CLONING, EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THERMOPHILIC ENZYMES CATALYZING PLANT BIOMASS DEGRADATION

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A dissertation submitted for the partial fulfilment of MS degree in Science

Indian Institute of Science Education and Research, Mohali

April 2022

To,

My parents and Nene

Certificate of Examination

This is to certify that the dissertation titled **'Cloning, expression, purification and characterization of thermophilic enzymes catalyzing plant biomass degradation'** submitted by **Aarushi Naskar** (Reg. No. MP19013) for the partial fulfillment of MS 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.

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Dated: 24.04.22

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.

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Dated: 24.04.22

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]
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Acknowledgements

First and foremost, I would like to thank my supervisor Prof. Purnananda Guptasarma, for introducing me to the world of Proteins, for guiding me throughout my thesis with his engaging discussions and his stimulating suggestions. I have been very fond of the way he interprets research data and life as well. His sheer intuition that he shared has affected how I think about science and especially about life.

I would also like to express my gratitude and appreciation for my lab members. Their constant motivation, support, love and care, played an indispensable role in my thesis. I will be forever grateful to Arpita Sarkar for all her help and support she has provided and for always having my back.

I will always be indebted to my friends for their unwavering support and for reminding me to take short breaks and long walks when things looked grim. Special thanks to Sayan, Yogita for making a difference and to Uttaran for always believing in me, when I didn't.

I am obliged to my family for their unconditional love and support. I am grateful to my parents for their constant belief in me and to my sister without whose support I won't be the person I am today.

Last, but not the least, I am thankful to the existence of music in my life which has kept me going in all times of pressure.

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ABSTRACT

With the ever-increasing demand for food and crops, which are the primary source of production of 1st generation biofuels, the world is moving towards 2nd generation biofuels. The most abundant constituent of biomass, cellulose, is a regenerating source of biomass energy, however, it is present in a meshwork of lignin and hemicellulose, which are complex polymers and hence rigid and recalcitrant. Thus, the most essential step in the generation of 2nd generation biofuels from biomass is efficient enzymatic depolymerization. Thermophilic and hyperthermophilic cellulases which are very advantageous, owing to their ability to survive high temperatures, could be the best candidates for cellulose depolymerization. Here, we discuss our efforts to clone, express, purify and characterize some of the most potent and effective thermostable cellulases from the genome of *Clostridium thermocellum* and *Pyrococcus furiosus*. Through structural characterization, most of the enzymes were observed to be thermostable as well as resistant to chemical denaturation by urea and guanidine chloride. We also describe the attempts to study their activity on the substrate carboxymethyl cellulose with varying temperatures.

Chapter 1.
Introduction

INTRODUCTION

As the demand for energy and its resources all around the world increases, humankind has been trying to discover and use new renewable and sustainable sources of energy. The limited amount, high demand and cost of fossil fuels in addition to the surplus of greenhouse gases have made them impractical and outdated as a source of energy. With this, new renewable sources of energy have become all the more important. (Ellabban, Abu-Rub, & Blaabjerg, 2014).

One of such renewable sources of energy is biofuel which is advised as a cost-efficient and environmentally admirable alternative as opposed to fossil fuels such as petroleum, coal and gas (Lehman, 2020). Biomass, the source of biofuel, can be replenished readily unlike fossil fuels and therefore has grabbed much attention.

1.1.1 Biomass and its composition

Any live or recently deceased organism more specifically plants and their by-products that can be used in the creation of energy is referred to as biomass. Biomass feedstocks comprise woody crops, agricultural crop residues, forestry residues, algae wood processing residues, municipal garbage and wet waste. These can either be combusted or transformed into another form of energy ("Biomass Resources"). Biomass resources offer a ubiquitous and regenerating resource for the generation of second-generation biofuels (Leis et al., 2018).

The plant biomass is a heterogenous composition of plant polysaccharides, which can be divided into two categories: storage polysaccharides and plant cell wall polysaccharides (Table 1.1). The second generation of biofuels attempts to exploit polysaccharides found in plant cell walls, such as celluloses, hemicelluloses, and pectin. Different forms of cellulose and hemicellulose monomers, as well as the aromatic polymer lignin, are bound together with different kinds of linkages to make plant cells tough and recalcitrant. The specific proportion of biomass varies greatly depending on geographical location, plant species, tissue type, and season (Benocci, 2018).

Plant polysaccharide	Polymer type	Polymer	Main monomers
Plant cell wall	Cellulose		D-glucose
	Hemicellulose	Xylan	D-xylose
		Arabinoglucuronoxylan	D-xylose, L-arabinose, D-glucuronic
		Galacto(gluco)mannan	D-glucose, D-mannose, D-galactose
		Mannan/galactomannan	D-mannose, D-galactose
		Xyloglucan	D-glucose, D-xylose, D-fructose, D-galactose
	Pectin	Homogalacturonan	D-galacturonic acid
		Xylogalacturonan	D-galacturonic acid, D-xylose
		Rhamnogalacturonan I	D-galacturonic acid, D-rhamnose, D-galactose, D-arabinose, ferulic acid, D-glucuronic acid
		Rhamnogalacturonan II	D-galacturonic acid, D-rhamnose, D-galactose, D-arabinose, L-fucose, D-glucose, D-mannooctulosomic acid, D-apiose, L-acetic acid
Storage	Inulin		D-fructose, D-glucose
	Starch	Amylose	D-glucose
		Amylopectin	D-glucose
	Various gums		D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose
	Lignin		Monolignols: p-courmaryl alcohol, coniferyl alcohol, sinapyl alcohol

Table 1.1: Plant biomass composition (Benocci, (2018))

The conversion of the plant cell wall polysaccharides into biofuels involves the depolymerization or hydrolytic step wherein, these polymers are converted to monomeric sugar units. These are then converted into biofuels such as ethanol through fermentation by yeast (Chandel, Chandrasekhar, Silva, & Silverio da Silva, 2012).

1.1.2 Depolymerization of biomass into fermentable sugars

The process of deconstruction of biomass into fermentable sugar monomeric units is the most critical step towards production of biofuel. Even after years of continuous attempts, it still remains as one of the limiting factors in the bio-refinery process (Leis et al, 2018)

Limiting factors of depolymerization –

1) Physicochemical treatments only partially open up the tough structure of tightly packed cellulose microfibrils, making it extremely difficult to metabolize the biomass due to limited access.
2) Furthermore, different species of fungi from the ascomycetes and basidiomycetes taxa, such as Myceliophthora heterothallica, have been utilised to break down pre-treated biomass for a long period.
3) Because biomass composition is highly complex and varied, a depolymerization strategy that includes thermostable (to endure unfavourable pretreatment conditions), highly adaptable (to adapt to various types of biomass), and a variety of biomass degrading enzymes are essential to breakdown the different constituents of biomass.

1.1.3 Use of thermophilic-hyperthermophilic enzymes-

Enzymatic degradation is be brought about by a potent enzyme cocktail consisting of a variety of potent biomass enzymes. It allows for selective degradation of biomass depending on the nature of the polysaccharides (Andlar et al., 2018). Since, the industrial production of biofuel requires harsh conditions like pH and temperature, enzymes that can tolerate these conditions

should be the ideal candidates. Thus, thermostable enzymes are desirable candidates for enzymatic cocktails.

1) Thermophilic enzymes have higher reaction rates and lower diffusional restrictions, which make the process of depolymerization faster. Due to higher stability, these enzymes can be reused to a great extent (Markoglou & Wainer, 2003).
2) Use of thermophilic enzymes allows for saccharification of biomass at elevated temperatures guaranteeing lesser contamination and more accessibility to substrate because of reduced substrate viscosity. (van den Brink et al., 2013).
3) Thermophilic enzymes, once expressed in mesophilic hosts can be easily purified by heat treatment.
4) Thermostable enzymes, along with their extra-ordinary stability, are also more resistant to chemical agents that allows them to be used in industries as pre-treatment of biomass is known to release some by-product that inhibit the lesser stable mesophilic enzymes (Lasa & Berenguer, 1993).

Efficient degradation of biomass calls for synergistic activity of a combination of enzymes that are highly adaptive to different substrates. In nature, there exists a cellular machinery in some cellulolytic organisms precisely for this function, called cellulosome. Cellulosome is an extracellular multienzyme cellulolytic complex that is present on cell surface of many anaerobic cellulolytic bacteria such as *C. thermocellum*, *Acetivibriocellulolyticus* (Lamed, Naimark, Morgenstern, & Bayer, 1987). First described my Lamed et al, the cellulosome was reported to be remarkably stable (Ljungdahl et al., 1988), the cellulosome displays extraordinary cellulolytic activity i.e. it can hydrolyse both amorphous and highly ordered crystalline cellulose (Felix & Ljungdahl, 1930).

Cellulosomes can prove to be extremely desirable for efficient degradation of biomass on an industrial scale. Studies have been carried to overexpress cellulosomes and purify them, only to get very poor yields (Lamed et al., 1987). Development of synthetic cellulosomes seems like a plausible alternative given its advantages in degrading biomass.

1.1.4 Most abundant biomass polysaccharide – Cellulose

Cellulose is the most abundant plant cell wall polysaccharide. It is made up of β-D glucose units linked by β -(1-4)-O-glycosidic bond organized in bundles called microfibrils. Its molecular formulae is $(C_6H_{12}O_6)_n$. The degree of polymerization, indicated by n, is broad, ranging from several thousand to several ten thousand (Kolpak & Blackwell, 1976).

Cellulolysis refers to the breakdown of cellulose into cellodextrins/glucose units. It is the hydrolysis of (1,4)- β -D glucosidic linkage in β -D-glucans and is brought about, by a class of enzymes called cellulases also called β -glucanases.

A general cellulase consists of a signal peptide (SP) followed by a catalytic domain (CD) connected to a carbohydrate-binding module (CBM) (Vuong & Wilson, 2010) by a short polypeptide sequence called a linker (Lie et al., 2009). Several types of cellulases are known to exist that differ in structure and mechanism of action. The process by which cellulose is depolymerized into glucose units involves a variety of cellulases-

a. Endoglucanases, which catalyse the endo-hydrolysis of cellulose

b. Exocellulase (also called Cellobiohydrolases), that perform hydrolysis at ends of cellulose to release successive glucose units

c. β -glucosidase, which release glucose units from dimer cellobiose (Gomez del Pulgar & Saadeddin, 2014).

Exocellulases are further classified – Type I act on reducing ends of cellulose and release glucose units on hydrolysis whereas Type II act on non-reducing ends releasing cellobiose on hydrolysis.

Chapter 2.

Materials and Methods

2.1 Materials

This section includes the materials used to carry out the experiments.

2.1.1 Bacterial strains and plasmids used for cloning of genes and expression of proteins.

Strains	Genotype	Used for
Ecoli XL I Blue	rec A l end A l gyr96(nal^R) thi-l hsdR l7 (rk-mk+) supE44 relA 1 lac [F'proABlacIqΔ(lacZ)M15 Tn1O(Tetr)] • Derived from E.coli K12 strain • Tetracycline resistant (from F plasmid)	• Used as cloning host for genes cloned in pET23a vector.
TOP10 E. coli	F- mcrA (mrr-hsdRMS-mcrBC) 80lacZ M15 lacX74 recA1 ara 139 (ara-leu)7697 galU galK rpsL (StrR) endA1 nupG	• Used as cloning host for genes cloned in pET28a vector.
E.coli BL21 (DE3)pLys*	F^-ompT[1on]gal dcmhsdS_B(rB^-mB^-) λ(DE3)pLysS(cm^R)rne 131 • An E.coli B strain • IPTG inducible lac UV5 promoter • pLysS plasmid encodes chloramphenicol resistant gene. • T7 phage lysozyme (inhibitor for T7 polymerase) which reduces expression from transformed T7 promoter containing plasmid when not induced.	• Used as expression host for some genes cloned in pET vectors.

Rosetta DE3	These cells are derived from E.coli B121 cells and are used for enhanced production of eukaryotic genes. The genotype of these cells is F^-ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm (DE3) pRARE (Cam^R).. • These strains supply tRNA for AGG, AGA, AUA, CUA, CCC, GGA on chloramphenicol plasmids.	Used as expression host for some genes cloned in pET vectors.
pET23a	• Size- 3666 bp • T7 promoter with pBR322 origin of replication. • Multiple cloning sites (BamH I – Xho) • Ampicillin resistance gene as selection marker • 6X His tag present at the C-terminal end for protein purification.	
pET28a	• Size- 5369 bp • T7 promoter with pBR322 origin of replication. • Multiple cloning sites (BamH I – Xho) • Kanamycin resistance gene as selection marker. • 6X His tag present at both N and C-terminal end for protein purification.	

2.1.2. Media

Luria Bertani Broth (LB)

Components	**Amount of each component per 1L**
Tryptone	10g
Yeast extract	5g
NaCl	10g
Agar (For LB agar plates)	2 %
Total volume	1L

The media was sterilized by autoclaving (15 psi and 121^{o}C for 15 minutes).

2.1.3 Antibiotics

Antibiotic	**1000X stock concentration**
Ampicilin	100 mg/ml in water
Chloramphenicol	35 mg/ml in methanol
Tetracycline	12.5 mg/ml in 70% ethanol
Kanamycin	25 mg/ml in water

Stock of each antibiotic were sterilized by filter sterilization using 0.22 μM Millipore filters and stocks were stored at -20^{o}C. The working concentration of antibiotics was 1X.

2.1.4. Buffers for molecular biology work

2.1.4.1. Buffer for making chemical competent cells

Calcium chloride	60 mM
Glycerol	15% v/v
PIPES	10 mM
pH	7.0

The solution was sterilized by passing through 0.22 μM filter followed by autoclaving. The solution was stored at 4^{o}C.

2.1.4.2. 6X DNA gel loading buffer (In deionized water)

Bromophenol blue	0.25%
Glycerol	30%

2.1.4.3. 50X TAE

Components	Amount per 1L
Tris.Cl	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
pH	8

2.1.4.4. 0.8% Agarose

Agarose	0.8gm
1x TAE	100 ml

2.1.4.5. Ethidium bromide stock solution (1% w/v)

Ethidium bromide	0.1 g
Deionized water	10 ml

The stock solution was stored in amber coloured MCT and stored at 4^{o}C.

2.1.4.6. TE buffer (Tris EDTA buffer in deionized water)

Tris.Cl (pH 8.0)	10 ml
EDTA	1 mM

2.1.5. Buffers and solutions for SDS-PAGE

2.1.5.1. Acrylamide

Acrylamide	30 g
N.N'-Methylene bis-acrylamide	0.8 g
Total Volume	100 ml

2.1.5.2. Upper Tris (4X), pH 6.8

Tris	6.06 g
10%SDS	4 ml
pH	6.8
Total volume	100 ml

2.1.5.3. Lower Tris (4X), pH 8.8

Tris	18.17 g
10% SDS	4 ml
pH	8.8
Total volume	100 ml

2.1.5.4. 5X sample loading buffer

Tris.Cl (pH6.8)	0.15 M
SDS	5%
Glycerol	25%
Beta-mercaptoethanol	12.5%
Bromophenol blue	0.06%
Total volume	100 ml

2.1.5.5. Laemmli buffer (Laemmli, 1970)

Tris buffer	3.00 g
Glycine	14.4 g
SDS	1 g
Total volume	1000 ml

2.1.5.6. Gel staining solution

Methanol	40%
Glacial acetic acid	10%
Coomassie brilliant Blue R-250	0.1%
Deionized water	50%
Total volume	100 ml

2.1.5.7. Gel destaining solution

Methanol	40%
Glacial acetic acid	10%
Deionized water	50%
Total volume	100ml

2.1.5.8. Native purification of 6X His-tagged proteins

Native Lysis Buffer (pH 8.0)

NaH_2PO_4	50 mM
Nacl	300 mM
Imidazole	10 mM

Native Wash Buffer (pH 8.0)

NaH_2PO_4	50 mM
Nacl	300 mM
Imidazole	20 mM

Native Elution Buffer (pH 8.0)

NaH_2PO_4	50 mM
Nacl	300 mM
Imidazole	250 mM

2.1.6. Tris-Nacl Buffer (pH 7.5)

Tris	20 mM
Nacl	100 mM
$CaCl_2$	2 mM

2.1.7. DNSA Reagent

DNSA	0.876g in 40 mL
Sodium Potassium Acetate	23.92g
NaOH	2 N of 16 mL
Deionized water	Upto 100 mL

2.2. Methods

2.2.1. Primer designing

Primer designing for all the potential genes was done manually using Gene Runner. All primers were made of appropriate length and GC content. Respective restriction sites and 4-5 bases of overhangs (for the restriction enzyme to get enough space to sit on the sequence and cut) were added to the correct position on the primers and some silent mutations were done in the sequence to get the optimum GC content. Primers were checked for primer dimer and secondary structure formation as well. The primers were ordered and appropriate amount of autoclaved distilled water was added to make primer stock concentration of 100µM concentration. The Tm for each of the primer was used to select different annealing temperature for each pair of primers.

The primers for the PCR amplification of the genes from *Clostridium Thermocellum* genome were already present in the lab. Detail of the gene length and the designed primers for each enzyme used for cloning is given in the table.

Gene Name (*Clostridium thermocellum*)	Nucleotide length (bp)	Restriction sites	Primers (5'-3')
Xylanase A	1971	Nde1	ATATATCATATGATGCTGAAGAAAAAACTGTTGACCCTC
		Xho1	ATATATCTCGAGAAGTTCTCTCAGAACGAGTTTTTTGAC
Cellulase A	1440	Nde1	ATATATCATATGGTGAAGAACGTAAAAAAAAAGAG
		Xho1	ATTAATCTCGAGATAAGGTAGGTGGGGTATG
Cellulase Q	2065	Nde1	ATATATCATATGGCAGGAAGCTATAACTATGCGGAA
		Xho1	TATATTCTCGAGTTCTACCGGAAATTTATCTATTATAC
Cellulase S	2160	Nde1	ATATATCATATGGGTCCTACAAAGGCACCTACAAAAGAT
		Xho1	TATATTCTCGAGGTTCTTGTACGGCAATGTATCTATTTC
BetaglucosidaseA	1350	Nde1	ATATATCATATGTCAAAGATAACTTTCCCAAAAGATTTC
		Xho1	TATTATCTCGAGGAAACCGTTGTTCTTGATTACTTCTTTG
Xylanase D	1857	Nhe1	ATATTAGCTAGCGCAGAAGGCAATTTACTTTTCAACCC
		Xho1	ATTAATCTCGAGACGTTTTAAAGGCAATTCATCTATTTC
BetaglucosidaseB	2277	Nhe1	ATATATGCTAGCATGGCGGTAGATATCAAGAAAATA
		Xho1	TATTATCTCGAGTTCCACGTTGTTTATTTTGTCAA

Gene Name (*Pyrococcus furiosus)*	Nucleotide length (bp)	Restriction sites	Primers (5'-3')
Glycoside Hydrolase	1843	Nco1	ATATATCCATGGATGTTTATGAAATTCACATATCACTG
		Xho1	ATATATCTCGAGTTCTTTCCCTTTATGCTCGCACCACTCCATG
Neopullunase	1977	Nde1	ATATATCATATGATGTATAAGCTCGTCAGTTTCAGAGATAG
		Xho1	ATATATCTCGAGGTACTTTATCAATTTCACTTTATCTTCATTAAT
Alpha-amylase	1422	Nco1	ATATATCCATGGGTGAACATAAAGAAATTAACACCACTCC
		Xho1	ATATATCTCGAGCCCAACACCACAATAACTCCATACGGAG

Table 2.1: Genes and their designed primers

2.2.2. Polymerase chain reaction (PCR)

Polymerase Chain Reaction is a technique used to amplify a gene. For amplification, a mixture of heat stable DNA polymerase, four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP), forward and reverse primers which are complimentary to the template are required. The PCR reactions were performed in Eppendorf PCR machine, USA. It involves the following steps:

Denaturation-In this step the reaction mixture is heated to 94-98^{o}C for 30-60 seconds. It helps in disrupting the hydrogen bonds between complimentary strands and rendering it single stranded.

Annealing- During this step the temperature is lowered to 50-65^{o}C for 20-40 seconds. It allows the binding of primers to the single stranded 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 the DNA fragment to be amplified. Generally, the temperature for extension step is 72^{o}C

Standard PCR reaction components

		Stock concentration	Final concentration
1	Template	Variable	1μg genomic DNA
2	Forward primer	10 μM	0.2 μM
3	Reverse	10 μM	0.2 μM
4	dNTPs	10 mM	200 μM
5	Polymerase (Taq.Vent,VegaPol,Phusion	2 units/ μl	0.02 units μl
6	Buffer	10 X for Taq, Vent, and 5X for Phusion	1 X
7	$MgSO_4$ (optional)	10 mM	2-14 mM
8	DMSO (optional)	25%	1%

All the PCR reactions were carried out in Eppendorf PCR machine with the following PCR reaction

	Steps	Temperature	
1.	Initial denaturation	95°C	5 minutes for colony PCR and 1-2 minutes for genomic DNA
2.	Denaturation	95°C	30 seconds
3.	Annealing	depends upon Tm of primers.	30-45 seconds for all polymerases.
4.	Extension	72°C	1kb/30 seconds for Phusion and 1kb/minute for other polymerases
5.	Final extension	72°C	10 minutes

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

Gene Name *(Clostridium thermocellum*)	Enzyme	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
Xylanase A	Phusion	98 ºC 3 minutes	98 ºC 30 seconds	67 ºC 30 seconds	72ºC 1 minute	72ºC 10 minutes	30
Cellulase A	Vent	95ºC 5 minutes	95ºC 45 seconds	65 ºC 45 seconds	72ºC 2 minutes	72ºC 10 minutes	24
Cellulase Q	Vent	95ºC 5 minutes	95ºC 45 seconds	60 ºC 45 seconds	72ºC 2 minutes	72ºC 10 minutes	24
Cellulase S	VegaPol	98 ºC 3 minutes	98 ºC 30 seconds	65 ºC 45 seconds	72ºC 2 minutes	72ºC 10 minutes	30
BetaglucosidaseA	Vent	95ºC 5 minutes	95ºC 45 seconds	63 ºC 45 seconds	72ºC 1 minute	72ºC 10 minutes	24
Xylanase D	VegaPol	98 ºC 3 minutes	98 ºC 30 seconds	68 ºC 45 seconds	72ºC 2 minutes	72ºC 10 minutes	35
BetaglucosidaseB	Vent	95ºC 5 minutes	95ºC 45 seconds	64 ºC 30 seconds	72ºC 2 minutes	72ºC 10 minutes	24
Glycoside Hydrolase	Phusion	98 ºC 3 minutes	98 ºC 30 seconds	68 ºC 40 seconds	72ºC 1 minute	72ºC 10 minutes	30
Neopullunase	Phusion	98 ºC 3 minutes	98 ºC 30 seconds	68 ºC 40 seconds	72ºC 1 minute	72ºC 10 minutes	30
Alpha-amylase	Phusion	98 ºC 3 minutes	98 ºC 30 seconds	67ºC 40 seconds	72ºC 1 minute	72ºC 10 minutes	30

Table 2.2. Optimized PCR conditions for the genes under study

2.2.3. Agarose gel electrophoresis

The separation of DNA fragments was done on agarose gel. The polysaccharide gel was formed by taking different concentrations (0.8 to 1.5%) of agarose for longer and shorter DNA. Gel was prepared by dissolving required amount of Agarose in 1X TAE buffer. Ethidium bromide (0.5 g/ml) was supplemented for visualizing under UV trans-illuminator. 6X loading was added to the sample with the final concentration of 1X. Electrophoresis was carried out in 1X TAE buffer at 8 90 volts. 1000 base pairs ladder were used for calculating sizes – L fragments.

2.2.4. Gel extraction of desired DNA bands from agarose gel

After electrophoresis, the DNA fragments were visualized under trans-illuminator and the desired band was excised from the agarose gel and weighed. Qiagen gel extraction kit was used to extract DNA from excised pieces of agarose gel. The steps for the protocol are as follows:

1. Solubilization: Excised gel was weighed and dissolved in Qiagen's solubilization and binding buffer, which is named as QG buffer (300 µl/100mg of gel piece). Incubation was done at 55^{o}C till the gel was completely dissolved with intermittent mild mixing. Furthermore, one gel volume of isopropanol was added and mixed by inverting the tube.
2. Binding: The dissolved agarose solution containing DNA was then poured onto the QIAquick spin column (provided with kits by the manufacturer) to allow the adsorption of DNA onto the silica gel matrix. Centrifugation was done at 13000x g for 1 minute.
3. Washing: The columns were washed with 750µl of an ethanol containing wash buffer (Buffer PE) to remove impurities and the flowthrough was discarded.
4. Elution: Finally, the absorbed DNA was eluted in (30-50µl) autoclaved water.

2.2.5 Restriction digestion

Restriction digestion is a method to cleave DNA molecules at specific restriction sites using special molecular scissors, enzymes called restriction endonucleases. These cleave the sugar-phosphate backbone of DNA. They commonly recognize recognition sequences that are mostly palindromes.

When the same restriction enzymes are used to cut both vector and insert, generation of similar sticky ends help in the ligation of the two into one recombinant plasmid. After quantitation, the gel extracted PCR product and the plasmid DNA were digested using specific restriction enzymes. The following reaction was set for restriction digestion:

1.	Template	200 ng of PCR product and 1000ng of plasmid DNA
2.	Fast digest buffer (10X)	To the final concentration of 1 X
3.	Restriction enzymes	1µl each for the given concentration of DNA
4.	Water	To make up final volume

The digestion reaction was incubated at 37^{o}C for 3 hours. The digested product was either PCR cleaned up or run-on agarose gel for purification.

2.2.6. PCR clean-up of digested insert

The PCR products double digested with restriction enzymes were directly purified and the over hangs removed from the mixture using Qiagen's PCT cleanup kit. This procedure includes the following steps:

1. Binding: 5 volumes of column binding buffer was added to PCR reaction and loaded onto the DNA binding columns.
2. Washing: washing was done with 750µl PE wash buffer containing ethanol to remove impurities.
3. Elution: The elution was done with autoclaved distilled water.

2.2.7 Quantification of DNA

The eluted DNA after gel extraction or plasmid purification or PCR cleanup was quantified visually after running on agarose gel and then visually comparing band intensities with the

various bands of the ladder or by using Nano drop spectrophotometer. The Nano drop absorbance was taken by putting 1 µl of DNA on probe after setting up a baseline with solution in which the DNA was eluted. The absorbance was measured at 250nm. The purity of eluted DNA was ascertained by looking at the ratio of OD_{260}/OD_{280}. Pure DNA has this ratio around 1.8 – 2.0.

2.2.8. Ligation

Ligation is a process of joining DNA fragments with T4 DNA ligase. It involves creating a phosphodiester bond between the 3'hydroxyl of one nucleotide and the 5' phosphate of another. For a particular amount of digested vector (generally 50ng), the amount of digested PCR product(insert) was calculated using this formula:

Amount of insert (ng)=

$$\frac{\text{Amt. of digested vector (ng) x molar ratio (insert:vector, 3:1) x size of insert}}{\text{Plasmid size (bp)}}$$

The digested vector and insert were ligated by taking vector to insert ration of 1:3 and the following reaction was set up:

S.No	Components	Volume (µl)
1.	Vector	50ng
2.	Insert	xng (Depend upon size)
3.	Buffer 10X for T4 DNA ligase	1X
4.	Ligase	1µl
5.	Water	For volume make up

The reaction was incubated at 25°C or 16°C for 2 and 12 hours, respectively.

2.2.9. Preparation of E.coli competent cells

In order to get multiple copies of the recombinant plasmid, it needs to be put into a cloning host. Cloning host used in this case was XL1-Blue and TOP10 strain of E.coli. The cells were made chemically competent to take up DNA using $Cacl_2$ method.

1. A primary culture of 5ml of the strain of the bacterial cells to be made competent was grown overnight at 37 ^oC.
2. On the next day, the secondary culture was set up by re-inoculating 200ml of LB to the final concentration of 1% and was grown till the early log phase (O.D. at 600nm of 0.5 – 0.5)
3. The cells were chilled on ice for 15 minutes and 50ml of these cells centrifuged at 160xg for 7 minutes at 4^oC in pre-chilled centrifuge bottles. Cells were kept on ice during subsequence competent cells preparation steps.
4. The cell pellet was re-suspended in 20ml of pre-chilled 60mM calcium chloride solution after which they were again centrifuged at 1100xg for 5 minutes at 4^oC.
5. Supernatant was again discarded and step 4 was again repeated.
6. Again, the pellet was dissolved in 20ml of calcium chloride solution but this time cells were left on ice for 30 minutes.
7. The cells were again centrifuged at 1100xg for 5 minutes at 4^oC and supernatant was discarded after which the cells were re-suspended in 4ml of ice-cold calcium chloride solution.
8. Finally the aliquots of 100µl were made and stored at -80^oC for further use.

2.2.10. Transformation

1. Competent cells were thawed on ice for 15 minutes.
2. Ligation mixture was added and left for adsorption on calcium chloride coated competent cells for 15-20 minutes on ice.
3. Heat shock was given by heating cells at 42^oC for 90 seconds in water bath.
4. After giving heat shock, 1ml of sterile LB was added to these cells and left for growing at 37^oC for 45-50 minutes in incubator shaker set at 220rpm.
5. After incubation, cells were pelleted down by centrifuging at 5000rpm for 3 minutes.

6. Pelleted cells were re-suspended in 100µl of fresh LB media and spread on LB agar plates containing desired antibiotics selection.

7. Plates were incubated at 37^{o}C overnight and observed for colonies on the next day.

2.2.11. Plasmid DNA purification

For plasmid DNA purification, Qiagen miniprep kit was used. Standard protocol given by the manufacturer was used. The steps are as following:

1. Cell growth: Few of the transformants from the plates were picked and inoculated into LB media overnight with appropriate antibiotics.
2. Pelleting and re-suspension: cells were pelleted in the same MCT by taking 5ml of grown bacterial culture and finally re-suspending in 250µl of PI buffer. PI buffer is kept at 4^{o}C as it contains RNase.
3. Lysis: Cells were lysed by adding 250µl of P2 buffer (lysis buffer) and gently mixed by inverting up and down.
4. Neutralization: 350µl of N3 buffer (neutralization buffer) was added and mixed gently. The precipitated solution was centrifuged at 13000 rpm for 15-20 minutes.
5. Loading: Clear supernatant containing plasmid DNA is loaded over DNA binding column and spun at 13000 rpm for 1 minute.
6. Washing: Column was washed with 750µl of PE wash buffer by spinning at 13,000 rpm for 1 minute. An empty spin of 2 minutes was given to dry off residual ethanol of PE buffer.
7. Elution: Finally, the DNA was eluted with autoclaved distilled water.

2.2.12. Screening of the transformants

Two step screening of transformants before sending for sequencing was done.

1. **Colony PCR**: This is done to check for successful clones after transformation. The colonies are used as template and vector specific and/or insert specific primers are used for amplification. Successful integration of insert has bigger fragment of amplified DNA.

Individual colonies were picked and suspended into the PCR reaction mixture to a desired final concentration of each PCR components in PCR tubes. For colony PCR, Taq polymerase (from NEB) was used. Vector specific primers (T7 promoter forward and T7 terminator reverse) flanking the MCS region for pET vectors were used for amplification. The amplified PCR product was run on 0.8% agarose gel and the size was measured by comparing with DNA ladder. The positive clones were further verified by restriction digestion.

S.No	Components	Amount(µl)
1.	Distilled water	11
2.	T7 Forward Primer	1
3.	T7 Reverse Primer	1
4.	dNTPs	0.8
5.	$MgCl_2$ (working concentration-0.25mM)	1.6
6.	Taq Flexi Buffer	4
7.	Taq Flexi enzyme	0.15
	Total	20

2. **Restriction digestion**: Following the plasmid isolation from the culture of selected clones, the plasmid was checked for the excision of the amplified DNA by digesting with the restriction enzymes. The digested plasmid was run on agarose gel and the fall out was compared with DNA ladder and the size of the gene inserted between those restriction sites.

2.2.13 Glycerol stock preparation

For glycerol stocks, 1500µl of overnight grown bacterial culture was mixed with 500µl of 60% glycerol and stored at -80^{o}C for further use.

2.2.14 Expression of recombinant protein in E.coli

For the expression of the gene from the pET series vectors, the plasmid was transformed either into the BL21star (DE3) plysS cells or were transformed into the *Rosetta* cells depending upon the toxicity of the protein. Protein expression check includes following steps:

1. Primary culture: cells were inoculated in 5ml of primary culture supplemented with required antibiotics and incubated at 37^{o}C and 220 rpm.

2. Secondary culture: 1% of the overnight grown primary culture was inoculated into the fresh LB media. Cells were allowed to grow till mid log phase (O.D. of 0.6 at 600nm) and were induced with 1mM IPTG. The cells were left for 6-8 hours after induction with IPTG.

3. SDS-PAGE: Harvested cells were boiled for 5 minutes in 50µl of SDS-PAGE sample loading buffer. Then the samples were analyzed by running on the SDS-PAGE. The over expressed band among thousands of proteins of E.coli with required size represents the protein of interest.

   To check whether the protein is coming into the soluble fraction, 1ml cells were pelleted and lysed in non-denaturing buffer. After sonication, cell debris was settled down by centrifuging at high speed. 10µl supernatant, after mixing in sample loading dye and boiling at 99^{o}C for 2 minutes, was run on SDS-PAGE and analyzed for desired band.

2.2.15 Protein purification by Ni-NTA agarose beads

All the proteins cloned contain either N or C terminal 6X histidine tag and were purified using Ni-NTA affinity chromatography with the steps described below:

1. Lysis: Secondary culture of the protein expressing cells were pelleted by spinning at 8000 rpm for 10 minutes at 4^{o}C.
2. Pellet was lysed in lysis buffer, to which lysozyme was added.

3. Loading: Cell debris was removed after centrifugation at 11500 rpm for 45 minutes at 4^{o}C and the clear supernatant was loaded on Ni-NTA column pre-equilibrated with Lysis Buffer.
4. Washing: In order to remove the non-specific proteins bound to the column, washing was done with a buffer containing very less concentration of imidazole.
5. Elution: Immobilized protein was eluted with elution buffer containing an imidazole concentration of 200 mM.
6. SDS-PAGE- Eluted fractions were analyzed for purity by running on SDS-PAGE.

2.2.16. Gel filtration chromatography

After purifying the proteins using Ni-NTA column, they were repurified using size exclusion chromatography. The Gel filtration chromatography was performed on GE's Akta Purifier 10 workstation connected to 500 µl loop using the column Superdex 200 increase, equilibrated with the Tris-NaCl buffer to remove salts and imidazole. The fractions of 1 ml were collected. The flow rate was set at 0.5 ml/min. To calculate the molecular weight of eluted protein, its elution volume ml was compared with the standard curve generated for the Superdex 200 increase column, after running known molecular weight markers. The calibration curves for superdex 200 are shown in the figure below.

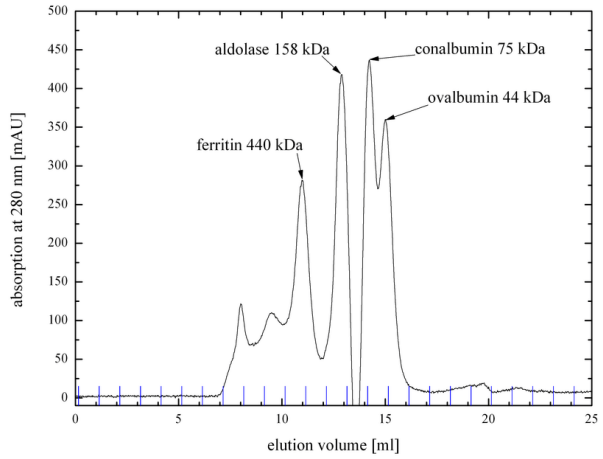


Figure: 2.1: Standard chromatogram for Superdex200

2.2.17. UV-Vis Absorption spectroscopy

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

2.2.18 Fluorescence spectroscopy

In general, the fluorescence emission maximum is a combination of contribution of all the tryptophan residues distributed over their entire structure of protein. Fluorescence spectroscopy was carried out on Cary Varian Eclipse Fluorimeter. The emission spectra were collected by exciting at 295 nm and collecting the spectra in the range of 300-400 nm. The slit width used for the experiments was 5 nm and the speed scan was set at100 nm/min. All the spectra were averages of 5 scans.

2.2.19. Circular Dichroism (CD) spectroscopy

Circular Dichroism is the difference in the absorption of left circularly polarised light and right circularly polarized light and occurs when a molecule contains one or more chiral molecules. CD spectra may exhibit positive as well as negative peaks. Major secondary structures observed are α-helix and β-sheet.

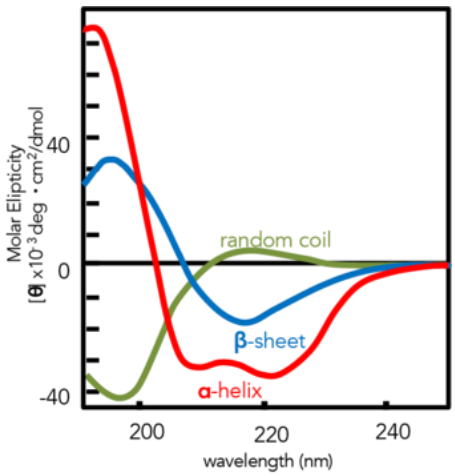


Figure 2.2: Characteristic curves for secondary structures in CD

Far UV-Circular dichroism spectra for proteins were collected on MOS -500 CD spectrophotometer (Biologic) using a quartz cuvette of 1mm path length (Sterna scientific, UK). The slit width was 2nm for most of the experiment depending upon the values of HV and the protein concentrations used for CD experiments ranged from 0.15-0.2 mg/ml. Data was collected in the form of raw ellipticity in millidegree (mdeg) and was converted into mean residual ellipticity (MRE) using following formula.

$$[\theta] = \frac{\theta\text{obs(mdeg)} \text{ x } 100 \text{ x MRW}}{1000 \text{ x concentration } \left(\frac{\text{mg}}{\text{ml}}\right) \text{ x pathlength (cm)}}$$

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

$[\theta]$ = Mean residual ellipticity (mdeg cm^2dmol^{-1})

θobs(mdeg) = Raw Ellipticity (deg)

2.2.20 DNSA Assay

3,5-Dinitrosalicylic acid (DNSA) is an aromatic compound that detects and reacts with reducing sugars. This involves the oxidation of the aldehyde functional group (in glucose) and the ketone functional group (in fructose). During this reaction DNSA is reduced to 3-amino5-nitrosalicylic acid (ANSA) which under alkaline conditions is converted to a reddish orange coloured complex which has an absorbance maximum of 540 nm.

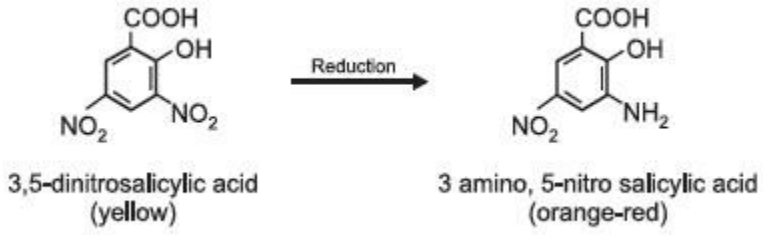


Figure 2.3: Chemical reaction of conversion of DNSA to ANSA

The enzyme activity for each purified protein was measured as follows.

1) A stock solution of 1% of the substrate CMC (Carboxymethyl cellulose) and Xylose was prepared.
2) 0.5 µM enzyme was incubated with 0.5% substrate at temperatures ranging from 20℃ to 80℃ for different time periods (2 hours and 6 hours). Total reaction volume was made upto 100 µL. The remaining volume was made up with Tris-Nacl buffer.
3) Blanks were prepared in the same way by adding Tris-Nacl Buffer instead of enzyme.
4) After the incubation period, 75 µL of DNS -reagent was added to each reaction and again incubated for 30 minutes at 80℃.
5) The reaction was stopped by putting the reaction tubes in ice and the absorbance was measured immediately at 540 nm using the plate reader.

Chapter 3.

Results and Discussion

3.1 Cellulase A

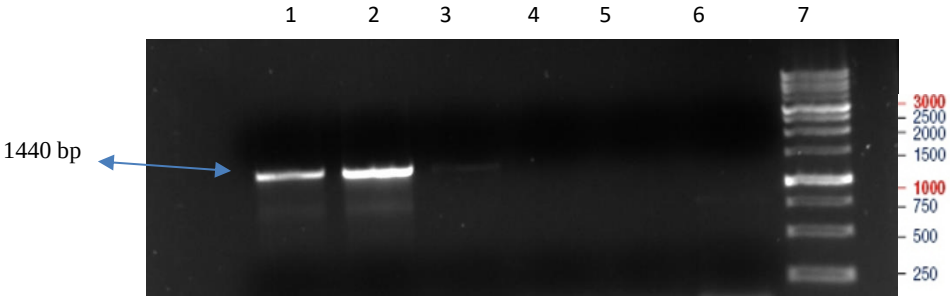
Cellulases, which catalyze the hydrolysis of glycosidic bonds in cellulose, can be classified into several different protein families. Cellulase A from *Clostridium thermocellum* is a thermophilic endoglucanase enzyme and is a member of glycosyl hydrolase family 8. The protein folds into a regular (α/α)6 barrel formed by six inner and six outer α helices.

The CelA gene was characterized and found to encode a polypeptide of 488 amino acid residues, consisting of a signal-peptide-like segment, a catalytic domain, and a C-terminal dockerin domain. CelA hydrolyzes carboxymethylcellulose but shows very low or no activity for Avicel (crystalline cellulose) or xylan.

3.1.1 CLONING

3.1.1.1 Amplification

Cellulase A was amplified from the *Clostridium thermocellum* genomic DNA. The conditions for gene amplification for PCR are mentioned in the materials and methods section. The desired band (1440 bp) was obtained at 65 ℃.



- Lane 1: at 63 ℃
- Lane 2: at 65 ℃
- Lane 3: at 67 ℃
- Lane 4: at 69 ℃
- Lane 5: at 71 ℃
- Lane 6: at 73 ℃
- Lane 7: 1kb DNA ladder

Figure 3.1 Amplicons of Cellulase A at different annealing temperature

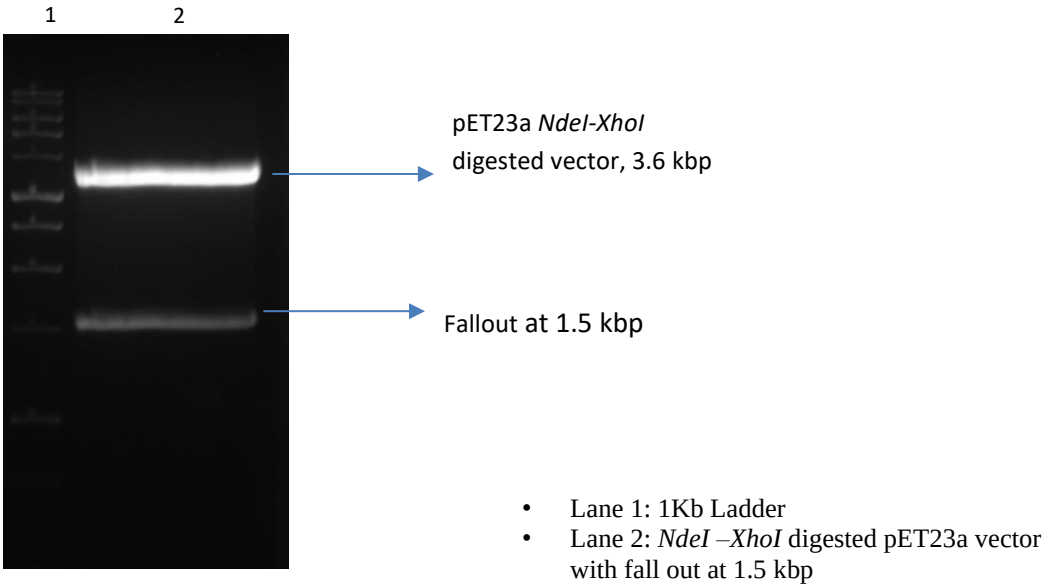


Figure 3.2 Digestion of vector pET23a

3.1.1.2 Digestion and Ligation

1. Further, the amplified insert was upscaled, the band excised and gel extracted. For digestion, the clone containing pET23a plasmid was inoculated and grown overnight at 37 ℃. Plasmid DNA was isolated the next day and both the plasmid and insert (Cellulase A) were double digested with *NdeI* and *XhoI* restriction enzymes by incubating them at 37 ℃ for 3 hours.
2. After 3 hours, PCR purification of the digested insert was carried out while the digested plasmid was run on 1% agarose gel for 3 hours at 60 V.
3. Double digested pET23a band was carefully excised and gel extraction was performed.
4. The concentration of both the digested vector and insert was checked using NanoDrop (Thermocientific) instrument.
5. Ligation reaction was incubated at 25 ℃ for 3 hours with T4 Ligase. For 50ng of digested pET23a, the amount of digested Cellulase A was calculated using the formula mentioned in the materials and methods section.
6. The ligation mixture was then transformed in the cloning host cells, XL1Blue. The colonies were obtained the next day.

3.1.1.3 Colony PCR

The band in the lane 2 and 5 show putative positive colonies. We proceeded with colony 4. The colony was inoculated in a fresh LB media grown at 37℃ overnight. Plasmid DNA was isolated and test digestion was done to check the integration of the insert in the vector. The cloning was confirmed by test digestion and was further confirmed by DNA sequencing.

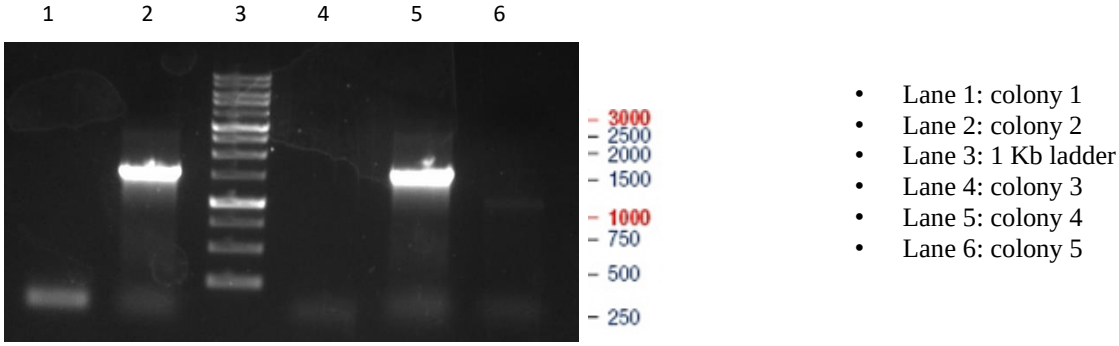


Figure 3.3 Colony PCR of Cellulase A

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

3.1.2 Ni-NTA Purification :

The purification was done using Ni-NTA column by native method. Cellulase A was expressed in soluble form and found to be present in the cytosol.

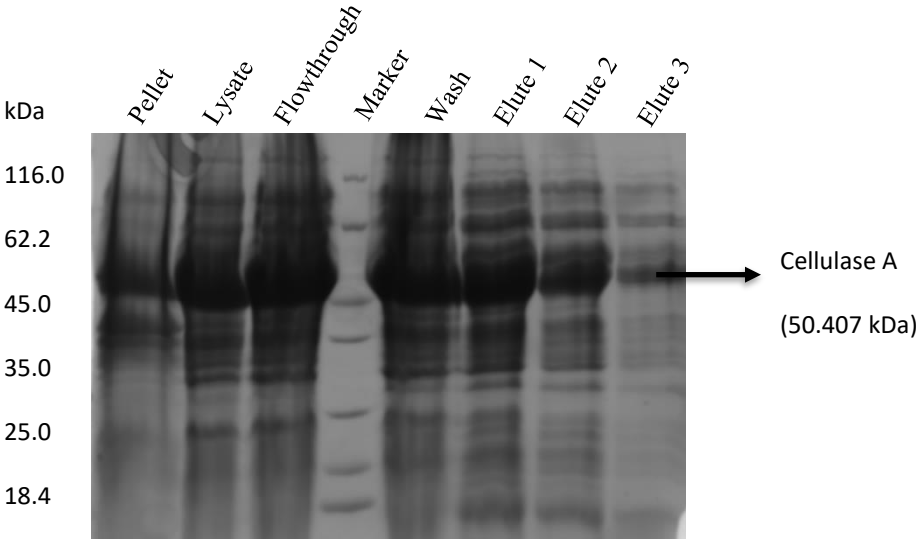


Figure 3.4. SDS –PAGE of Ni-NTA purified Cellulase A

3.1.3 Biophysical Characterization

3.1.3.1 Gel Filtration

Gel Filtration chromatography was done as the second step purification and to know the oligomeric status of the protein. The protein was run on the column Superdex 200 10/300 GL (24 mL). The protein was equilibrated with 20 mM Tris.

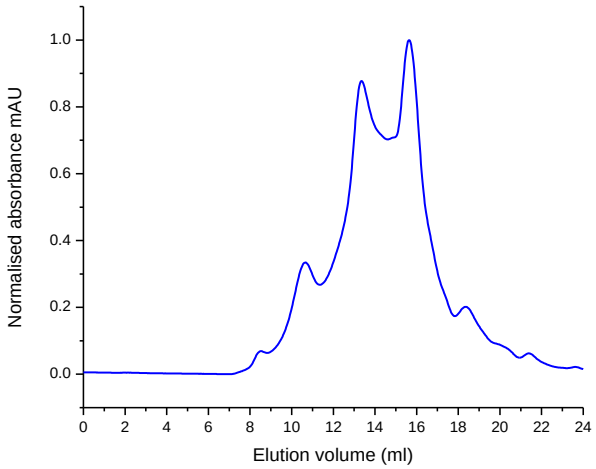


Figure 3.5. Gel filtration chromatogram of cellulase A

The elution volumes were compared with the elution profiles of the standards run on the same column. The fractions of 1.5 mL were collected and run on 12% SDS gel and the fractions containing the protein was concentrated and was used for further analysis.

3.1.3.2 Circular Dichroism

Far UV-Circular dichroism spectra for Cellulase A were collected on MOS-500 CD spectrophotometer (Biologic) using a quartz cuvette of 1 mm path to look at the secondary structure of the protein. Concentration of 0.2 mg/ml of the protein was used. The figure represents the far UV-CD spectrum of the protein and it shows that the spectral shape is dominated by α-helix while the intensity is contributed by both α-helix and β-sheets.

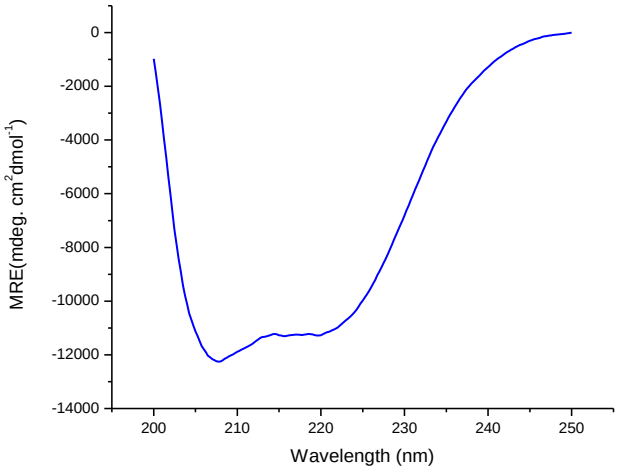


Figure 3.6. CD spectrum of Cellulase A dominated by alpha helix

3.1.3.3 Tryptophan fluorescence

Fluorescence emission spectra was collected by setting the excitation at 295 nm and collecting the spectra at a range of 300-400 nm. The slit width used for the experiment was 5 nm for both excitation and emission and the protein concentration used was 2 mg/ml. Since, the λ_{max} emission is at 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.

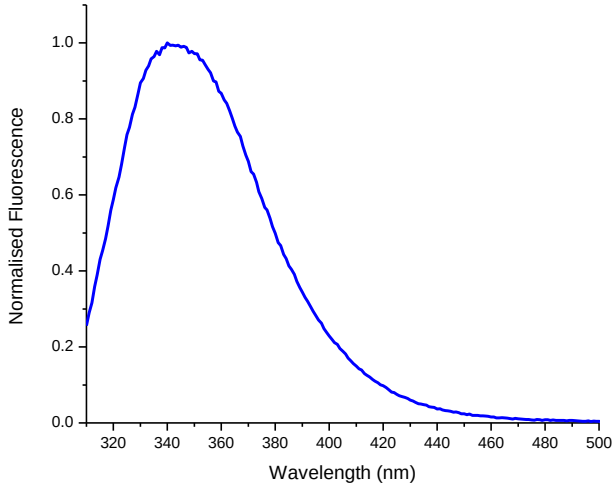


Figure 3.7. Intrinsic fluorescence emission spectrum of Cellulase A

3.1.3.4 Chemical stability monitored using tryptophan fluorescence

The protein was checked for stability against guanidine hydrochloride (GdmCl) by subjecting the protein (0.2 mg/ml) to chemical treatment. The protein was incubated overnight at 25 ℃ with different concentrations of guanidine hydrochloride (0 M, 1 M, 2 M, 3 M, 4 M, 5 M, 6 M). Fluorescence emission spectra was collected at different concentrations by setting the excitation at 295 nm and collecting the spectra at a range of 300-400 nm. The unfolding of Cellulase A was studied by measuring the intrinsic fluorescence intensity at a wavelength of excitation corresponding to tryptophan's fluorescence. The maxima emission (λmax) of Cellulase A at 0 M GdmCl concentration was 340 nm, however, in a solution of 6 M GdmCl, the λmax shifted to 360. A shift in the maximum of emission (red shift), which reflected unfolding of protein, was observed. Differences in the fluorescence behavior of the protein is attributed to disruption of the structure of proteins by denaturants. Also, the overall fluorescence intensity decreases as the protein unfolds and buried tryptophans become exposed to hydrophilic solvent.

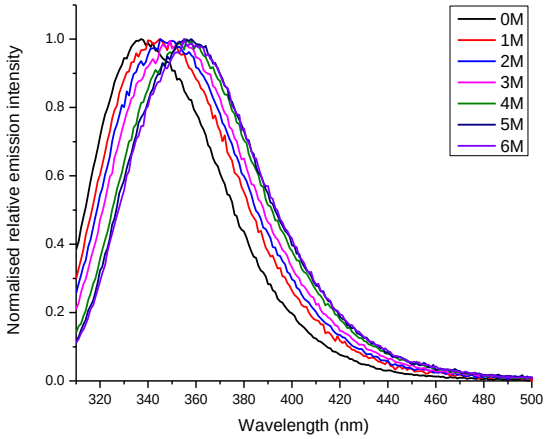


Figure 3.8.

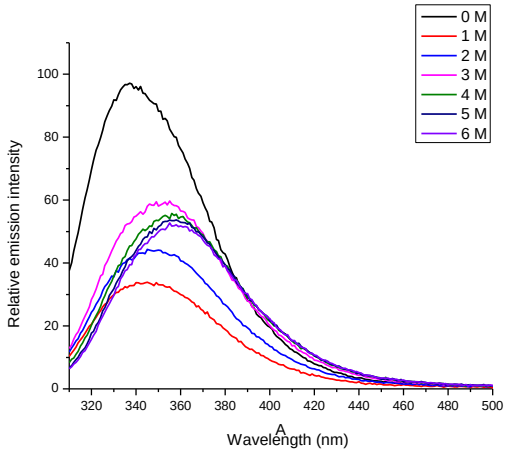


Figure 3.9.

Relative florescence intensities of Cellulase A with increasing concentration of GdmCl

3.1.3.5 Chemical stability monitored using Circular- Dichroism

Far UV CD spectra were acquired in Tris-Nacl buffer (pH 8.0) and the spectra were monitored from 210 to 250 nm. The path length was 1 mm and spectra were recorded as an average of 8

scans. The molar ellipticity starts degrading from 1 M concentration itself and disappears above 5 M, implying that Cellulase A denatures completely.

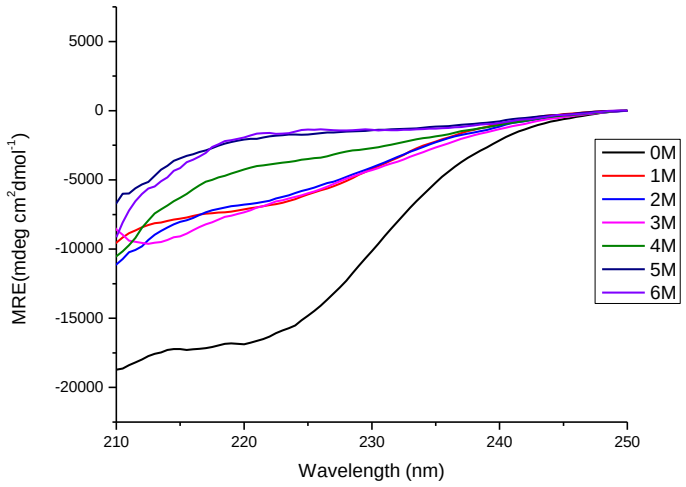


Figure 3.10. CD spectrum of Cellulase A with increasing concentration of GdmCl

3.1.4 DNSA activity assay with carboxymethyl cellulose (CMC)

The DNSA enzyme activity assay was conducted in triplicates by incubating the protein Cellulase A (0.5 µM), for 2-hours and 6-hours respectively, with 0.5% substrate CMC (Carboxymethyl cellulose). Plotting the mean absorbance at 540nm at different temperatures ranging from 20 ℃- 90 ℃ at 10 ℃ intervals, revealed that the enzyme is most active at 50 ℃ and 60 ℃ and starts dropping after that.

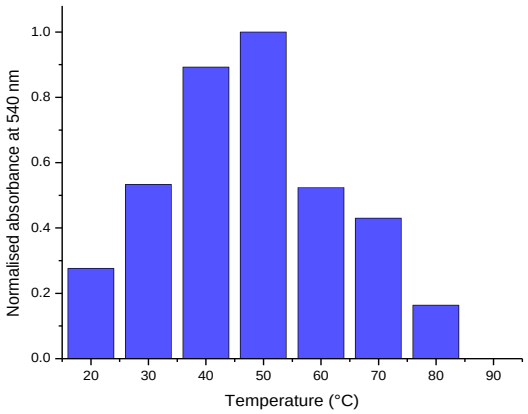


Figure.3.11. Temperature dependent DNSA activity assay of Cellulase A with CMC for 2-hours

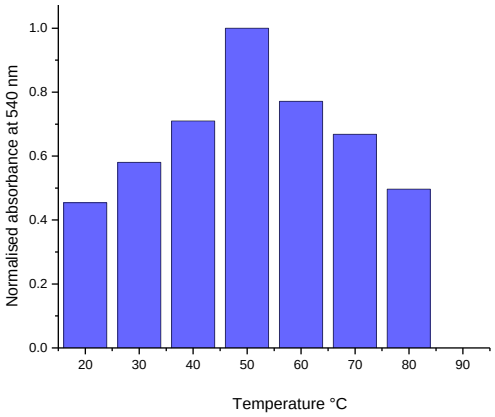


Figure.3.12. Temperature dependent DNSA activity assay of Cellulase A with CMC for 6-hours

3.2 Cellulase Q

Cellulases, which catalyze the hydrolysis of glycosidic bonds in cellulose, can be classified into several different protein families. Cellulase Q from *Clostridium thermocellum* is a thermophilic endoglucanase enzyme and is a member of glycosyl hydrolase family 9. The crystallographic asymmetric unit contains two copies of the CelQ molecule that form a homodimer. The catalytic domain has an (α/α)6 -barrel topology, and the CBM has an antiparallel b-sandwich fold. These two domains are separated by a 17-residue linker which is essential for the endoglucanase activity. The CelQ gene was characterized and found to encode a polypeptide of 709 amino acid residues, consisting of a type 3c carbohydrate-binding module (CBM), a catalytic domain, and a type-I dockerin domain.

3.2.1 CLONING

3.2.1.1 Amplification

Cellulase Q was amplified from the *Clostridium thermocellum* genomic DNA. The conditions for gene amplification for PCR are mentioned in the materials and methods section. The desired band (2133 bp) was obtained at 60℃.

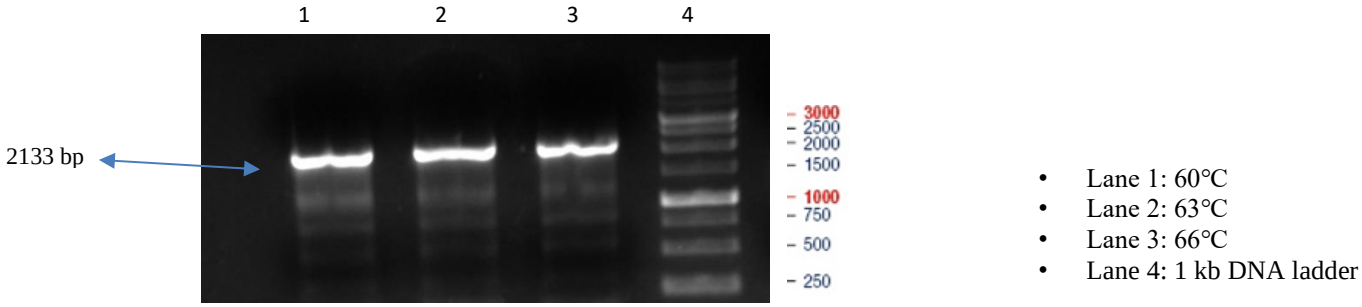
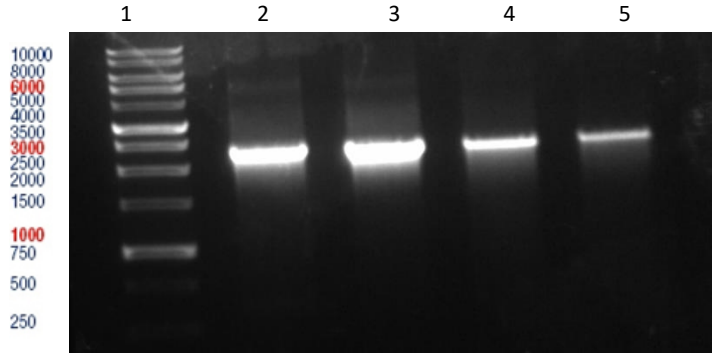


Figure 3.13. Amplicons of Cellulase Q at different annealing temperatures

3.1.1.3 Colony PCR

The band in all the lanes show putative positive colonies. We proceeded with colony 3 and it was confirmed by test digestion and DNA sequencing.



- Lane 1: 1 kb ladder
- Lane 2: colony 1
- Lane 3: colony 2
- Lane 4: colony 3
- Lane 5: colony 4

Figure 3.14. Colony PCR of Cellulase Q

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

3.2.2 Ni-NTA Purification

The purification was done using Ni-NTA column by native method. Cellulase Q was expressed in soluble form and found to be present in the cytosol.

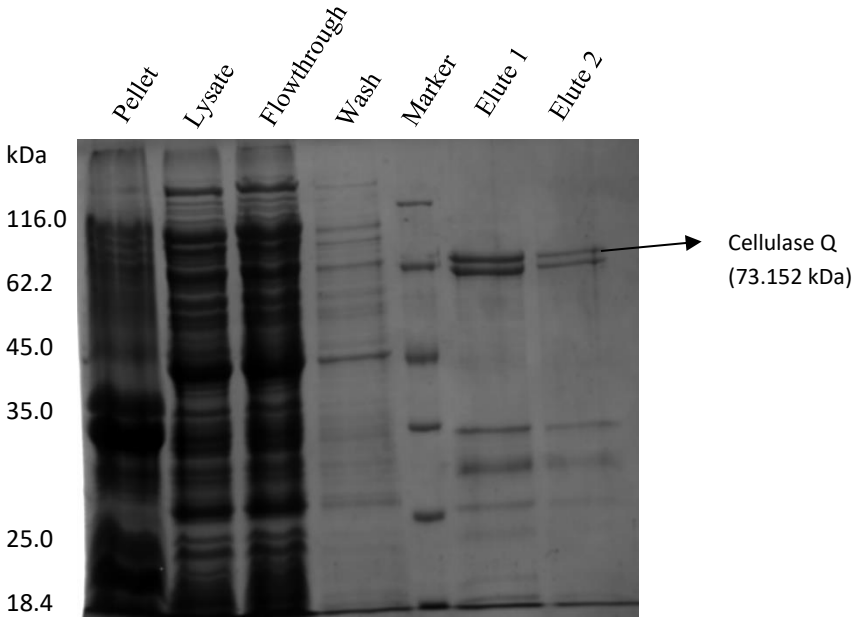


Figure 3.15. SDS-PAGE profile of Cellulase Q

3.2.3 Biophysical Characterization

3.2.3.1 Gel Filtration

The protein was run on the column Superdex 200 10/300 GL(24 mL). The protein was equilibrated with 20 mM Tris buffer and 500 µl of the protein was loaded for analysis. The protein is being eluted as a monomer and as a dimer.

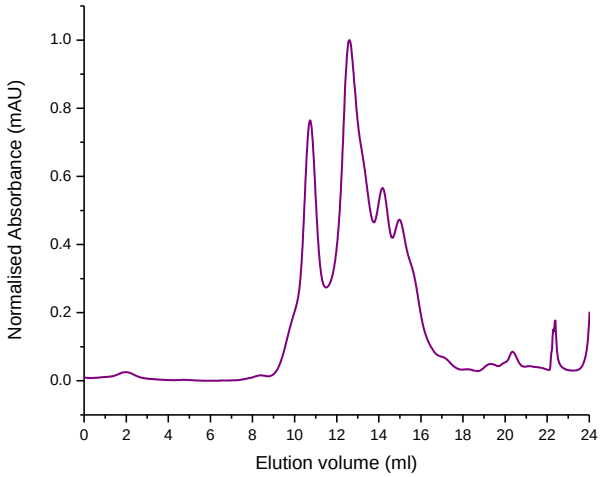


Figure 3.16. Gel filtration chromatogram of cellulase Q

The elution volumes were compared with the elution profiles of the standards run on the same column and the fractions of 1.5 mL were collected and run on 12 % SDS gel.

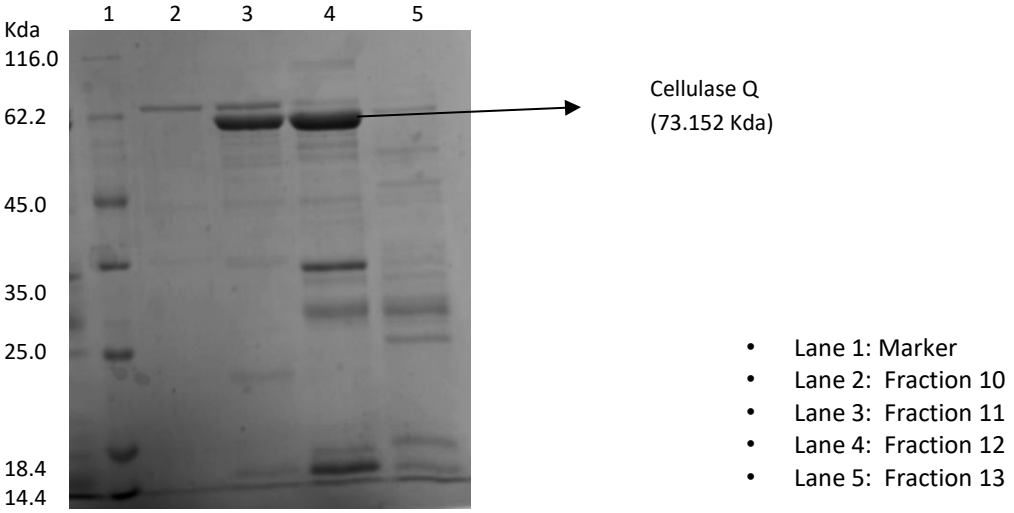


Figure 3.17. Collected fractions of cellulase Q

3.2.3.2 Circular Dichroism

Concentration of 0.2 mg/ml of the protein was used to study the far UV-Circular dichroism spectra for Cellulase Q. The figure shows that the spectral shape is dominated by α-helix but the spectral intensity is also contributed by β-sheets.

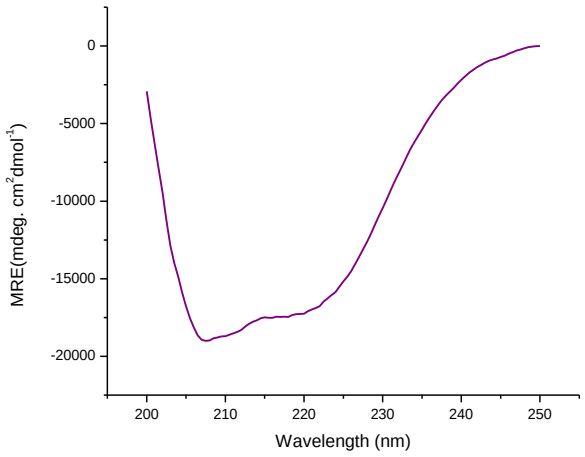


Figure 3.18 CD spectrum of Cellulase Q consisting of α-helix and β-sheets

3.2.3.3 Tryptophan fluorescence

The protein concentration used was 0.8 mg/ml to study the intrinsic fluorescence of Cellulase Q. Since, the λ_{max} emission is at 338 nm in the emission spectra, we can say that the protein seems to be well folded and the tryptophan is buried and not exposed.

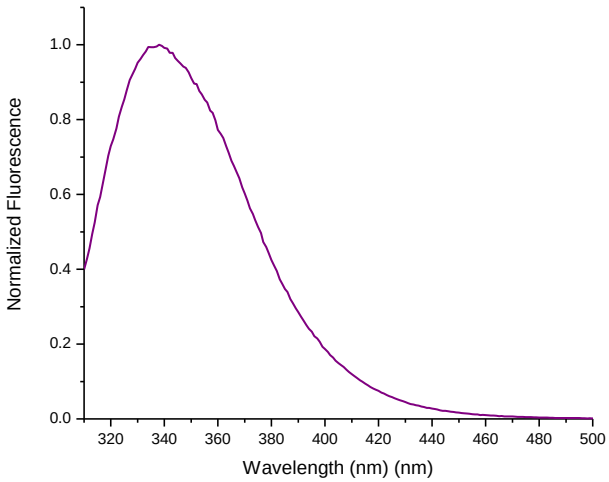


Figure 3.19. Intrinsic fluorescence emission spectrum of Cellulase Q

3.2.3.4 Chemical stability monitored using tryptophan fluorescence

The protein was checked for stability against guanidine hydrochloride (GdmCl) by subjecting the protein (0.2 mg/ml) to chemical treatment. The maxima emission (λmax) of Cellulase Q at 0 M GdmCl concentration is 338 nm, however, in a solution of 6 M GdmCl, the λmax shifted to 365. Thus, a red shift, which reflected the unfolding of protein was observed. Also, there is an increase in intensity in fluorescence emission which is due to the reason that the fluorescence of the tryptophan in its buried state was getting quenched.

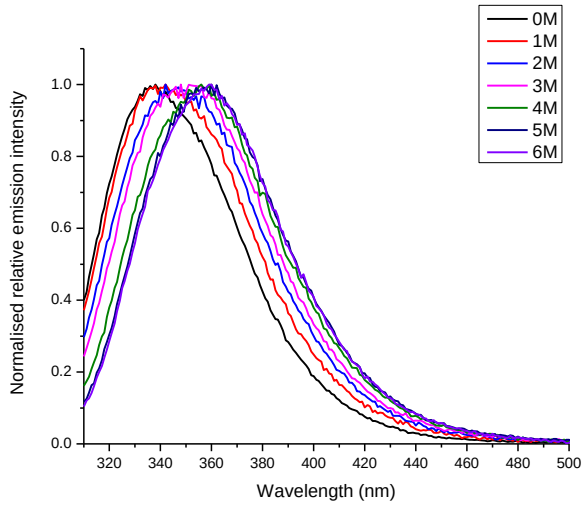


Figure 3.20.

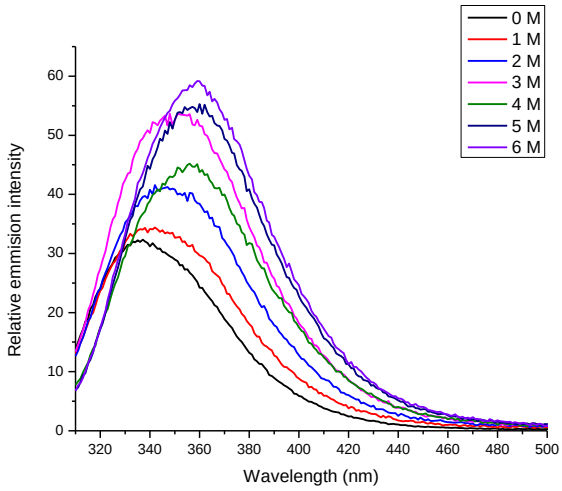


Figure 3.21.

Relative florescence intensities of Cellulase A with increasing concentration of GdmCl

3.2.3.5 Chemical stability monitored using Circular- Dichroism

Far UV CD spectra were acquired in Tris-Nacl buffer (pH 8.0) and the spectra were monitored from 210 to 250 nm. The molar ellipticity starts degrading from 1 M concentration itself and remains stable till 3 M but disappears above 5 M, implying that Cellulase Q denatures completely.

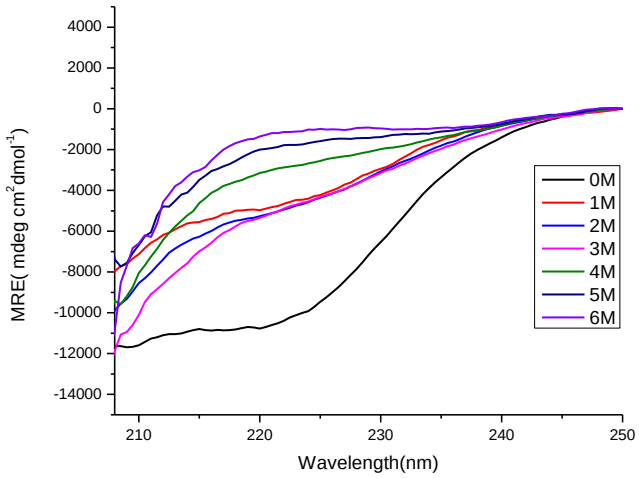


Figure 3.22. CD spectrum of Cellulase Q with increasing concentration of GdmCl

3.2.4 DNSA activity assay with carboxymethyl cellulose (CMC)

The DNSA enzyme activity assay was conducted in triplicates by incubating the protein Cellulase Q for 2-hours and 6-hours respectively, with the substrate CMC (Carboxymethyl cellulose). Plotting the mean absorbance at 540 nm at different temperatures ranging from 20 ℃- 90 ℃ at 10 ℃ intervals, revealed that the enzyme is most active at 80 ℃.

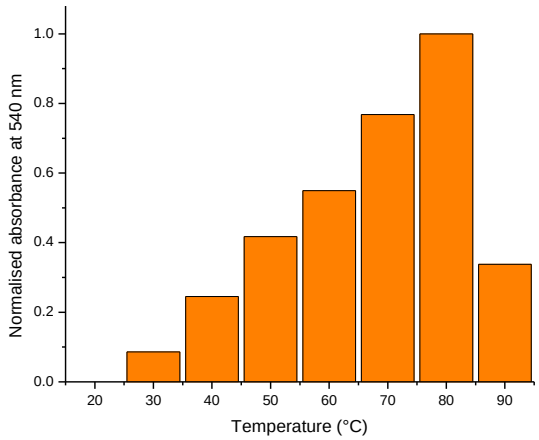


Figure 3.23. Temperature dependent DNSA activity assay of Cellulase Q with CMC for 2-hours

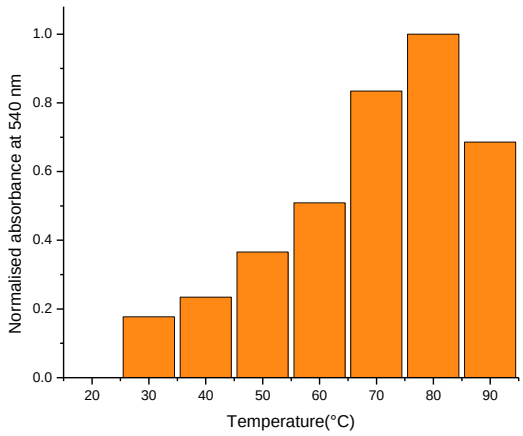


Figure 3.24. Temperature dependent DNSA activity assay of Cellulase Q with CMC for 6-hours

3.3 Xylanase A

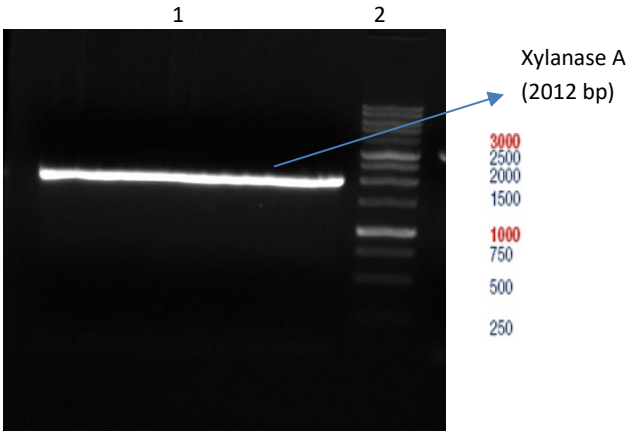
β1,4-Xylan, which has a backbone of β1,4-linked xylopyranosyl residues with various substituent side-groups such as acetyl, is a major component of the hemicellulose fraction of plant cell walls. Enzymes involved in hydrolysis of the main chain of xylan are endoxylanase (EC 3.2.1.8) and β -xylosidase (EC 3.2.1.37) (Biely 1985). On the basis of amino acid sequence homology, xylanases can be essentially divided into two groups, i.e., families 10 and 11, both of which have catalytic domains of glycosyl hydrolases. (Henrissat and Bairoch 1996).

Xylanase A gene from the organism *Clostridium thermocellum*, encoding the xylanase XynA is a modular enzyme composed of a typical N-terminal signal peptide and four domains in the following order: a family-11 xylanase domain, a cellulose-binding domain, a dockerin domain, and a NodB domain. The enzyme is highly active toward xylan.

3.3.1 CLONING

3.3.1.1 Amplification

Xylanase A was amplified from the *Clostridium thermocellum* genomic DNA. The conditions for gene amplification for PCR are mentioned in the materials and methods section. The desired band (2012 bp) was obtained at 67°C.



- Lane 1: Upscale of amplicon at 67°C
- Lane 2: 1Kb DNA ladder

Figure 3.25. Upscale amplification of Xylanase A

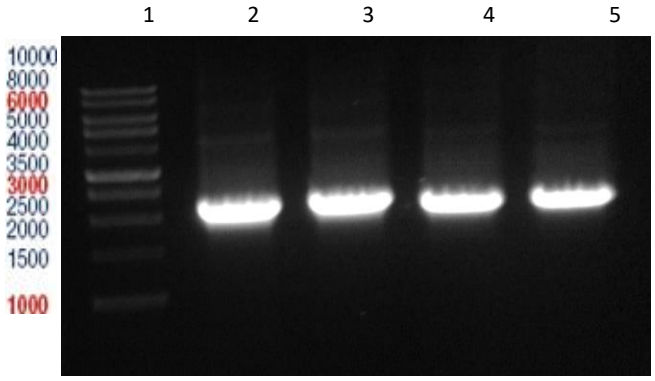
3.3.1.2 Digestion and Ligation

Digestion and Ligation

1. The band was then excised and gel extracted. Further, the gel extracted PCR product and the vector were double digested with Nde1-Xho1.
2. Ligation reaction was incubated at 25℃ for 3 hours with T4 Ligase. For 50ng of digested pET23a, the amount of digested Xylanase A was calculated using the formula mentioned in the materials and methods section.
3. The ligation mixture was then transformed in the cloning host cells, XL1Blue. The colonies were obtained the next day.

3.1.1.3 Colony PCR

The band in all the lanes show putative positive colonies. We proceeded with colony 3 and the cloning was confirmed by test digestion and DNA sequencing.



- Lane 1: 1Kb ladder
- Lane 2: colony 1
- Lane 3: colony 2
- Lane 4: colony 3
- Lane 5: colony 4

Figure 3.26. Colony PCR of Xylanase A

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

3.3.2 Ni-NTA Purification

The purification was done using Ni-NTA column by native method. Xylanase A was expressed in soluble form and found to be present in the cytosol.

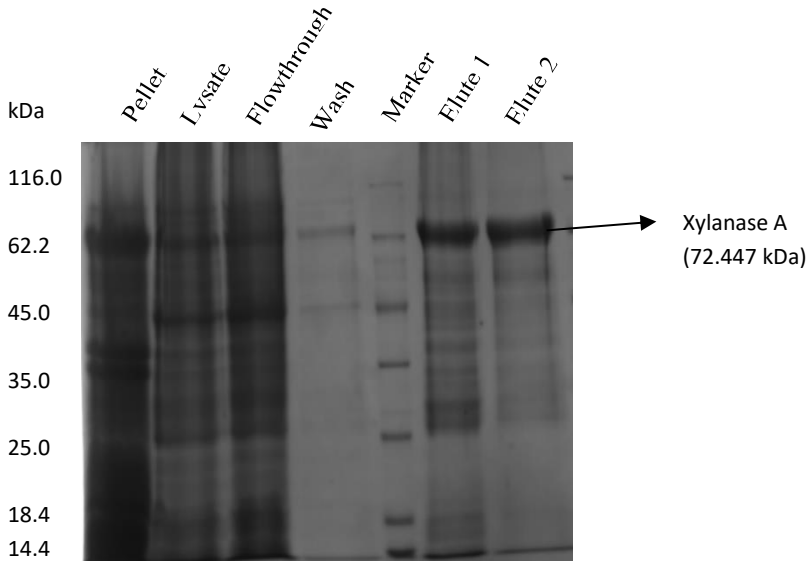


Figure 3.27. SDS-PAGE profile of Xylanase A

3.3.3 Biophysical Characterization

3.3.3.1 Gel Filtration

The protein was run on the column Superdex 200 10/300 GL(24 mL). The protein was equilibrated with 20 mM Tris buffer and 500 µl of the protein was loaded for analysis. The chromatogram shows that the protein is getting eluted as a monomer.

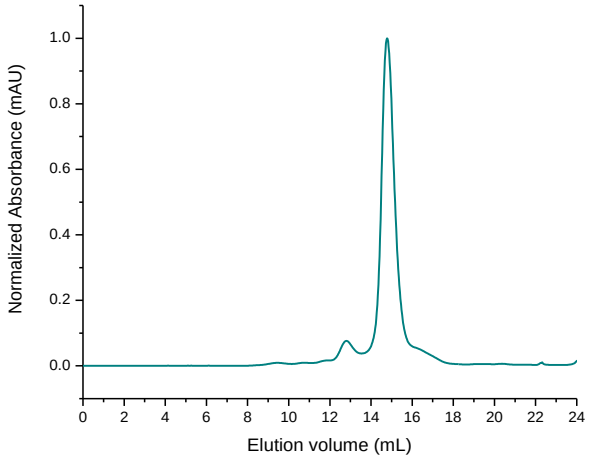


Figure 3.28. Gel filtration chromatogram of Xylanase A

The elution volumes were compared with the elution profiles of the standards and the fractions of 1.5 mL were collected and run on 12% SDS gel.

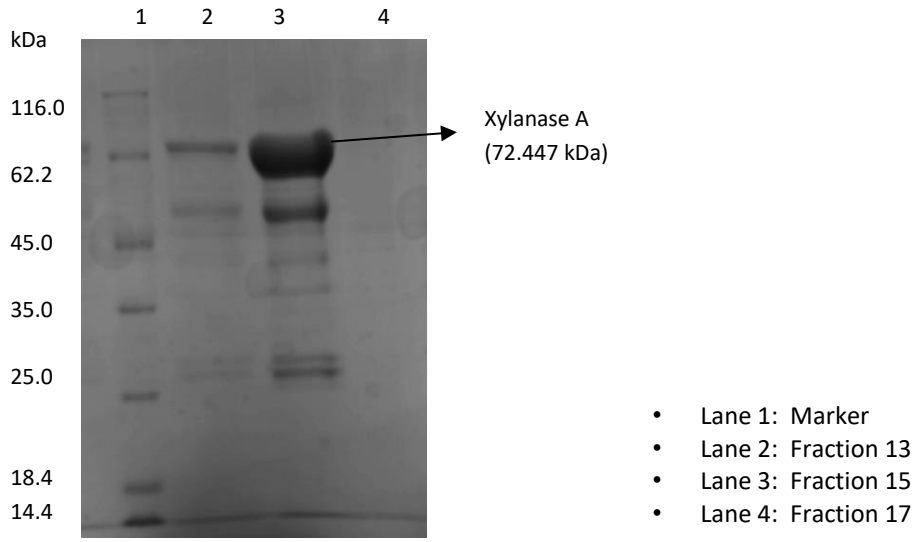


Figure 3.29. Collected fractions of Xylanase A

3.3.3.2 Circular Dichroism

Far UV-Circular dichroism spectra for Xylanase A shows that the protein's spectral shape was dominated by β-sheets, which supports the literature.

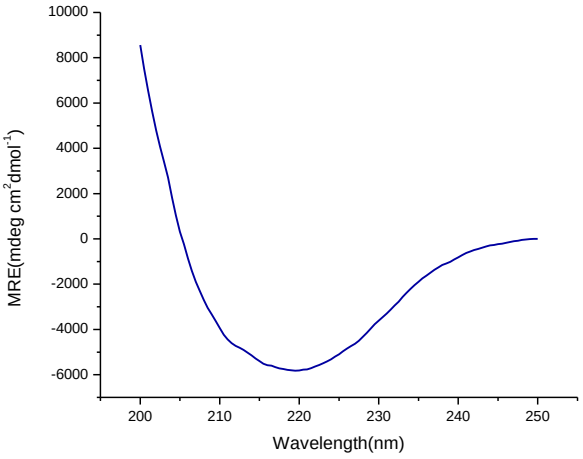


Figure 3.30. CD spectrum of Xylanase A

3.3.3.3 Tryptophan fluorescence

The protein concentration used was 2.5 mg/ml to study the intrinsic fluorescence of Xylanase A. Since, the λ_{max} emission is at 348 nm in the emission spectra, we can say that the protein seems to be well folded.

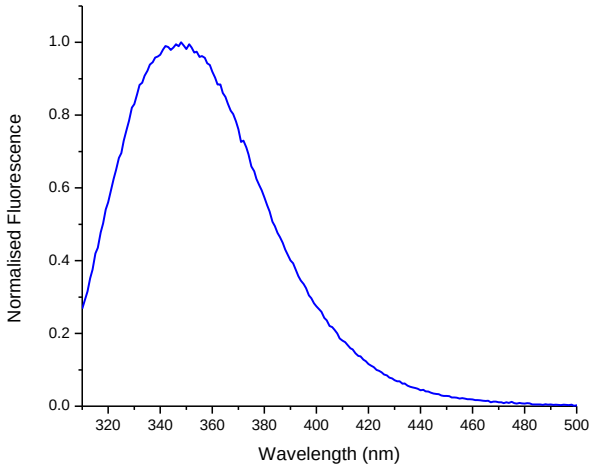
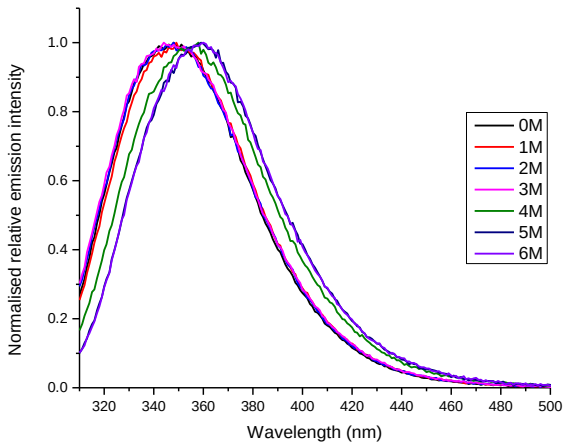


Figure 3.31. Intrinsic fluorescence emission spectrum of Xylanase A

3.3.3.4 Chemical stability monitored using tryptophan fluorescence

The protein was checked for stability against guanidine hydrochloride (GdmCl) by subjecting the protein (0.2 mg/ml) to chemical treatment. The maxima emission (λmax) of Xylanase A till 3 M GdmCl concentration remained 348 nm, however, in a solution of 6M GdmCl, the λmax shifted to 360 nm. Thus, a red shift, which reflected unfolding of protein, was observed only after 3M.



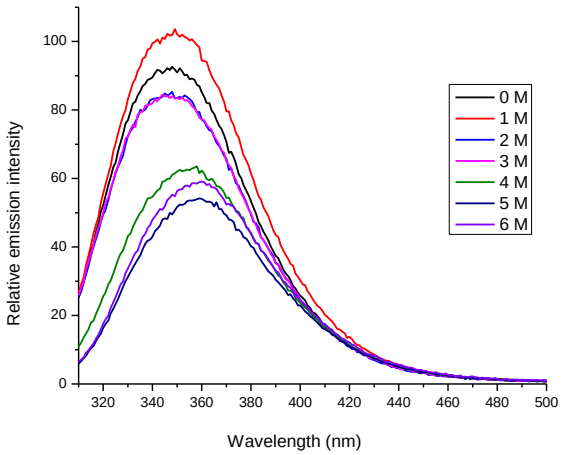
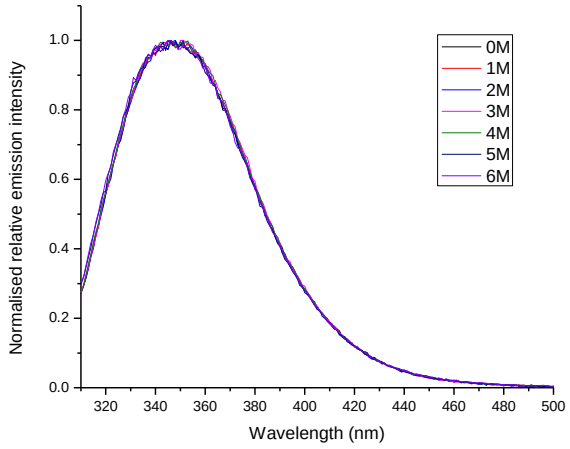


Figure 3.32. & 3.33. Relative florescence intensities of Xylanase A with increasing concentration of GdmCl

The intensity also decreases with the increasing concentration of GdmCl.

The protein was also checked for stability against Urea by subjecting the protein (0.2 mg/ml) to chemical treatment by incubating the protein overnight at 25℃ with different concentrations of Urea (0 M, 1 M, 2 M, 3 M, 4 M, 5 M, 6 M). Fluorescence emission spectra revealed that the maxima emission (λmax) of Xylanase A remained at 348 nm even at 6 M Urea concentration which shows that the structure of the protein is very stable against urea. The intensity also slightly increases which could be again due to the reason that the tryptophan in its buried state was getting quenched.



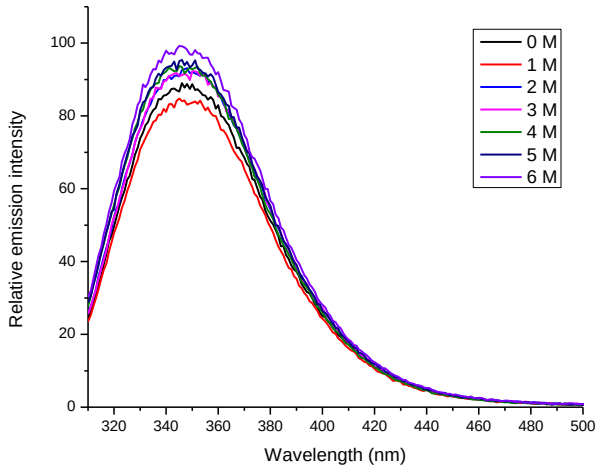


Figure 3.34 **Figure 3.35**

Relative florescence intensities of Xylanase A with increasing concentration of Urea

3.3.3.5 Chemical stability monitored using Circular- Dichroism

The protein was checked for stability against guanidine hydrochloride (GdmCl) and Urea by subjecting the protein (0.2 mg/ml) to chemical treatment and its far UV CD spectra were monitored from 210 to 250 nm. For GdmCl, the molar ellipticity doesn't change at 1 M concentration but starts degrading from 3 M and rapidly degrades after that, implying that Xylanase A has a stable structure.

For Urea denaturation, the molar ellipticity starts increasing at first, suggesting gain in structure and then starts decreasing slowly. The enzyme still maintains its structure even at 6 M urea, implying that the protein has only partially unfolded.

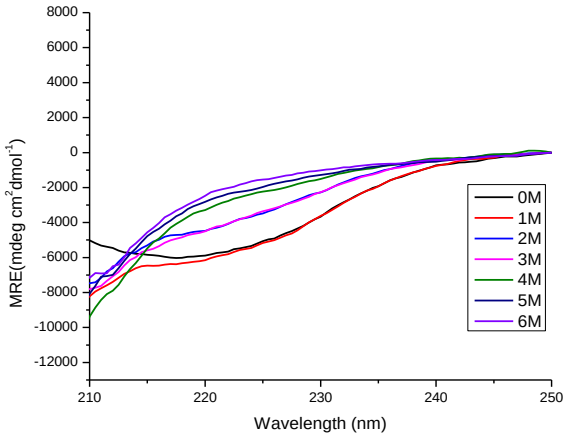


Figure 3.36. CD spectrum of Xylanase A with increasing concentration of GdmCl

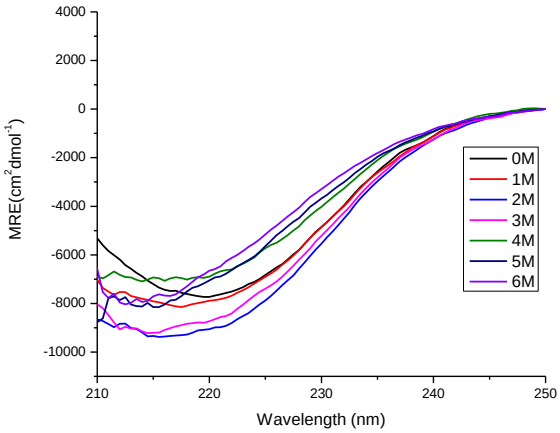


Figure 3.37. CD spectrum of Xylanase A with increasing concentration of Urea

3.3.4 DNSA activity assay with Xylose

The DNSA enzyme activity assay was conducted in triplicates by incubating the protein Xylanase A (0.5µM) for 2-hours, with the substrate xylose. Plotting the mean absorbance at 540nm at different temperatures ranging from 20℃- 90℃ at 10℃ intervals, revealed that the enzyme has optimum activity at 60℃

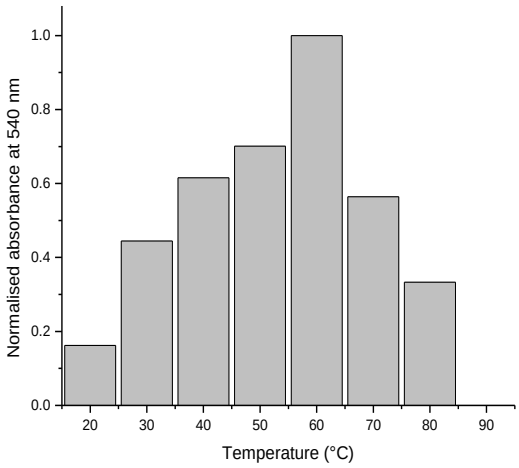


Figure 3.38. Temperature dependent DNSA activity assay of Xylanase A with xylose for 2-hours

3.4 Cellulase S

Cellulases, which catalyze the hydrolysis of glycosidic bonds in cellulose, can be classified into several different protein families. Cellulase S from Clostridium thermocellum is a thermophilic exoglucanase or cellobiohydrolases enzyme and is a member of glycosyl hydrolase family 48. Cellulase is a modular enzyme composed of a typical N-terminal signal peptide and one dockerin domain. It is highly and preferrable active towards crystalline cellulose than Carboxymethyl cellulose.

3.4.1 CLONING

3.4.1.1 Amplification

Cellulase S was amplified from the *Clostridium thermocellum* genomic DNA. The conditions for gene amplification for PCR are mentioned in the materials and methods section. The desired band (2333 bp) was obtained at 65℃.

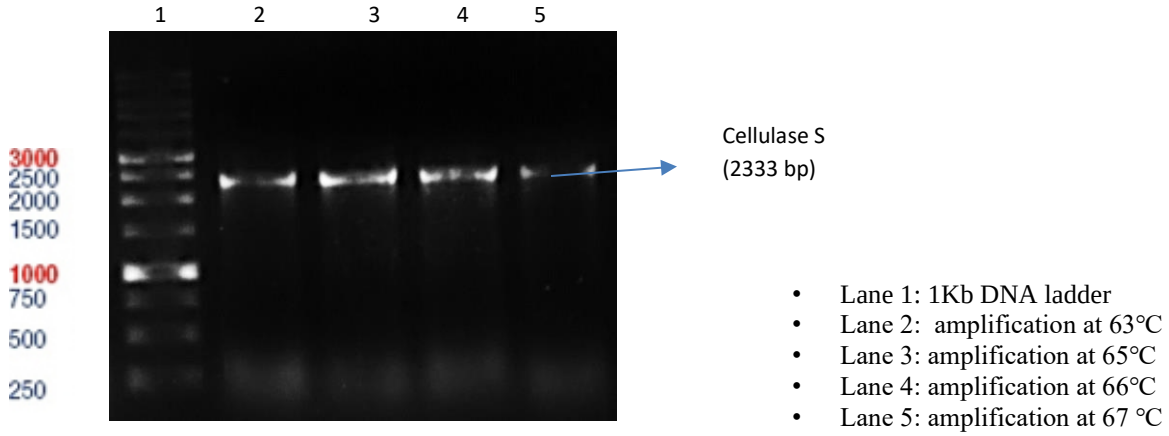


Figure 3.39. PCR amplification of Cellulase S

3.4.1.2 Digestion and Ligation

1. The band was then excised and gel extracted. Further, the gel extracted PCR product and the vector were double digested with *NdeI-XhoI*.

2. Ligation reaction was incubated at 25°C for 3 hours with T4 Ligase. For 50ng of digested pET23a, the amount of digested Cellulase S was calculated using the formula mentioned in the materials and methods section.
3. The ligation mixture was then transformed in the cloning host cells, XL1Blue. The colonies were obtained the next day.

3.4.1.3 Colony PCR

The band in the lanes (2,3,4) show putative positive colonies. We proceeded with colony 3 and the cloning was confirmed by test digestion and DNA sequencing.

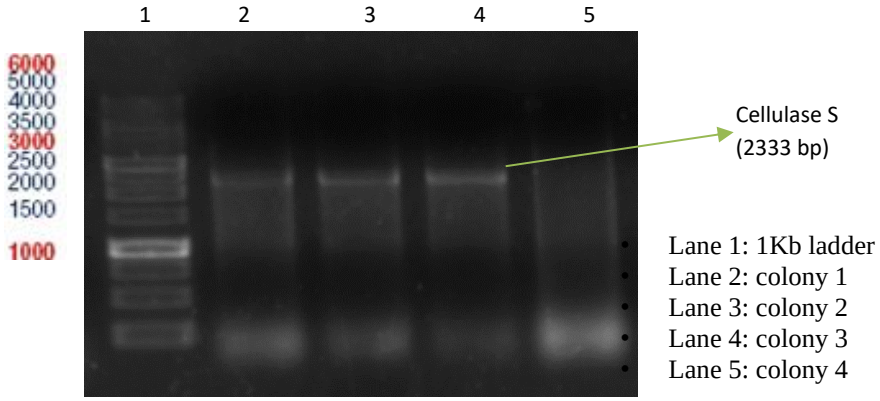


Figure 3.40. Colony PCR of Cellulase S

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

3.4.2 Ni-NTA Purification

The purification was done using Ni-NTA column by native method. Cellulase S was expressed in soluble form and found to be present in the cytosol.

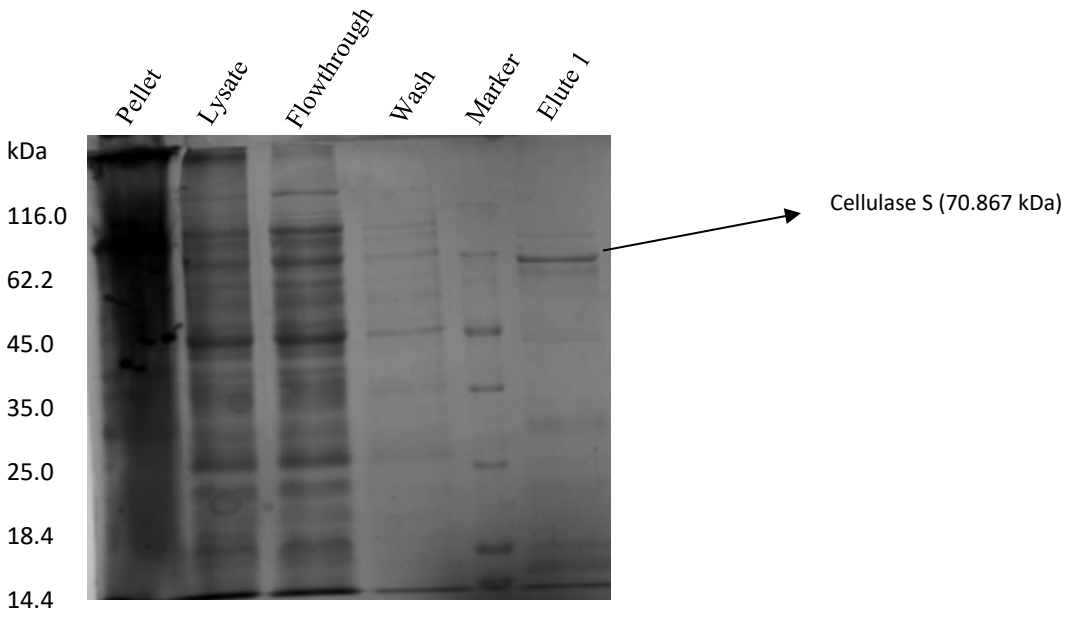


Figure 3.41. SDS-PAGE profile of Cellulase S

3.4.3 Biophysical Characterization

3.4.3.1 Gel Filtration

The protein was run on the column Superdex 200 10/300 GL(24 mL). The protein was equilibrated with 20 mM Tris buffer and 500 µl of the protein was loaded for analysis. The chromatogram shows that the protein is getting eluted as a monomer and a dimer.

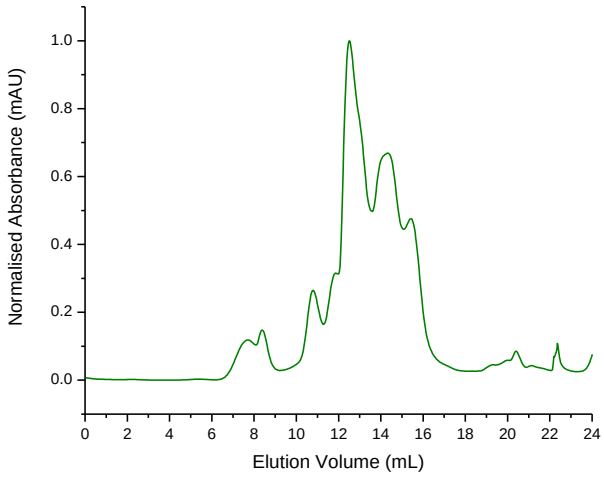


Figure 3.42. Gel filtration chromatogram of cellulase S

The elution volumes were compared with the elution profiles of the standards and the fractions of 1.5 mL were collected and run on 12% SDS gel.

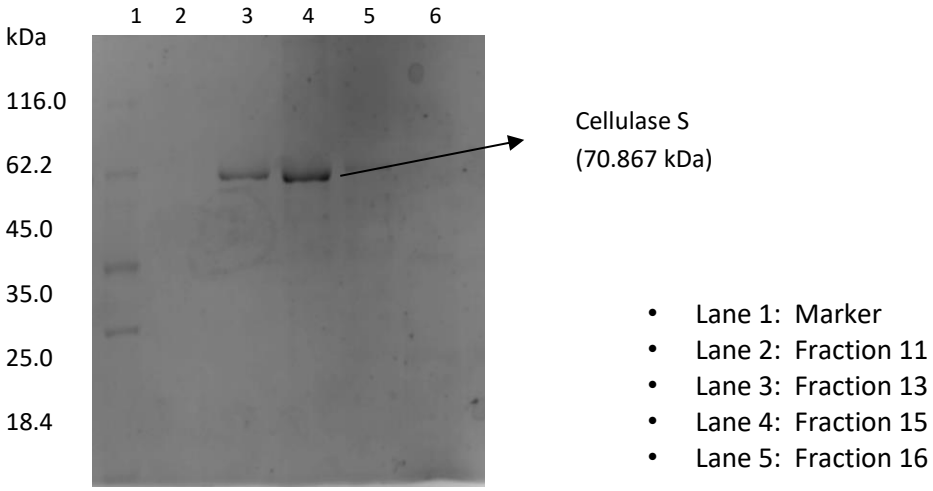


Figure 3.43. Collected fractions of Cellulase S

3.4.3.2 Circular Dichroism

Far UV-Circular dichroism spectra for Cellulase S shows that the protein's spectral shape was dominated by α-helix, which supports the literature. A concentration of 0.2 mg/ml of the protein was used.

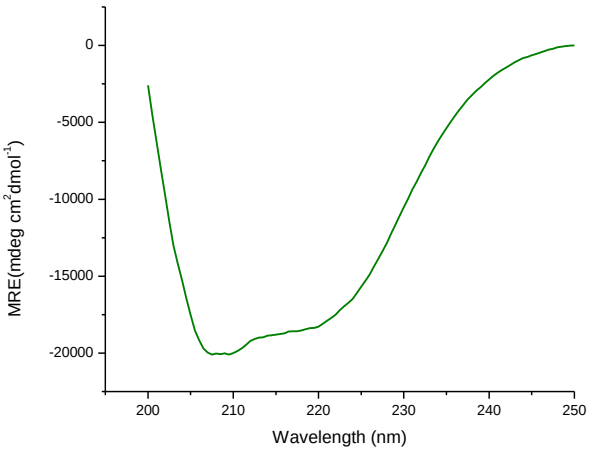


Figure 3.44. CD spectrum of Cellulase S

3.4.3.3 Tryptophan fluorescence

The protein concentration used was 0.7 mg/ml to study the intrinsic fluorescence of Cellulase S. Since, the λ_{max} emission is at 336 nm in the emission spectra, we can say that the protein seems to be well folded.

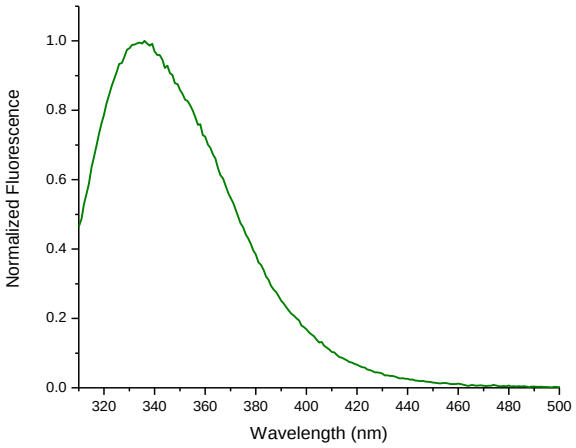


Figure 3.45. Intrinsic fluorescence emission spectrum of Cellulase S

3.4.4 DNSA activity assay with carboxymethyl cellulose (CMC)

The DNSA enzyme activity assay was conducted in triplicates by incubating the protein Cellulase S (0.5µM) for 2-hours and 6-hours respectively, with the substrate CMC (Carboxymethyl cellulose). Plotting the mean absorbance at 540nm at different temperatures ranging from 20℃- 90℃ at 10℃ intervals, revealed that the enzyme was mostly active at 50℃-60℃.

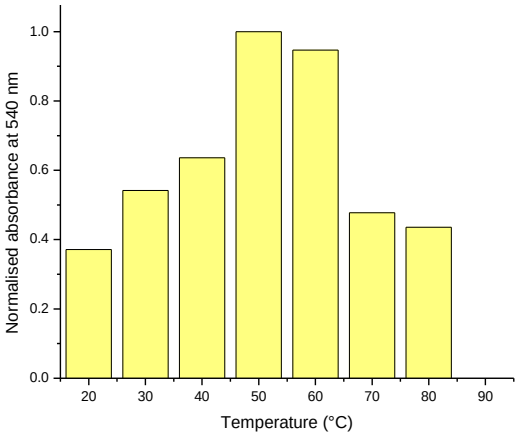


Figure 3.46. Temperature dependent DNSA activity assay of Cellulase S with CMC for 2-hours

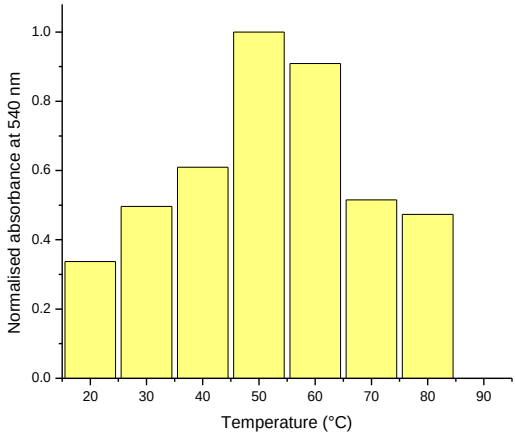


Figure 3.47. Temperature dependent DNSA activity assay of Cellulase S with CMC for 6-hours

3.5 Beta-glucosidase A

Beta-glucosidases or (cellobiases) play a crucial role in the enzymatic degradation of cellulose by converting cellobiose and cellodextrins formed by cellulase action to fermentable glucose. From the cellulolytic thermophile Clostridium thermocellum two Beta-glucosidase genes, designated bglA and bglB, have been extensively studied in literature. They differ markedly in their substrate specificities. BglA hydrolyzes both beta-glucosides and beta-galactosides, whereas BglB also exhibits beta-xylosidase activity.

3.5.1 CLONING

3.5.1.1 Amplification

BglA was amplified from the *Clostridium thermocellum* genomic DNA. The conditions for gene amplification for PCR are mentioned in the materials and methods section. The desired band (1344 bp) was obtained at 63℃.

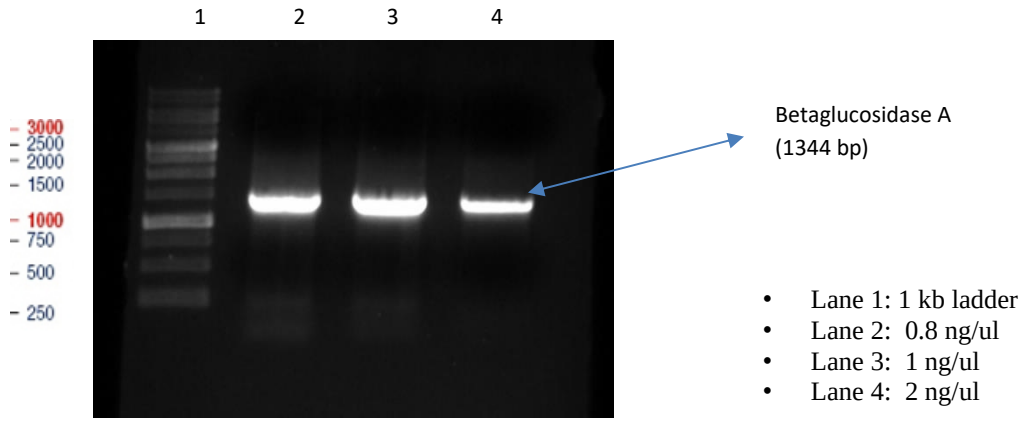


Figure 3.48. Amplicons of BglA at different genomic concentrations

3.5.1.2 Digestion and Ligation

1. The band was then excised and gel extracted. Further, the gel extracted PCR product and the vector were double digested with *NdeI-XhoI*.

2. Ligation reaction was incubated at 25°C for 3 hours with T4 Ligase. For 50ng of digested pET23a, the amount of digested BglA was calculated using the formula mentioned in the materials and methods section.
3. The ligation mixture was then transformed in the cloning host cells, XL1Blue. The colonies were obtained the next day.

3.5.1.3 Colony PCR

The band in all the lanes, except lane 3, show putative positive colonies. We proceeded with colony 1 and the cloning was confirmed by test digestion and DNA sequencing.

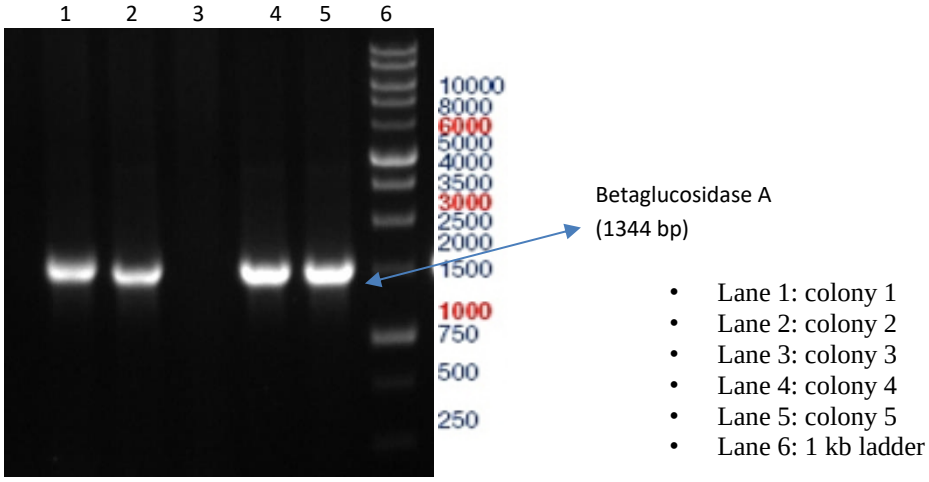


Figure 3.49. Colony PCR of BglA

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

3.5.2 Ni-NTA Purification

The purification was done using Ni-NTA column by native method. Cellulase S was expressed in soluble form and found to be present in the cytosol.

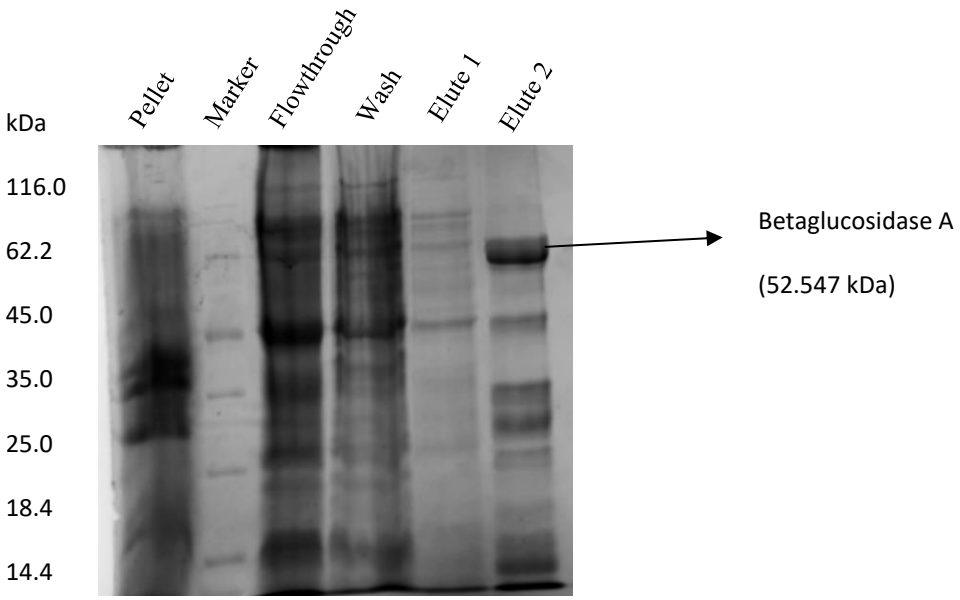


Figure 3.50. SDS-PAGE profile of BglA

3.5.3 Biophysical Characterization

3.5.3.1 Gel Filtration

The protein was run on the column Superdex 200 10/300 GL(24 mL). The protein was equilibrated with 20 mM Tris buffer and 500 µl of the protein was loaded for analysis. The chromatogram shows that the protein is getting eluted as a monomer, dimer and a trimer.

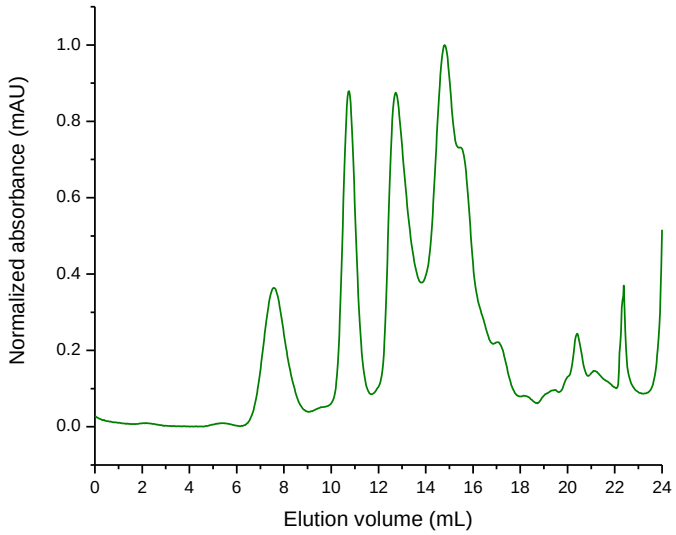


Figure 3.51. Gel filtration chromatogram of Betaglucosidase A

3.5.3.2 Circular Dichroism

Far UV-Circular dichroism spectra for Beta-glucosidase A shows that the protein's spectral shape is dominated by α-helix while the spectral intensity was also contributed by β sheets.

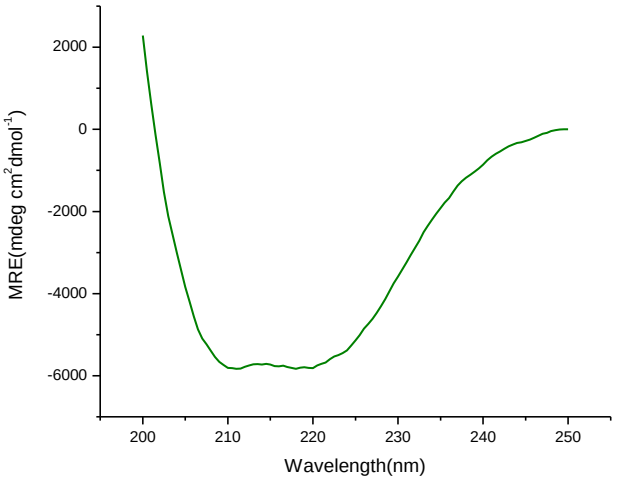


Figure 3.52. CD spectrum of Betaglucosidase A consisting of α-helix and β-sheets

3.5.3.3 Tryptophan fluorescence

Fluorescence emission spectra was collected by setting the excitation at 295 nm and collecting the spectra at a range of 300-400 nm. The slit width used for the experiment was 5nm for both excitation and emission and the protein concentration used was 0.5 mg/ml. Since, the λ_{max} emission is at 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.

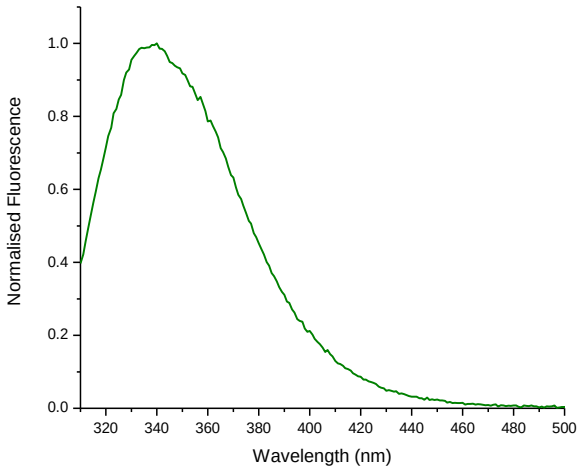


Figure 3.53. Intrinsic fluorescence emission spectrum of Betaglucosidase A

3.5.4 DNSA activity assay with carboxymethyl cellulose (CMC)

The DNSA enzyme activity assay was conducted in triplicates by incubating the protein Beta-glucosidaseA (0.5µM) for 2-hours and 6-hours respectively, with the substrate CMC (Carboxymethyl cellulose). Plotting the mean absorbance at 540nm at different temperatures ranging from 20℃- 90℃ at 10℃ intervals, revealed that the enzyme was mostly active at 50℃-60℃.

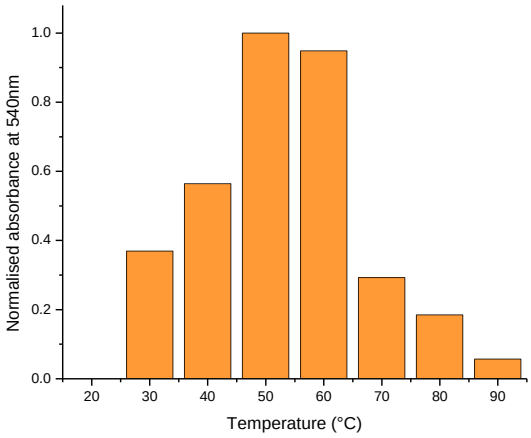


Figure 3.54. Temperature dependent DNSA activity assay of Beta-glucosidaseA with CMC for 2-hours

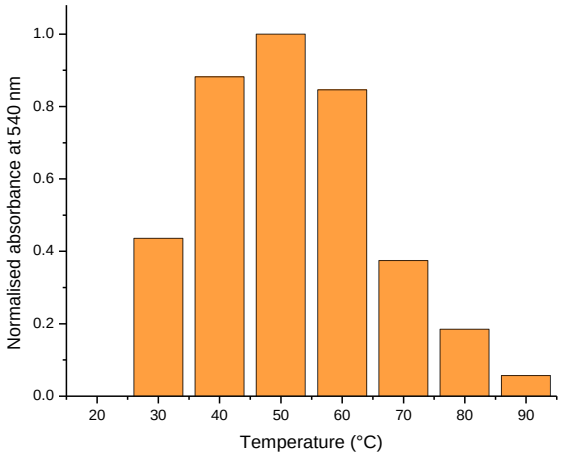


Figure 3.55. Temperature dependent DNSA activity assay of Beta-glucosidase A with CMC for 6-hours

3.6 Glycosyl Hydrolase

3.6.1 CLONING

3.5.1.1 Amplification

Glycoside hydrolase was amplified from the hyperthermophilic organism, *Pyrococcus furiosus* genomic DNA. The conditions for gene amplification for PCR are mentioned in the materials and methods section. The desired band (1843 bp) was obtained at 68℃

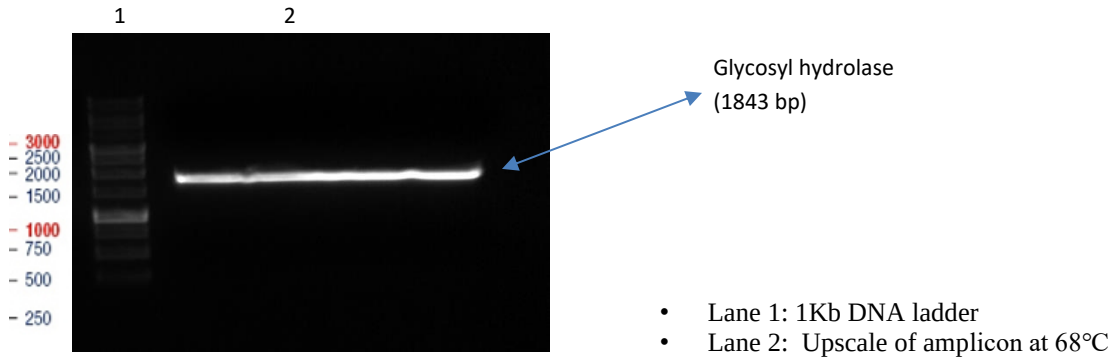


Figure 3.56. Upscale of PCR amplified Glycosyl hydrolase

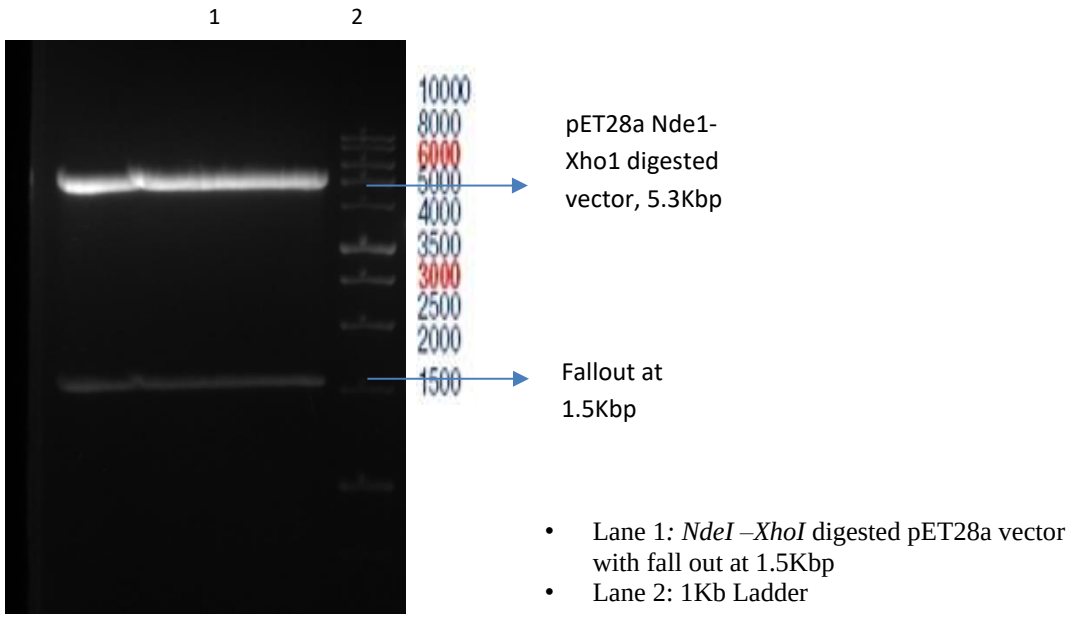


Figure 3.57. Digestion of pET28a with *NdeI-XhoI*

3.5.1.2 Digestion and Ligation

4. Further, the bands was excised and gel extracted. For digestion, the clone containing pET28a plasmid was inoculated and grown overnight at 37℃. Plasmid DNA was isolated the next day and both the plasmid and insert were double digested with *NdeI* and *XhoI* restriction enzymes by incubating them at 37℃ for 3 hours.
5. After 3 hours, PCR purification of the digested insert was carried out while the digested plasmid was run on 1% agarose gel for 3 hours at 60V to resolve it.
6. Double digested pET28a band was carefully excised and gel extraction was performed.
7. The concentration of both the digested vector and insert was checked using NanoDrop (Thermocientific) instrument.
8. Ligation reaction was incubated at 25℃ for 3 hours with T4 Ligase. For 50ng of digested pET28a, the amount of digested Glycosyl hydrolase was calculated using the formula mentioned in the materials and methods section.
9. The ligation mixture was then transformed in the cloning host cells, TOP10. The colonies were obtained the next day.

3.5.1.3 Colony PCR

The band in lane 2 and 4, show putative positive colonies. We proceeded with colony 4 and the cloning was confirmed by test digestion and DNA sequencing.

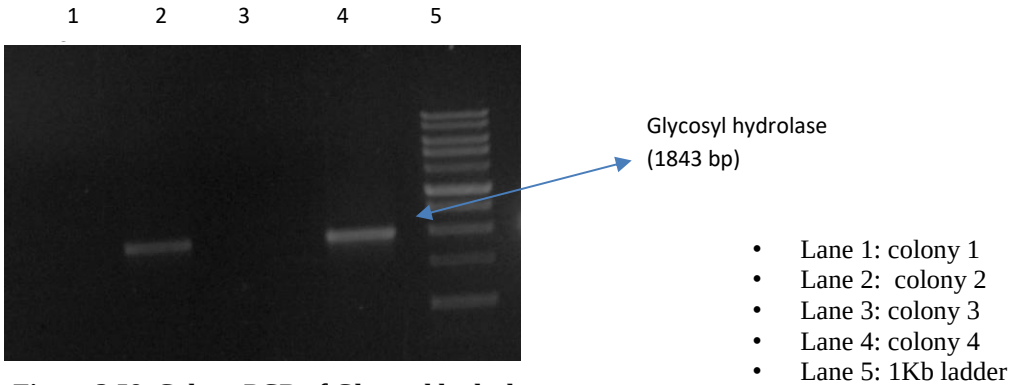


Figure 3.58. Colony PCR of Glycosyl hydrolase

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

3.6.2 Ni-NTA Purification

The purification was done using Ni-NTA column by native method. Glycoside Hydrolase was expressed in soluble form and found to be present in the cytosol.

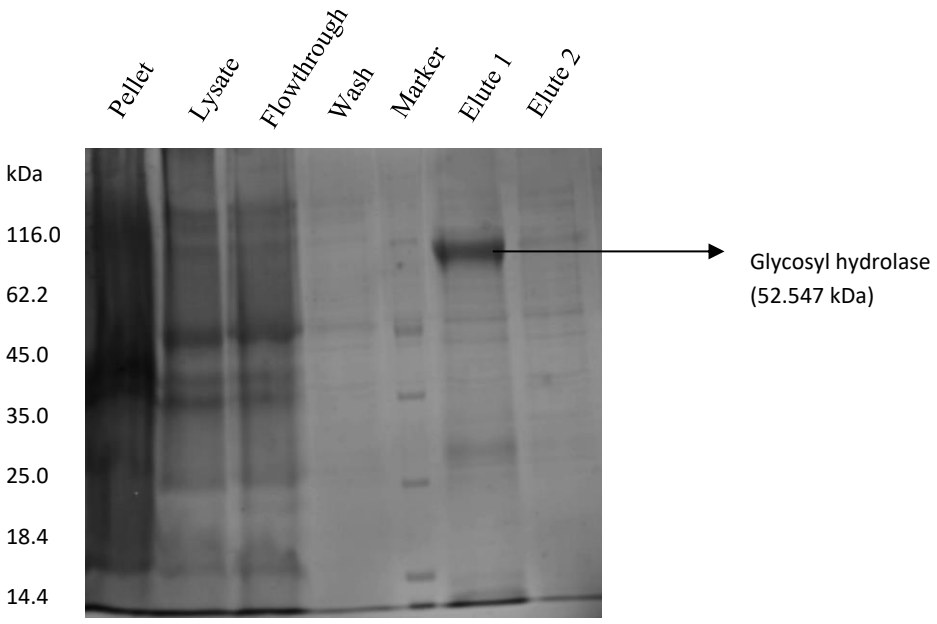


Figure 3.59. Ni-NTA Purification of Glycosyl hydrolase

3.6.3 Biophysical Characterization

3.6.3.1 Gel Filtration

The protein was run on the column Superdex 200 10/300 GL(24 mL). The protein was equilibrated with 20 mM Tris buffer and 500 µl of the protein was loaded for analysis.

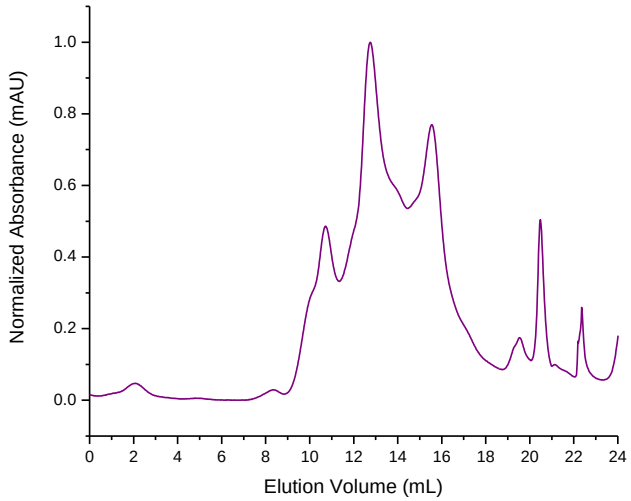


Figure 3.60. Gel filtration chromatogram of Glycosyl hydrolase

The eluted fractions were then run on 12% SDS gel.

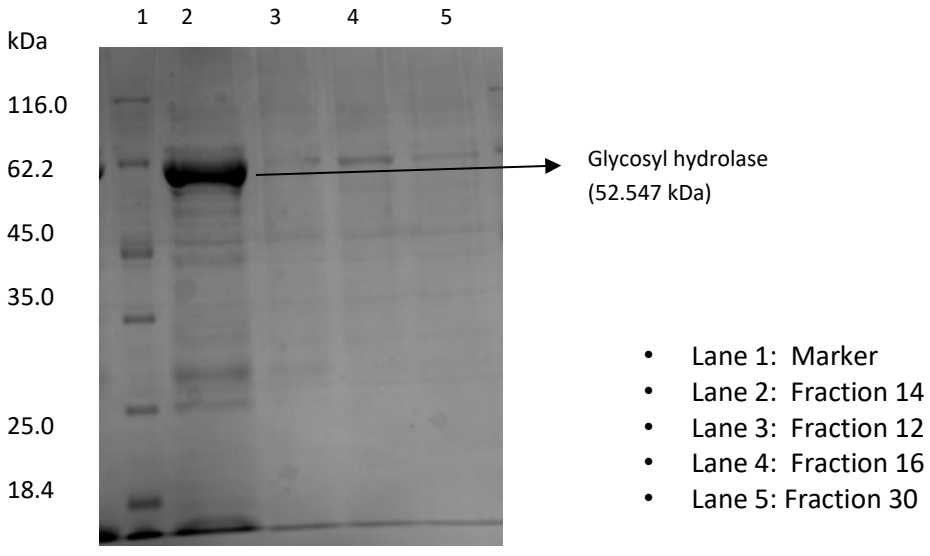


Figure 3.61. Collected fractions of Glycosyl hydrolase

3.6.3.2 Circular Dichroism

Far UV-Circular dichroism spectra for Glycosyl hydrolase were collected to look at the secondary structure of the protein. Concentration of 0.2 mg/ml of the protein was used. The figure shows a mix spectrum of alpha helix and beta-sheets.

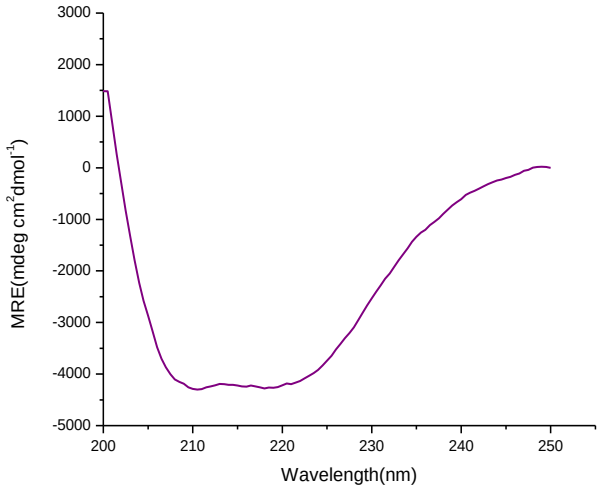


Figure 3.62. CD spectrum of Glycosyl hydrolase

3.6.3.3 Tryptophan fluorescence

The protein concentration used was 0.8 mg/ml to study the intrinsic fluorescence of Glycosyl hydrolase. Since, the λ_{max} emission is at 335 nm in the emission spectra, we can say that the protein seems to be well folded.

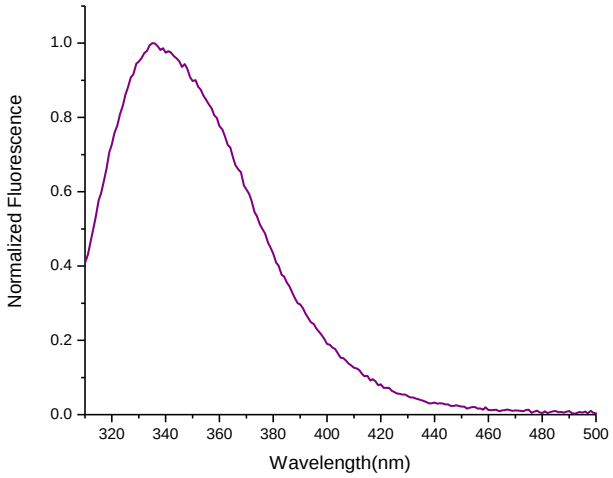


Figure 3.63. Intrinsic fluorescence emission spectrum of Glycosyl hydrolase

3.6.4 DNSA activity assay with carboxymethyl cellulose (CMC)

The DNSA enzyme activity assay was conducted in triplicates by incubating the protein Glycoside Hydrolase (0.5µM) for 2-hours and 6-hours respectively, with the substrate CMC (Carboxymethyl cellulose). Plotting the mean absorbance at 540nm at different temperatures ranging from 20℃- 90℃ at 10℃ intervals, revealed that the enzyme was mostly active at 50℃-60℃.

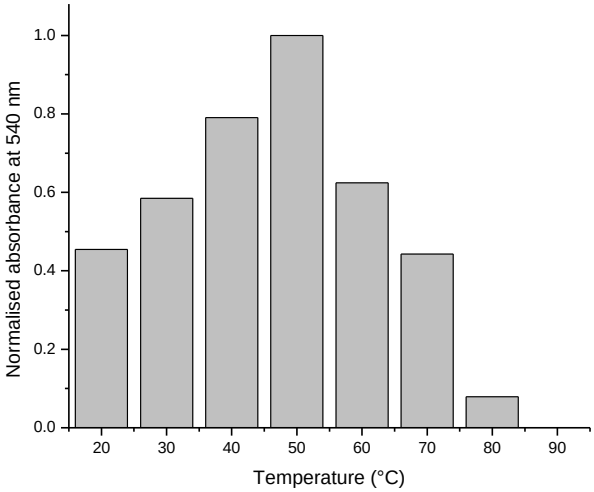


Figure 3.64. Temperature dependent DNSA activity assay of Glycosyl hydrolase with CMC for 2-hours

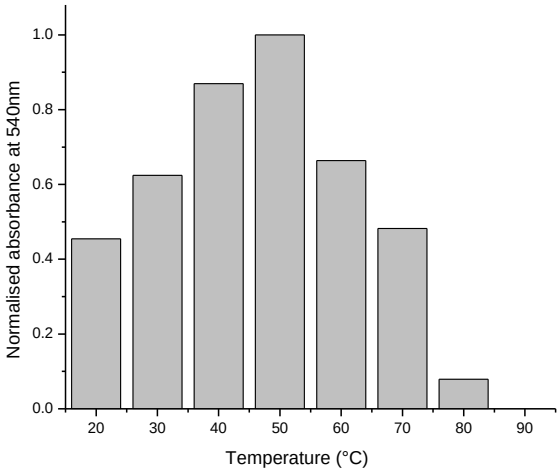


Figure 3.65. Temperature dependent DNSA activity assay of Glycosyl hydrolase with CMC for 6-hours

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