Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Plasticware/Glassware

All plasticwares like pre-sterilized Petri dishes and Polystyrene tubes were purchased from Tarson, India; glassware like Erlenmeyer flasks, beakers and test tubes were purchased from Borosil, Mumbai, India.

3.1.2 Chemicals and Reagents

All chemicals and reagents used were of analytical grade and purchased from Merck India Ltd., SRL, Qualigens, Himedia, Sigma Aldrich and Bangalore Genei.

3.1.3 Microbiological grade culture media

Commercial microbiological grade media were purchased from HiMedia, Mumbai, India for the investigation.

3.1.4 Bacterial strains and plasmids used

The bacterial strains and plasmids used in the study are presented in Table 3.1.

Table 3.1 List of bacterial strains and plasmids used in the study

Strains/plasmids	Genotype/Description	Source
E. coli DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG pu rB20 φ80d*lac*ZΔM15 Δ(*lac*ZYA-argF)U169, hsdR17($r_K^-m_K^+$), λ^-	Invitrogen (CA, USA)
E. coli BL21(DE3)	F^- ompT gal dcm lon hsdS_B($r_B^-m_B^-$) λ(DE3[*lac*I *lac*UV5-T7 gene 1 ind1 sam7 nin5]) [$malB^+$]$_{K-12}$(λ^S)	Novagen (CA, USA)
pUC19	Amp^r; cloning vector, *lacZ*α	NEB (Essex)
pET28a(+)	Kan^r; Expression vector, T7 promoter	Novagen (CA, USA)

3.1.5 Molecular Biology grade chemicals and kits

Molecular Biology grade chemicals and kits used in the present investigation are presented in Table 3.2.

Table 3.2 List of Molecular Biology grade chemicals/kits

Chemical Name	**Common Name**	**Company**
5-bromo-4-chloro-3-indolyl-D-galactopyranoside	X-Gal	Thermo Scientific, USA
Agarose		Sigma-Aldrich, USA
Ampicillin, Kanamycin		Sigma-Aldrich, USA
Ethidium bromide	EtBr	Thermo Scientific, USA
Isopropyl-β-thio-galactopyranoside	IPTG	Sigma-Aldrich, USA
Low melting point agarose		
Proteinase K, RNase A		
Sodium dodecyl sulphate	SDS	
*Eco*RI, *Bam*HI, *Hin*dIII, *Nde*I		
Calf intestine alkaline phosphatase	CIAP	Fermentas, USA
Bovine serum albumin	BSA	
polymerase chain reaction dNTP mix	PCR dNTP mix	Thermo Scientific, USA
10X Taq buffer		
DNA ladders (1 kb plus)		
Taq DNA polymerase	Taq Pol	Bangalore Genei, India
Protein molecular weight marker	PM2510	Smobio, Taiwan
PCR primers		Integrated DNA technologies, USA
NucleoSpin PCR clean-up kit		Macherey-Nagel, USA
NucleoSpin Gel extraction kit		
NTA-Nickel Column for protein purification		Bio-Rad, USA
Plasmid DNA miniprep purification kit		HiMedia, India.

3.1.6 Equipment used for the study

The equipment used during the study is listed in Table 3.3.

Table 3.3 List of the equipment used in the present study

Equipment Name	**Company Name**
Autoclave	Daihan Labtech, India
Laminar Hood	Rico Scientific Industries, India
Deep Freezer	Eppendorf, USA
Centrifuge	REMI, India
Cooling Centrifuge	REMI, India
Lyophilizer	Delvac pumps Pvt Ltd, India
Digital Weighing balance	Mettler Toledo, India
Water bath	Allied Scientific products, India
Hot air oven	REMI, India
Microwave oven	LG, India
Incubator	Scigenics Biotech, Orbitech, India
Incubator shaker	Scigenics Biotech, Orbitech, India
Heating mantle	JSGW, India
pH Meter	CyberScan 500, Germany
Vortexer	JSGW, India
Sonicator	Ultrasonic homogenizer, OMNI International, India
UV Spectrophotometer	ThermoScientific, UV-10, UV-VIS, USA
Polymerase Chain Reaction thermal cycler	Applied Biosystems, USA
Electrophoresis system for DNA	Genaxy Scientific Pvt. Ltd, India
DNA Sequencer	Applied Biosystems, USA
Electrophoresis system for protein	BioRad, USA

3.2 Methods

3.2.1 Buffers

Most of the buffers were prepared as described by Sambrook and Russel[123]. The pH was adjusted with 1N NaOH or 1N HCl.

3.2.2 Media composition

Different media used during the investigation are listed below.

3.2.2.1 Media used for inoculation

The inoculum medium provides appropriate biochemical and biophysical environment for the cultivation of bacteria. Luria Bertani (LB) broth medium was used as the inoculum medium in our study.

Composition of inoculum medium (LB broth)

Constituents	Amount (g L^{-1})
Tryptone	10.0
NaCl	10.0
Yeast Extract	5.0
pH	7.0

3.2.2.2 Maintenance medium

The medium employed for the maintenance of the bacterial cells was Luria Bertani Agar (LBA). LBA medium was sterilized by autoclaving at 121°C, for 20 min and 15 psi. All thermolabile solutions and components such as antibiotics stocks were prepared by filter sterilization (0.22 µm). Bacterial medium was supplemented with ampicillin (100 µg/ml) and kanamycin (50 µg/ml) when used for growth of transformed *E. coli* cells.

Composition of maintenance medium (LBA)

Constituents	Amount (g L^{-1})
Tryptone	10.0
NaCl	10.0
Yeast Extract	5.0
Agar	15.0
pH	7.0

3.2.2.3 Media used for cellulase production

The medium used for the cellulase production was carboxymethyl cellulose (CMC) that is specific for the growth of cellulolytic bacteria.

Composition of selective medium (CMC agar):

Constituents	Amount (g L^{-1})
Carboxymethyl cellulose	10.0
Sodium nitrate	2.0
K_2HPO_4	1.0
$MgSO_4.7H_2O$	0.5
Potassium chloride	0.5
Peptone	2.0
Agar	15.0
pH	6.5

3.2.3 Sterilization

Glasswares such as flasks and test tubes were sealed in autoclavable polypropylene bags before sterilization. Media used for the various experiments were sterilized in flasks/tubes plugged with non-absorbent cotton at 121°C and 15 psi for 20 min. Heat sensitive materials such as antibiotics were subjected to filter sterilization prior to the use.

3.3 Collection of sample for metagenomic study

The goat rumen digesta was sampled from a slaughter house located at Napaam, Tezpur, Assam, India. The collected sample was transported to laboratory in a sterile plastic bag for storage at –80ºC until the DNA extraction was performed**.**

3.4 Metagenomic study

3.4.1 Extraction of metagenomic DNA from goat rumen digesta

Metagenomic DNA (mgDNA) from goat rumen digesta sample was isolated using five different methods so as to assess the best one. The methods P1, P2, P3, and P4 were performed according to protocols of Sharma *et al.*[124], Yu and Morison[125], Krause *et al.*[126] and Popova *et al.*[127], respectively. P5 is a modified

CTAB extraction protocol developed in our laboratory (Bashir *et al.*[128]) as presented below:

1. Weighed 0.5 g goat rumen digesta.
2. Placed the sample in a 15-mL Falcon tube and added 5 mL CTAB extraction buffer [1% (w/v) CTAB; 100 mM Tris-HCl, pH 8.0; 50 mM EDTA; 1.5 M NaCl; and 100 μg/mL proteinase K]. Incubated at 65°C for 2 h with occasional mixing.

   Proteinase K was added to the buffer after pre-warming it to 65°C.
3. Centrifuged the lysate at 10000× g for 5 min and retained the supernatant. Added 500 μL of 25:24:1 phenol:chloroform:isoamyl alcohol and 100 μL of 35% polyethylene glycol (PEG, M_r = 4000) and centrifuged at 15 000× g for 10 min at 4°C.
4. Precipitated the DNA from the recovered aqueous phase with 0.6 volumes of isopropyl alcohol. Recovered the DNA by centrifugation at 10000× g for 15 min at 4°C.
5. Measured the DNA concentration spectrophotometrically or by gel electrophoresis.

Methods P1, P2, P3, P4 and P5 were compared on the basis of DNA yield, purity, processing time, and suitability for PCR and restriction digestion.

3.4.2 Agarose gel electrophoresis of mgDNA

Reagents required: 0.8% agarose, 50X and 1X Tris-Acetate-EDTA (TAE) buffer, loading dye and ethidium bromide (EtBr) solution.

DNA sample preparation: 2.0 μl of loading dye was added to 5.0-10.0 μl of the isolated mgDNA.

Composition of loading dye

Constituents	Amount (%)
Bromophenol blue	0.25
Xylene cyanol	0.25
Glycerol	30

Procedure

- 0.8 % agarose was added to 1X TAE buffer and heated to dissolve the agarose.
- It was allowed to cool to around 45ºC and then EtBr (10 mg/ml stock) was added to make the final concentration to 0.5 mg/ml.
- Agarose gel was poured into the gel caster sealed with adhesive tape and fitted with comb.
- After solidifying the gel, the comb and adhesive tape were removed.
- The gel-containing caster was placed in the electrophoresis tank filled with 1X TAE buffer.
- DNA samples were loaded in the gel wells.
- The samples were run at 75 volts/cm till the loading dye reached 75% of the gel.
- The gel was removed from the electrophoresis tank and examined on UV trans-illuminator.

3.4.3 Quantification of mgDNA

Quantification of mgDNA is commonly performed to determine the average DNA concentration and its purity in a solution. Generally, absorbance at 260 nm (A_{260}) is used to determine the concentration of DNA and $A_{260/280}$ and $A_{260/230}$ ratio is calculated to assess purity. Polyphenols such as tannins absorb illumination at 230 nm, proteins at 280 nm, whereas DNA at 260 nm. $A_{260/280}$ ratio below 1.7 indicates the protein contamination whereas $A_{260/230}$ ratio below 2.0 indicates tannin contamination. If the $A_{260/280}$ ratio is more than 1.7, DNA can be quantified based on an A_{260} value of 1.0 equivalent to 50 µg/ml. DNA concentrations were measured spectrophotometrically (A_{260} nm, Cecil 7400, Cambridge). Absorbance ratios at 260/230 nm (DNA/humic acid) and 260/280 nm (DNA/protein) were used to evaluate the purity of the goat rumen mgDNA.

3.4.4 Use of P5 protocol in the isolation of genomic DNA from the culturable bacteria

The protocol P5 was also used to isolate genomic DNA from *E. coli* (MTCC 40) to validate the utility of the extraction protocol for the culturable bacteria as well.

3.4.5 Suitability of mgDNA for PCR amplification

The purity of mgDNA for downstream applications was checked by the amplification of 16S rRNA gene using the universal primers {(F27, AGA GTT TGA TCC TGG CTC AG and R1492, ACG GTT ACC TTG TTA CGA CTT); (UD Scientific) [Weisberg *et al.*[129]]}. Amplification was carried out in a 20 µl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Fermentas, USA), 10 pmole of each primers, 10 ng DNA template and 1 U Taq polymerase (Bangalore Genei, India) with reaction buffer supplied by the manufacturer. PCR was performed with a thermal cycler (Applied Biosystems, USA) by using the following program: 95ºC for 3 min; 30 cycles consisting of 95ºC for 30 sec; 55ºC for 30 sec; 72ºC for 1 min; and a final extension step consisting of 72°C for 5 min. The amplification was determined by electrophoresis of reaction product in 1% agarose gel.

3.4.6 Restriction digestion of mgDNA by *Eco*RI and *Bam*HI

To examine the suitability of mgDNA for restriction digestion, 0.25 µg of mgDNA was digested separately with 2.5 units of *Eco*RI and *Bam*HI (MBI Fermentas, Germany) restriction enzymes in a 25 µl reaction mixture (Sagar *et al.*[130]). The mixtures were incubated at 37°C for 3 h followed by inactivation of the restriction enzyme by heating at 70°C for 10 min. The digested products were resolved on 0.8% agarose gel.

3.5 Construction of mgDNA library

mgDNA library was constructed using pUC19 as the cloning vector. Purified mgDNA was partially digested using *Bam*HI (MBI Fermentas, Germany) restriction enzyme by incubation at 37ºC for 20 min. The digested product was resolved in 0.8% agarose gel and DNA fragments ranging about 0.5–2.0 kb were fractionated by agarose gel purification using NucleoSpin gel extraction kit (Macherey-Nagel, USA). Subsequently the purified mgDNA fragments were ligated to *Bam*HI digested and CIAP dephosphorylated pUC19 cloning vector using T4 DNA ligase (MBI Fermentas, Germany) at 16°C overnight. The ligated mixture was then transferred to *E. coli* DH5α by electroporation (200 Ω, 25 µF and 2.5 kV) using Gene Pulser (Biorad, USA). The undigested pUC19 cloning vector was transferred to *E. coli* DH5α competent cells as the positive control to confirm

the transformation efficiency. Transformed cells were cultured on LBA plates supplemented with ampicillin (100 µg/ml), IPTG (0.1 mM) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (50 µg/ml). The recombinants were scored by blue–white screening after overnight incubation at 37°C. The resulting library was stored in 15% glycerol at −80°C.

3.5.1 Partial restriction digestion of mgDNA

*Bam*HI recognizes the sequence GGATCC and generates fragments with 5'-cohesive termini that contain the pentanucleotide sequence GATCC.

mgDNA (5µg) was partially digested with *Bam*HI. The partial restriction digestion reaction was set up as described below:

Components	Volume
mgDNA	15.0 µl (5 µg)
Cut smart 10X buffer	5.0 µl
*Bam*HI (10 U/µl)	1.5 µl
Nuclease free water	29.5 µl
Total volume	50.0 µl

The reaction mixture was incubated at 37°C for 20 min and then the enzyme was inactivated by heating at 70°C for 10 minutes. The digested DNA fragments were resolved on 0.8 % agarose gel in 1X TAE.

3.5.2 Extraction of DNA fragments from agarose gel

After running the *Bam*HI-digested mgDNA on agarose gel, it was examined under UV trans-illuminator and the gel slice containing DNA fragments ranging about 0.5-2.0 kb was separated using sterile scalpel. The mgDNA fragments were purified using using NucleoSpin gel extraction kit (Macherey-Nagel, USA). Briefly, the excised gel was mixed with 3 volumes of A1 buffer, incubated at 50°C until the gel slice got dissolved completely. For binding of DNA, the sample was applied to NucleoSpin column, and centrifuged for 1 min at 11,000 x g. The flow-through was discarded and the column was placed back in the same collection tube. 500 µl of A2 buffer was added to the column and centrifuged for 1 min at 11,000 x g. A volume of 600 µl of A3 buffer was added to the column for washing followed by centrifugation at 11,000 x g for 1 min. The

column was centrifuged for an additional 1 min at 11,000 x g to remove the residual buffer. The NucleoSpin column was placed into a clean 1.5 ml microfuge tube. To elute DNA, 40 µl of deionized water (preheated to 55°C) was added to the center of the NucleoSpin column membrane. The column was allowed to stand for 1 min and then centrifuged at 11,000 x g for 1 min. The recovered DNA fragments were used for library construction.

3.5.3 Isolation of pUC19 plasmid DNA from *E. coli* DH5α cells

Natural *E. coli* strains often carry plasmids specifying resistance to antibiotics. All plasmids used as cloning vectors contain three common features; an origin of replication (Ori), a selectable marker gene and a multi-cloning site (MCS). The pUC19 cloning vector was used to construct the mgDNA library. The map of pUC19 cloning vector is presented in Fig 3.1.

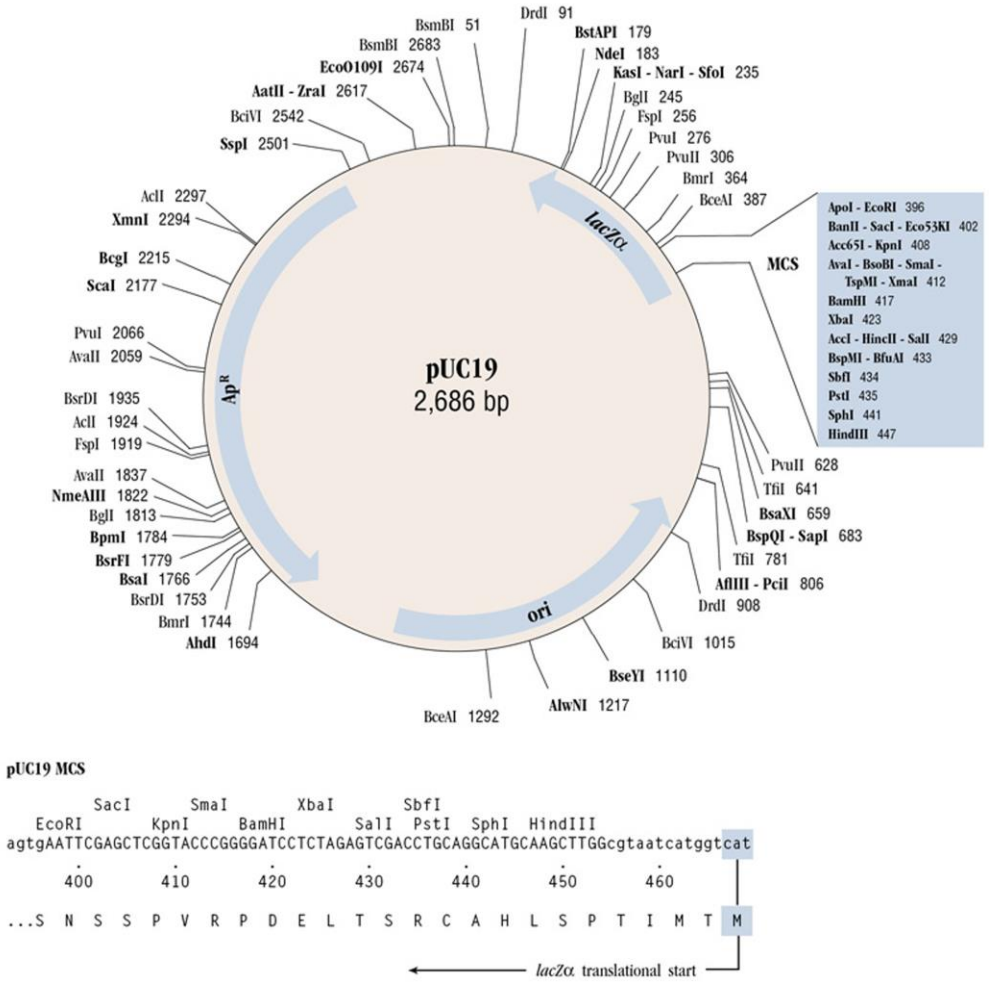

Fig. 3.1 pUC19 cloning system

The plasmid pUC19 (New England Biolabs, Essex) was earlier mobilised into *E. coli* DH5α competent cells and the glycerol stock of the same was preserved at -80 ºC for long term storage in the laboratory of Prof. Bolin Kumar Konwar, Dept. of Molecular Biology and Biotechnology, Tezpur University. For the present study, 5 µl of the *E. coli* DH5α glycerol stock harbouring the pUC19 plasmid was inoculated into 50 ml of fresh LB medium supplemented with ampicillin (100 µg/ml) and incubated at 37 ºC overnight. The pUC19 plasmid DNA was isolated from the overnight grown culture by the alkaline lysis method (Sambrook *et al*.[131]).

Reagents:

Solution I

Glucose	50 mM
Tris HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	10 mM

Solution II (Freshly prepared)

NaOH	0.2 N
SDS	1%

Solution III

Potassium acetate	5 M
Glacial acetic acid	11.5 ml
Distilled water	28.5 ml

The protocol

- The overnight grown culture of bacterial cells, supplemented with ampicillin (100 µg/ml), was pelleted by centrifugation at 10,000 rpm for 5 min in a refrigerated Beckman Centrifuge (UK) and supernatant was decanted.
- The cell pellet was suspended in 100 µl of ice-cold Solution I with vigorous vortexing.
- An aliquot of 200 µl of freshly prepared alkaline lysis solution II was added to each bacterial suspension.

- The contents were mixed by inverting the microfuge tubes gently and incubated on ice for 5 min.
- 150 µl of ice-cold Solution III was added to each microfuge tube and dispersed through the viscous bacterial lysate by gently inverting the tube 5-6 times and incubated on ice for 15 min.
- The bacterial lysate was centrifuged at 13,000 rpm for 10 min at 4°C.
- The supernatant was transferred to the fresh tube and an equal volume of isopropanol was added to precipitate the DNA. The mixture was allowed to stand for 20 min at room temperature.
- The tube was centrifuged at 10,000 rpm for 10 min at 4°C. Following the removal of supernatant the pellet was washed with 500 µl of 70% ethanol.
- The plasmid DNA was recovered by centrifugation at 13,000 rpm for 5 min at 4°C.
- After carefully draining the supernatant, each tube was kept open at room temperature until the ethanol got evaporated and no fluid remained visible in the tube.
- The pellet was dissolved in 50 µl of nuclease free water and stored at -20°C.

3.5.4 Restriction digestion of pUC19 plasmid DNA with *Bam*HI

*Bam*HI is a type II restriction endonuclease which recognizes a short sequence of 6 bp of DNA (5'-GGATCC-3'). The restriction enzyme binds at this recognition sequence and cleaves just after the 5'-Guanine on each strand. This cleavage results in the production of sticky ends (5'-GATCC-3') of 5 bp length.

The plasmid pUC19 (3 µg) was digested with *Bam*HI. The setting of the digestion reaction is presented below:

Components	Volume
pUC19 plasmid DNA	15.0 µl (3 µg)
Cut smart 10X buffer	5.0 µl
*Bam*HI (10 U/µl)	0.5 µl
Nuclease free water	29.5.0 µl
Total volume	50.0 µl

The reaction mixture was incubated at 37°C for 3h and the enzyme was subsequently inactivated by heating at 70°C for 10 min. The linearized plasmid DNA was dephosphorylated by adding 1 unit of Calf intestinal alkaline phosphatase (CIAP) (Fermentas, USA) and incubated at 37°C for 1h to prevent self-ligation. Digested pUC19 plasmid DNA was resolved on 1% agarose gel in 1x TAE buffer. The digested and dephosphorylated plasmid DNA was purified using NucleoSpin gel extraction kit (Macherey-Nagel, USA).

3.5.5 Ligation of mgDNA fragments into pUC19 plasmid DNA

Purified mgDNA fragments ranging from 0.5-2.0 kb were mixed with the BamHI-digested and CIAP dephosphorylated pUC19 plasmid vector DNA in 3:1 ratio. The ligation reaction was set as described below:

Components	Volume
10X T4 ligase buffer	2.0 µl
pUC19 vector	3.0 µl
mgDNA fragments	14.0 µl
T4 DNA ligase (3 U/µl)	1.0 µl
Total volume	20.0 µl

The reaction mixture was incubated at 16°C overnight.

3.5.6 Transformation

3.5.6.1 Preparation of *E. coli* DH5α competent cells

Competent cells are capable of taking up foreign DNA. The treatment of bacterial cells with $CaCl_2$ induces a transient state of competence in which temporary pores are formed in the cell wall of bacteria enabling them to take up foreign DNA. The *E. coli* DH5α competent cells were prepared by following the method of Sambrook *et al*.[131] with some modifications.

The protocol

- A volume of 0.5 ml of *E. coli* DH5α overnight bacterial culture was inoculated aseptically into 50 ml of LB antibiotic free medium.
- The liquid bacterial culture was allowed to grow upto early log phase at 37°C in a shaker at ~180 rpm until OD_{600} reached 0.6 (3-3.5 h).

- The bacterial cell suspension was transferred to 50 ml sterile ice cold Oak Ridge tube and kept in ice for 30 min.
- Each tube was centrifuged at 3,500 rpm for 5 min at 4°C.
- The supernatant was discarded and the pellet was completely resuspended in 35 ml of sterile ice cold 100 mM $CaCl_2$ solution by pipetting gently.
- The tubes were rested in ice for 30 min.
- The tubes were then centrifuged at 3,500 rpm for 5 min at 4°C. The pellet obtained was resuspended carefully in 3.5 ml of sterile ice cold FT buffer (200 mM $CaCl_2$ and 15% glycerol).
- Aliquots of 100 µl of bacterial cells were pipetted to pre-chilled 1.5 ml eppendorf tubes using the end cut tips.
- The tubes were kept frozen on ice for a few minutes and stored at -80°C until further use.

3.5.6.2 Transformation of *E. coli* DH5α competent cells with recombinant pUC19 plasmid DNA

Transformation is the process by which the recombinant DNA enters into the host cells and then proliferates. The presence of antibiotic resistance gene in the plasmid enables selection of the cells having an incorporated recombinant DNA molecule. The recombinant DNA thus transferred also multiplies along with the host cells to produce as many identical copies of the clone.

The transformation of recombinant plasmid DNA was done using electroporation according to manufacturer's guidelines. Competent *E. coli* DH5α transformed with non-recombinant pUC19 DNA and *E. coli* DH5α cells were used as positive and negative control, respectively.

The protocol

- The vials containing competent bacterial cells were thawed on ice. The ligated product was then added to the competent *E. coli* DH5α vials in case of pUC19 + mgDNA fragments. Positive (*E. coli* DH5α + pUC19 DNA) and negative (*E. coli* DH5α) controls were also prepared. The contents were mixed gently and kept on ice for 30 min.

- The ligated mixture was transformed into *E. coli* DH5α host cells by electroporation (200 Ω, 25 μF and 2.5 kV) using gene pulser (Biorad, USA).
- 1 ml of SOC medium was added to each tube in a laminar hood (sterile condition) and the contents were then mixed by pipetting. The tubes were incubated at 37°C for 1 h with shaking. The tubes were centrifuged at 5,000 rpm for 10 min.
- The supernatant was decanted and the cells were resuspended in the remaining SOC medium.
- An aliquot of 100 μl of transformed cells was plated on LBA + ampicillin (100 μg/ml) plates overlaid with IPTG (0.1 mM) and X-gal (50 μg/ml) and incubated at 37°C overnight.

3.5.6.3 Transformation efficiency of pUC19 plasmid DNA

The normal and recombinant pUC19 plasmid DNAs were transferred to *E. coli* DH5α. The transformation efficiency was calculated by the following equation:

$$\text{Transformation efficiency (no. of transformants/μg DNA)} = \frac{\text{No of colonies}}{\text{pg pUC19 DNA}} \times \frac{10^6}{\mu g} \times \frac{\text{volume of transformants}}{\text{volume plated (μl)}} \times \text{dilution factor}$$

3.5.7 Blue-white screening

A cloning experiment with pUC19 plasmid involves selection of transformants on ampicillin agar plates followed by screening for β-galactosidase activity to distinguish recombinants from non-recombinants. The screening for the presence or absence of β-galactosidase, encoded by *lac*Z' gene, can be done by incorporating the lactose analog X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) which is broken down by β-galactosidase to a deep blue coloured product. If X-gal and isopropyl-β-D-thiogalactopyranoside (IPTG, an inducer of the β-galactosidase enzyme) is added to the nutrient agar plate along with ampicillin, then the non-recombinant colonies synthesizing β-galactosidase will be coloured blue whereas recombinants with a disrupted *lacZ′* gene will be unable to make β-galactosidase and thus will remain white.

In the present investigation, blue-white screening was followed to distinguish recombinants from non-recombinant clones. The recombinant clones were picked, sub-cultured and preserved at -80°C.

3.5.8 Assessment of mgDNA library

3.5.8.1 Isolation of recombinant pUC19 plasmid DNA from randomly selected clones

The recombinant pUC19 plasmid DNA was isolated from randomly selected *E. coli* DH5α clones from the mgDNA library by alkaline lysis method (Sambrook *et al.*[131]). The shift in the size of the recombinant pUC19 plasmid DNAs was compared to normal pUC19 plasmid DNA (2,686 bp).

3.5.8.2 Restriction digestion of recombinant pUC19 plasmid DNA with *Kpn*I

Restriction digestion of recombinant pUC19 plasmid DNA isolated from randomly selected *E. coli* DH5α clones from the mgDNA library was performed using *Kpn*I restriction enzyme to check the presence of DNA inserts of different sizes. The restriction digestion reaction was set up as presented below:

Components	Volume
Recombinant pUC19 plasmid DNA	10.0 µl
Cut smart 10X buffer	2.0 µl
Enzyme (*Kpn*I) (10 U/µl)	1.0 µl
Nuclease free water	7.0 µl
Total volume	20.0 µl

3.5.9 Screening of mgDNA library for cellulase producing clone(s)

The functional screening of mgDNA libraries is a powerful technique to identify and assess the function of novel genes without any prior sequence knowledge. The recombinants were screened for cellulolytic activity on carboxymethyl cellulose (CMC) agar plates supplemented with 1% (v/v) CMC and ampicillin (100 mg/ml) and incubated at 37°C for 24–36 h. Cellulolytic clones were selected based on the formation of distinct yellow halos around the colonies on staining with Grams iodine. The putative transformant colonies were divided into groups of thousands, named as T1, T2, T3 and T4.

3.5.10 Isolation and restriction digestion of recombinant pUC19 plasmid from cellulase producing clone

A loopful of the cellulase producing recombinant bacterial colony was inoculated into 5 ml of LB broth medium supplemented with ampicillin (100 µg/ml) and incubated overnight at 37°C.

The recombinant plasmid was isolated from the cellulolytic clone by Plasmid DNA miniprep purification kit (HiMedia, India). The restriction digestion of recombinant plasmid was done using *Bam*HI restriction enzyme. The restriction digestion reaction was set up as presented below:

Components	Volume
Recombinant plasmid	15.0 µl (4 µg)
Cut smart 10X buffer	3.0 µl
*Bam*HI (10 U/µl)	1.0 µl
Nuclease free water	11.0 µl
Total volume	30.0 µl

3.5.11 Sequencing of cloned mgDNA fragment

The recombinant pUC19 plasmid DNA harbouring the cellulolytic mgDNA fragment was subjected to sequencing using M13 forward and reverses primers. The sequencing was done using ABI PRISM-3100 sequencer (Applied Biosystems, USA). Sequencing reaction was performed by using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Using the M13 forward and reverse primers, a PCR reaction was set up in a 0.2 ml PCR tube containing 2.0 µl of target mgDNA fragment to be sequenced (50 ng/µl of plasmid DNA), 1.5 µl of 5x dilution buffer (supplied in the kit), 0.5 µl of each sequencing primer (10 pmol/µl), 1.0 µl of 2.5X Ready reaction mixture (containing DNA polymerase, dNTPs, and dye-labelled ddNTPs) and 5.0 µl of Nuclease free water.

The primers used in DNA sequencing are given below:

Primer Name	**Primer sequence (5' to 3')**
M13 Forward (-20)	GTAAAACGACGGCCAGT
M13 Reverse (-24)	AACAGCTATGACCATG

PCR conditions used for the sequencing are given below:

Steps	Conditions
Initial denaturation	96°C for 1 min
Denaturation	96°C for 10 sec
Hybridization	50°C for 5 sec
Elongation	60°C for 4 min
Cycle	25

After PCR, the tubes were stored at 4°C till further purification. To remove the non-incorporated dye terminators and the primer, 12 µl of master mix-I (2.0 µl of 125 mM EDTA, 10 µl water), followed by 52 µl of master mix-II (2.0 µl of 3M Sodium acetate, pH 4.6; 50 µl of 95% ethanol) were added to the sequencing product. The contents were mixed by gentle tapping, incubated at room temperature for 15 min, and centrifuged at 10,000 rpm for 15 min. The supernatant was discarded, the pellet washed twice with 100 µl of 70% ethanol. The pellet was air-dried for 5 min and then dissolved in 24 µl Hi-Di-formamide (Applied Biosystems, USA). The purified product (12 µl) was loaded into the 96-well plate of the auto-sampler in the sequencer.

3.5.12 Analysis of the sequenced mgDNA fragment

The full length gene sequence alignment was carried out using different tools available at National Center for Biotechnology Information (NCBI) and European Bioinformatics Institute (EBI) for protein and nucleotide analysis. Full length gene (complete open reading frame) was identified by ORF finder of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/), Blastn and Blastp were performed at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) to search homology of the cellulase gene against the database. Multiple sequence alignment was carried out using Clustal W (http://www.ebi.ac.uk/clustalW/). The signal peptide present in amino acids sequence of the protein was determined using signalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/).

3.5.13 PCR amplification of DNA sequence for cellulase gene

The gene conferring cellulolytic activity on the clone, named as CelT3, was amplified from T3 clone harbouring the recombinant pUC19 plasmid DNA using the following set of primers designed using Primer designing tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/):

Forward primer: 5'- GGGAATTCCATATGACTACA -3'

Reverse primer: 5'-CCCAAGCTTCTAGACCTTCT -3'

The *Nde*I and *Hin*dIII sites were added to the 5' ends of the forward and reverse primers, respectively. The PCR conditions used for the same are given below:

Steps	Conditions
Initial denaturation	94°C for 5 min
Denaturation	94°C for 1min
Annealing	59°C for 45 sec
Elongation	72°C for 1 min
Final Elongation	72°C for 10 min
Cycles	30

The PCR amplified product was electrophoresed on 1% agarose gel prepared in 1X TAE buffer and gel purified using NucleoSpin PCR-clean up kit (Macherey-Nagel, USA).

3.5.14 GenBank submission

The nucleotide sequence of CelT3 was deposited in the GenBank database.

3.5.15 Analysis of the cloned cellulase gene

The nucleotide sequence of CelT3 gene was translated into amino acid sequence using ExPASy translate tool. Multiple sequence alignment for cellulase enzyme was performed with the other known cellulase sequences in the NCBI database using BLASTp algorithm.

3.5.16 Subcloning of CelT3 cellulase gene into pET28a(+) expression vector

The vector used for the expression of CelT3 cellulase gene was pET28a(+). It contains a kanamycin resistance gene (Kan^R) as a selectable marker, T7 promoter sequence, N-terminal His-tag and a multiple cloning site. The map of pET28a(+) expression vector is presented in Fig 3.2.

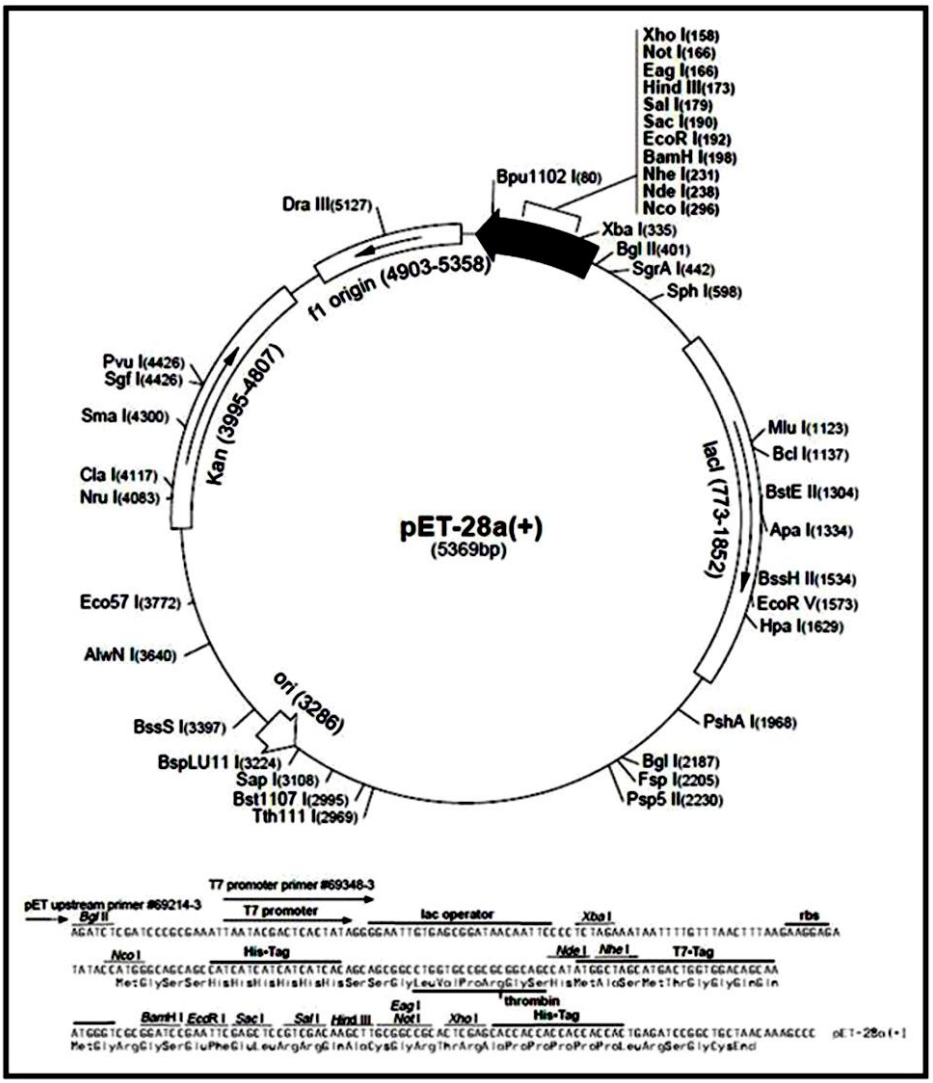

Fig. 3.2 pET28a(+) expression system

3.5.16.1 Isolation of pET28a(+) plasmid DNA from *E. coli* DH5α cells

The pET28a(+) expression vector (Novagen, USA) was earlier mobilised into *E. coli* DH5α competent cells and the glycerol stock of the same was preserved at -80 ºC for long term storage in the laboratory of Dr. Gurvinder K. Saini, Indian Institute of Technology Guwahati, India. For the present study, 5 µl of the *E. coli* DH5α glycerol stock harbouring the pET28a(+) plasmid was

inoculated into 50 ml of fresh LB medium supplemented with kanamycin (50 µg/ml) and incubated at 37 ºC overnight. The pET28a(+) plasmid DNA was isolated from the overnight grown culture by the alkaline lysis method (Sambrook *et al.*[131]).

3.5.16.2 Restriction digestion of pET28a(+) plasmid DNA and CelT3 cellulase gene

The pET28a(+) plasmid DNA and PCR amplified CelT3 cellulase gene were separately digested by two restriction enzymes *Nde*I and *Hin*dIII, each. The separate restriction digestion reactions were carried out overnight at 37°C. The enzymes were inactivated by incubating the reaction mixtures at 70°C for 10 min. The restriction digestion reaction was set up as presented below:

Components	Volume
pET28a(+) plasmid DNA/ CelT3	10.0 µl
Cut smart 10X buffer	2.0 µl
Enzymes (*Nde*I and *Hin*dIII) (10 U/µl)	1.0 µl
Nuclease free water	7.0 µl
Total volume	20.0 µl

3.5.16.3 Ligation of the CelT3 cellulase gene into pET28a(+) plasmid DNA

Purified cellulase encoding gene CelT3 was mixed with the digested and dephosphorylated pET28a(+) vector DNA in the ratio of 3:1. The ligation reaction was set as described below:

Components	Volume
10X T4 ligase buffer	2.0 µl
pET28a(+) vector	4.0 µl
CelT3 gene	13.0 µl
T4 DNA ligase (3 U/µl)	1.0 µl
Total volume	20.0 µl

The reaction mixture was incubated at 16°C overnight. The construct was named as pET28CT3.

3.5.17 Transformation of *E. coli* DH5α with the recombinant plasmid pET28CT3

The recombinant plasmid pET28CT3 [pET28a(+) + CelT3] was transferred into competent cells of *E. coli* DH5α cells as discussed in the section 3.5.6.2.

3.5.18 Retransformation of pET28CT3 into *E. coli* BL21(DE3)

On being established in *E. coli* DH5α cloning host, the CelT3 protein expression was initiated by transferring the pET28CT3 recombinant plasmid into *E. coli* BL21(DE3) expression host containing a chromosomal copy of the T7 RNA polymerase gene. This gene is inserted into the chromosome of *E. coli* BL21 by lysogenizing it with T7 phage. T7 RNA polymerase gene is transcribed under the *lac* promoter; therefore, it will be expressed only if IPTG is added to the culture medium. The T7 RNA polymerase will then transcribe the CelT3 gene cloned into the pET28a(+) vector (Fig. 3.3).

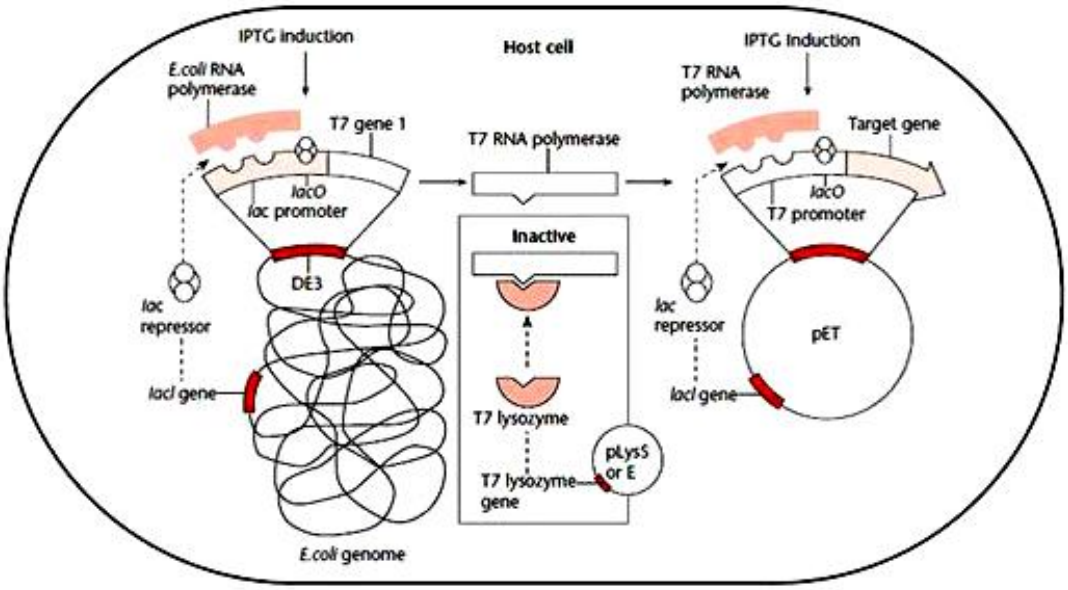

Fig. 3.3 Strategy for regulating the expression of genes cloned in a pET vector. (Adopted from the Novagen Catalog, p. 24, Novagen, Madison, Wisconsin, 1995)

3.5.18.1 Isolation of recombinant plasmid pET28CT3 from *E. coli* DH5α

The recombinant plasmid was isolated from *E. coli* DH5α cells using the plasmid DNA miniprep purification kit (HiMedia, India) according to manufacturer's instructions.

3.5.18.2 Transformation of pET28CelT3 into *E. coli* BL21(DE3) cells

The recombinant plasmid pET28CT3 containing the cellulase gene was transferred into the competent cells of *E. coli* BL21(DE3) cells as discussed in the section 3.5.6.2.

3.5.19 Preservation of recombinant plasmid pET28CelT3

Since *E. coli* BL21(DE3) has fully functional endonuclease and recombinase pathways, the plasmids that are stored in *E. coli* BL21(DE3) get damaged or rearranged after some time. Therefore, the isolated recombinant plasmid pET28CelT3 was transferred to *E. coli* DH5α cells and preserved at -80°C. The isolated and purified recombinant plasmid DNA was also stored at -20°C.

3.5.20 Confirmation of cellulase production by transformed *E. coli* BL21(DE3) cells

A loopful of transformed *E. coli* BL21(DE3) colony was grown overnight in LB medium containing kanamycin (50 µg/ml) in orbital shaker. The cells were harvested and subjected to sonication (Sonic Ruptor, OMNI International, India). The supernatant was then added to wells (6 mm) punctured out of CMC agar plate supplemented with 1% (v/v) CMC and incubated at 37°C for 24–36 h. Cellulolytic activity was confirmed based on the formation of distinct yellow halo around the well on staining with Grams iodine.

3.6 Phylogenetic tree construction of CelT3 cellulase

To determine the evolutionary relationship of the metagenome-derived CelT3 cellulase with the established cellulolytic enzymes, the cellulase gene sequence was translated into a protein sequence using Translate tool of Expasy server (http://web.expasy.org/translate/). Subsequently the amino acid sequence was subjected to protein BLAST (BLASTp) analysis of NCBI. Sequences that showed similarity to the query sequence were then used to construct a phylogenetic

tree using Neighbor-Joining method. One thousand bootstrap replications were performed using the program MEGA 5.2.

3.7 Overexpression of the recombinant protein

E. coli BL21(DE3) cells carrying the recombinant plasmid pET28CT3 with CelT3 gene insert were initially cultured overnight at 37°C in 5 ml liquid LB medium containing kanamycin (50 µg/ml) in orbital shaker. Next day 1% (v/v) of the overnight grown culture was inoculated in a flask containing 500 ml of LB medium supplemented with kanamycin (50 µg/ml). The culture was grown at 37°C in orbital shaker till the optical density (OD) reached the log phase i.e. $\mathbf{A_{600}}$**~0.5**. The culture was then induced with 0.1-0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5h at 37°C. The cells were harvested by centrifugation at 10,000 rpm for 10 min in a cold centrifuge.

3.8 Recombinant protein purification

Reagents used:

- Lysis buffer (pH 8.0)
  50 mM Sodium phosphate (NaH_2PO_4)
  300 mM NaCl
- 100 µl Ni-NTA Agarose beads

- Equilibration buffer (pH 8.0)
  50 mM sodium phosphate buffer
  300 mM NaCl

- Washing buffer (pH 8.0)
  50 mM sodium phosphate buffer
  300 mM NaCl
  10 mM imidazole

- Elution buffer (pH 8.0)
  50 mM sodium phosphate buffer
  300 mM NaCl
  20, 40, 80, 120, 160 mM, 200 mM imidazole

The protocol

Bacterial cells containing the recombinant protein were washed in 1 ml lysis buffer, vortexed for homogeneity, and subsequently disrupted by sonication (Sonic Ruptor, OMNI International, India). The supernatant was centrifuged at 13,000 rpm for 30 min at 4ºC and then purified by immobilized-metal affinity chromatography (IMAC) using Ni^{2+}-NTA agarose matrix (Bio-Rad, USA).

Prior to adding the protein for purification, 2 ml Ni^{2+}-NTA (Nickel-Nitrilotriacetic acid Agarose) gel was added to the Ni-NTA column and equilibrated with two volumes of the equilibration buffer. The supernatant was then loaded into Ni-NTA column. The column was kept at 4°C for 2 h after adding the supernatant to the column to allow the protein to bind to the beads. The column was washed with buffer containing 10 mM imidazole. After washing off the background proteins, a homogeneous protein was eluted with a gradient of (20, 40, 80, 120 and 160 mM and 200 mM) imidazole. The eluted and purified protein was analysed by electrophoresis in 12% SDS polyacrylamide gel.

3.9 Determination of molecular weight of the protein by SDS-PAGE

Reagents used:

12% resolving gel composition

Components	Volume
Distilled Water	3.3 ml
30% Acrylamide soln.	4.0 ml
1.5 M Tris.HCl (pH 8.8)	2.5 ml
10% SDS	100 µl
10% Ammonium per sulfate	80 µl
TEMED	2.0 µl
Total volume	10.0 ml

5% stacking gel composition

Components	Volume
Distilled Water	2.7 ml
30% Acrylamide soln.	0.67 ml

1.5 M Tris.HCl (pH 6.8)	0.5 ml
10% SDS	40 µl
10% Ammonium per sulfate	40 µl
TEMED	4.0 µl
Total volume	2.0 ml

1x SDS gel loading buffer:

50 mM Tris.HCl (pH 6.8)
100 mM dithiothreitol
2% SDS
0.1% Bromophenol blue
10% Glycerol

The protocol

Denaturing PAGE was performed according to the method of Laemmli[132]. Each of the samples collected viz. uninduced, induced, and purified protein were resuspended in 1X gel loading buffer. The samples were heated for 5-6 min in a boiling water bath and kept on ice for 2 min prior to loading. Electrophoresis was carried out with a constant current of 15 mA until the dye front (bromophenol blue) reached the bottom of the gel. The gel was removed from the glass plates and then soaked overnight in coomassie brilliant R-250 in methanol:acetic acid:water (4:1:5 v/v/v) for staining. On the next day, the gel was destained using distilled water and then scanned in a Biospectrum 500 Imaging system, India. Mobility of the purified protein was compared with the PM2510 ExcelBand Protein ladder.

3.10 Quantification of total protein

For the assessment of different parameters viz. fold purification, yield (%) and specific activity, the recombinant protein was purified at a large scale followed by concentration using Centricon.

Cellulase activities were expressed as micromoles of glucose equivalent liberated per minute per mL of enzyme solution (U ml^{-1}). Specific activities were expressed as U mg^{-1} protein.

The total protein content was quantified by Folin-Lowry method[133] using Bovine Serum Albumin (BSA) as the protein standard. The BSA concentration

ranging from 50-750 μg/ml was used to make the standard curve for the protein. The protein content of the sample was calculated from the standard curve obtained by plotting of absorbance vs concentration of BSA (1.0 mg ml^{-1}). The absorbance was measured at 562 nm.

3.11 Homology model and validation for protein structure prediction

For the structure prediction of the recombinant cellulase, the CelT3 nucleotide sequence was translated into amino acid sequence using Translate tool of ExPASy online server. The homology modelling was performed using sequence alignment of the amino acid sequence against Protein Data Bank (PDB) by BLAST algorithm. The template was selected on the basis of maximum sequence identity with the target sequence. Homology model was constructed using Swiss-PDB viewer v. 4.0.1 program. The stereo-chemical quality of the protein was evaluated using Ramachandran plot obtained from the RAMPAGE server[134]. Three dimensional ribbon model for protein was generated using Molegro-Molecular Viewer v.2.5.0.

3.12 Biochemical characterization of the purified cellulase

All the assays were performed in triplicate with proper substrate and enzyme controls.

3.12.1 Determination of specific activity of the purified cellulase

The 3,5-dinitrosalicylic acid (DNS) method[135] was used to quantify the amount of reducing sugars produced after enzymatic reaction with some modifications. The reaction is based on the principle that DNS is reduced to 3-amino-5-nitrosalicylic acid in the presence of reducing sugars.

Reagents used:

- 1% Dinitrosalicylic acid solution

  Dinitrosalicylic acid: 10 g

  Sodium sulfite: 0.5 g

  Sodium hydroxide: 10 g

  Add water to: 1 litre
- 40% Sodium potassium tartrate solution (Rochelle salt)

The protocol

The purified recombinant cellulase (1 ml) was added to 1 ml of 1% CMC and the tubes were incubated for 5 min at 45ºC in a water bath. 2 ml of dinitrosalicyclic acid (DNS) was then added and the tubes were subsequently kept in a boiling water bath for 5 min. Thereafter, 1 ml of sodium potassium tartrate was added. After cooling the tubes, the absorbance was recorded at 540 nm using a UV-Vis spectrophotometer (Cecil 7400, Cambridge).

3.12.2 Substrate specificity

To determine the substrate specificity of the purified recombinant cellulase, various substrates such as CMC, Avicel, filter paper and laminarin were used at a final concentration of 1% (v/v) and incubated at 45°C for 30 min. The cellulolytic activity was assayed by DNS method under the standard conditions.

3.12.3 Determination of K_m and V_{max} for the enzyme catalysed reactions

A constant amount of enzyme was incubated with graded concentration (0.1-2.0%) of CMC for 10 min at 45°C. The Michaelis constant (K_m) and maximum rate of reaction (V_{max}) catalysed by the purified enzyme was calculated using Lineweaver-Burk plot[136]. Lineweaver-Burk plot was drawn using Graph Pad Prism 6 software. The cellulase enzyme activity was estimated using DNS method under the standard conditions.

3.12.4 Effect of pH on the catalytic activity of cellulase

To determine the optimum pH for the catalytic activity of the purified cellulase, the enzyme assay was carried out by using buffers at different pH values ranging from 4.0-10.0. Buffers used to maintain the respective pH were Sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–8.0), Tris-HCl (pH 8.0–9.0) and glycine-NaOH (pH 9.0–10.0).

3.12.5 Effect of temperature on the catalytic activity of cellulase

In order to assess the effect of temperature on the catalytic activity of cellulase, the enzyme was incubated with the substrate at varying degrees of temperature ranging from 15 to 75°C followed by the estimation of enzyme activity under the standard conditions. Each reaction mixture was pre-incubated for 30 min at the designated temperature prior to the assay.

3.12.6 Effect of metal ions on the catalytic activity of cellulase

To investigate the effect of divalent cations on the activity of cellulase enzyme, the assay was performed with the purified protein in the presence of different metal ions like Ca^{2+}, Fe^{2+}, Ni^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+}. The activity of the enzyme without the addition of metal ions served as the control and was considered as 100%.