Chapter 4: Results

Over the years, the demand for more efficient cellulases has increased owing to the cost-intensive deconstruction of cellulosic biomass. Moreover, the limitations related to the cellulase enzymes from culturable bacteria such as slow activity and inability to stand extremes of temperature and pH make it inevitable to look for new cellulase enzymes that are more efficient and cost-effective. In the light of the stated facts, there is a need to look for cellulase enzymes beyond the culturable domain with the exploitation of genomes of unculturable bacteria through metagenomics.

Results obtained following the Materials and Methods described in Chapter 3 are presented below:

4.1 Extraction of metagenomic DNA (mgDNA)

The mgDNA from the goat rumen digesta was isolated using the method P5 developed in the laboratory (Bashir *et al.*[128]). The isolated mgDNA was electrophoresed on 0.8% agarose gel along with GeneRuler 1 kb plus DNA ladder (Thermo Scientific, USA) which showed high-molecular-weight and quality DNA. The size of the extracted mgDNA was found to be > 23 kb and the agarose gel electrophoresis image of the same is shown in Fig. 4.1.

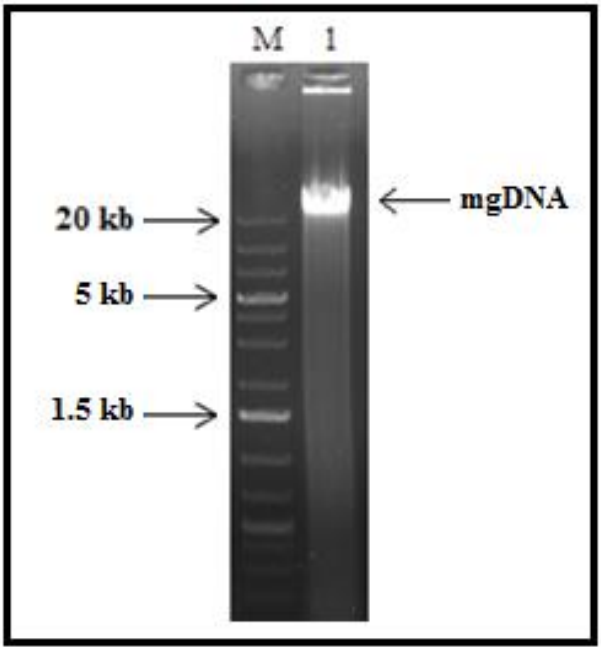

Fig. 4.1 mgDNA from goat rumen digesta. Lane 1: mgDNA isolated from goat rumen digesta; lane M: GeneRuler 1 kb plus DNA ladder

4.1.1 Spectrophotometric assessment of yield and purity of mgDNA

DNA was quantified spectrophotometrically (A_{260} nm, Cecil 7400, Cambridge) and the purity of DNA was assessed from A_{260}/A_{230} and A_{260}/A_{280} ratios spectrophotometrically to check possible contamination of DNA with tannins and proteins, respectively. Yield and purity of the isolated mgDNA are presented in Table 4.1.

Table 4.1 Yield and purity of mgDNA

Sample	**Crude DNA (µg/g digesta)**	**A_{260}/A_{280} ratio**	**A_{260}/A_{230} ratio**
Goat rumen digesta	240.6±8.1	1.75	0.9

4.1.2 Comparison of mgDNA extraction methods

The mgDNA isolated by five different methods (P1–P5) was compared on the basis of yield, purity, processing time, suitability for PCR and restriction digestion by *Eco*RI and *Bam*HI restriction endonucleases. The purity and yield of the isolated mgDNA was calculated measuring absorbance at 230, 260 and 280 nm in the UV/VIS spectrophotometer (Cecil 7400, Cambridge). The highest DNA yield was obtained in the case of P5, followed by P4. The mgDNA extracted using P5 method represented both high yield with low contamination of proteins and tannins in comparison to the other methods. Data thus obtained are presented in Fig 4.2, Table 4.2, and Fig 4.3.

Genomic DNA was isolated from *E. coli* (MTCC 40) using the method P5 (section 3.4.1) to confirm the suitability of this method for the isolation of genomic DNA from the culturable bacteria. The method gave high yield of DNA from *E. coli* (MTCC 40) as shown in agarose gel electrophoresis image (Fig. 4.2), thus expanding the scope of usage for P5 extraction method to culturable bacteria as well.

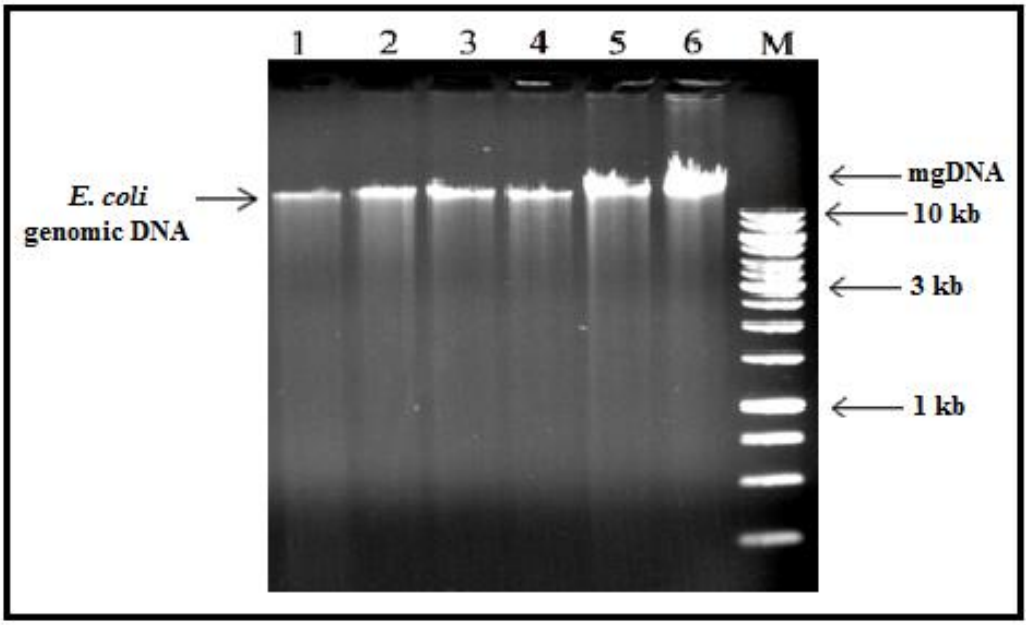

Fig. 4.2 mgDNA extracted from goat rumen digesta using different methods. Lane 1: genomic DNA extracted from *E. coli* (MTCC 40) using method P5; lanes 2 to 6: mgDNA extracted using methods P1–P5, respectively; lane M: 1 kb DNA ladder

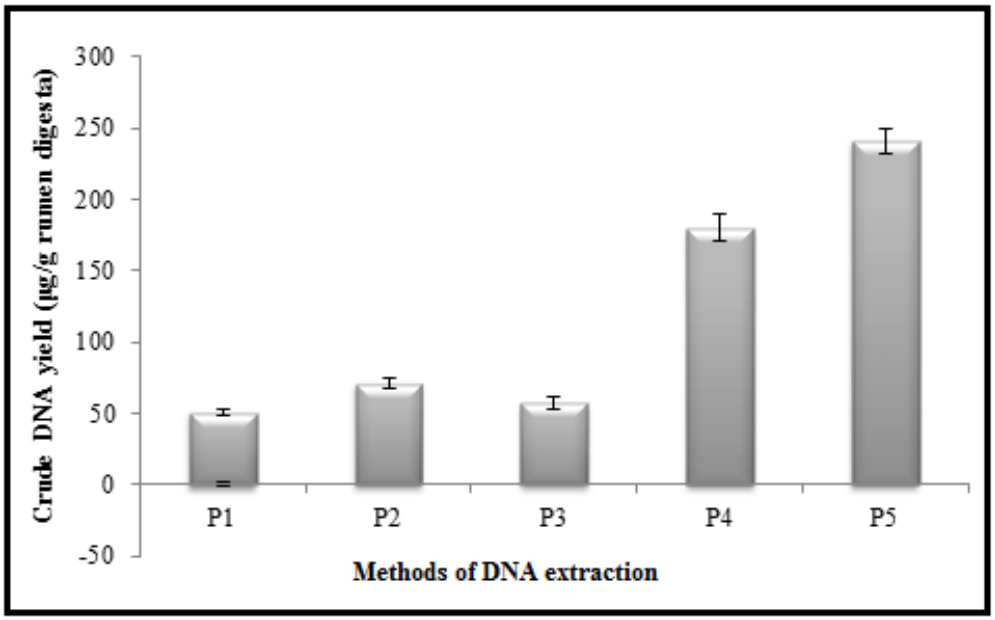

Fig. 4.3 Bar graph plots on the comparative analysis of five different extraction methods of DNA (P1–P5)

Table 4.2 Yield, purity and other useful parameters of crude mgDNA isolated by five different methods

Method	Crude DNA yield (µg/g rumen digesta)	Absorbance ratios		Processing time (h)	16S rDNA amplification (PCR)	Endonuclease activity		References
		A_{260}/A_{280}	$A_{260/230}$			*Eco*RI	*Bam*HI	
P1	50±2.4	1.6	0.5	5	–	–	–	124
P2	70.2±3.7	1.2	0.8	3	–	–	–	125
P3	56.8±3.9	1.3	0.6	2.5	–	–	–	126
P4	180±9.7	1.65	0.7	>8	+	+	+	127
P5	240.6±8.1	1.75	0.9	3.5	+	+	+	This study

Values are mean ± standard error of three replicates.

+: methods are showing suitability for PCR and endonuclease activity

–: methods are not showing suitability for PCR and endonuclease activity

4.1.3 Assessment of quality of mgDNA for PCR amplification

All five methods of mgDNA extraction (P1-P5) were evaluated to confirm the suitability of the extracted mgDNA for the subsequent molecular analyses. The PCR amplification of 16S rDNA from mgDNA isolated by five methods is shown in agarose gel electrophoresis image below (Fig. 4.4).

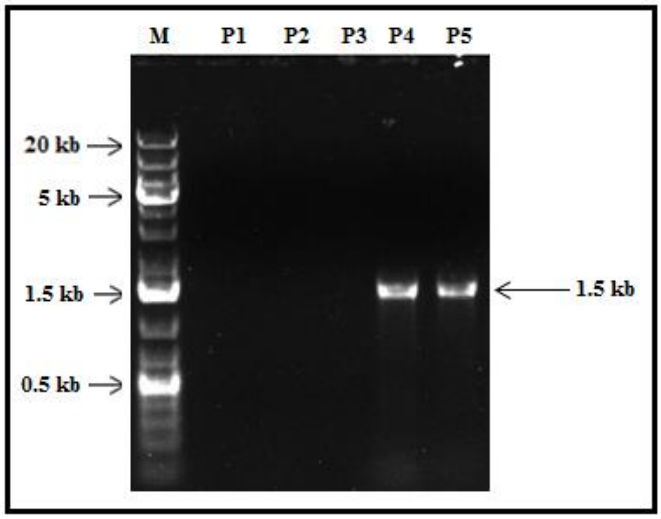

Fig. 4.4 PCR amplification of 16S rRNA gene. Lane M: 1 kb plus DNA ladder; lanes 1 to 3: methods P1 to P3, respectively (no PCR product); lanes 4 and 5 represent methods P4 and P5, respectively, yielding the amplified product (~1,500 bp)

4.1.4 Testing for restriction digestion of mgDNA

The mgDNA was digested with *Eco*RI and *Bam*HI restriction enzymes. The digested mgDNA was electrophoresed and the same is presented in Fig. 4.5.

Fig. 4.5 Restriction digestion of mgDNA isolated by P5 method. Lane M: λ-*Hin*dIII DNA marker; lanes 1 and 2: restriction digestion of mgDNA with *Eco*RI and *Bam*HI, respectively

4.2 Construction of mgDNA library

The mgDNA library was constructed successfully using the pUC19 cloning vector. Further, the recombinants were selected on the basis of blue–white screening after overnight incubation at 37°C temperature.

4.2.1 Partial restriction digestion of mgDNA

The mgDNA was partially digested with *Bam*HI restriction enzyme. It was resolved on 0.8% agarose gel electrophoresis. The mgDNA fragments ranging from 0.5–2.0 kb were fractionated from the agarose gel and used for mgDNA library construction because the size of cellulase gene falls within this range. The agarose gel electrophoresis image of the same is represented in Fig. 4.6.

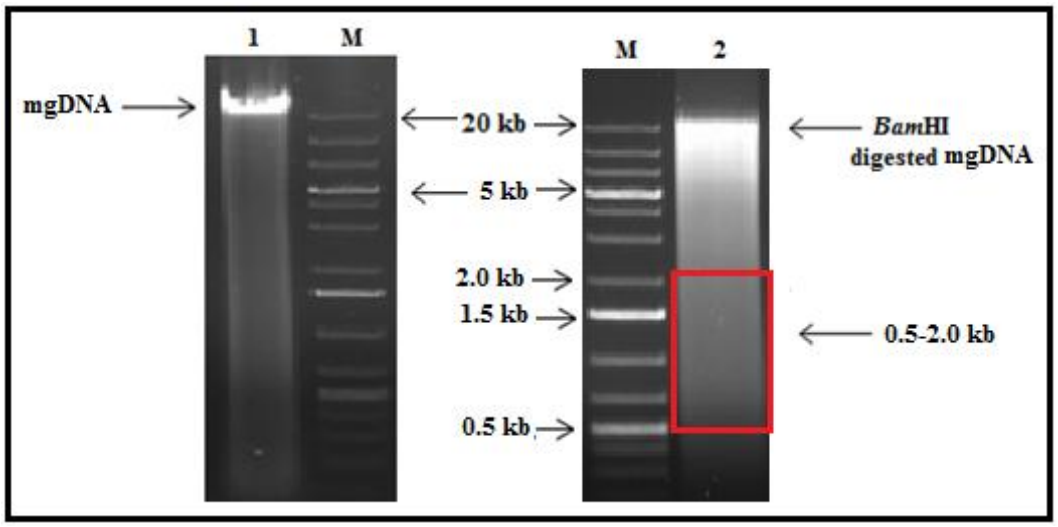

Fig. 4.6 mgDNA partially digested with *Bam*HI. Lane 1: mgDNA; lane M: 1 kb plus DNA ladder; lane 2: partial restriction digestion of mgDNA with *Bam*HI. mgDNA ranging from 0.5 kb-2.0 kb (highlighted in red) were eluted from the agarose gel

4.2.2 Isolation of pUC19 plasmid DNA from *E. coli* DH5α

The cloning vector pUC19 plasmid DNA was isolated from the host *E. coli* DH5α cells and electrophoresed along with 1.0 kb plus DNA ladder. Size of the isolated plasmid pUC19 DNA was 2,686 bp. The agarose gel electrophoresis image of the same is presented in Fig. 4.7.

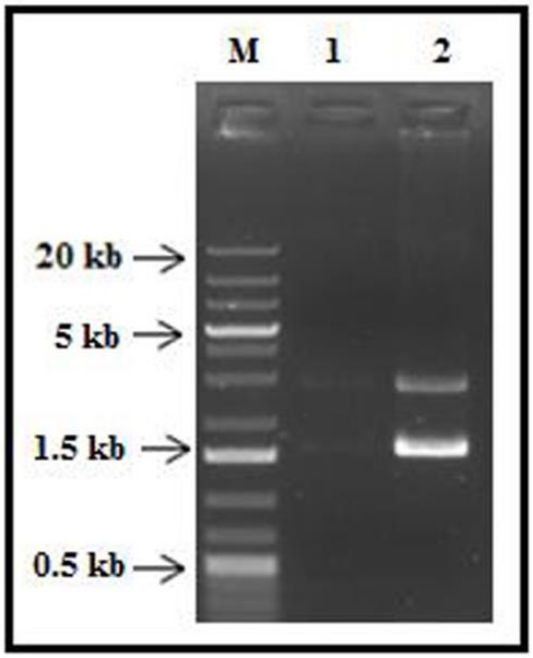

Fig 4.7 pUC19 plasmid DNA**.** Lane M: 1 kb plus DNA ladder; lanes 1 and 2: pUC19 plasmid DNA (2,686 bp)

The purity of pUC19 plasmid DNA was calculated by measuring absorbance at 260 and 280 nm in a UV/VIS spectrophotometer (Cecil 7400, Cambridge). The $A_{260/280}$ ratio of the isolated plasmid DNA was found to be 1.8. The DNA yield was calculated by measuring absorbance at 260 nm and the same is presented in Table 4.3.

Table 4.3 Yield and purity of pUC19 plasmid DNA

Sample	A_{260}/A_{280}	DNA yield (µg/ml)
pUC19 plasmid DNA	1.8	200

4.2.3 Linearization of pUC19 plasmid DNA

The pUC19 plasmid DNA was linearized with *Bam*HI restriction enzyme. The linearized pUC19 plasmid DNA was run on 0.8% agrose gel and the agarose gel electrophoresis image of the same is presented in Fig. 4.8.

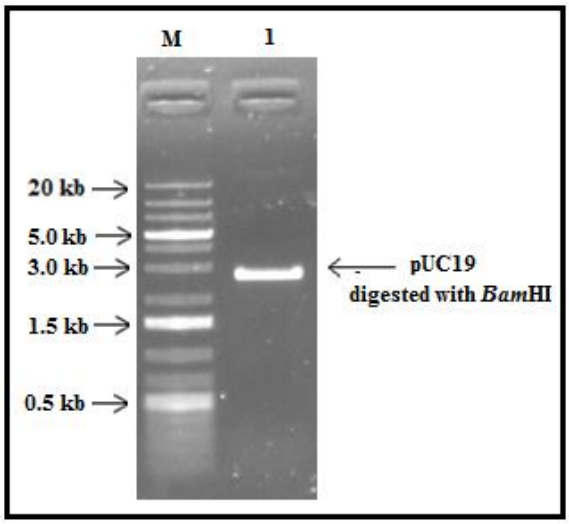

Fig. 4.8 *Bam*HI-digested pUC19 plasmid DNA on 0.8% agarose gel. Lane M: 1 kb plus DNA ladder; lane 1: linearized pUC19 plasmid DNA

4.2.4 pUC19 as the potential cloning vector

The normal pUC19 plasmid was transferred to *E. coli* DH5α bacterial cells so as to assess its transformation efficiency. The plasmid was diluted upto 0.25 µg/µl prior to the transformation experiment. The transformation efficiency was found to be 1 x 10^6 cfu/µg DNA.

4.2.5 Ligation of mgDNA and pUC19 plasmid DNA

The gel eluted mgDNA fragments ranging from 0.5-2.0 kb in size were ligated into the linearized pUC19 cloning vector using T4 DNA ligase. The ligated mixture was transferred into *E. coli* DH5α host cells by electroporation (200 Ω, 25 µF and 2.5 kV) using gene pulser (Biorad, USA). The transformation reaction mixture was plated on LBA+ ampicillin (100 µg/ml) + IPTG (0.1 mM) + X-gal (50 µg/ml) plates and incubated overnight at 37°C overnight. The library consisted of approximately 24,000 clones. Therefore, mgDNA library was constructed successfully using the DNA extracted by the method P5.

4.2.6 Assessment of mgDNA library

4.2.6.1 Isolation of recombinant pUC19 plasmid from the recombinant bacterial colonies

The mgDNA library was assessed by isolating the recombinant plasmid DNA from randomly chosen *E. coli* DH5α recombinant colonies (white colonies). All the plasmid DNAs showed a shift in their band size as compared to the control

pUC19 plasmid DNA (2,686 bp). The agarose gel electrophoresis image of the same is presented in Fig. 4.9.

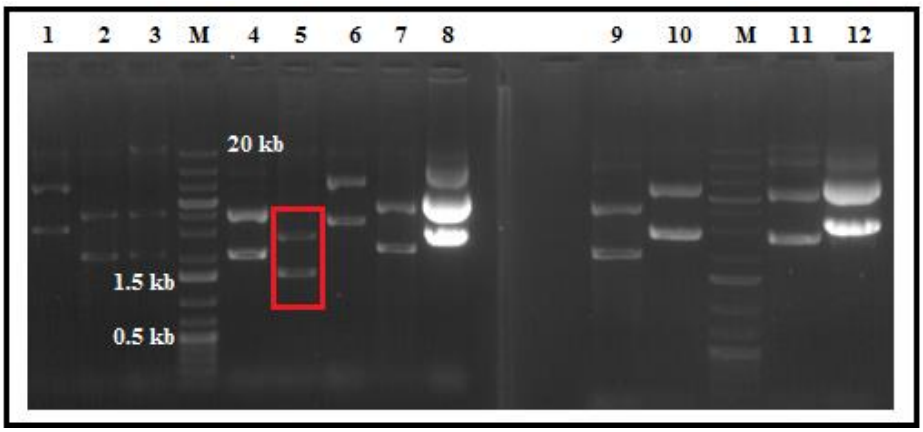

Fig. 4.9 Recombinant pUC19 plasmid DNA from randomly selected *E. coli* DH5α recombinant colonies. Lanes 1-4 and 6-12: recombinant plasmid pUC19 DNA isolated from randomly chosen transformants; lane 5: control pUC19 DNA without DNA insert (highlighted in red); lane M: 1 kb plus DNA ladder

4.2.6.2 Linearization of recombinant pUC19 plasmid DNA

The recombinant pUC19 plasmid DNA isolated from *E. coli* DH5α recombinant colonies was linearized with *Kpn*I restriction enzyme to confirm the presence of DNA inserts. All the bands showed a shift as compared to linearized non-recombinant pUC19 DNA (2,686 bp). The agarose gel electrophoresis image of the same is presented in Fig. 4.10.

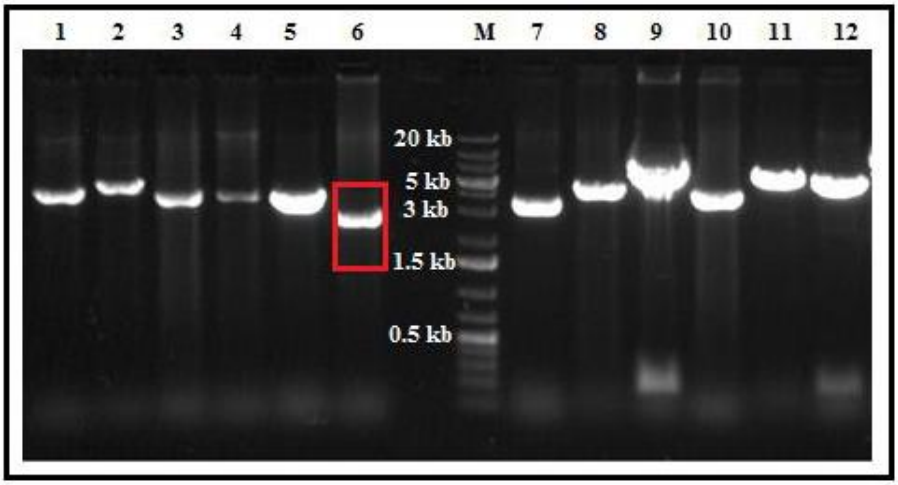

Fig. 4.10 Linearization of recombinant pUC19 plasmid DNA from randomly selected *E. coli* DH5α recombinant colonies using *Kpn*I. Lanes 1-5 and 7-12: *Kpn*I digested recombinant plasmid pUC19 DNA isolated from transformants; lane 6: control linearized pUC19 DNA without DNA insert (highlighted in red); lane M: 1 kb plus DNA ladder

4.3 Functional screening of mgDNA library for cellulolytic clone(s)

Approximately 2,000 recombinant bacterial colonies were screened for cellulolytic activity on CMC agar plates supplemented with 1% carboxymethyl cellulose (CMC) + 100 µl of ampicillin (100 mg/ml). The plates were incubated for 24-36 h. After screening the recombinant colonies for cellulolytic activity, only one was found to exhibit yellow halo around the colony after 36 h of incubation at 37°C and the same was designated as "T3". The recombinant bacterial colony showing the cellulolytic activity is presented in Fig. 4.11.

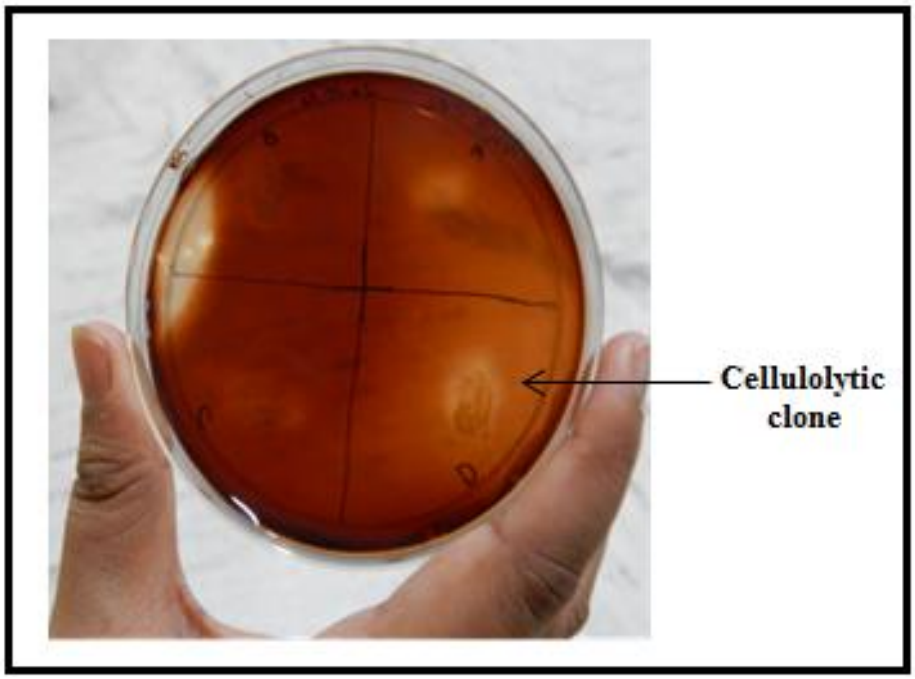

Fig. 4.11 Screening of mgDNA library for cellulolytic clones on CMC agar. Grams iodine staining showing the formation of yellow halo around cellulolytic clone 'T3'

4.4 Isolation of recombinant plasmid DNA from the cellulolytic T3 clone

The recombinant clone "T3" showing the cellulolytic activity was grown overnight in LB medium containing ampicillin (100 µg/ml) at 37°C. The recombinant pUC19 plasmid DNA was isolated from the clone and the agarose gel electrophoresis image of the same is presented in Fig. 4.12.

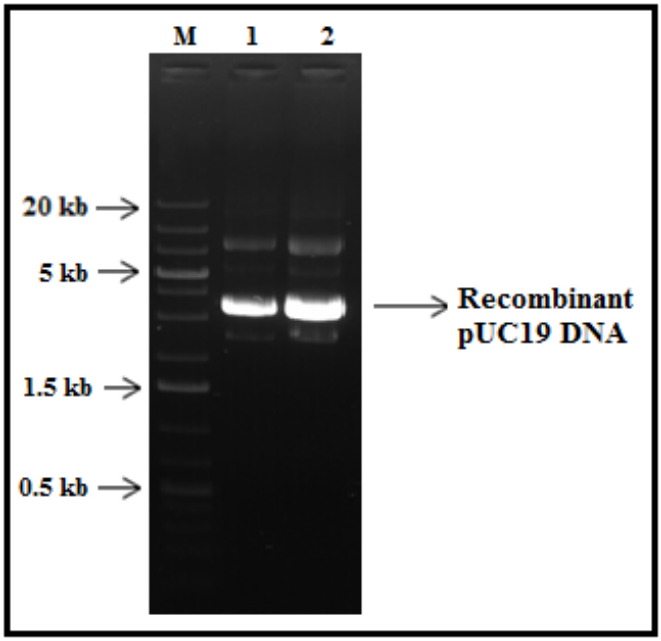

Fig 4.12 Recombinant pUC19 plasmid DNA from the cellulolytic T3 clone. Lane M: 1 kb plus DNA ladder; lanes 1 and 2: recombinant pUC19 plasmid DNA from T3 cellulolytic clone

The yield and purity of the isolated recombinant plasmid DNA was measured by taking absorbance readings at A_{260} and A_{280}. The same is presented in Table 4.4.

Table 4.4 Yield and purity of the recombinant pUC19 plasmid DNA

Sample	A_{260}/A_{280}	DNA yield (µg/ml)
Recombinant pUC19 DNA	1.75	266

4.4.1 Restriction digestion of recombinant plasmid DNA from the T3 clone

The recombinant plasmid DNA was digested with *Bam*HI in order to determine the size of the DNA insert expressing cellulolytic activity. The digested product was electrophoresed in 0.8% agarose gel along with 1 kb plus DNA ladder. The size of the DNA insert was observed to be ~1.5 kb. The agarose gel electrophoresis image of the same is presented in Fig. 4.13.

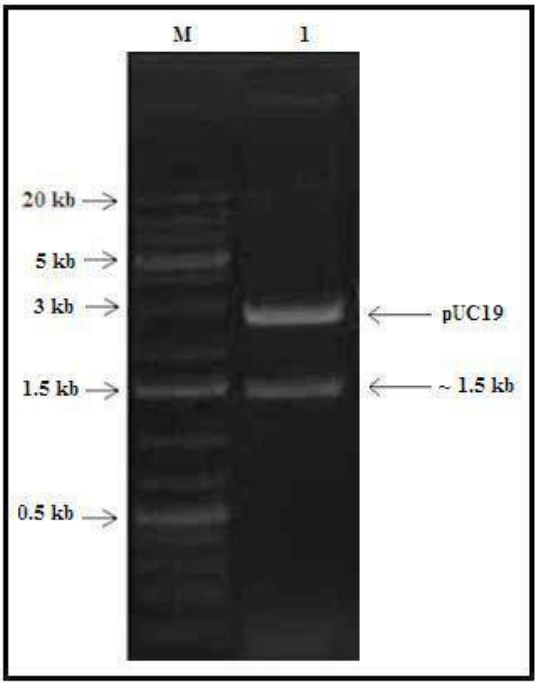

Fig. 4.13 *Bam*HI-catalysed digestion of the recombinant pUC19 plasmid containing CelT3 cellulolytic clone. Lane 1: cellulolytic DNA insert (~1.5 kb) released from pUC19 DNA; lane M: 1 kb plus DNA ladder

4.5 Sequencing and sequence analysis of cloned cellulase gene

The recombinant plasmid from cellulolytic bacterial clone T3 was sequenced using M13 Forward (-20) and M13 Reverse (-24) sequence specific primers. The sequence of recombinant pUC19 plasmid consisted of a DNA insert of 1,501 bp.

On subjecting the full sequence to the open reading frame (ORF) Finder of NCBI, an ORF of 1,122 bp was observed encoding a cellulase with 373 amino acids. This cellulase gene was named as 'CelT3' and is shown in Fig 4.14.

```
>CelT3
ATGACTACAGCAAAAACTCTCCGACGCCTGCTGGGCGCCGTGTTGGTGCTGCTATTAAGCAGCGCTGCTTTGGCC
GACAACGCCTGGGAAACCACCAGCGGCTGGTGGAATGCCAGCGATATTCCCGCCTTCGACAAAAGCAAAATCACT
CGCCAACTTCCGCTGATCAAGGTGGAAGGTAATCGCTTTGTGGATGAGCAGGGCAAGACAATCGTGTTTCGCGGC
GTTGATATTTCCGATCCCGACAAGATCGACAAAGACAAACGCTTTAGCAAAAAGCACAAAGAGGTGATTCGCAGC
TGGGGCGCCAATGTAGTTCGCGTACCGGTGCACCCACGTGCGTGGAAAGAGCGCGGCGTAAAAGGCTATTTAGAG
CTGCTGGATCAAGTGGTTGCCTGGAACAACGAGTTGGGCATCTACACCATCCTCGACTGGCACTCCATCGGCAAT
CTCAAGTCCGAAATGTTCCAAAACAATTCCTATCACACCACCAAAGGCGAAACCTTTGATTTGTGGCGCCGTGTG
TCCGAGCGCTACAACGGCATCAACTCTGTGGCCTTCTACAAAATTTTCAACGAGCCAACGGTATTCAATGGTCGC
TTGGGCATAGCCACCTGGGGGGAGTGGAAAGCCATCAATGAAGAGGCCATCACTATTATCCAAGCCCACAACCCC
AAGGCCATCGCTCTGGTCGCCGGTTTTAACTGGGCCTACGAGCTGAAAGAAGCTGCCGCCAACCCCATCGATCGC
CAGAACATCGCCTACGTCAGCCACCCCTACCCACAAAAAGTAGGCGCGCCATACCAAGCCAACTGGGACCGTGAT
TTTGGTTTTATAGCCGATCACTACCCGGTATTCGCCACTGAAATCGGCTACCAGCGCGCCACCGACAAAGGCGCC
CATATCCCCGTTATCGACGACGGCAGCTACGGCCCGCGCATCACCGACTACTTCAACAGCAAGGGCATCAGCTGG
GACGACTACTTCGACCCCGACTGGTCACCGCAACTCTTTACCGACTACCAAACCTACACCCCAACCATGCAGGGT
GAACATTTCCGCAAGGTGATGTTGCAGGACAATAAGAAGTATTATGAAGGTGATGCGGCCAAGAAGGTCTAG
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Fig: 4.14 DNA sequence of the CelT3 gene

4.6 GenBank submission

The cloned cellulase CelT3 gene sequence from the goat rumen digesta metagenome was deposited in the GenBank under the accession number KX226390.

4.7 Subcloning of CelT3 gene into pET28a (+) expression vector

4.7.1 PCR amplification of CelT3 gene from T3 clone

The cellulase encoding CelT3 gene was amplified using the primers mentioned in the section 3.5.13. The agarose gel electrophoresis image of the same is presented in Fig. 4.15.

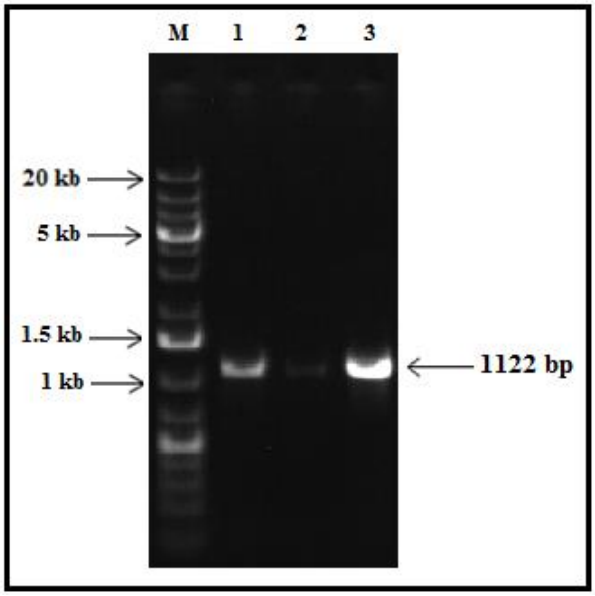

Fig. 4.15 PCR amplification of CelT3 gene. Lane M: 1 kb plus DNA ladder; lanes 1, 2 and 3: amplified CelT3 gene (1,122 bp)

4.7.2 Isolation of pET28a(+) plasmid DNA and its digestion with *Nde*I and *Hin*dIII

The pET28a(+) plasmid DNA (5,369 bp) was isolated from the *E. coli* DH5α bacterial cells and double digested with two restriction enzymes, *Nde*I and *Hin*dIII. The agarose gel electrophoresis image of the same is presented in Fig. 4.16.

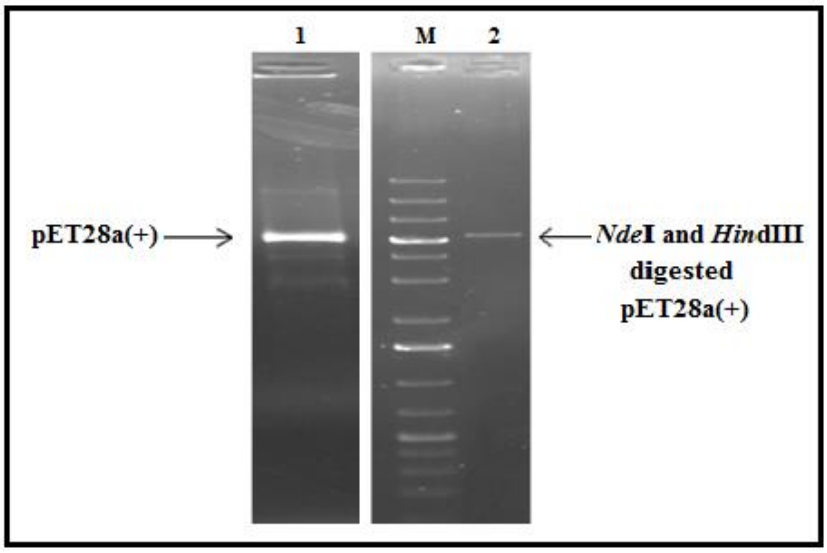

Fig. 4.16 pET28a(+) plasmid DNA and its double digestion with *Nde*I and *Hin*dIII. Lane 1: pET28a; lane M: 1 kb plus DNA ladder; lane 2: double digestion of pET28a(+)

4.7.3 Double digestion of CelT3 DNA fragment with *Nde*I and *Hin*dIII

The amplified CelT3 DNA fragment was double-digested with two restriction enzymes *Nde*I and *Hin*dIII. The agarose gel electrophoresis image of the same is presented in Fig. 4.17.

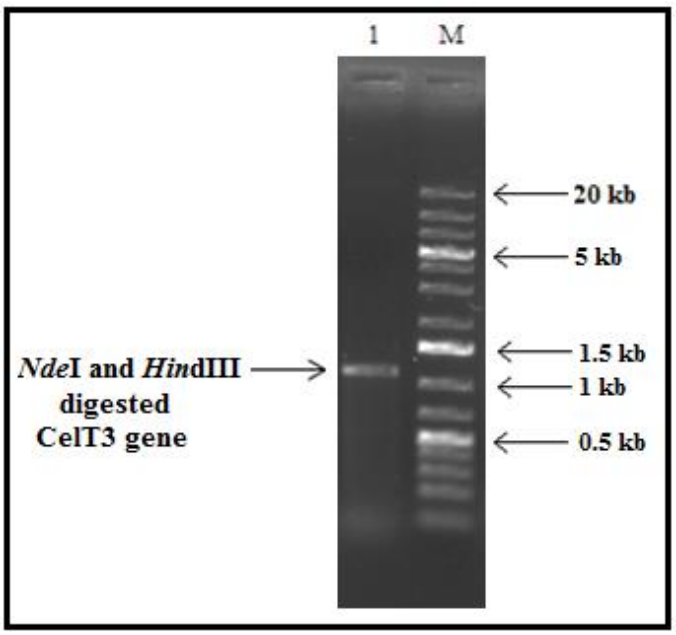

Fig. 4.17 Double digestion of CelT3 gene with *Nde*I and *Hin*dIII. Lane 1: double-digested CelT3 gene; lane M: 1 kb plus DNA ladder

4.7.4 Ligation of double-digested pET28a(+) DNA and CelT3 gene

The double digested pET28a(+) DNA and CelT3 gene were ligated in 1:3 ratio, respectively, at 16ºC overnight. The ligated product was named as pET28CT3.

4.7.5 Transformation of *E. coli* DH5α cells by pET28CT3

The ligated product (pET28CT3) was transferred into *E. coli* DH5α host cells by electroporation (200 Ω, 25 μF and 2.5 kV) using gene pulser (Biorad, USA). The transformation reaction mixture was plated on LBA+ kanamycin (50 μg/ml) medium and incubated overnight at 37°C. The transformation efficiency was high and the same is presented in Fig 4.18.

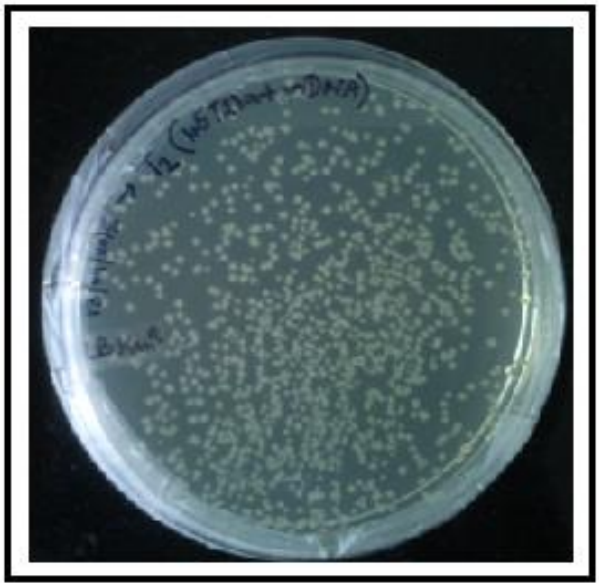

Fig. 4.18 Transformation of *E. coli* DH5α cells by pET28CT3

4.7.6 Restriction digestion of pET28CT3 with *Nde*I and *Hin*dIII enzymes

The plasmid DNA pET28CT3 isolated from *E. coli* DH5α cells was double digested with *Nd*e and *Hin*dIII restriction enzymes. A DNA insert of 1,122 bp was released from the pET28a(+) plasmid DNA confirming the presence of the DNA insert. The agarose gel electrophoresis image of the same is presented in Fig. 4.19.

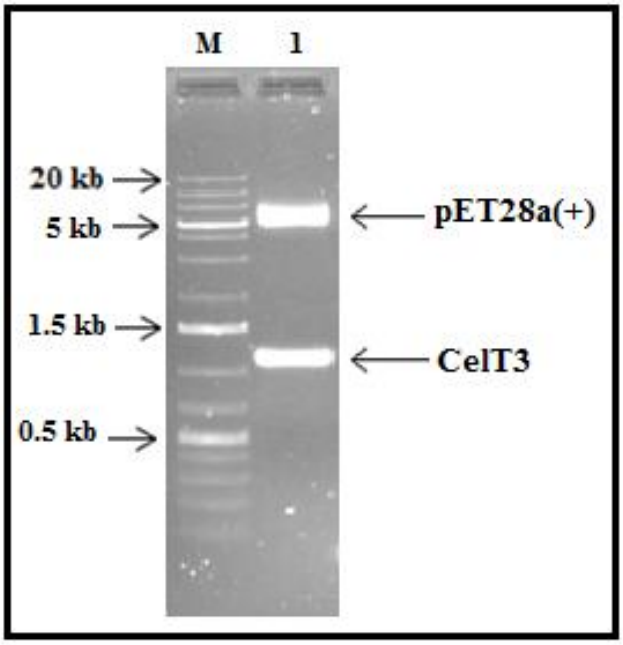

Fig. 4.19 Recombinant pET28a(+) DNA digestion with *Nde*I and *Hin*dIII. Lane M: 1 kb plus DNA ladder; lane 1: double digestion of pET28a(+) with *Nde*I and *Hin*dIII releasing 1,122 bp CelT3 gene

4.7.7 Isolation of pET28CT3 from *E. coli* DH5α cells

The recombinant plasmid pET28CT3 was isolated from *E. coli* DH5α colonies. The agarose gel electrophoresis image of the same is presented in Fig. 4.20.

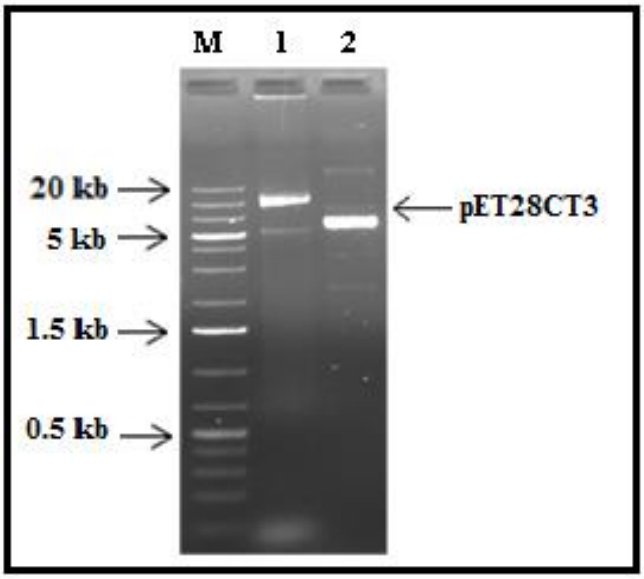

Fig. 4.20 pET28a(+) plasmid DNA from *E. coli* DH5α cells. Lane M: 1 kb plus DNA ladder; lane 1: pET28a(+); lane 2: recombinant pET28a(+) containing 1,122 bp DNA insert

4.7.8 Preservation of recombinant plasmid pET28CT3

The recombinant plasmid pET28CT3 was transferred into *E. coli* DH5α and then the recombinant bacterial colonies preserved at -80°C. The isolated and purified recombinant plasmid DNA was preserved at -20°C.

4.7.9 Transformation of *E. coli* BL21(DE3) cells by pET28CT3

The recombinant pET28CT3 was retransferred into *E. coli* BL21(DE3) cells by electroporation (200 Ω, 25 μF and 2.5 kV) using gene pulser (Biorad, USA). The transformation reaction mixture was plated on LBA+ kanamycin (50 μg/ml) medium and incubated overnight at 37°C.

4.7.10 Confirmation of cellulolytic activity in pET28CT3 transformed *E. coli* BL21(DE3) cells

The pET28CT3 transformed *E. coli* BL21(DE3) were grown overnight on LB medium. The cell free extract was then added to the CMC agar plate for confirming the expression of CelT3 gene and same is presented in Fig. 4.21.

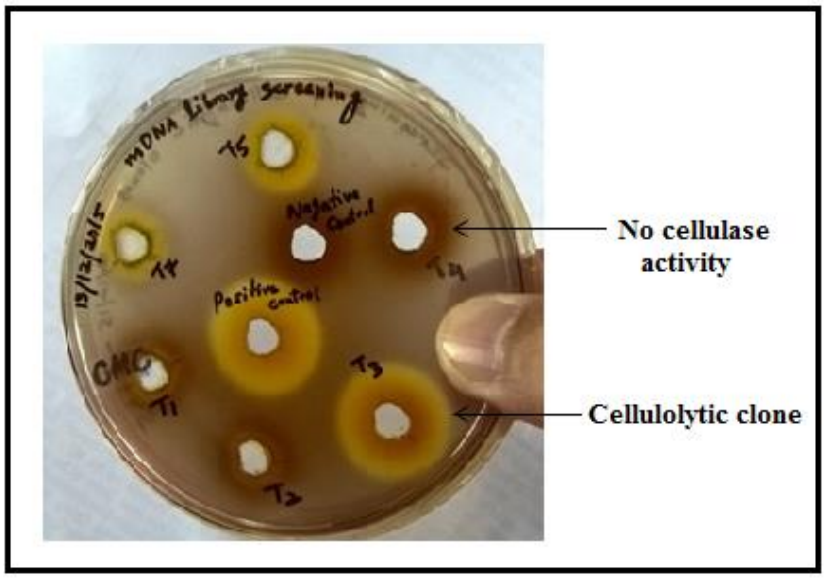

Fig. 4.21 Cell free extract of recombinant clone ["pET28a(+) with CelT3" in *E. coli* BL21(DE3)] showing cellulolytic activity on CMC agar plate. Positive control: Cellulase from *Trichoderma reesei* (ATCC 26921); Negative control: cell free extract of *E. coli* BL21(DE3) containing only pET28a(+) plasmid DNA

4.8 Recombinant protein expression

IPTG at 0.5 mM concentration was found to be optimum for the over-expression of CelT3 recombinant protein. On running the protein on 12% SDS-

PAGE, the protein was found to be ~45 kDa in size. The expressed and purified protein is shown in Fig. 4.22.

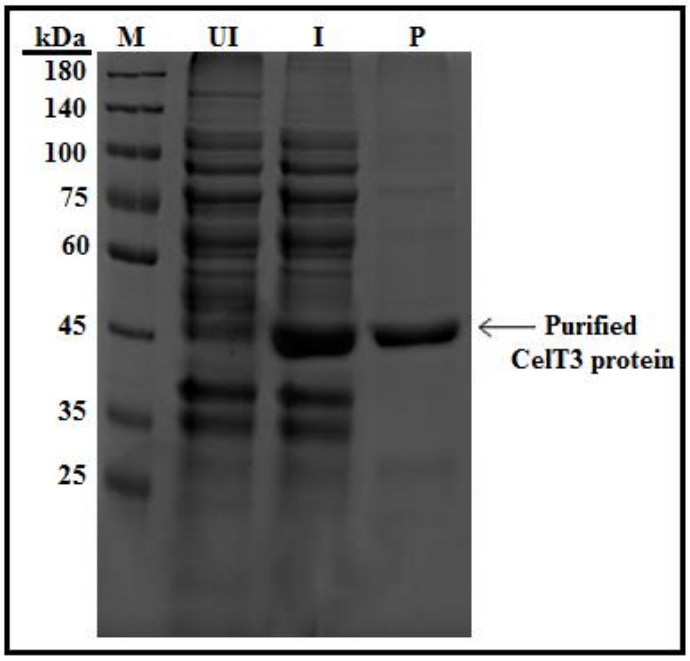

Fig. 4.22 SDS-PAGE of recombinant protein CelT3. Lane M: PM2510 ExcelBand Protein ladder; lane UI: pET28a(+) with CelT3 gene uninduced; lane I: pET28a(+) with CelT3 induced with 0.5mM IPTG; lane P: cellulase enzyme purified using Ni-NTA column

4.9 Protein sequence of CelT3 protein

The nucleotide sequence of CelT3 cellulase gene was translated into the amino acid sequence using ExPASy translate tool (http://expasy.org/translate). The amino acid sequence of the CelT3 protein was found to contain 373 amino acid residues as shown in Fig. 4.23.

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MTTAKTLRRLLGAVLVLLLSSAALADNAWETTSGWWNASDIPAFDKSKITRQLPLIK
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WKERGVKGYLELLDQVVAWNNELGIYTILDWHSIGNLKSEMFQNNSYHTTKGETFDL
WRRVSERYNGINSVAFYKIFNEPTVFNGRLGIATWGEWKAINEEAITIIQAHNPKAI
ALVAGFNWAYELKEAAANPIDRQNIAYVSHPYPQKVGAPYQANWDRDFGFIADHYPV
FATEIGYQRATDKGAHIPVIDDGSYGPRITDYFNSKGISWDDYFDPDWSPQLFTDYQ
TYTPTMQGEHFRKVMLQDNKKYYEGDAAKKV
```

Fig. 4.23 Protein sequence of cloned cellulase gene

The amino acid sequence of the CelT3 cellulase enzyme showed 96% sequence homology with the cellulase produced by an uncultured bacterium AFJ05146 (Table 4.5).

Table 4.5 Homologous search result of the cellulase gene (CelT3) using the Basic Local Alignment Tool (BLASTp) from NCBI

S. No.	Accession	Description	Max Score	Total Score	Query coverage	E-Value	Identity
1	AFJ05146	cellulase [uncultured bacterium]	720	720	97%	0.0	96%
2	4HTY.A	Chain A, Crystal structure of a metagenome-derived cellulase Cel5a	662	662	92%	0.0	93%
3	AAB61461	cellulase A [*Cellvibrio mixtus*]	642	642	97%	0.0	84%
4	WP007639596	glycoside hydrolase [*Cellvibrio* sp. BR]	639	639	92%	0.0	88%
5	WP012488945	glycoside hydrolase [*Cellvibrio japonicus*]	601	601	97%	0.0	77%
6	WP053094260	glycoside hydrolase [*Cellvibrio* sp. *pealriver*]	565	565	92%	0.0	76%
7	WP052417544	glycoside hydrolase [*Cellvibrio mixtus*]	562	562	95%	0.0	74%
8	ABA02176	cellulase [uncultured bacterium]	561	561	95%	0.0	74%
9	ACR23659	cellulase [bacterium enrichment culture clone CelA24]	561	561	95%	0.0	73%
10	WP062064832	glycoside hydrolase [*Cellvibrio* sp. OA-2007]	557	557	97%	0.0	72%
11	ACY24829	endo-1,4-beta-D-glucanase [uncultured organism]	462	462	83%	5e-161	68%
12	WP028875775	glycoside hydrolase [*Teredinibacter turnerae*]	368	368	94%	5e-123	52%
13	ADB80110	endoglucanase [uncultured microorganism]	301	301	75%	4e-97	50%
14	CAP07661	beta-glucanase [uncultured rumen bacterium]	299	299	80%	1e-96	47%
15	ADX05732	putative carbohydrate-active enzyme [uncultured organism]	299	299	80%	1e-96	46%
16	AAA27599	beta-endoglucanase [*Xanthomonas albilineans*]	258	258	97%	5e-80	43%

4.10 Phylogeny of CelT3 protein

The phylogenetic tree of CelT3 protein was generated using the Neighbor-Joining method. The data set was re-sampled 1,000 times by using the bootstrap option and the percentage values are presented at the nodes. The same is presented in Fig. 4.24.

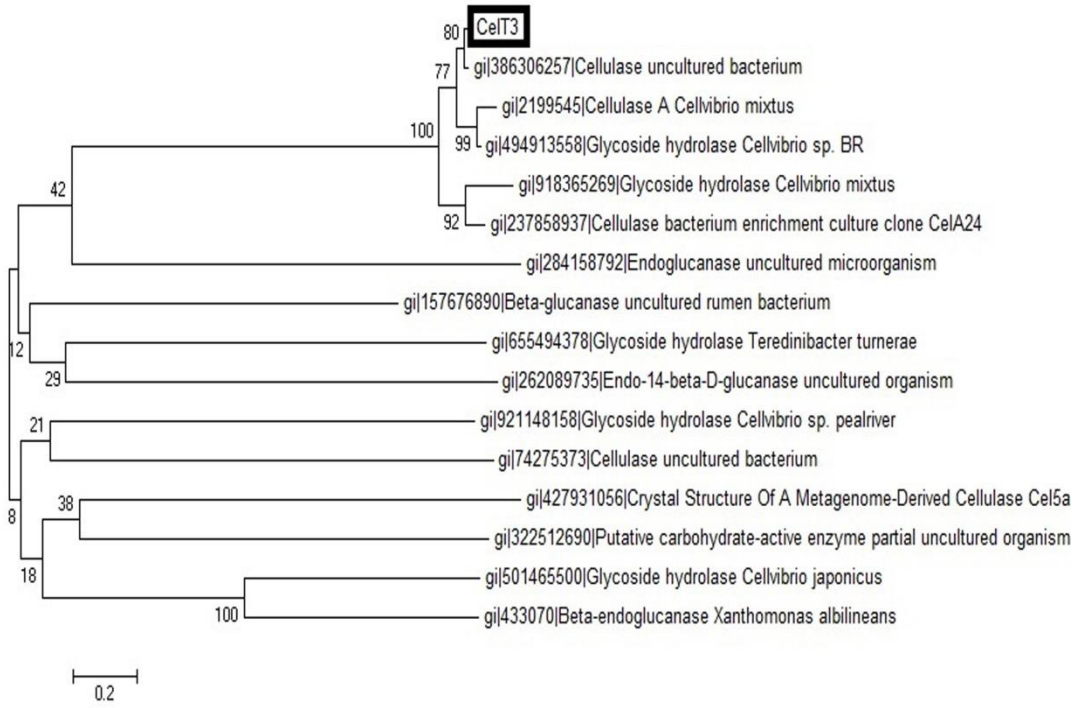

Fig. 4.24 Phylogenetic tree of CelT3 protein. Bootstrap values for 1000 replications and Poisson correction were performed. The bar represents 0.2 changes per amino acid

4.11 Multiple sequence alignment

On BLASTp analysis, the CelT3 cellulase enzyme showed similarity to cellulases belonging to glycosyl hydrolase family 5 (GH5). Multiple sequence alignment of GH5 cellulases with CelT3 recombinant cellulase was done to check the presence of conserved sequences and the same is represented in Fig. 4.25.

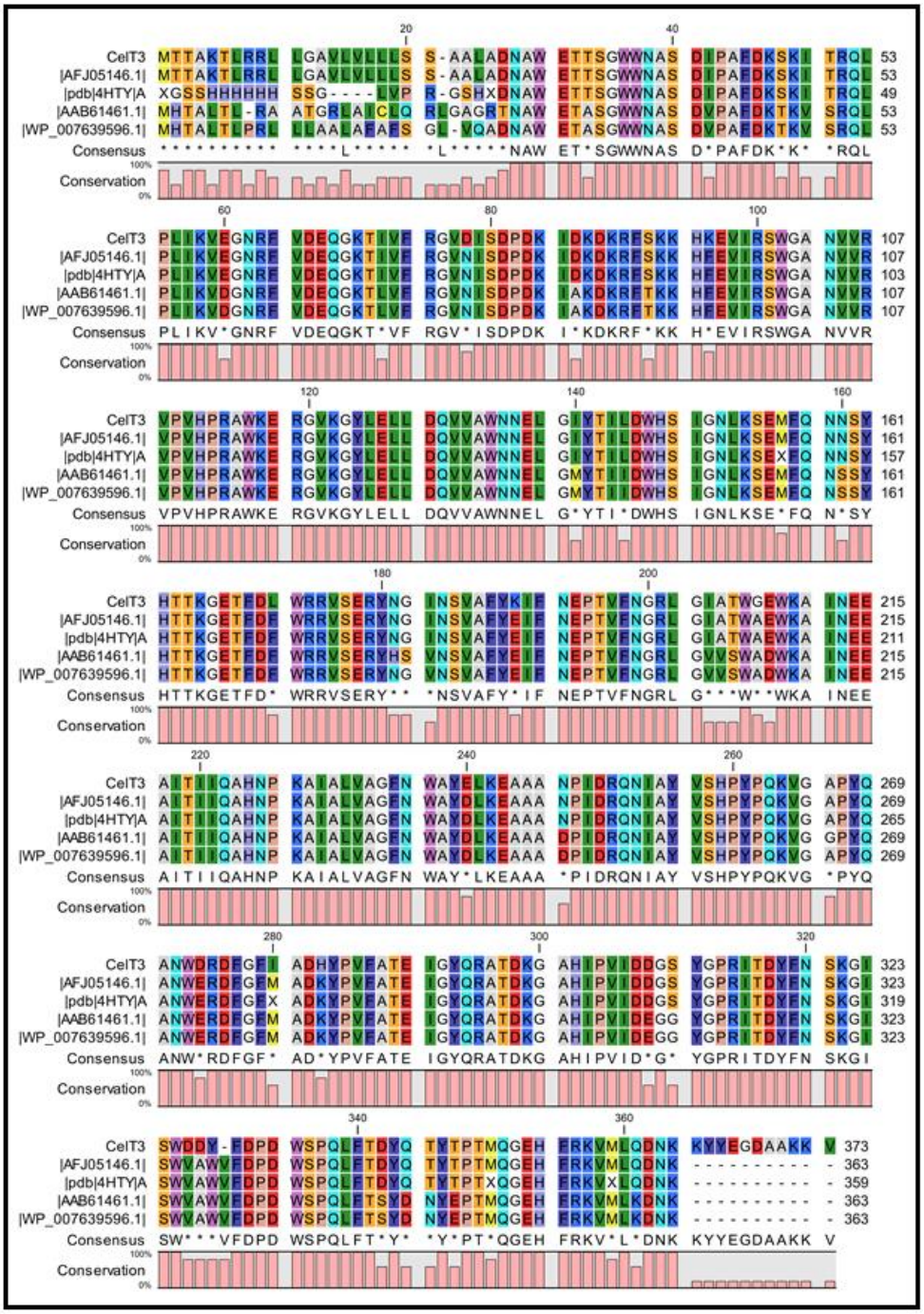

Fig. 4.25 Multiple sequence alignment of CelT3 with GH5 cellulases

4.12 Homology modeling and protein structure prediction

To determine the protein structure, the CelT3 gene sequence was translated into amino acid sequence using ExPASy online server. The protein sequence contained 373 amino acids. The homology modelling was done using the sequence alignment of the amino acid sequence of CelT3 against the protein data bank. Based on the alignment, a model of CelT3 was made using a metagenomic

cellulase (PDB ID: 4HTY) as the template. The modelled enzyme consists of 21 coils, 11 β-strands and 10 α-helices. The ribbon model was generated using Molegro-Molecular Viewer v. 2.5.0 and the same is shown in Fig. 4.26.

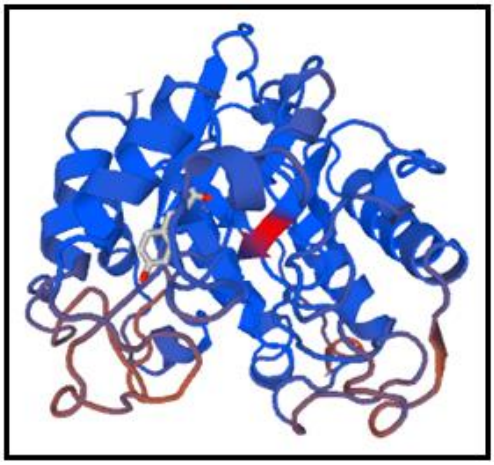

Fig. 4.26 Ribbon representation of three dimensional structure of CelT3 cellulase

The Ramachandran plot indicated the region of possible angle formations by ϕ (phi) and ψ (psi) angles, the torsion angles on either side of the α-carbon in peptides. The Ramachandran plot was divided into three regions: Favoured (96.6%), allowed (2.8%) and outlier (0.6%). The result is significant as there was >96% percentage of residues in the favoured region. The Ramachandran plot is shown in Fig. 4.27.

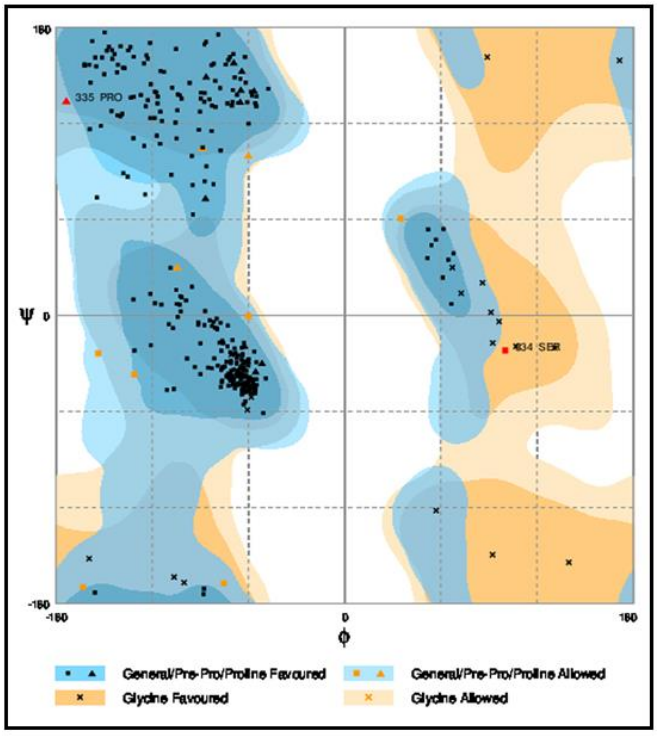

Fig. 4.27 Ramachandran plot for validation of CelT3 structure prediction

4.13 Quantification of protein

After observing expression at small scale, the CelT3 protein expression was carried out at large scale in 1,000 ml production medium for purification and biochemical assays. The cellulase protein produced by the pET28CT3 transformed *E. coli* BL21(DE3) cells was estimated using Lowry's method. Cell lysis was carried out using sonication and total protein concentration and specific activity of the cellulase enzyme in cell lysate was calculated to be 0.746 mg ml^{-1} and 4.01 U mg^{-1}, respectively.

4.14 Determination of specific activity, percentage yield and protein fold purification

The activity of the CelT3 recombinant protein purified using Ni-NTA column was determined by the standard method of Miller (1960). The purified protein exhibited a maximum activity of 2.38 U ml^{-1} and the specific activity of 17.74 U mg^{-1}.

The results obtained after purification of the recombinant CelT3 cellulase enzyme are presented in Table 4.6.

Table 4.6 Specific activity, percentage yield and purification fold of the recombinant CelT3

Samples	Total protein (mg ml^{-1})	Total activity (U ml^{-1})	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude	0.74632	3.00	4.01	100	1
Ni-NTA purification	0.13414	2.38	17.74	79.33	4.42

4.15 Biochemical characterization of the purified CelT3 cellulase enzyme

4.15.1 Substrate specificity

To determine the substrate specificity of the CelT3 recombinant cellulase enzyme, various substrates with the final concentration of 1.0% were used and the enzyme assay was performed. The CelT3 enzyme showed the maximum activity in the presence of carboxymethyl cellulose and same is presented in Table 4.7.

Table 4.7 The specific activity of the purified cellulase

Substrates	Specific activity (U mg^{-1})	Relative activity (%)[a]
Carboxymethyl cellulose	17.74±1.19	100
Avicel	0.025±0.01	0.14
Filter paper	0.021±0.02	0.11
Laminarin	0.016±0.01	0.09

[a]The relative enzyme activity is the ratio of activity of each sample to the maximum activity of the sample. The relative activity was calculated from the specific activity of the enzyme.
Results are expressed as mean ± S.D. of three experiments

4.15.2 Effect of pH on the catalytic activity of cellulase

The effect of pH on enzyme activity was determined using buffers ranging from 4.0 to 10.0. The CelT3 cellulase displayed relatively high activity in a broad pH range. The maximum enzyme activity was observed at pH 6.0. The relative enzyme activity was retained significantly at pH 5.0-7.0 varying from 72% to 88%, respectively. A sharp decline in enzyme activity to only 2% was observed at pH 4.0.

The CelT3 cellulase enzyme also showed significant activity in the alkaline pH. The relative enzyme activity at pH 8.0 and 9.0 were 70% and 47%, respectively. A decline in enzyme activity to 25% was observed at pH 10.0. The enzyme displayed the highest activity at pH 6.0 and this pH was used for standard enzymatic assays. Data obtained are presented in Fig. 4.28.

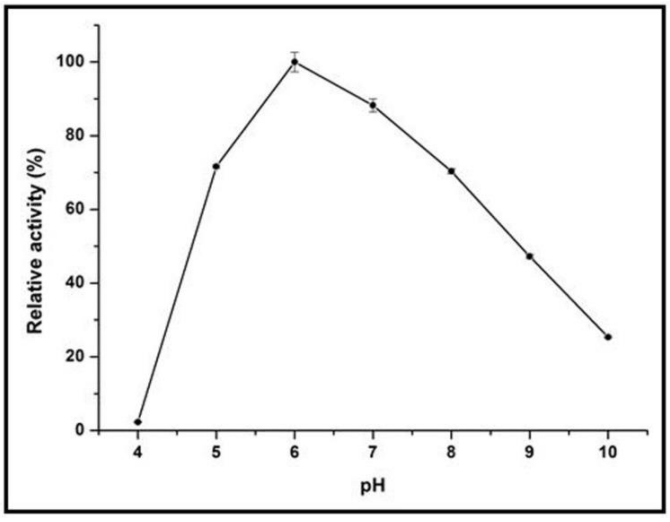

Fig. 4.28 Effects of pH on the activity of CelT3. Results are expressed as mean ± S.D. of three experiments

4.15.3 Effect of temperature on the catalytic activity of cellulase

The CelT3 enzyme showed activity at a broad temperature range. The optimum temperature for the enzyme activity was determined to be 45°C. The relative activity was 44% even at a temperature as low as 25°C. Similarly at 55°C and 65°C, the enzyme displayed relative enzyme activity of 60% and 27%, respectively. After this there was a sharp decline in enzyme activity to 2.5% at 75°C. The enzyme displayed the highest activity at 45°C and this temperature was used for the standard enzymatic assays. Data obtained are shown in Fig. 4.29.

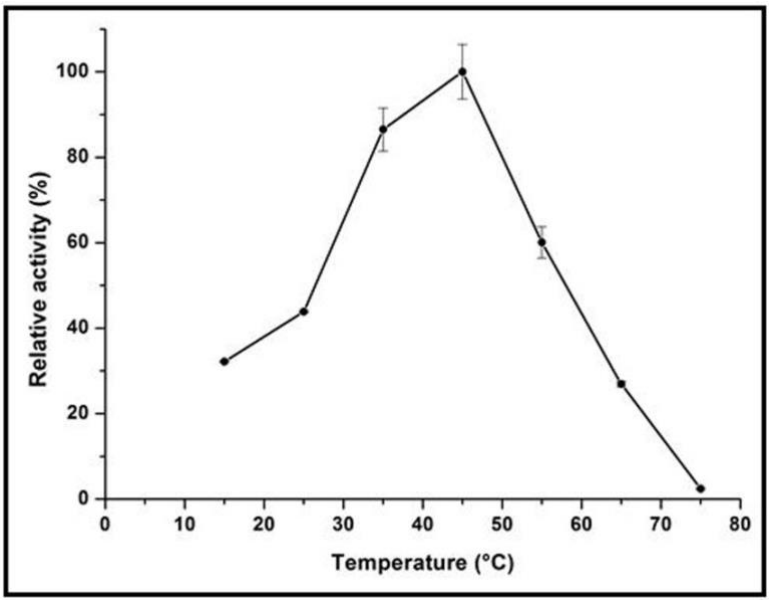

Fig. 4.29 Effect of temperature on the activity of CelT3. Results are expressed as mean ± S.D. of three experiments

4.15.4 Effect of metal ions on the catalytic activity of cellulase

The CelT3 recombinant cellulase showed the maximum activity in the presence of Ca^{2+} followed by Fe^{2+}. Ni^{2+} mildly inhibited the activity whereas ions such as Mg^{2+}, Co^{2+}, Zn^{2+} and Cu^{2+} significantly inhibited the activity of CelT3. Data obtained are presented in Fig 4.30.

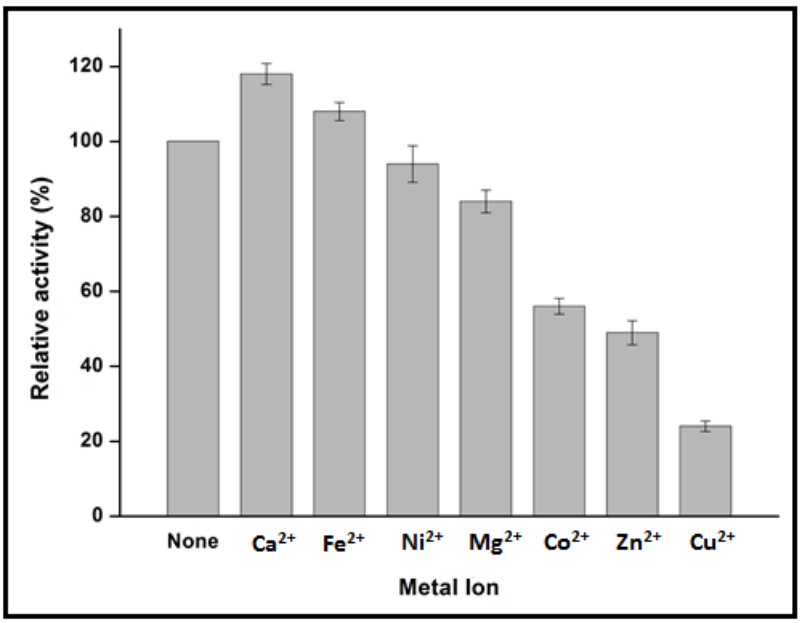

Fig 4.30 Effect of metal ions (1 mmol L^{-1}) on the activity of CelT3. The activity of cellulase without any additive was considered as 100%. Results are expressed as mean ± S.D. of three experiments

4.15.5 K_m and V_{max} for the CelT3 catalysed reactions

The values for K_m and V_{max} were obtained using Lineweaver-Burk plot. Initial rates of the purified cellulase enzyme on the different concentrations of CMC were determined under the standard assay conditions at pH 6.0. At relatively low concentrations of CMC, initial rate increased almost linearly. Upto 1% of CMC rate increased and beyond this concentration saturation was observed. Data obtained are shown in Fig. 4.31.

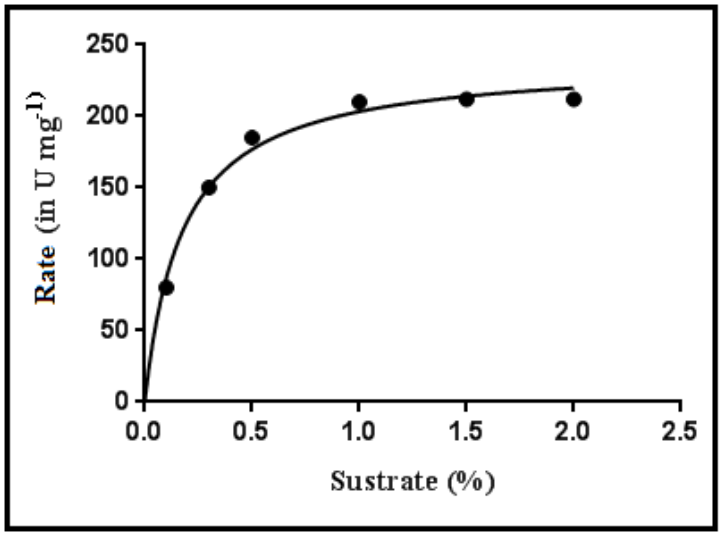

Fig. 4.31 Effect of substrate concentration on the initial rate of CelT3 cellulase catalyzed reaction

By plotting the values of 1/[V] as a function of 1/[S], a straight line was obtained that intersected the vertical line at a point which is the $1/V_{max}$ (since 1/[S] = 0, therefore $1/[V] = 1/V_{max}$). Extension of the straight line results in intersecting the horizontal axis (1/[S]) at the point which is $-1/K_m$. Lineweaver-burk plot was constructed and V_{max} and K_m values were calculated using CMC as the substrate. The calculated V_{max} and K_m values of the CelT3 catalysed reactions are 222.22 U mg^{-1} and 0.35 mg ml^{-1}, respectively. Data obtained are presented in Fig. 4.32.

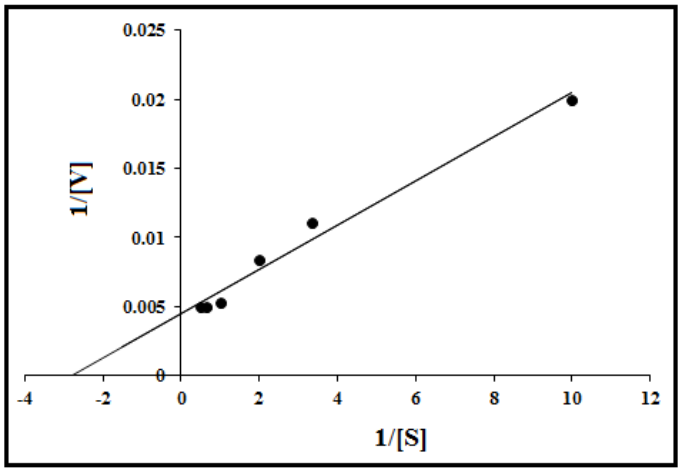

Fig. 4.32 Determination of K_m and V_{max} of the CelT3 recombinant cellulase using Lineweaver-Burk plot. The Lineweaver-Burk plot was plotted using GraphPad Prism 6 software