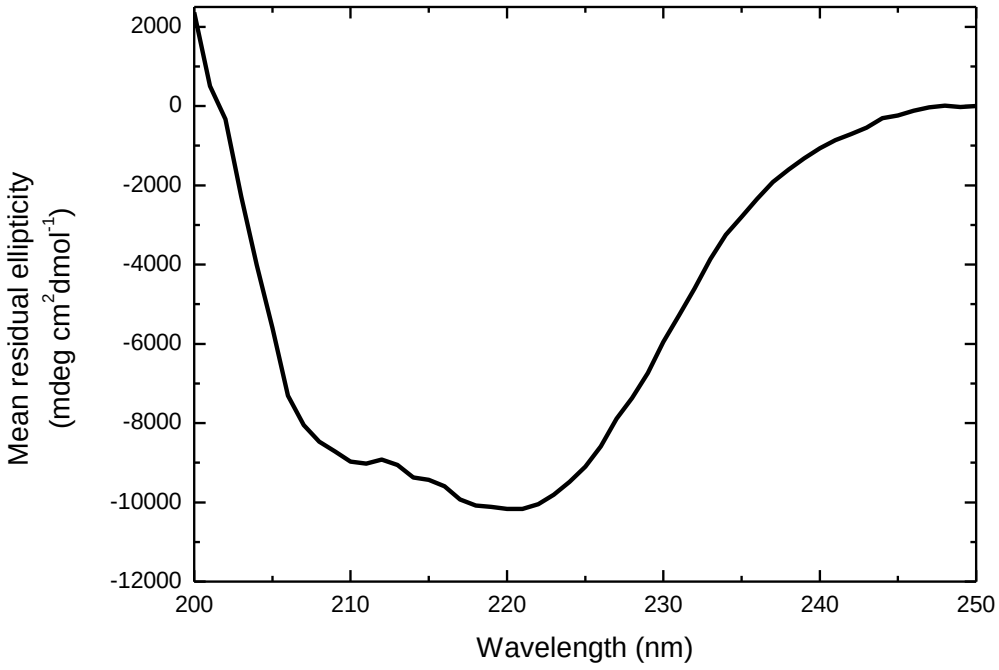



Figure3.11: Far UV CD-spectrum of chimeric xylanse refolded on PD10 (desalting) column

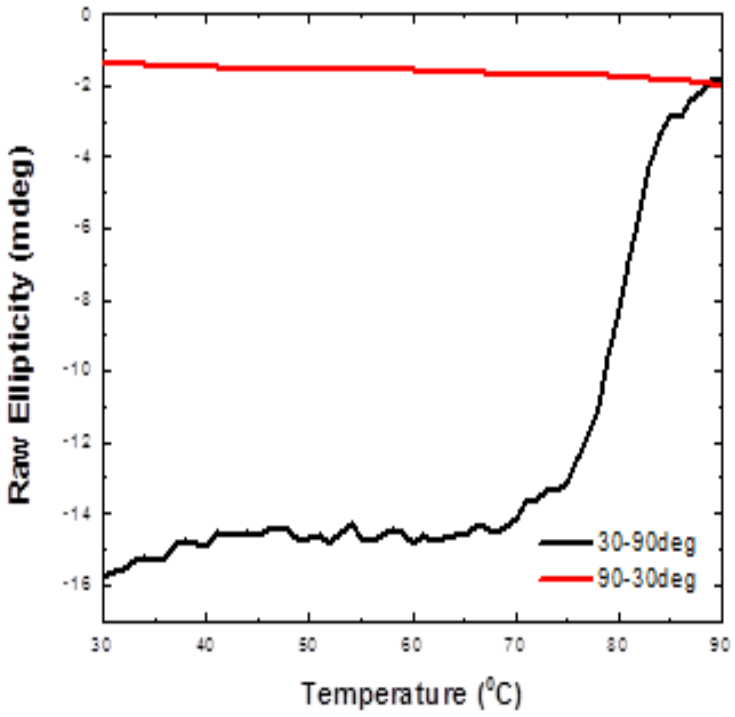



Figure 3.12: Thermal denaturation of PD10 refolded protein

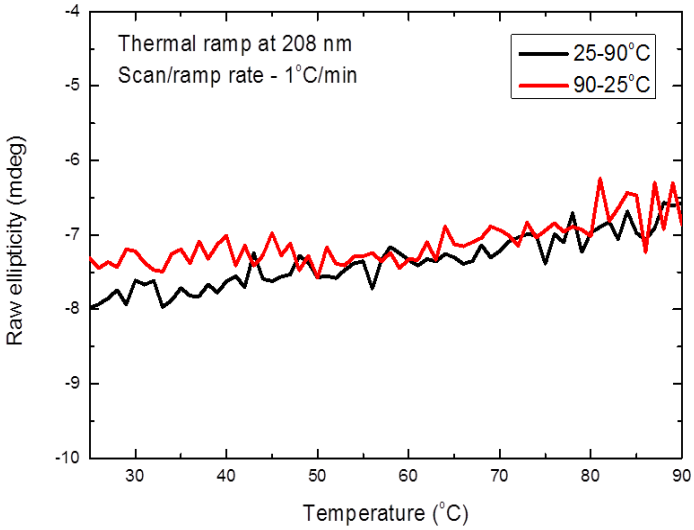



Figure 3.13 a: Thermal denaturation of protein refolded by dialysis.

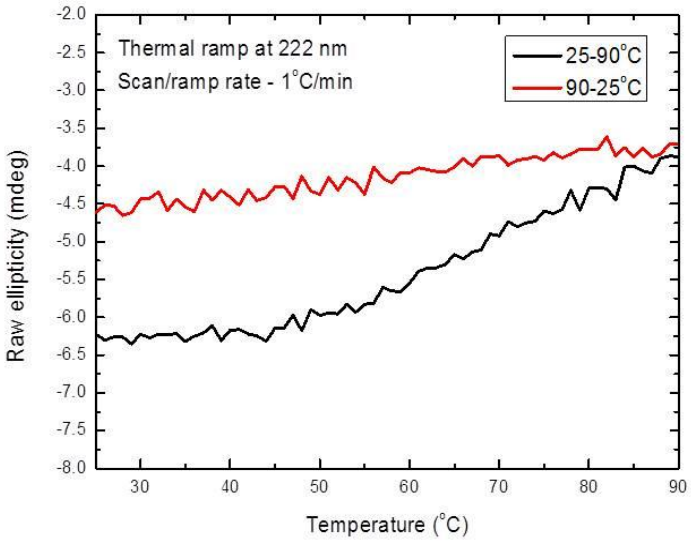



Figure3.13 b: Thermal denaturation of protein refolded by dialysis.

During thermal denaturation, the desalted protein displayed a cooperative melting profile above 70 deg C, whereas the dialyzed sample displayed no clear melting. This is consistent with the fact that the former had a large content of dimeric species, whereas the latter was largely a soluble aggregate eluting in the void volume of the gel filtration column, and expected to display great resistance to thermal

## 3.4 Activity assays

The desalted protein was examined at two temperatures (~30 and ~80 deg C), while the dialyzed protein was examined at about 8 different temperatures. None of the reactions performed showed any change in color implying that this enzyme was not active at these temperatures. However, scope remains for working further with the desalting-based sample and examining it at other temperatures and over longer durations of incubation in assays to see if there is any trace activity which can be improved through further protein engineering. This possibility remains to be explored. Also, in the plate based assay, in which the plate containing the refolded protein kept for overnight incubation at 37ºC, we could not see any activity.

# Conclusions:

1) We were successful in making a chimeric xylanase in which β/α loops of *C. adeliae* xylanase have been transplanted onto NG-27 xylanase scaffold.

2) The best expressing conditions for protein is 0.1 mM IPTG and incubation at 37°C after induction.

3) Because the protein was present in cell pellet ,protein was purified under denaturing conditions and the minimum concentration of the denaturant required for extracting out protein from cell pellet was 8 M urea or 6M GuHCl.

4) All methods (dialysis, desalting) used for refolding resulted in refolded protein. The desalting method produced some dimeric populations, while the dialyzed sample produced soluble aggregates.

5) Whether as a dimer, or as a soluble aggregate, the refolded protein is structured and shows the characteristic peaks of alpha helix and beta sheet.

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