Studies of biomass converting enzymes and engineering design of bi-functional fusion construct

Sreelakshmi C

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

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Certificate of Examination

This is to certify that the dissertation titled "**Studies of biomass converting enzymes and engineering design of bi-functional fusion constructs**" submitted by **Ms. Sreelakshmi C** (Reg. No. MS13142) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr Kausik Chattopadhyay Dr.A.Mukhopadhaya Prof. Purnananda Guptasarma
(Supervisor)

Dated: _____

Declaration

The work presented in this desertion has been carried out by me under the guidance of Prof. Purnananda Guptasarma at 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 it 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

Sreelakshmi C

(Candidate)

Dated: _____

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

(Prof. Purnananda Guptasarma)

(Supervisor)

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Notations

μ :	Micro
APS:	Ammonium persulphate
ATP:	Adenosine triphosphate
bp:	Base pair
DNA:	Deoxyribonucleic acid
DW:	Distilled water
EDTA:	Ethylene diammine tetra acetate
IPTG:	Isopropyl-beta-D-thio galactopyranoside
Kb:	Kilo base pair
kDa:	kilo dalton
L:	Litre
LB:	Luria Bertani
M:	Molar
Mg:	Milligram
mL:	Millilitre
mM:	milli molar
NTP(s):	Nucleotide triphosphate
ºC:	Degree Celsius
PAGE:	Polyacrylamide gel electrophoresis
PCR:	Polymerase Chain Reaction
rpm:	Rotations per minute
SDS:	Sodium dodecyl sulphate
TAE:	Tris-acetate-EDTA
TEMED:	Tetramethyl ethylene diamine
O/N:	Over Night
min:	Minute
hr:	Hour

Contents

Abstract

Biomass includes all of the earth's living matter, plants and animals, and the remains of this living matter. One of the most abundant organic materials on earth is plant biomass. There is a wide range of microbial enzymes that has achieved different actions for biomass degradation in nature. Some of those enzymes which break down the plant biomass are endoglucanases and cellulases that act synergistically for subsequent hydrolytic reactions. The enzymes that have garnered relatively more attention are those that can withstand high temperatures, i.e., are thermostable. Using seven thermostable enzymes of different origin, we aim to do a green approach for biomass degradation. Sweet corn and rice are widely distributed crops that generate agricultural wastes like corn cob and rice husk without significant commercial value. Their cell wall mainly consists of cellulose, hemicelluloses and lignin, which are complex polymers difficult to process. We show that these thermostable enzymes hydrolyze these biomass components into simple sugars. The successful visualization of these simple sugars was achieved by Thin Layer Chromatography (TLC). Colorimetric method like 3, 5-Dinitrosalicylic acid assay was carried out to check the presence of reducing sugars. Later, we are creating a fusion construct using these thermostable enzymes for better degradation of biomass. In this study we are genetically fusing BSX XYLANASE-ThtLAC, using the flexible linker having a sequence of (Gly- Gly- Gly- Gly-Ser). This construct will have a potential role in improving delignification of hydrolyzed plant biomass, and for bleaching of pulp for paper industry.

Introduction

1.1 Proteins

"Sometimes progress is slow. But then there does come a time when a lot of people accept a new idea and see ways in which it can be exploited. And because of the larger number of workers in the field, progress becomes rapid. That is what happened with the study of protein structure."

— Linus Pauling

Proteins are large, complex molecules that form an essential part of every living being. They are macromolecules consist of one or more chains of amino acid.

There are 20 different types of amino acids, which combine in different ways to make protein. The biological function of a protein is dictated by the arrangement of their atoms in the 3-dimensional space. The protein structure provides a greater level of understanding on the way a protein works, the ways in which we can control and modify it.

Protein structure is mainly classified into four organizational levels viz., primary (consist of the sequence of amino acid), secondary (consist of fixed arrangement of polypeptide chains like alpha helix and beta sheet), tertiary (consist of unique three-dimensional shape of protein as a whole) and quaternary structure (multiply-folded polypeptide chains). [1]

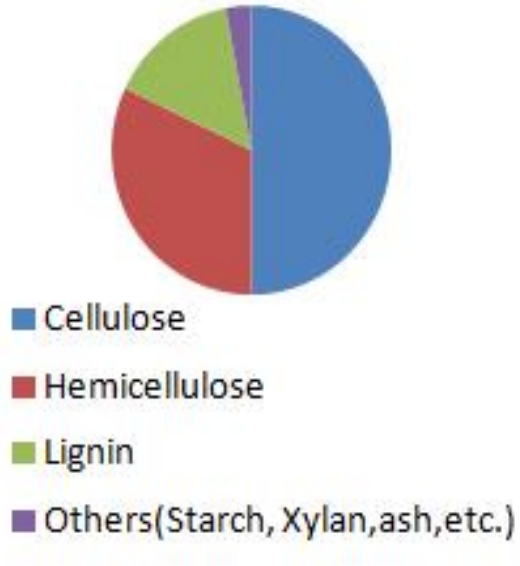
Proteins are assorted in nature, and different proteins behave differently under varied conditions. There are some proteins, which can withstand higher temperature, higher pressure, high salt concentration etc. Here, we are trying to study some proteins from organisms that thrive in the extremes of nature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

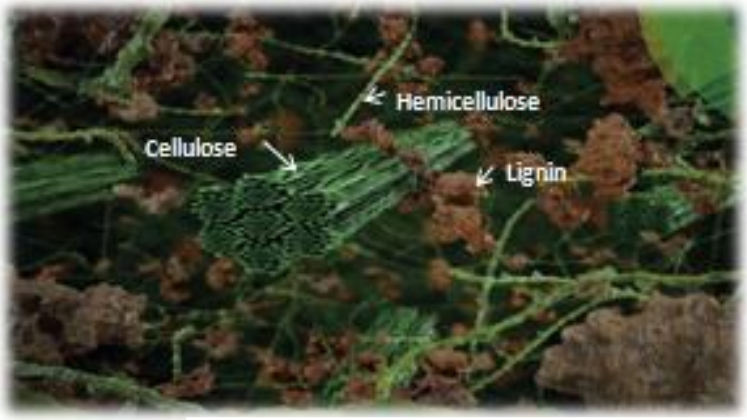
In the present thesis, studies of seven such enzymes involved in biomass degradation are discussed. Further, engineering design of fusion constructs involving these enzymes are discussed for improved catalytic function.

1.2 Biomass

Biomass incorporates the greater part of the earth's living matter, plants animals, and the remaining parts of these living things. Plant biomass has a lot of industrial applications. The pulp and paper industry produces a lot of energy rich plant biomass as waste. Because of the complexity of their structure, there are many chemical treatments carried out to generate the simple components like simple sugars. As an alternative to these chemical treatments, different enzymes involved in biomass degradation can be used.

Biomass consists of cellulose, hemicelluloses, lignin, lipids, starch etc. Two larger carbohydrate categories that have significant value are cellulose and hemicelluloses. The lignin fraction consists of non-sugar type molecules. Cellulose is a homopolymer of glucose units, hemicellulose is a heteropolymer of glucose and other sugar units and lignin is a complex organic polymer. [2]





(Brian H. Davison et al. 2014)

Figure 1.1(a): Different components in biomass, (b): Cartoon schematic of plant biomass

Due to higher temperature and pH extremes involved in the biomass to biofuel conversion, the use of thermostable cellulases and endoglucanases are advantageous. Cellulases are those enzymes, which does the hydrolysis of cellulose polymers into simple sugars. And there are three

distinct classes of cellulases: endoglucanase, cellobiohydrolase, and β-glucosidases. Endoglucanases act by cleaving the β-glycosidic bonds in the cellulose chain, thereby making chain ends accessible to cellobiohydrolase and it produce end product like cellobiose. The end product cellobiose is further broken down to sugar units by β-glucosidase.

α-amylases and endoglucanases are the enzyme categories, which attained more attention in the degradation of plant biomass. Both are involved in the conversion of plant material into simple sugars like glucose, maltose, galactose etc, can further be used as a fuel. α -amylases degrade the starch, whereas endoglucanase breakdown plant cellwall cellulose. Endoglucanases are also capable of making random internal cleavages, and their hydrolysis products can be used as substrate by other enzymes. [3]

In general, thermostability is a desired quality for proteins that have industrial and therapeutic significance. Introducing thermostability has been one of the major focuses of protein engineering studies.

1.3 Organisms under study

Thermophilic organisms are those that survive at higher temperature and can perform their optimal activities at temperature above 80 ˚C. This thesis describes proteins or enzymes involved in biomass degradation sourced from different organisms described below.

1.3.1 *Pyrococcus furiosus*

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

It has been shown that *Pyrococcus furiosus* has modified its method of metabolizing sugars. It also has a distinct way to regenerate ATP. The bacterium has enzymes that contain tungsten that is very rarely found in biological molecules. [4]

1.3.2 *Clostridium cellulolyticum*

Clostridium cellulolyticum is a gram-positive, rod-shaped, anaerobic, mesophilic cellulolytic bacterium. The bacterium was first isolated from compost containing decayed grass. Studies have confirmed that this microbe is not a part of any ruminant system.

The ability of *Clostridium cellulolyticum* to degrade cellulose is an active area of research. [5]

1.3.3 *Sulfolobus solfataricus*

Sulfolobus solfataricus is a species of thermophilic archaeon. The species grows best in temperatures around 80 ˚C, a pH level between 2 and 4, and enough sulfur for *solfataricus* to metabolize in order to gain energy. It usually has a spherical cell shape and it makes frequent lobes. Being an autotroph it receives energy from growing on sulfur or even a variety of organic compounds. [6]

1.3.4 *Thermus thermophilus*

Thermus thermophilus is a Gram negative eubacterium used in a range of biological applications. The bacterium is extremely thermophilic with an optimal growth temperature of about 65-82 ˚C. *Thermus thermophilus* was originally isolated from a thermal vent within a hot spring in Izu, Japan by Tairo Oshima and Kazutomo Imahori. The organism has also been found to be important in the degradation of organic materials in the thermogenic phase of composting. [7]

1.3.5 *Bacillus* sp. NG27

Alkalophilic *Bacillus* sp. strain NG-27 is an endoxylanase active at 70 ˚C and at a pH of 8.4. [8]

1.3.6 *Rhodothermus marinus*

Rhodothermus marinus is a species of bacteria. It is obligately aerobic, moderately halophilic, thermophilic, Gram-negative and rod-shaped. *R. marinus* has been isolated from several similar but distantly located geothermal habitats, many of which are subject to large fluctuations in environmental conditions. This presumably affects the physiology of *R. marinus*. Many of its enzymes show optimum activity at temperatures considerably higher than 65 ˚C, the optimum for growth, and some are active over a broad temperature range. [9]

1.4 Enzymes under study

There studies have been carried out in the thermostable and mesostable proteins. In this thesis, we will first provide a short description of thermostable and mesostable enzymes having a role in biomass degradation. And the later part will discuss their biomass degradation studies.

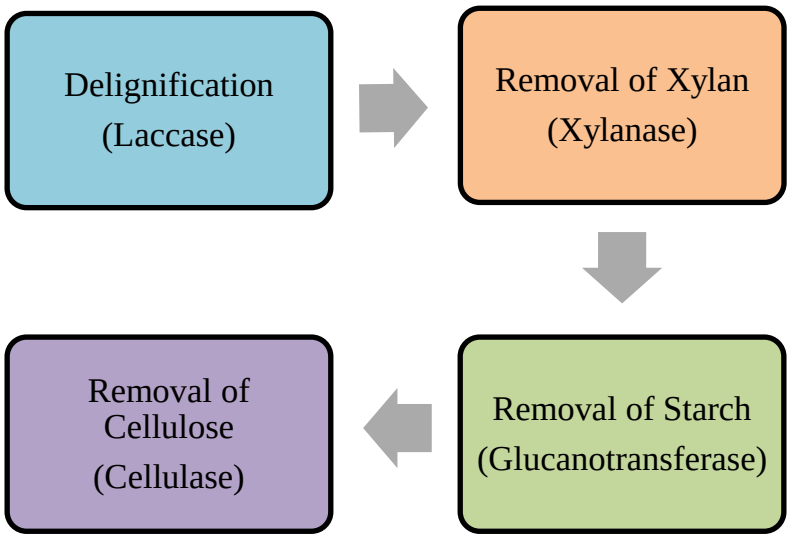


Fig 1.2: Flowchart showing stages of biomass degradation.

The seven enzymes used in study have different role in biomass degradation. The enzymes ThtLAC and BSX XYLANASE have function in delignification and removal of xylan

respectively. Whereas, cellulases and glucanotransferases degrades cellulose, hemicellulose and starch.

List of constructs (for the gene encoding the following enzymes) available in our lab for biomass degradation study:-

Protein	**Size**	**Cloned vector**	**Antibiotic resistance**	**Enzyme specificity**
CceCelCCA (*Clostridium cellulolyticum*)	31 kDa	*BamH1, Hind III* XL1 Blue, pQE30	Tetracycline Ampicillin	Endoglucanase /cellulase
SsoLacS (*Sulfolobus sulfataricus*)	39.7 kDa	*BamH1, Hind III* XL1 Blue, pQE30	Tetracycline Ampicillin	(β-galactosidase)
ThtLAC (*Thermus thermophilus*)	53 kDa	*Nde1, EcoR1* XL1 Blue, pET28a	Kanamycin Tetracycline	Multi copper containing phenol oxidase.
BSX XYLANASE (*Bacillus sp. NG27*)	42 kDa	*BamH1, Hind III* XL1 Blue, pQE30	Tetracycline Ampicillin	Hydrolyze the hemicellulose of plant cellwall
PfuCelB (*Pyrococcus furiosus*)	39.3 kDa	*BamH1, Hind III* XL1 Blue, pQE30	Tetracycline Ampicillin	Endoglucanase /cellulase (β-glucosidase)
PfuAmyGT (*Pyrococcus furiosus*)	77 kDa	*Nde1, Xho1* XL1 Blue, pET23a	Chloramphenicol Ampicillin	Glucanotransferase (Degrade Starch)
RMCel12A (*Rhodothermus marinus*)	28 kDa	XL1 Blue, pQE30	Tetracycline Ampicillin	Cellulase

Table 1.1: Details of enzymes under study

1.4.1 CceCelCCA

The biological conversion of cellulose to glucose generally requires three types of hydrolytic enzymes: (a) Endoglucanases which cut internal β-1,4-glucosidic bonds; (b) Exocellobiohydrolases that cut the disaccharide cellobiose from the non-reducing end of the cellulose polymer chain; (c) β-1,4-glucosidases which hydrolyze the cellobiose and other short cello-oligosaccharides to glucose. CceCelCCA is an endoglucanase/cellulase, which does the endohydrolysis of (1-4)-β-D-glucosidic linkages in cellulose.

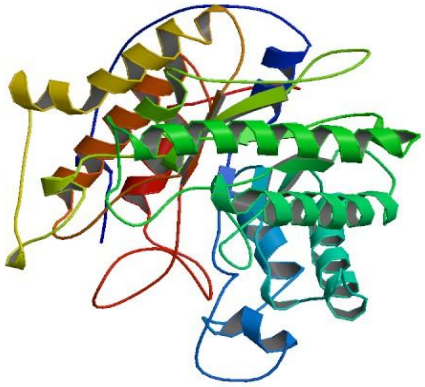


Fig 1.3: Cartoon representation of CceCelCCA (Ref: PDB Id: 1EDG)

1.4.2 PfuCelB

PfuCelB is inolved in the degradation of complex natural cellulosic substrates. It does the endohydrolysis of (1-4)-β-D-glucosidic linkages in cellulose.

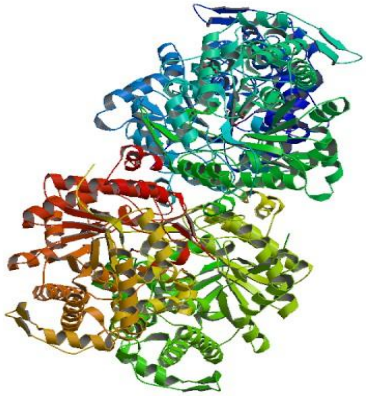


Fig 1.4: Cartoon representation of PfuCelB (Ref: PDB Id: 3APG)

1.4.3 SsoLacS

It is a β-galactosidase (glycoside hydrolase) which catalyzes the hydrolysis of β-galactosidase into monosaccharides through the breaking of glycosidic bond.

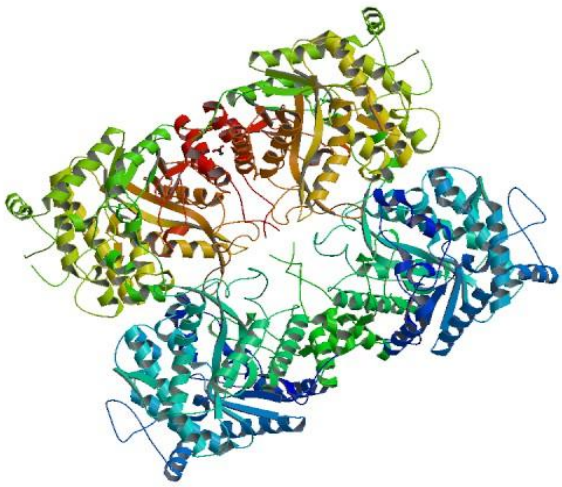


Fig 1.5: Cartoon representation of SsoLacS (Ref: PDB Id: 1GOW)

1.4.4 ThtLAC

ThtLAC is a multi copper-containing phenol oxidase which catalyzes the oxidation of a wide range of phenolic/non-phenolic and aromatic substrates.

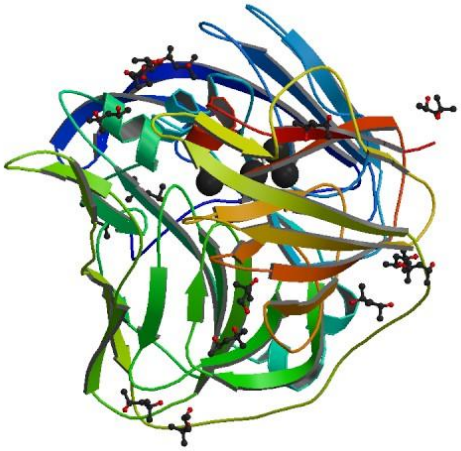


Fig 1.6: Cartoon representation of ThtLAC (Ref: PDB Id: 2XU9)

1.4.5 BSX XYLANASE

Xylanase enzyme is mesophilc in origin and possessing extremely thermostable properties. It is used to hydrolyze the hemicellulose of plant cell wall, bio-bleaching the kraft pulp. [10]

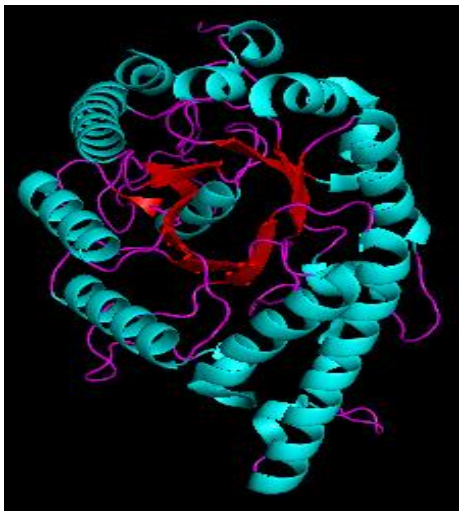


Fig 1.7: Cartoon representation of BSX XYLANASE (Ref: Ghosh A. et al, Protein Science (2006))

1.4.6 RMCel12A

RMCel12A is a cellulase, that catalyzes the hydrolysis of cellulose into smaller oligosaccharides, specifically, the hydrolysis of the 1, 4-β-D-glycosidic linkages. Conversion of food and agricultural wastes to valuable sugars are the important uses of cellulase enzymes.

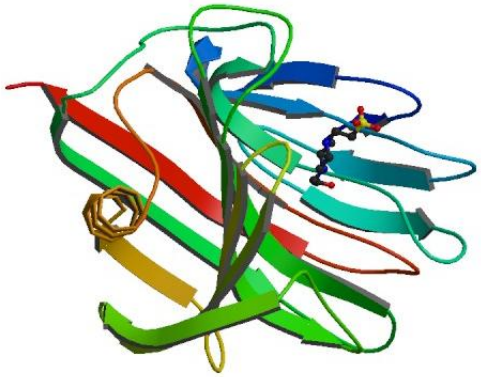


Fig 1.8: Cartoon representation of RMCel12A (Ref: PDB Id: 1HOB)

1.4.7 PfuAmyGT

PfuAmyGT endohydrolyses the (1-4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1-4)-alpha-linked D-glucose units.

Materials and methods

This section provides a general outline of the materials used and methodologies followed to perform report in the thesis.

2.1 Materials

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

2.1.1 Bacterial Strains and Plasmids

Strains	**Genotype**
E.coli BL21(DE3)pLysS	*F ⁻ompT gal dcm lon hsdS$_B$(rB ⁻mB⁻) λ(DE3) pLysS(cm^R)*
E. coli DH5α	*F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–*
E. coli XL1Blue	*endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[Tn10 proAB+ lacIq Δ(acZ) M15] hsd17(rK - mK -)*
pQE30	T5 promoter, PDS56/RBSII and PDS781/RBSII-DHFRS ColE1 origin, for ssDNA production, ampicillin resistance gene (beta-lactamase), N-terminal histidine tag

Table 1.2: Bacterial strains and plasmids

2.1.2 Media (For Bacterial Cultures)

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

Table 1.3: Medias for bacterial cultures

LB agar contains 2% agar in LB medium.

2.1.3 Chemicals and Kits

Reagents used in the study are obtained from commercial sources. Restriction/modification enzymes and molecular biology reagents are obtained from New England Biolabs (NEB), USA or Thermo Scientific. Plasmid isolation kits, PCR purification kits, Plasmid miniprep kits, Gel extraction kits, Ni-NTA agarose spin column and Ni-NTA Agarose/ Super flow used in the studies were obtained from Qiagen, USA.

The compositions of buffers for plasmid isolation miniprep kit are given as follows:

Buffers	Composition	Storage
Buffer P1 (Resuspension buffer)	50mM Tris-Cl, pH 8.0, 10mM EDTA, 100µg/ml RNase A	2-8 ˚C after addition of RNase
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS (w/v)	15-25 ˚C
Buffer N1 (Neutralization buffer)	3.0 M potassium acetate, pH 5.5	15-25˚C
Buffer PE (Colum binding/ Wash buffer)	1.0 M NaCl, 50 mM MOPS, pH 7.0, 70 % ethanol	15-25˚C
Buffer EB (Elution buffer)	10 mM Tris, pH 8.0	15-25˚C

Table 1.4: Composition of buffers for plasmid isolation miniprep

2.1.4 Antibiotics

Ampicilin, Kanamycin, Chloramphenicol and Tetracycline used in the studies were obtained from Sigma Chemicals, USA, and their stocks (1000X) were prepared as follows:

Antibiotics	Stock concentration (1000 X)
Ampicillin	100 mg/ml in water
Kanamycin	25 mg/ml in water
Tetracycline	12.5 mg/ml in 70 % ethanol
Chloramphenicol	35 mg/ml in methanol

Table 1.5: Antibiotics used in the study

Stock solutions of antibiotics were sterilized through 0.22 µM filters (Millipore) and stored in aliquots at -20˚C. These were used at 1000 fold dilution to obtain the working concentrations.

2.1.5 TLC

Mobile phase (Solvent)

Solvent	**Amount used**
Butanol	5 mL
Ethanol	3 mL
Water	10 mL

Table 1.6: TLC mobile phase components

Spraying Solution

Methanol	19 mL
Conc.H_2SO_4 (98%)	1 mL

Table 1.7: TLC spraying solution components

2.1.6 DNSA Reagent

DNSA	0.876 g in 40 mL
Sodium potassium acetate	23.92 g
NaOH	2 N of 16 mL
Deionized water	Up to 100 mL

Table 1.8: DNSA components

2.1.7 Buffers and Solutions

50X TAE buffer (pH 8)

Tris HCl	242 g
Glacial acetic acid	57.1 mL
0.5M EDTA	100 mL
Deionized water	Up to 1 L

Table 1.9: 50x TAE components

6X DNA loading dye (In deionized water)

Bromophenol blue	0.25 %
Glycerol	30.0 %

Table 2.0: 6X loading dye composition

Agarose gel

Agarose	1g
Deionized water	100 mL

Table 2.1: 1% Agarose gel components

Ethidium bromide stock solution (1% w/v)

Ethidium bromide	0.1g
Deionized water	10 ml

Table 2.2: EtBr components

Ammonium persulfate

APS	100 mg
Deionized water	Up to 1 mL

Table 2.3: APS components

Lower Tris (4X), pH 8.8

Tris	18.17 g
10% SDS	1 ml
Deionized water	Up to 100 ml

Table 2.4: Lower Tris components

Upper Tris (4X), pH 6.8

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

Table 2.5: Upper Tris components

5X Sample loading buffer

Tris. Cl (pH 6.8)	0.15 M
10% SDS	5 %
Deionized water	25 %
β-mercaptoethanol	12.5 %
Bromophenol blue	0.06 %

Table 2.6: 5X sample loading dye components

Laemmli buffer (Laemmli, 1970)

Tris buffer	3.0 g
SDS	1.0 g
Glycine	14.4 g
Deionized water	Up to 1 L

Table 2.7: laemmli buffer components

Gel staining solution

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

Table 2.8: Gel staining solution components

Gel destaining solution

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

Table 2.9: Gel destaining solution components

Native purification of 6X His-tagged proteins

Native lysis buffer (pH 8.0)

NaH_2PO_4	0.05 M
NaCl	0.3 M
Imidazole	0.01 M

Table 3.0: Native lysis buffer components

Native wash buffer (pH 8.0)

NaH_2PO_4	0.05 M
NaCl	0.3 M
Imidazole	0.03 M

Table 3.1: Native wash buffer components

Native elution buffer (pH 8.0)

NaH_2PO_4	0.05 M
NaCl	0.3 M
Imidazole	0.25 M

Table 3.2: Native elution buffer components

2.2 METHODS

2.2.1 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) and forward and reverse primers which are complementary to the template are required. The PCR reactions were performed in vapo.protect from Eppendorf, USA. It involves the following steps:

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

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

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

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

The PCR programme used is mentioned as follows:

Steps	**Phusion Polymerase (Thermo/NEB)**	**DeepVent/ Vent Polymerase**	**Taq Polymerase**	
Initial denaturation	98 ˚C, 5min	95˚C, 5min	95˚C, 5min	
Denaturation	98 ˚C, 30 sec	95 ˚C, 30 sec	95 ˚C. 30 sec	**25-30 cycles**
Annealing	55-68˚C, 40 sec (depends on primer)	45-66 ˚C, 40 sec (depends on primer)	47-64 ˚C, 40 sec (depends on primer)	
Extension	72 ˚C, 30 sec/kb	72˚C 1min/kb	68˚C 1min/kb	
Final Extension	72 ˚C, 10min	72 ˚C, 10min	68 ˚C, 10min	

Table 3.3: Different PCR conditions

2.2.1.1 SOE PCR

This technique was used for joining smaller fragments of DNA together to stitch together a large desired gene. This method employs two rounds of PCR. Generating DNA fragments with overlapping ends. To splice two DNA molecules, special primers are designed with 5’ overhang having a complementarity with 3’ overhang of the other primer. Once the two templates are being amplified, they are further added together and will run for 5-10 cycles without primer. After the first few cycles, primers were added into the PCR tubes and again PCR will run for another 25 cycles.

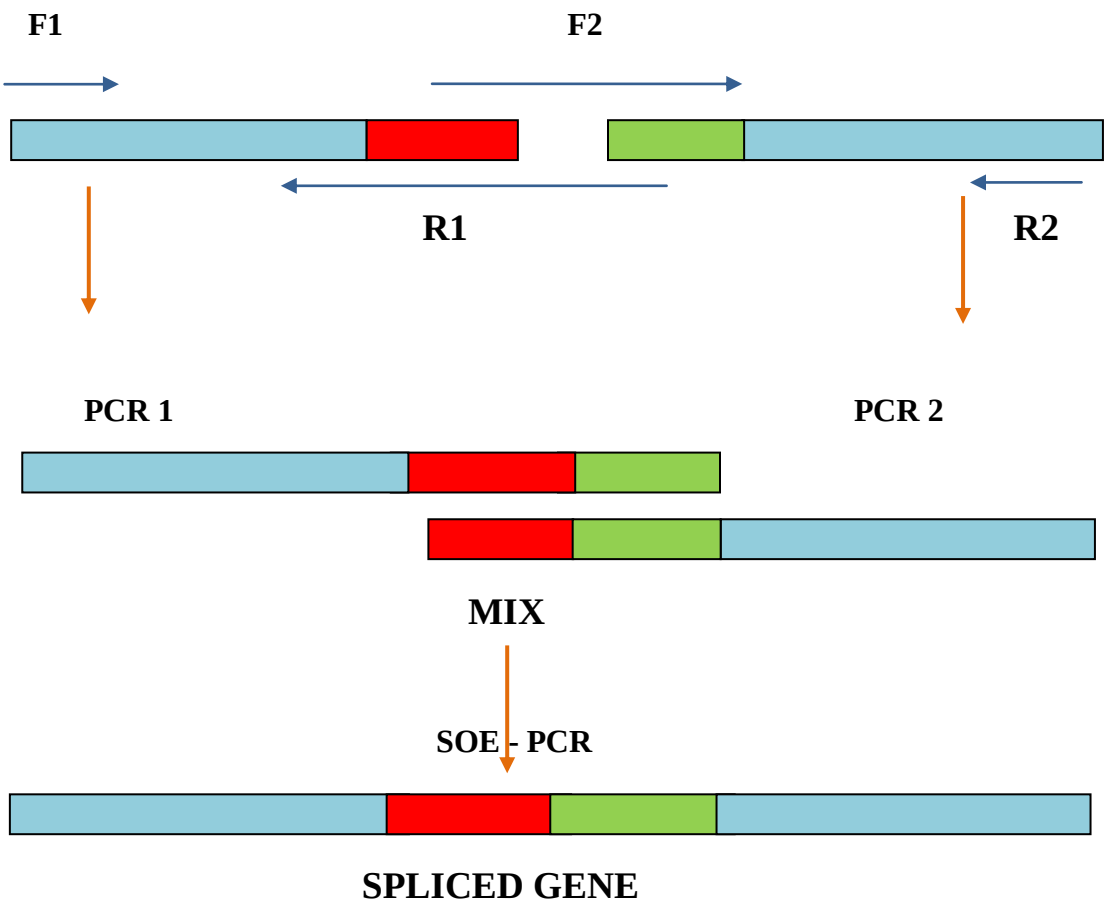


Fig 2.1: Cartoon schematic of SOE PCR

The list of primers used during the study:

S.no	Name of primer	Length	Sequence (5'----------3')
1.	BSX F BamH1	32-mer	ATTAGGATCCGTTCAACCGTTCGCGTGGCAGG
2.	Laccase NheIR	33-mer	AATATTGCTAGCTCAACCCACCTCGAGGACTCC
3.	LinkLacF	57-mer	TCAGGTGGAGGAGGTTCAGGAGGAGGTGGCAGCC AAGGACCTTCATTCCCCGAGCCC
4.	LinkBSX R	53-mer	GCTGCCACCTCCGCCTGAACCTCCTCCACCTGAGT CGATGATACGCCAGAACG

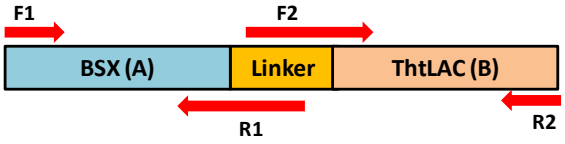
Table 3.4: Primers used during the study

The gene encoding the following enzymes **(a) Xylanase (BSX XYLANASE) (b) ThtLAC (ThtLAC)** has been previously cloned (in our lab) and the individual constructs are available to design (used as template) the bi-functional fusion constructs; using a flexible glycine serine

linker (SG_4SG_4S) (which will join the two proteins with certain degree of movement or interaction)

We attempt here to design novel "Fusion Construct"

BSX XYLANASE-ThtLAC Double fusion construct



PCR NO:	Base pairs (kb)
PCR-1 (BSX XYLANASE+linker)	1062+33 =1095
PCR-2 (linker+ThtLAC)	33+1323 =1356
PCR-3 (Total fusion)	1062+33+132 =**2418**

Fig 2.2

F1- Forward primer for region A

R1- Reverse primer for overlapping linker region and region A

F2- Forward primer for overlapping linker region and region B

R2- Reverse primer for region B

For all the three bi-functional fusion constructs (1, 2, 3) the complete gene can be thought of as a of two smaller fragments with the overlapping linker region.

- The first part (A)/ or gene will be PCR amplified using forward primer F1 and a reverse primer R1 having overlapping linker region **PCR-1**
- The second part (B)/ or gene will be PCR amplified using forward primer F2 having overlapping linker region and reverse primer R2. **PCR-2**
- These two fragments having overlapping regions will be assembled/spliced by overlapping extension using PCR (SOE-PCR) to produce the final DNA sequence using primers F1 and R2. **PCR-3**

2.2.2 PCR purification

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

1. Five volumes of PB buffer was added to one volume of PCR reaction and mixed.
2. To bind DNA, the sample was applied to the column and centrifuged at 13000 rpm for 1 minute. The flow through was discarded and column was placed back in same tube.
3. To wash, 750 µL PE buffer was added to the column. Centrifugation at 13000 rpm for 1 minute was carried out. The flow through was discarded.
4. Centrifuged once more to remove residual wash buffer.
5. To elute DNA, 20 µL deionized water was added to the center of the column and was allowed to stand for 1 minute. Then, centrifugation at 13,000 rpm for 1 minute was carried out.

2.2.3 Agarose Gel Electrophoresis

Electrophoresis through agarose is a standard method to separate, identify and purify nucleic acids. An electric field is applied to move the negatively charged DNA through the gel. The percentage of agarose in the solidified gel determines the pore size of the matrix. The molecules travel through the pores in the gel at a speed that is inversely proportional to their size. Thus, a small DNA molecule will travel a greater distance than a large DNA molecule. Here, 1 % agarose gels were used throughout the study.

2.2.4 Purification of DNA fragment(s) from agarose gels

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

2.2.5 Quantification of DNA

The purified DNA fragments were quantified (concentration estimation) either by running on the gel and visually comparing them with the various bands of the ladder depicted a definite amount of DNA in a definite amount of ladder being loaded, or by putting 1 µL of DNA on the analysis slot of a nano drop spectrophotometer after setting up the baseline with solution in with the DNA was eluted. In the latter, the absorbance was measured at 260 nm. The purity of DNA was confirmed by taking a ratio of OD_{260}/OD_{280}. Purified DNA should have an OD_{260}/OD_{280} ratio of around 1.8-2.0.

2.2.6 Restriction Digestion

Restriction digestion is a method to cleave DNA molecules at specific restriction sites using special enzymes called restriction endonucleases. These cleave the sugar-phosphate backbone of DNA. They commonly recognize recognition sequences that are mostly palindromes. Digestion reactions were carried out for 30 minutes (using 1 µL of enzyme/ µg plasmid DNA or per 200 ng of PCR product) at 37 ˚C. The DNA (plasmid/ insert) to be digested is mixed with appropriate buffer and restriction enzyme/ enzymes of interest (generally 1unit/reaction) and kept at 37 ˚C. After digestion, DNA samples were separated on agarose gel, gel purified and quantitated. For all the genes mentioned above, *BamH1* and *Nhe1* restriction enzymes were used for digestion.

2.2.7 Ligation

Ligation is a process of joining DNA fragments with T4 DNA ligase or Quick Ligase. It involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. Ligation reactions were incubated at 24 ˚C for two hours in case of T4 DNA ligase while it was set at 25 ˚C for 10-15 minutes in case of Quick Ligase. For all the ligation reactions, insert:vector ratio used was 3:1. For a particular amount of digested vector (generally 50 ng), amount of digested PCR product (insert) required can be calculated using the following formula:

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

General scheme for ligation reaction of 10 µl reaction volume is shown below:

S.No.	Components	Volume (µL)
1.	10 buffer (T4)	1.0 µL
2.	Digested vector (50 ng)	'X' µL
3.	Digested insert (Calculated from formula)	'Y' µL
4.	T4 DNA Ligase	0.4 µL
5.	Deionized water	To mak eup the vol. to 10 µL

Table 3.5: Ligation reaction components

2.2.8 Preparation of *E.coli* competent cells

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. The competent cells were prepared by following the steps given below:

1. A single colony of *E.coli* was inoculated in LB media and grown to saturation.
2. The culture was re-inoculated into 100 mL fresh LB medium at a dilution of 1:100 and grown to early log phase (A_{600} of 0.3-0.4).
3. The cells were chilled on ice for 15 minutes, centrifuged at 3000 x g for 15 minutes a 4 ˚C in pre-chilled centrifuge tubes. Cells were kept on ice at all subsequent steps.
4. The supernatant was discarded and cells were resuspended in 20 mL of ice cold CaCl2 solution (filtered and autoclaved) after which they were again centrifuged as in step 3.
5. The supernatant was again discarded and step 4 repeated.
6. The cell pellet obtained in step 5 was re-suspended in 2 mL of ice cold CaCl2 solution.
7. Finally aliquots of 80 or 100 µL were made from the suspension obtained above and these were used immediately or stored at -80°C till further use.

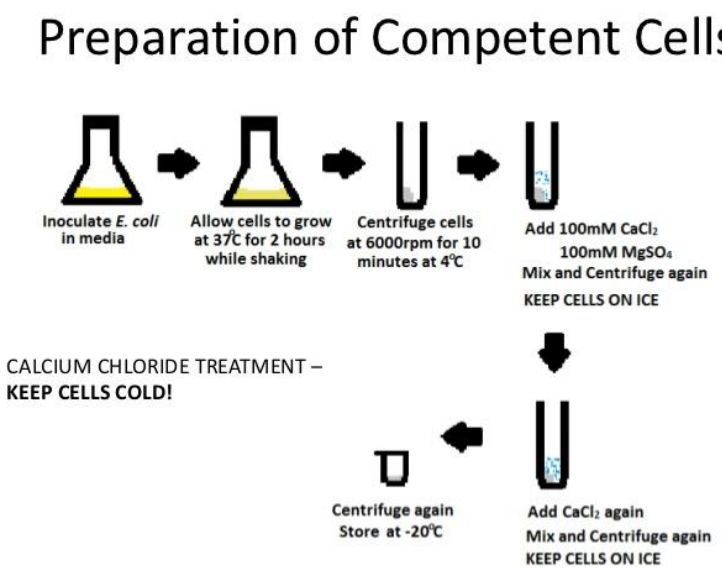


Figure 2.3: Schematic or cartoon representing the steps involved in competent cell preparation (https://www.slideshare.net/gUL90/transformation-13946722)

2.2.9 Transformation

After ligation, the ligation mixture was transformed into the cloning host's cells (XL1-Blue) (6). The protocol for transformation is as follows:

1. The chemically competent cells were thawed on ice for 15 minutes.

2. Further, the ligation mixture was added to the cells and mixed gently. They were then incubated on ice for 15-20 minutes.

3. Heat shock was given to the cells at 42 ˚C for 90 seconds by incubating the samples in water bath.

4. 1 mL of sterile LB media was added to the cells and was kept in an incubator shaker at 37 ˚C for 1 hour.

5. The cells were centrifuged at 5000 rpm for 5 minutes and the supernatant was discarded.

6. The pelleted cells were resuspended in 100 µL of fresh media then plated on the LB agar plates with appropriate antibiotics. The plates were kept inverted for overnight in an incubator set at

37 ˚C and the transformants were observed the next day.

2.2.10 Screening for transformants

The plasmid DNA was isolated from the cultures of transformants/clones and the clones were checked for the integration of insert into the vector. Restriction endonuclease digestion was performed with respective enzymes (Nde1 and Xho1) and was checked by running it on agarose gel. If the insert of expected size is seen on the gel, the clone is confirmed. Alternative method is by performing a colony PCR, in which colonies were picked up and resuspended in the PCR reaction master mix aliquoted in various tubes. This utilizes vector specific primers which will amplify the gene of interest if it integrates between the restriction sites in the multiple cloning sites. The plasmid from confirmed clone was first confirmed by sequencing and then transformed into an expression host (BL21 (DE3)pLysS) .

2.2.11 Glycerol stock (15%)

For making glycerol stocks 1500 µL of an overnight grown culture (37 ˚C) obtained by inoculating a single colony into sterile LB media supplemented with appropriate antibiotics was mixed with 500 µL of 60 % glycerol (autoclaved). The stock was stored at -80 ˚C.

2.2.12 Plasmid DNA isolation

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

1. Bacterial cells (3-5 mL) were pelleted by centrifugation at 13,000 rpm. The supernatant was discarded.
2. Pellet was resuspended in 250 µL of P1 buffer and mixed by pipetting.
3. Then 250 µL of P2 buffer was added and mixed gently, by inversion for lysis of the cells.
4. 350 µL of Neutralization buffer (Buffer N3) was then added and mixed by inversion.
5. The mixture was centrifuged at 13,000rpm for 10-15 minutes.
6. Then, the supernatant was loaded onto the columns and it was spun at 13,000 rpm for 1 minute.
7. Flow through was discarded and it was washed with 750 µL of wash buffer (Buffer PE) by centrifuging at 13,000rpm for 1 minute.
8. Centrifuged once more to remove residual wash buffer.

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

2.2.13 Purification of 6x His tagged proteins

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

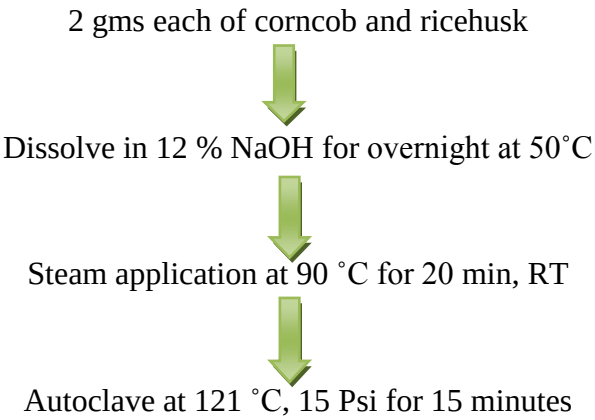
2.2.14 Dialysis

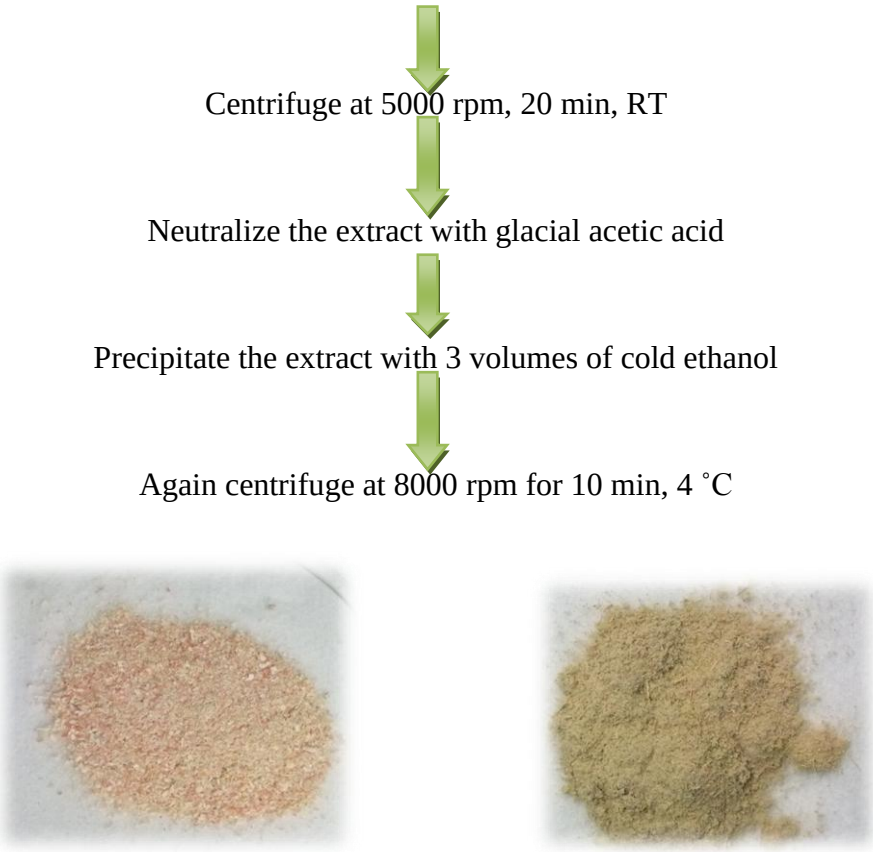
The eluted protein was dialysed against 20 mM Tris, pH 8.0 to remove the salts and imidazole. For this process certain semipermeable membranes was used.

2.2.20 Biomass Pretreatment

Sweet corn and rice are widely distributed crops that generate agricultural wastes like corn cob and rice husk without significant commercial value. Their cell wall mainly consists of cellulose, hemicelluloses and lignin, which are complex polymers difficult to process. The following are the biomass processing steps used before enzymatic treatment:

(a)





b) **c)**

Fig 2.4: a) Flow chart of biomass pretreatment, b) Ricehusk biomass, c) Corn cob biomass

The processed biomass was incubated with the enzymes for different time points like: zero hour, 30 minute, 2 hours, 5 hours and over night. Reduced sugar samples were further analyzed using TLC, DNSA Assay

2.2.15 UV-Vis Absorption spectroscopy

The absorption spectra were collected in the range of 200-600 nm using a Cary 50UV-Vis spectrophotometer. The concentrations of the protein were 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).

2.2.16 Fluorescence spectrum

If a molecule first absorbs energy, for instance a photon is called excitation. Very shortly (in the order of nanoseconds) after excitation it emits a photon of a longer wavelength and come back to the ground state.

This is called as fluorescence. The three amino acid residues that are primarily responsible for the inherent fluorescence of proteins are tryptophan, tyrosine and phenylalanine. These 3 aromatic amino acids have distinct excitation and emission wavelengths. Tryptophan is much more fluorescent than tyrosine and phenylalanine. Tyrosine can be excited at a wavelength similar to that of tryptophan, but emits at a distinctly different wavelength. Whereas phenylalanine is only observed in the absence of both tryptophan and tyrosine because of its weak fluorescence. Because of typtophan's greater absorptivity, the fluorescence spectrum of protein containing all 3 amino acids resembles usually hat of tryptophan.

Aromatic amino acid	**Absorption (nm)**	**Emission (nm)**
Tryptophan	282	340
Tyrosine	272	303
Phenylalanine	260-262	282

Table 3.6: Fluorescent characteristic of amino acid

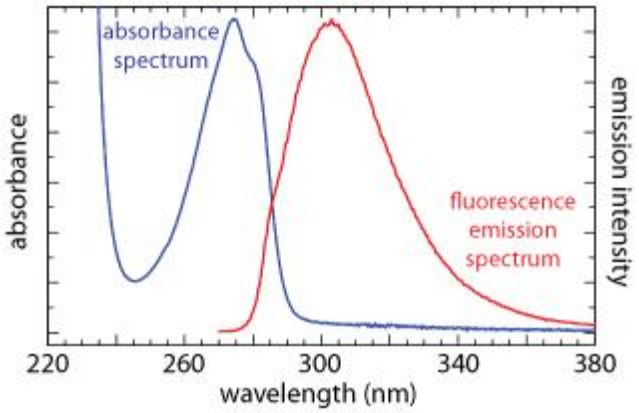


Fig 2.5: Emission and excitation spectrum of tyrosine (asdlib.org/imageandvideoexchangeforum)

The emission spectra were collected on a Cary Eclipse fluorimeter by exciting at 295 nm and collecting the spectra in the range of 300-400 nm. The slit width used for the experiment was 5 nm, 10 nm and 20 nm depending on the concentration of proteins for both excitation and emission. Scan speed was set at 100 nm/min and all spectra were averages of 5 scans.

2.2.17 Circular Dichroism (CD)

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

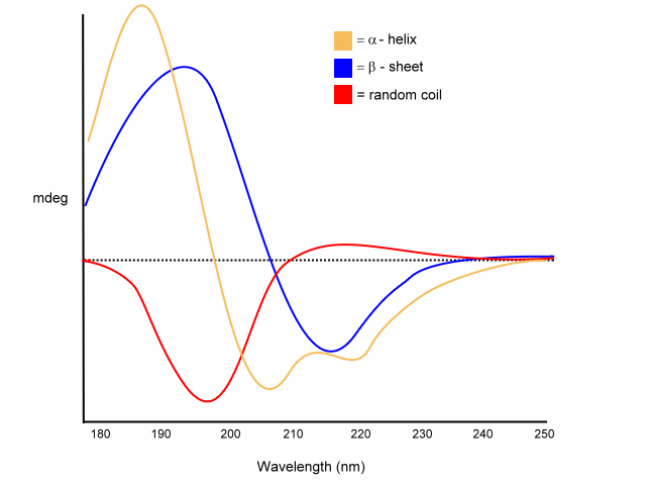


Fig 2.6: CD spectrum (Veronica et. Al, Biomolecular studies by circular dichroism, January 2011 Frontiers in Bioscience 16(1):61-73)

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

$$[\theta]+ = \frac{\theta \textbf{ obs (in mdeg) x100 x MRW}}{\textbf{1000 X concentration } \left(\frac{\textbf{mg}}{\textbf{ml}}\right) \textbf{ x pathlength(cm)}}$$

2.2.18 Thin Layer Chromatography

Similar to other chromatographic methods, thin layer chromatography is also based on the principle of separation. The separation depends on the relative affinity of compounds towards stationary and the mobile phase. The compounds under the influence of the mobile phase (driven

by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is achieved. Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or characters are identified by means of suitable detection techniques.

TLC system components consist of:

1. **TLC plates,** preferably ready made with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.
2. **TLC chamber.** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.
3. **Mobile phase.** This comprises of a solvent or solvent mixture the mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.

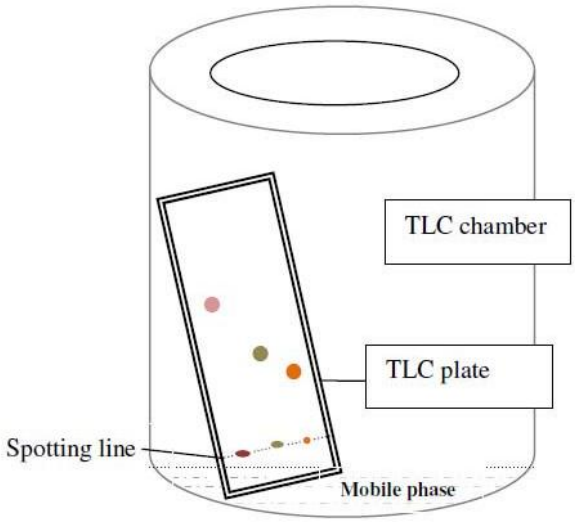


Fig 2.7: TLC system componenets (owlcation.com/stem/tlc-thin-layer-chromatography-Principle-Procedure)

2.2.19 DNSA Assay

3, 5-Dinitrosalicylic acid (DNS or DNSA, IUPAC name 2-hydroxy-3,5-dinitrobenzoic acid) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which strongly absorbs light at 540 nm. The enzyme activity to produce reducing sugars is checked using DNSA stopping assay.

For this, 100 µL of protein and 100µL of enzyme were added together to a tube and incubated at 50 ˚C for time intervals like: zero hour, 30 minute, 2 hours, 5 hours and overnight. Following are the convertions that happen during DNSA stopping assay.

Oxidation

aldehyde group ----------> carboxyl group

Reduction

3, 5-dinitrosalicylic acid ----------> 3-amino,5-nitrosalicylic acid

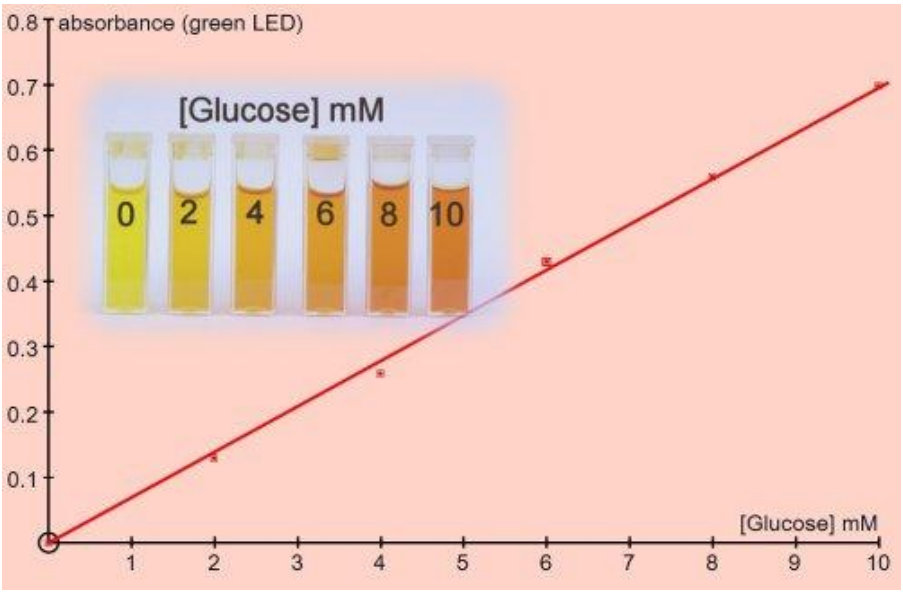


Fig 2.8: DNSA colour gradient (Ref: www.mystrica.com/Applications/DNSA)

Results

3.1 Protein Purification

The process of protein purification involves many steps like; pelleting, sonication, Ni-NTA purification and dialysis. Glycerol stocks of the recombinant clones were picked up separately to inoculate 10 mL of Luria Broth (LB) media. Media was supplemented with of 10 µl of respective antibiotics (as shown in Table 1.5) and grown overnight at 37 ˚C at 220 rpm. 10 mL of this pre-culture was used to inoculate 1 L of sterile LB media containing 1mL of respective antibiotics. After the cells reached an OD_{600} of 0.6- 0.8 they were induced with 1 mM of isopropyl -D- 1-thiogalactopyranoside (IPTG). The culture was grown overnight at 37 ˚C, 220 rpm and the cells were harvested by centrifugation at 8,000 rpm for 5 minute at 4 ˚C. The cell pellet obtained (for all the proteins) was used for the expression analysis on SDS-PAGE.

Protein purification was done by native method for all the proteins. The harvested cell pellet was resuspended in lysis buffer (10 mM imidazole, 50mM NaH_2PO4 pH 8.0, 300 mM NaCl), and then the cells were lysed by sonication followed by centrifugation at 14,000 rpm for 30 min. For, RMCel12A, PfuAmyGT and PfuCelB cells were heated at 80 ˚C for 20 min to remove the contaminating proteins; whereas ThtLAC was heated at 65 ˚C for 20 min. The supernatant was then applied to 2.5 mL of Ni-NTA resin (Qiagen), which was pre-equilibrated with lysis buffer. For SsoLacS and PfuCelB the native wash solution containing 30 mM imidazole were used, whereas 50 mM imidazole was used for CceCelCCA, RMCel12A, BSX XYLANASE, and PfuAmyGT in the native wash solution to remove the contaminants in a better manner. The elution was carried out at 250 mM imidazole and the fractions were collected in a 2 ml micro centrifuge tubes. Fraction no. 2 of all the proteins was run in a 12 % SDS-PAGE gel to see the purity of the protein. Purified proteins samples were dialyzed against 50 mM NaH_2PO4 buffer pH 8.0, and protein concentration was determined for further biomass degradation and structural studies.

SDS PAGE analysis

The Ni-NTA purified proteins were run on the SDS PAGE and bands approximate to 42 kDa, 28 kDa, 77 kDa, 54 kDa, 43k Da, 52 kDa and 56 kDa were obtained for BSX XYLANASE, RMCel12A, PfuAmyGT, PfuCelB, CceCelCCA, ThtLAC and SsoLacS respectively (Figure 2.9)

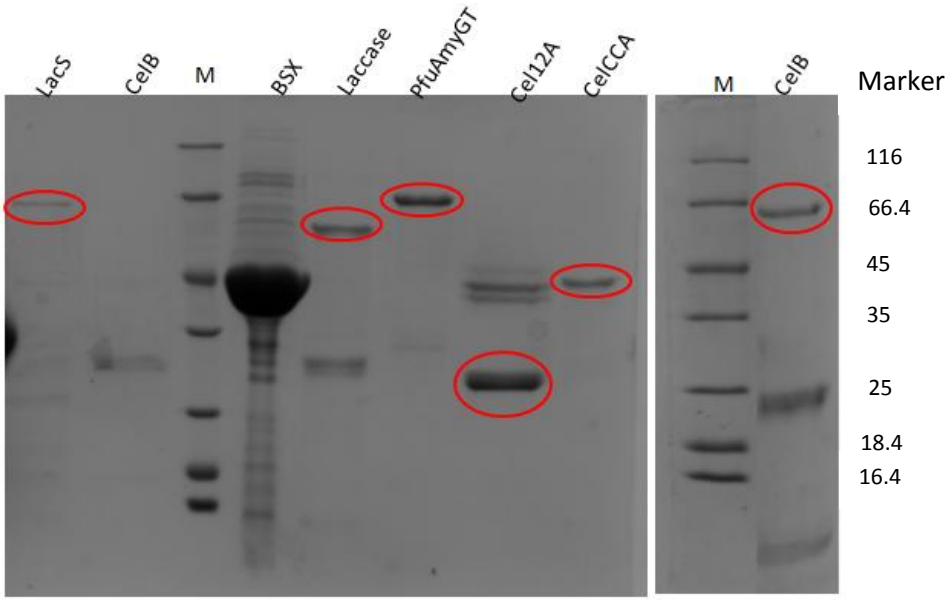


Fig 2.9: SDS-PAGE profile of purified proteins

3.2 Biophysical characterization

3.2.1 UV-Visible absorbance spectroscopy

UV–visible spectra showed distinct peaks around 280 nm for BSX XYLANASE, RMCel12A, Pfu AmyGT, PfuCelB, CceCelCCA, ThtLAC and SsoLacS. The concentration of the proteins were 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) (Figure 3). Table 1 showed the calculated concentration for all the proteins under study.

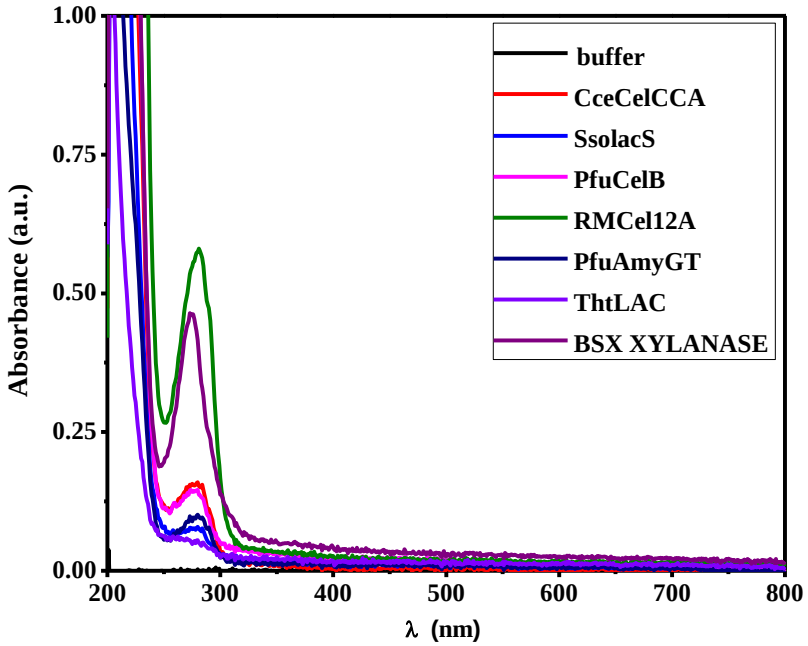


Fig 3: UV-Visible absorption spectrum

Protein	**Concentration at OD_{280}=1 (mg/ml)**	**Molar extinction coefficient (ε)**	**Protein concentration**
CceCelCCA	0.49	87750	0.2 mg/ml
PfuCelB	0.43	128280	0.09 mg/ml
RMCel12A	3.515	92150	0.6 mg/ml
BSX XYLANASE	1.92	81360	0.2 mg/ml
PfuAmyGT	0.5		0.3 mg/ml
ThtLAC	0.865	46535	0.04 mg/ml
CceCelCCA	0.55	140370	0.08 mg/ml

Table 3.1: Calculation of protein concentration as per the predicted molar extinction coefficient using ProtParam tool

3.2.2 Trypophan fluorescence

In general, the fluorescence emission maximum is a combination of contribution of all the tryptophan residues distributed over their entire structure of a protein. Fluorescence emission spectra were collected by setting the excitation at 295 nm and collecting the spectra in the range of 300-400 nm. The slit width used for this experiment for excitation and emission is shown in Table 3.2. Peak maxima were in the range of 332-345 nm, for all the protein; which suggested that all are well folded with its tryptophans buried (Figure 3.1). Further, CceCelCCA and SsoLacS showing peak maxima at 345 nm and 342 nm respectively, which suggests that they are less structured compared to the other protein.

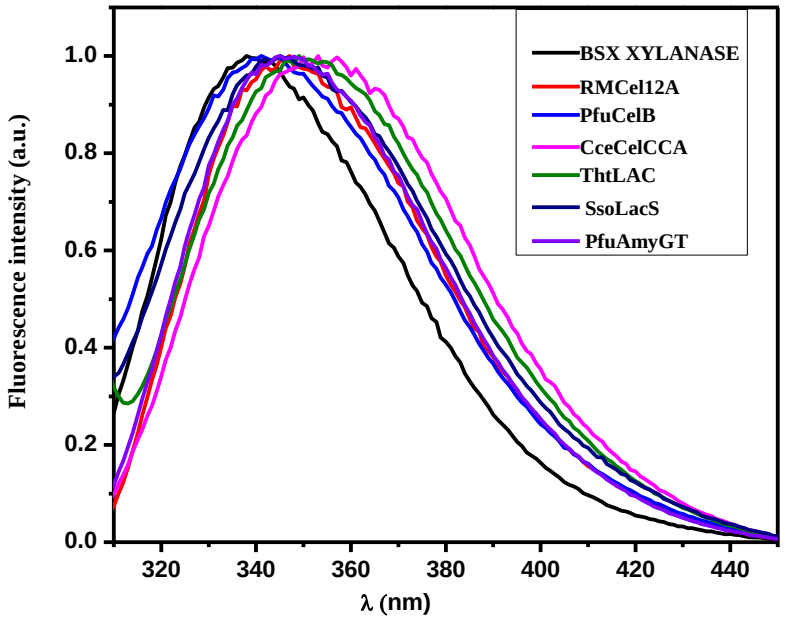


Fig 3.1: Fluorescence emission spectrum

Protein	λ_{max} (nm)	Excitation Slit width	Emission Slit width
CceCelCCA	345	5	10
PfuCelB	335	10	10
RMCel12A	341	5	5
BSX XYLANASE	332	5	10
PfuAmyGT	339	5	10

ThtLAC	330	10	20
SsoLacS	343	10	20

Table 3.2: Maximum Fluorescence emission wavelength

3.2.3 Circular Dichroism (CD) spectroscopy

The far UV-CD spectrum was collected to see the secondary structure of the proteins. Far UV CD spectra of all the seven proteins, under study showed a distinct peak characteristic of α-helix and β strands. The obtained CD spectra of BSX XYLANASE, PfuAmyGT, CceCelCCA, PfuCelB is implicative of a mixture of α-helix and β strands (Figure 3.2). The CD spectrum of SsoLacS showing substantial α-helical structure having mean residue ellipticity (MRE) of -35000 deg cm^2dmol^{-1}; however the far-UV CD spectrum of for RMCel12A, and ThtLAC shows a distinct negative band at ~218 nm wavelength confirms it to be a β-sheet-based secondary structure.

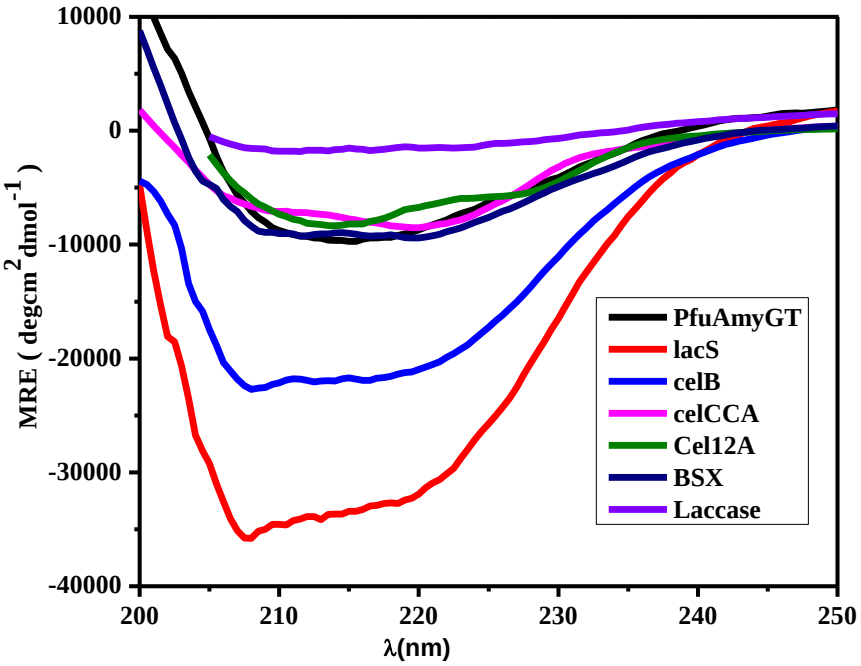


Fig 3.2: CD spectrum

3.3 Enzymatic degradation of Biomass

The pre-treated biomasses (as described in material and methods (page no. 28) of corn cob and rice husk were used as substrate for enzymatic degradation. Further, after the hydrolysis of these biomasses the enzyme activity of all the enzymes (as described below) was checked. Colorimetric method like DNSA assay was carried out to check the presence of reducing sugars. Since, this method is used to estimate the reducing sugar only, so we have also done Thin Layer Chromatography (TLC) for the successful visualization of various components generated after enzymatic treatment.

3.3.1CceCelCCA

3.3.1.1 DNSA Assay

Pretreated biomasses (100 µL) of corn cob and rice husk were suspended with 100 µL of (concentration as shown in table 3.1) Ni-NTA purified enzyme and was incubated at 50 ˚C on a thermo-mixer. For, enzymatic activity assay, aliquots were taken out at different time interval of 0 hr, 30 min, 2 hr, 5 hr and overnight time points. 100 µL of DNSA reagent was added to the sample tube to stop the reaction in each case. The reaction mixture was boiled in a beaker on a hot plate for 5 minutes. Absorbance was measured at 540 nm.

To our excitement CceCelCCA is able to degrade both the biomasses, giving intense brown color after enzymatic degradation of the same into simple sugars (Figure 3.3 a). Maximum enzyme activity was observed at 5 hr for both the biomasses. Further, this was also visualized by Thin Layer Chromatography as described in next section.

Fig 3.3: a) Estimation of hydrolyzed sugar products with CceCelCCA using biomass of corncob and rice husk using DNSA method (B represents Blank i.e. without enzyme).

3.3.1.2 Thin Layer Chromatography

Figure 3.3 b shows the TLC profile of the overnight hydrolyzed sugar products of biomass corncob and rice by CceCelCCA. A negative control (-); i.e biomass without enzymatic treatment, positive control (+); i.e 0 hr sample and a marker (M) was ran in the TLC plate. TLC result suggests that the CceCelCCA was able to hydrolyze corn cob and rice husk into simple sugars (may be into cellobiose or cellotriose sugar) which will be further confirmed by mass spectroscopy.

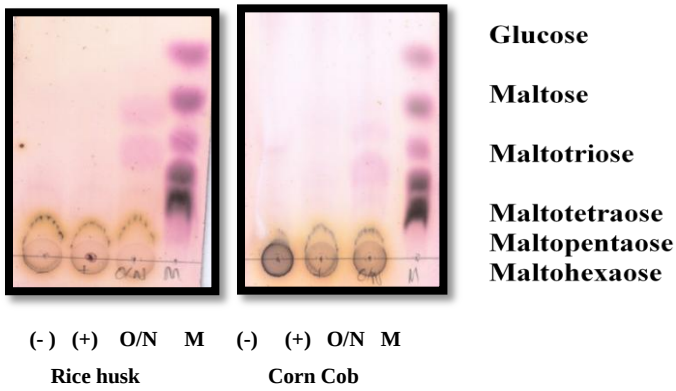


Fig 3.3: b) Estimation of hydrolyzed sugar products with CceCelCCA using biomass of corncob and rice husk using TLC.

3.3.2 RMCel12A

3.3.2.1 DNSA Assay

Under similar experimental conditions as explained above RMCel12A was incubated with pretreated biomass. RMCel12A is also able to degrade both the biomasses, giving intense brown color after enzymatic degradation of the same into simple sugars (Figure 3.4 a). Maximum enzyme activity was observed at 2 hrs for both the biomasses. Further, this was also visualized by Thin Layer Chromatography as described in next section.

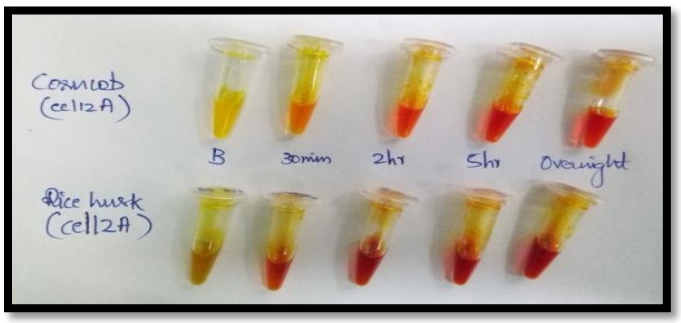


Fig 3.4.a): Estimation of hydrolyzed sugar products with RMCel12A using biomass of corncob and rice husk using DNSA method (B represents Blank i.e. without enzyme).

3.3.2.2 Thin Layer Chromatography

Figure 3.4 b shows the TLC profile of the overnight hydrolyzed sugar products of biomass corncob and rice by RMCel12A. A negative control (-); i.e biomass without enzymatic treatment, positive control (+); i.e 0 hr sample and a marker (M) was ran in the TLC plate as explained above. TLC result suggests that the RMCel12A was able to hydrolyze corn cob and rice husk into simple sugars (may be into cellobiose) which will be further confirmed by mass spectroscopy.

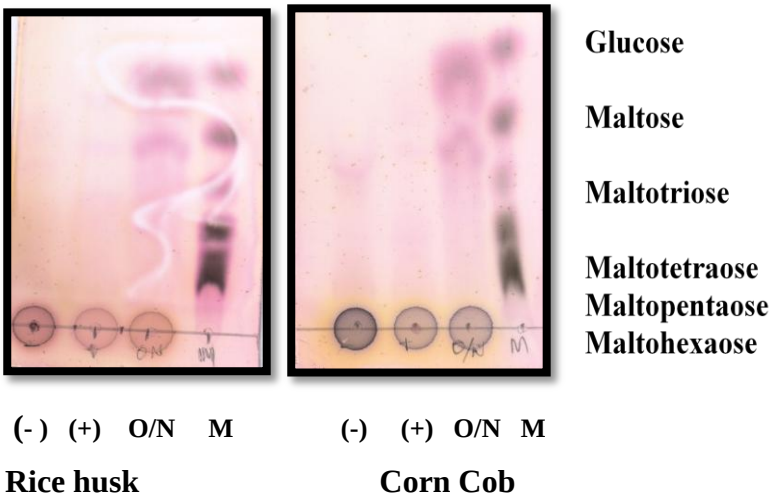
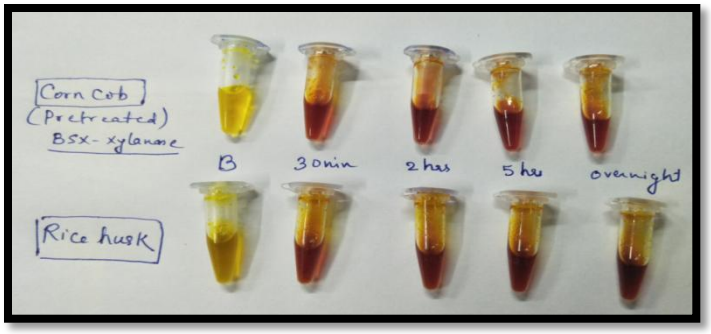


Fig 3.4.b): Estimation of hydrolyzed sugar products with RMRMCel12A using biomass of corncob and rice husk using TLC.

3.3.3 BSX XYLANASE

3.3.3.1 DNSA Assay

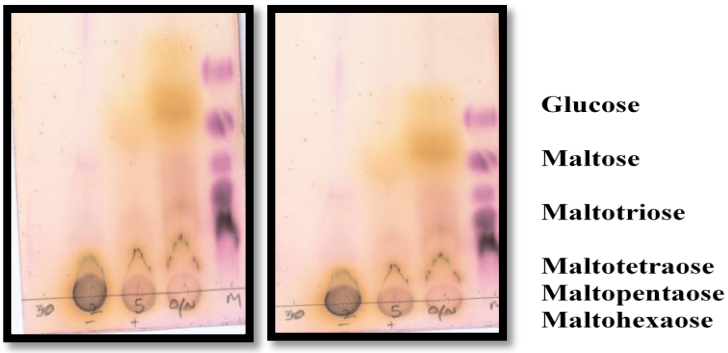
In case of BSX XYLANASE even at shorter incubation time, there was an intense colour change seen on addition of DNSA reagent. This suggests that BSX XYLANASE is very efficient in degrading biomass and saturation occurs very fast compared two other two enzymes as explained above.



3.5 a): Estimation of hydrolyzed sugar products with BSX XYLANASE using biomass of corncob and rice husk using DNSA method (B represents Blank i.e. without enzyme).

3.3.3.2 Thin Layer Chromatography

TLC result suggests that the BSX XYLANASE was able to hydrolyze corn cob and simple into simple sugars (may be into xylose or xylobiose) (Figure 3.5 b) which will be further confirmed by mass spectroscopy.



3.5. b): Estimation of hydrolyzed sugar products with BSX XYLANASE using biomass of corncob and rice husk using TLC.

2.3.6 PfuCelB

2.3.6.1 DNSA Assay

Under similar experimental conditions as explained above PfuCelB was incubated with pretreated biomass. PfuCelB is also able to degrade both the biomasses in a very slow manner, giving slightly brown color after enzymatic degradation of the same into simple sugars (Figure 3.8 a). Maximum enzyme activity was observed only for overnight incubated samples for both

the biomasses. Further, this was also visualized by Thin Layer Chromatography as described in next section.

Fig 3.6 a): Estimation of hydrolyzed sugar products with PfuCelB using biomass of corncob and rice husk using DNSA method (B represents Blank i.e. without enzyme).

2.3.6.2 TLC

TLC result suggests that the PfuCelB was able to hydrolyze corn cob and rice husk simple into simple sugars (Figure 3.6 b) which will be further confirmed by mass spectroscopy.

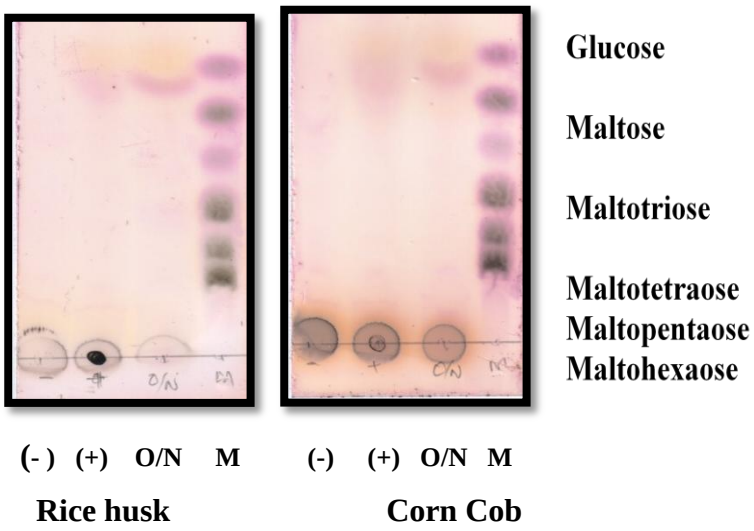


Fig 3.6. b): Estimation of hydrolyzed sugar products with PfuCelBA using biomass of corncob and rice husk using TLC.

3.3.4 PfyAmyGT

3.3.4.1 DNSA Assay

Under similar experimental conditions as explained above PfuAmyGT was incubated with pretreated biomass. PfuAmyGT was not able to degrade both the biomasses, as no colour change was seen on addition of DNSA (Figure 3.7 a). This shows that PfuAmyGT is very slow in degrading biomasses which will be further confirmed by mass spectroscopy.

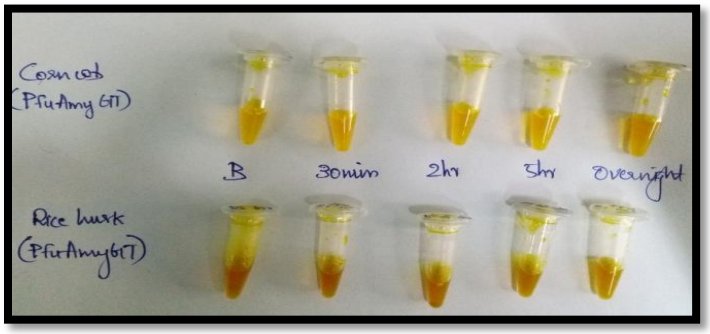
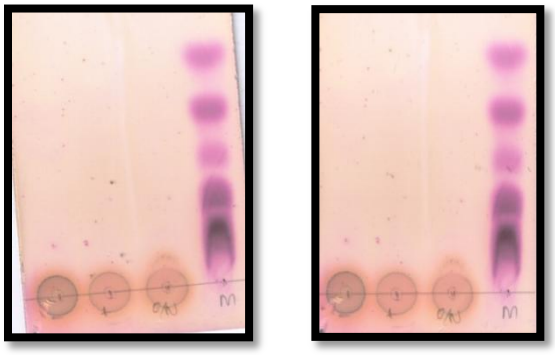


Fig 3.7 a): Estimation of hydrolyzed sugar products with PfuAmyGT using biomass of corncob and rice husk using DNSA method (B represents Blank i.e. without enzyme).

3.3.4.2 TLC

A band of higher molecular mass (higher than maltohexose) was observed (Figure 3.7 b). As, this a glucanotransferases that can excise and transfer small sugar units from a donor substrate to an acceptor molecule (so as to shorten long carbohydrate chains and lengthen short carbohydrate chains).



(-) (+) O/N M **(-) (+) O/N M**

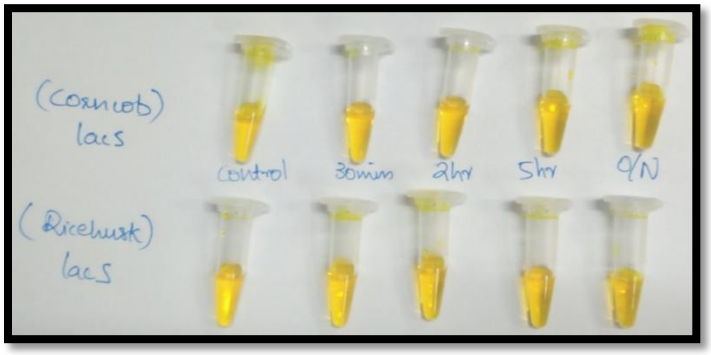
Rice husk **Corn Cob**

Fig 3.7 b) Estimation of hydrolyzed sugar products with PfuAmYGT using biomass of corncob and rice husk using TLC.

3.3.5 SsoLacS

3.3.5.1 DNSA Assay

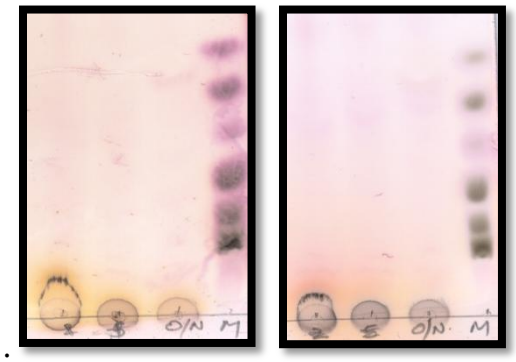
There were no color change was observed for both the biomasses even for overnight incubated samples (Figure 3.8 a) Further, this method also not suitable for SsoLacS assay



3.8 a): Estimation of hydrolyzed sugar products with SsoLacS using biomass of corncob and rice husk using DNSA method (B represents Blank i.e. without enzyme).

2.3.5.2 TLC

No band was observed on TLC (Figure 3.9 b)



(-) (+) O/N M (-) (+) O/N M

Rice husk Corn cob

Fig 3.8 b): Estimation of hydrolyzed sugar products with SsoLacS using biomass of corncob and rice husk using TLC.

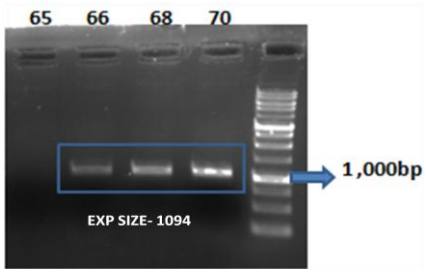
3.4 Engineering design of bi-functional enzyme constructs for degradation of biomass:-

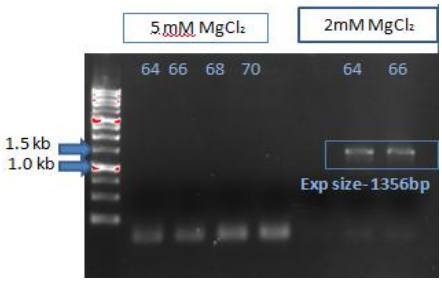
The concept of making bi-functional fusion constructs is an attempt to engineer a macromolecular machine in order to act synergistically and cooperatively and to have a higher efficiency of conversion of biomass and to lower requirements of replenishment during treatment. SOE-PCR approach will be used to make final constructs for the delignification of plant biomass, bleaching of pulp and for the paper industry.

For, BSX XYLANASE and ThtLAC fusion construct, the two separate templates have been made with overhang of linker (Figure 3.10). Both this templates is designated as BSX XYLANASE-linker and linker-ThtLAC. We have used these overlapping fragments as template to make the total gene. To make the complete gene we have used Phusion polymerase and 10 cycles of PCR cycles were done without addition of forward and reverse primers (in reaction mixture) as both templates contains overlapping region. Then for the next 25 cycles, we have added primers. We got the total gene as shown in the (Figure 3.11) after that it was digested using enzymes *Bam HI1 and Nhe I,* restriction sites The digested gene was ligated into the vector pQE 30 and further, transformed into its cloning/expression host XLI Blue strain of *E.coli* competent cells. Unfortunately we haven't got any positive colony. For, other two constructs also we are trouble shooting to get the complete gene; therefore, this work needs to be continued…

Round I- Amplification of Primary fragments with linker

BSX XYLANASE with linker (PCR-1) **Linker with _ ThtLAC (PCR-2)**





Round 2- Splicing reactions of primary fragments to get the complete gene

complete gene (PCR-3)

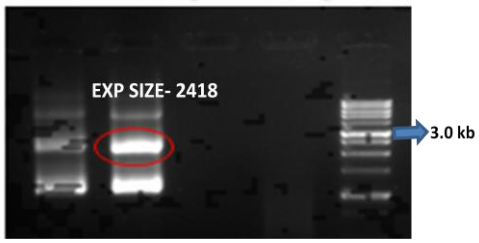


Fig. 3.11Amplification of Primary fragments with linker (shown as Round I,) and splicing reactions of primary fragments to get the complete gene (shown as Round II).

4. Summary

Thermostable enzymes have drawn the interests of many researchers pertaining to their high thermal and structural stability. They help the organism withstand harsh and extreme conditions. These enzymes have also been put to various industrial uses. In the current thesis, we reported various thermostable enzymes having potential role in biomass degradation. Further, DNSA assay and Thin Layer Chromatography was carried out to visualize them. Our results suggest that these enzymes are efficient in biomass degradation and can be used in various industrial and biotechnological applications. In later part, we have proposed and tried to make double fusion constructs with a glycine-serine linker in between. These are flexible linkers so; they will not creat any hinderance for the functioning of the enzymes when they are fused together. We were successful to get the complete gene for BSX XYLANASE and ThtLAC fusion construct. Further, this work need to be continues in order to get the constructs.

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