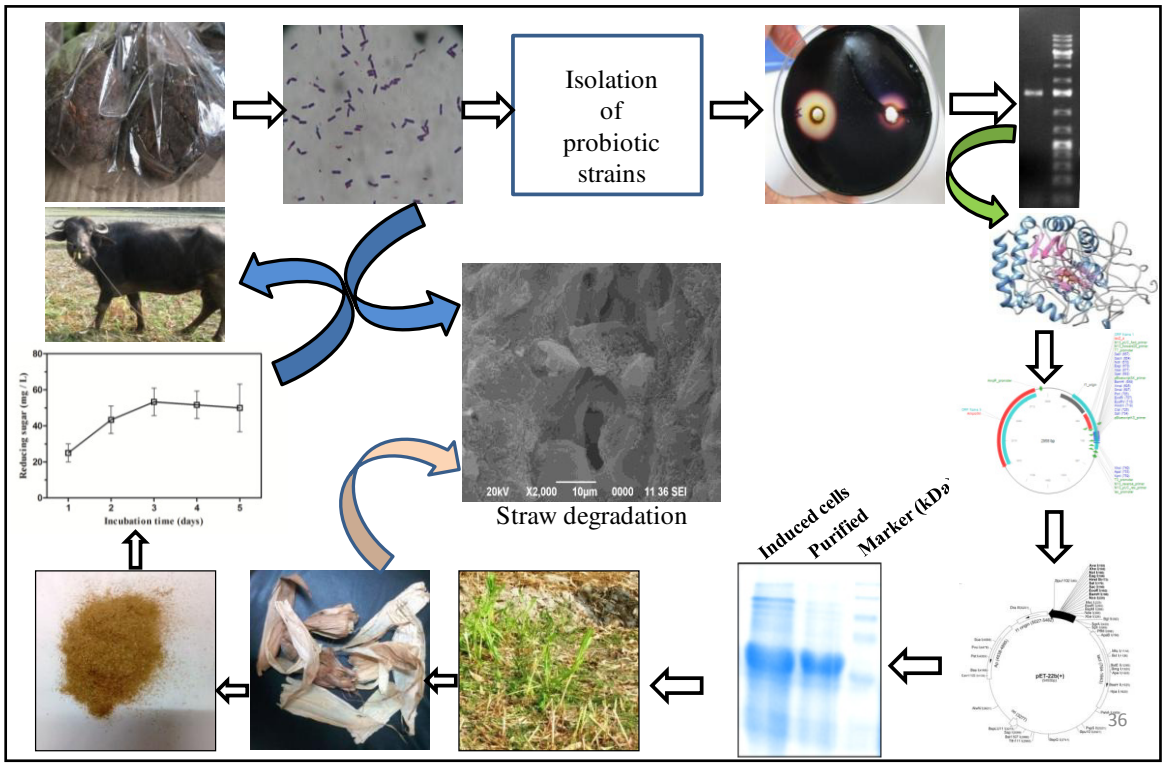
CHAPTER 6

IDENTIFICATION AND CHARACTERIZATION OF THERMO ACTIVE CELLULASE FROM POTENTIAL PROBIOTIC *BACILLUS AMYLOLIQUEFACIENS* AMS1 AND ITS APPLICATION ON HYDROLYSIS OF AGRO-CELLULOSIC WASTE MATERIAL TO BE USED AS ANIMAL FEED ADDITIVE



Graphical Abstract

IDENTIFICATION AND CHARACTERIZATION OF THERMO ACTIVE CELLULASE FROM POTENTIAL PROBIOTIC *BACILLUS AMYLOLIQUEFACIENS* AMS1 AND ITS APPLICATION ON HYDROLYSIS OF AGRO-CELLULOSIC WASTE MATERIAL TO BE USED AS ANIMAL FEED ADDITIVE

6.1. Abstract

This study reports the identification of cellulase encoding gene from potential probiotic *Bacillus amyloliquefaciens* AMS1 and its *in-silico* studies. Further cloning of cellulase gene and its heterologous expression in *E. coli* BL21(DE3) has been investigated. The purified enzyme was characterized and utilized for saccharification of acid pretreated maize straw. A full length cellulase gene of AMS1 was amplified and yielded approximately 1500 base pairs. The ORF encoded for a protein of 499 amino acid residues with the molecular weight (Mw) and isoelectric point (pI) predicted to be 55.23 kDa and 7.65, respectively. The amino acid sequence consists of a Glycosyl hydrolase family 5 domain (50–296 bp); Imm51 core (Immunity protein; 110–166 bp) and fused to a CBM-3 (cellulose binding domain; 356–436 bp) at its C terminal. Docking result showed that CMC binds to cellulase AMS1 with a binding energy of -7.97648 kJ mol^{-1} and showed the presence of 12 putative residues of cellulase AMS1 that contact with CMC through hydrophobic interaction (Asn134, Lys33, His131, Gln297, Lys296 and Trp69) and hydrogen bonding with Ala98, Asp99, Thr97, Ala36, His65. The purified recombinant cellulase protein was characterized and shows maximum activity within a pH (4.0-7.0) with an optimum at pH 5.0 (0.41 U/ml). It also showed maximum activity within a broad range of temperature (10 °C - 90 °C) with an optimum at 50 °C (0.43 U/ml). The purified cellulase enzyme was found to be thermo active and enhanced activity was observed in the presence of manganese salt. The enzyme showed potential for agri-biotechnological processes and could be employed as an animal feed additive.

6.2. Introduction

Cellulose, the major structural component of plant cell wall, is the most abundant polysaccharide on earth. Although cellulose is being digested by animals, it

is not completely utilized and 20–70 % partially digested cellulose is detected in discharged waste matter[1]. Microbial fermentation and enzymatic hydrolysis are the most efficient methods to convert complex cellulose and hemicellulose into glucose monomers which play a vital role in energy supply for forage animals. Cellulase is the key to hydrolyze cellulose and it is produced by different microorganisms including bacteria and fungi[2, 3]. In general, three enzymes are responsible for breakdown of complex cellulose to simple sugars: endoglucanase (EC 3.2.1.4), cleave the internal bonds of cellulose, and disrupt its crystalline structure, creating the individual polysaccharide chain ends; exoglucanase (EC 3.2.1.91), able to cleave the ends of the crystalline cellulose, resulting in production of cellobiose; and β-glucosidase (EC 3.2.1.21), able to break cellobiose and cello-oligosaccharide into glucose[4]. Among these, endoglucanase is the most extensively applied cellulolytic enzyme product[5]. Endoglucanases are more advantageous than exoglucanases in modifying cellulose fibers without damaging its properties[6, 7] and reported to have applications in the detergent, textile and pulp and paper industries[8, 9]. In addition, thermo active endoglucanases have the additional benefits to degrade lignin and hemicellulose in the pretreatment process which involves high temperatures, such as 70 °C with aqueous ammonia[10]. Cellulase cannot effectively adhere to cellulose in nature because cellulose is enveloped by lignin, hemicellulose and pectin[11, 12], therefore, high temperature pretreatment is a pre-requisite to alter cellulosic biomass structure and make cellulose more accessible to the hydrolyzing enzymes for converting the complex polymers into simple glucose monomers. Whereas, high chemical concentration employed in pretreatment is cost intensive and leads to the environmental pollution, and adversely affects animal health. It is also reported that acid and alkali pretreatments of straw are undesirable for feed processing since the presence of chemical ion contents (Na^+, Ca^{2+} and Cl^-) are beyond animal feeding standards[13, 14] . Therefore, searching for thermo active endoglucanases with greater efficacy from cellulolytic probiotic microorganisms are essential research prospect for pretreatment of straw and thereby achieving better utilization of animal feeds.

In the previous chapter 5, cellulolytic probiotic *Bacillus amyloliquefaciens* AMS1 isolated from traditional fermented food has been reported. The present study

was conducted to identify the cellulase encoding gene and its *in-silico* studies. Further cloning of cellulase gene and its heterologous expression in *E. coli* BL21(DE3) has been investigated. The purified enzyme was characterized and utilized for saccharification of acid pretreated maize straw.

6.3. Materials and Methods

6.3.1. Cellulolysis and identification of cellulase gene in *B. amyloliquefaciens* AMS1

Preliminary thermophilic cellulase stability of the isolate was assessed on carboxymethylcellulose (CMC) agar plate. The dialyzed culture supernatant was loaded on wells prepared on CMC agar plate, and incubated at psychrophilic condition (15 °C) and thermophilic condition (60 °C) in incubator (ORBITEK, Scigenics Biotech) and then flooded with Gram's iodine to observe the zone of hydrolysis. The gene encoding cellulase was amplified using primers (CF 5'-ACAGGATCCGATGAAACGGTCAATTTCTATTTT-3' and CR 5'-ACTCTCGAGATTGGGTTCTGTTCCCAAAT-3'). Cellulase gene was amplified by colony PCR method. The *B. amyloliquefaciens* AMS1 isolate was grown in Luria-Bertani agar for overnight at 37 ˚C. The template DNA was obtained by lysing a single bacterial colony using 20 mM NaOH at 95 ˚C for 10 min followed by centrifugation at 10,000 rpm for 10 min. The PCR reaction was carried out in a total volume of 30 µl containing 2 µl of each forward (CF) and reverse primers (CR), 15 µl of Dream Taq PCR Master Mix (2X, Thermo Scientific, #K1071), 10 µl nuclease free water (Thermo Scientific, #R0581) and 1 µl DNA template. The PCR reaction was carried out in a thermal cycler (Eppendorf Mastercycler, Germany) by programming the cycling profile consisting of an initial denaturation step of 5 min at 94 ˚C followed by an amplification for 35 cycles with denaturation for 40 sec at 94 ˚C, annealing for 40 sec at 55 ˚C, initial extension of 2 min at 72 ˚C and final extension of 10 min at 72 ˚C for 1 cycle. The PCR amplified DNA fragments were resolved by agarose gel (1%) electrophoresis and stained with ethidium bromide (0.5 mg/ml) followed by visualization under UV light. The amplified product was purified using QIAquick Gel Extraction Kit and sequenced using DNA sequencing facility (Applied Biosystems) at Tezpur University.

6.3.2. Sequence analysis, homology modeling and docking studies

The nucleotide sequence obtained after sequencing was translated using translate tool of EXPASY (http://expasy.org/) and the deduced amino acid sequence obtained was further subjected to BLASTp programme (http://www.ncbi.nlm.nih.gov) search tool in order to retrieve the homologous sequences in Genbank. The obtained sequences were aligned using CLC sequence viewer 7.5, phylogenetic tree was constructed by Neighbor-Joining (NJ) method using MEGA 5.05; signal peptide sequence was predicted using SignalP 4.1 server (www.cbs.dtu.dk/services/SignalP/) and domain structure was prepared using DOG 1.0. Protein models were obtained from three meta-servers I-TASSER[15]. Swiss model and HHPred and all the predicted structures were assessed using ANOLEA, RAMPAGE, SOLVX, QMEAN and DFIRE. Finally assessed and selected I-TASSER predicted cellulase structure was further minimized using GROMACS 5.0.3[16]. The loop regions of the final model were rebuilt using Modloop. In addition, molecular docking studies was performed to evaluate the binding affinity of Carboxymethylcellulose (CMC; CID:24748) towards the cellulase AMS1 using *Molegro Virtual Docker 6.0* and ligand binding analysis was performed using *LigPlot+ version 1.4.5*. Subsequently, active site residues were analyzed based on high quality ligand interactions with the receptor by calculating the MoleDock Scoring function and number of hydrogen bonds for each interaction. The MolDock scoring function E_{score} is defined by the following energy terms:

$E_{score} = E_{intra} + E_{inter}$

Where, E_{inter} is the ligand- protein interaction energy, and

$E_{intra} = \sum_{i \in ligand} \sum_{j \in ligand} E_{PLP}(r_{ij}) + \sum_{flexible\ bonds} A[1 - \cos(m.\theta - \theta_o)] + E_{clash}$

Where, PLP= Piecewise Linear Potential and $E_{clash}$is the clash potential[17]. 2D diagrammatic representation of ligand- protein interaction was automatically generated using LigPlot+ program.

6.3.3. Cloning, heterologous expression and purification of recombinant cellulase

The amplified PCR product of cellulase gene AMS1 was purified using QIAquick PCR Purification Kit (QIAGEN, Cat No./ID: 28104). Then, the purified amplified PCR product was cloned into pBluescript KS(+) and inserted between

*Bam*HI and *Xho*I restriction sites. The desired fragments of cellulase gene was released by double digestion of recombinant clones with *Bam*HI and *Xho*I and further sub-cloned into pET 22b (+) by following protocols described in previous chapter 4 (Section 4.3.5). The recombinant cellulase plasmid DNA was transformed into *E. coli* BL21 (DE3) cells and the culture at OD ~0.6 was induced with 0.1- 0.4 mM IPTG for 4 h at 37 ºC. The expression of recombinant protein was analyzed on 12 % SDS-PAGE followed by staining with Coomassie brilliant blue. Since the recombinant protein was mainly found in inclusion bodies, the proteins were solubilized with 8 M Urea and purified by Ni^{2+} -NTA affinity chromatography (QIAGEN). The recombinant cellulase bound to the column was eluted with 10 mM Tris, 300 mM NaCl, 8M Urea, 250 mM Imidazole, pH 7.4. The concentration of purified protein was measured using Bradford method (Fermentas #R1271). The urea denatured cellulase protein was refolded by gradient dialysis method as described in previous chapter 4 (Section 4.3.5.7).

6.3.4. Cellulase activity assay

Cellulase activity was estimated by measuring the reducing sugar released from the carboxymethyl cellulose (1%) by 3,5-dinitrosalicylic acid (DNS) method[18]. 100 µl of purified enzyme was incubated with 200 µl of substrate (1 % CMC dissolved in citrate buffer, pH 5.0) and the volume was made up to 1ml using distilled water. The reaction was incubated at 50 ºC for 15 min followed by addition of 3 ml DNS reagent. Then, the tubes containing reaction volume was heated in boiling water bath for 15 min. The tubes were cooled and the absorbance was measured at 545 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1µmol of reducing sugar as glucose per minute under assay condition.

6.3.5. Characterization of purified enzyme

The optimum temperature for enzyme activity was measured in citrate buffer (50 mM, pH 5), by incubating the purified enzyme at various temperatures from 5 to 90 °C. The reducing sugar released was then estimated by DNS method. The optimum pH was examined by measuring the enzyme activity at various pH buffer (50 mM). The buffers used were, citrate buffer (pH 4–6), sodium phosphate buffer (pH 7-8) and

glycine NaOH buffer (pH 9-10). The effect of various metal ions and other reagents on the activity of the purified enzyme was also studied. Metals used were Fe^{+2}, Ca^{+2}, Mn^{+2}, Mg^{+2}, Cu^{+2}, Zn^{+2} as sulphate salts and Ni^{+2} as chloride salts at a concentration of 5mM. Other reagents tested were 1 mM dithiothreitol (DTT), 5 mM EDTA, 0.5 M urea, 0.5% Triton X-100, Tween 80 and 0,25% sodium dodecyl sulfate (SDS). The additive effects of these reagents were studied by pre-incubating with enzyme solution at room temperature for 1 h followed by estimating the reducing sugar release by DNS method. The activity measured without any reagent was considered as 100% enzyme activity.

6.3.6. Glucose production from enzymatic hydrolysis of maize straw

Acid pre-treated maize straw was evaluated for enzymatic saccharification. For acid pre treatment, milled maize straw [1 % (w/v)] was treated with dilute H_2SO_4 (0.5% v/v) at 121 °C for 30 minutes in autoclave. After hydrolysis, the hydrolysate was passed through a muslin cloth and the solid fraction obtained was washed three times with distilled water. The solid fraction was further dried and studied for enzymatic hydrolysis. For enzymatic hydrolysis, 1% (w/v) maize straw was incubated at 50 °C in citrate buffer 50mM (pH 5) with an enzyme load of 2.5 ml (0.45 U/ml). The reducing sugar in the form of glucose released was then estimated.

6.4. Results and discussion

6.4.1. Cellulolysis

The preliminary screening of cellulase activity of dialyzed culture supernatant on CMCase agar plate incubated for 12 hr at 15 °C and 60 °C showed ~ 13 mm and ~ 20 mm zone of hydrolysis (Fig. 6.1).

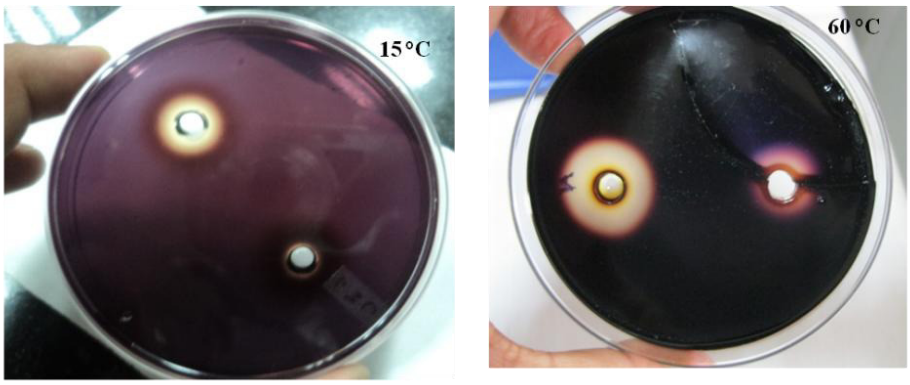


Fig. 6.1 Preliminary screening of cellulase activity of *B. amyloliquefaciens* AMS1 on 1% CMC agar plate: dialyzed culture supernatant incubated at psychrophilic condition (15 °C) and thermophilic condition (60 °C).

This indicates the stability of *B. amyloliquefaciens* AMS1 cellulolytic potential under broad range of temperature.

6.4.2. Identification of cellulase gene in *B. amyloliquefaciens* AMS1, Sequence analysis and homology modeling

The gene encoding cellulase was amplified using CF and CR primers yielded approximately 1500 base pairs (Fig. 6.2A). The sequencing of the amplicon resulted in a sequence of 1499 nucleotides and sequence analysis using the online software ExPASY (http://web.expasy.org) showed that the gene encoded a protein of 499 amino acids. The cellulase encoding gene was named as cellulase AMS1 and subsequently the nucleotide sequence of the cellulase gene was submitted into the NCBI GenBank database as *Bacillus amyloliquefaciens strain* AMS1 endoglucanase gene under accession number KP723365. The phylogenetic analysis of cellulase gene (Fig. 6.2B) showed maximum identity to endoglucanase *Bacillus amyloliquefaciens* (WP_017417894).

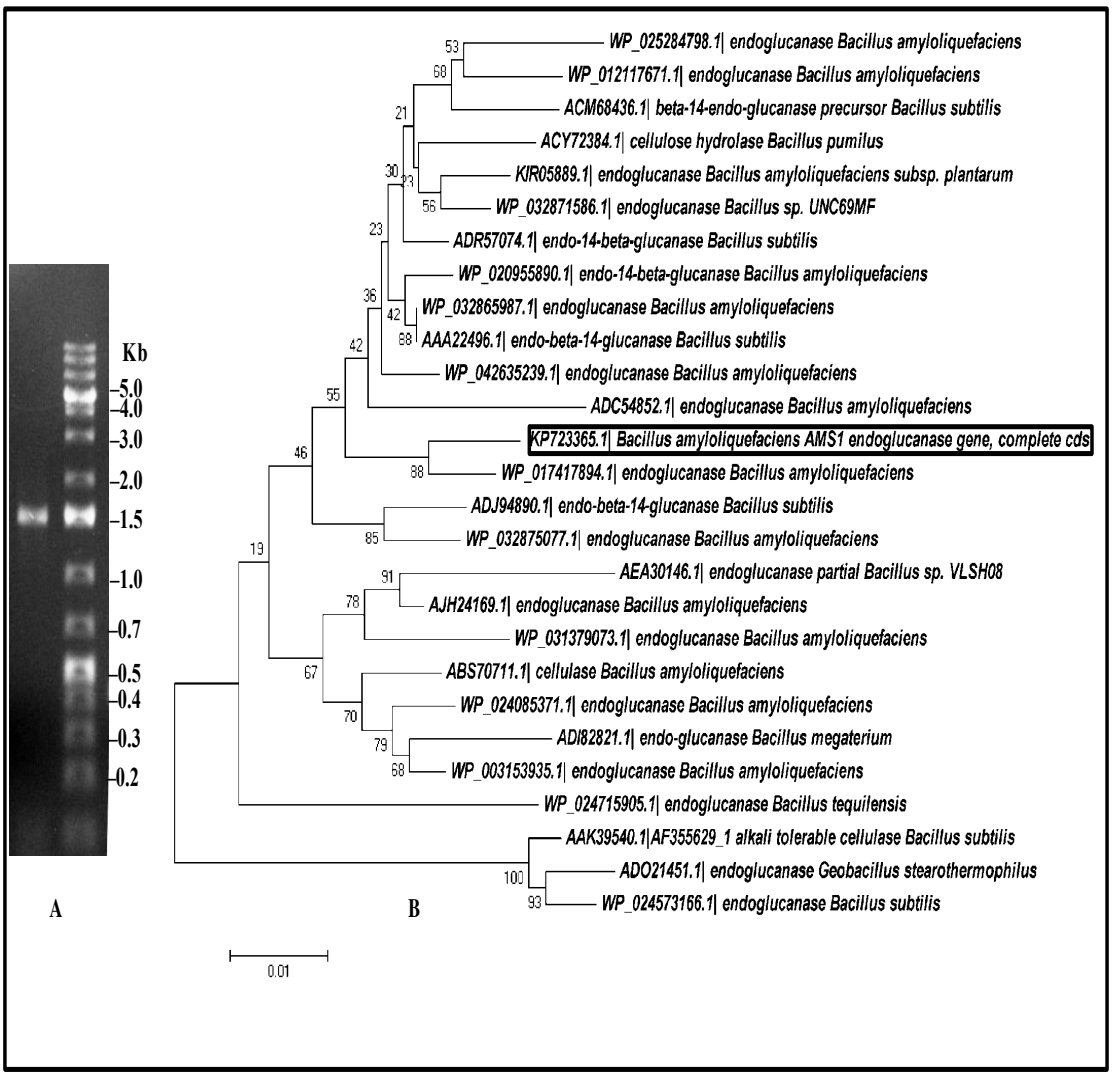


Fig. 6.2 (A) Gel electrophoresis showing amplification of cellulase gene of ~1.5 Kb size. **(B)** Phylogenetic tree showing the relationship of strain *B. amyloliquefaciens* AMS1 cellulase gene sequence with closely related species. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

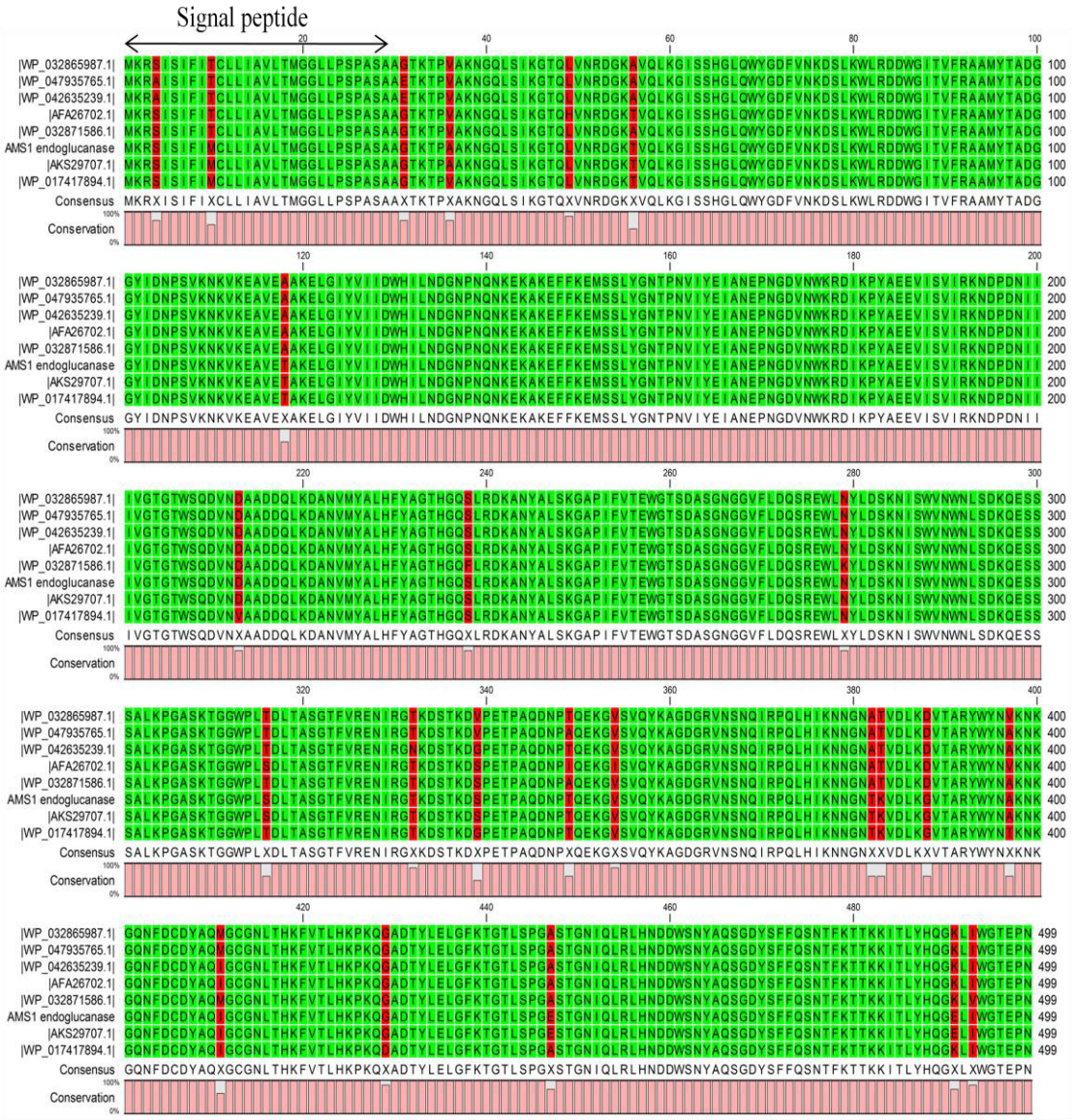


Fig. 6.3A Multiple sequences alignment of *B. amyloliquefaciens* AMS1 cellulase gene with highly similar sequences obtained from GenBank database. Highlighted green and red color indicates conserved sequence and un-conserved sequences respectively; Arrow indicates signal peptide sequence.

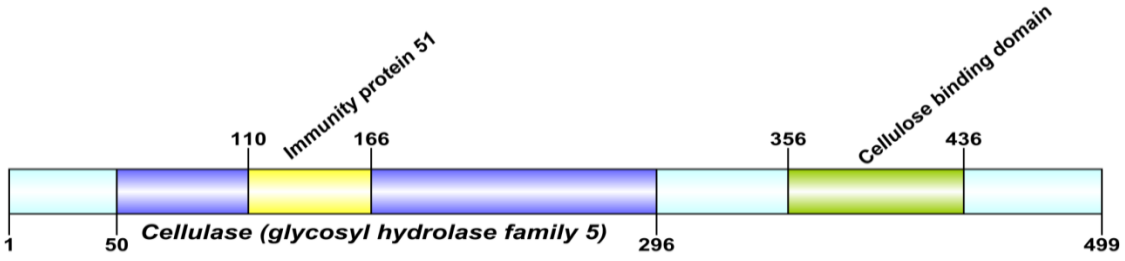


Fig. 6.3B Sequence analysis using Motif Finder online tool

The molecular weight (Mw) and isoelectric point (pI) of AMS1 cellulase gene was predicted to be 55.23 kDa and 7.65, respectively using ExPASy online server. Conserved and un-conserved sequence were identified using multiple sequence alignment of *B. amyloliquefaciens* AMS1 cellulase gene with highly similar sequence obtained from GenBank database as shown in highlighted green and red color (Fig. 6.3A). The SignalP 4.1 programme computed a 29 amino acid signal peptide at N-terminal region as depicted in Fig. 6.3A. The sequence analysis performed using Motif Finder online tool (http://www.genome.jp/tools/motif/) revealed that the amino acids sequence consists of a Glycosyl hydrolase family 5 domain (50–296 bp); Imm51 core (Immunity protein; 110–166 bp) fused to a CBM-3 (cellulose binding domain; 356–436 bp) at its C terminal (Fig. 6.3B). The catalytic domain along with CBM-3 contributes a large planar surface for cellulose detection, which regulates the substrate in a suitable conformation to the active site and enhances enzymatic efficiency. In conclusion, the cellulase secreted by the probiotic strain of *B. amyloliquefaciens* AMS1 belongs to glycosyl hydrolase family 5.

To further characterize this thermoactive enzyme, the AMS1 cellulase were compared with the previously reported amino acid sequences of thermostable cellulases of *Bacillus* spp. The conserved sequences were depicted in Fig. 6.4. AMS1 differs at 42 amino acid residues (37 in KFC32497.1, 37 in P10475; 37 in AID18514.1; 39 in ABV45393.1). Interestingly, AMS1 contains 29 Isoleucine (I) and 24 Valine (V) conserved residues in these 499 amino acid residues. Both Valine and Isoleucine have one extra strong hydrophobic substituent as compared with other amino acids which contain only one. Previous studies reported that hydrophobic interactions play role in the thermal stability of an enzyme[19, 20] . The secondary structure analysis with the primary protein sequence of the cellulase AMS1 using PROMOTIF server revealed the presence of sixteen beta sheet with percentage of 12.8% amino acid residues, eleven alpha helices with percentage of 21.8% amino acid residues, and 47 β-turns with 31.2% of amino acid residues were observed.

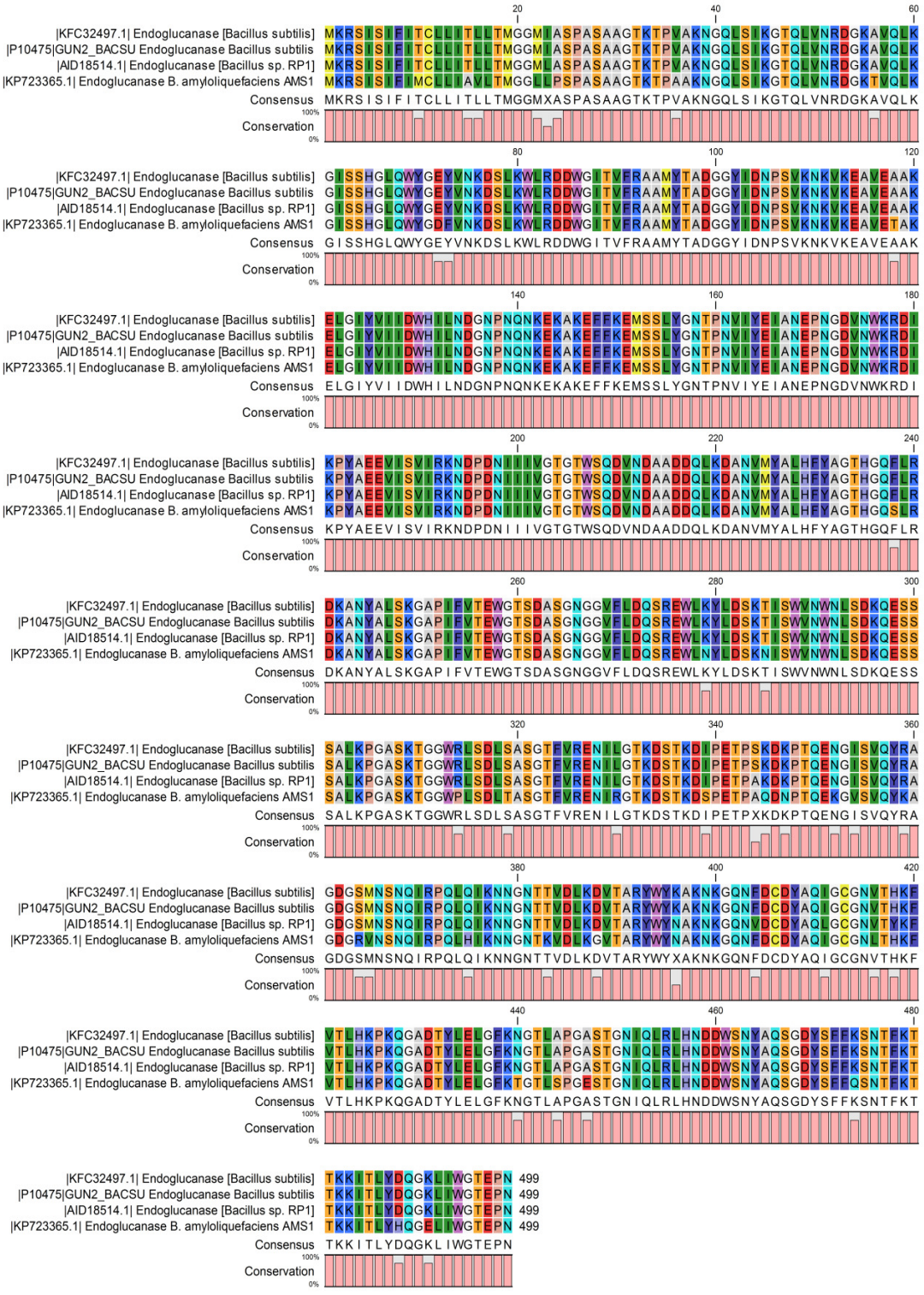


Fig. 6.4 Multiple sequence alignment of thermophilic cellulase from *Bacillus* spp.

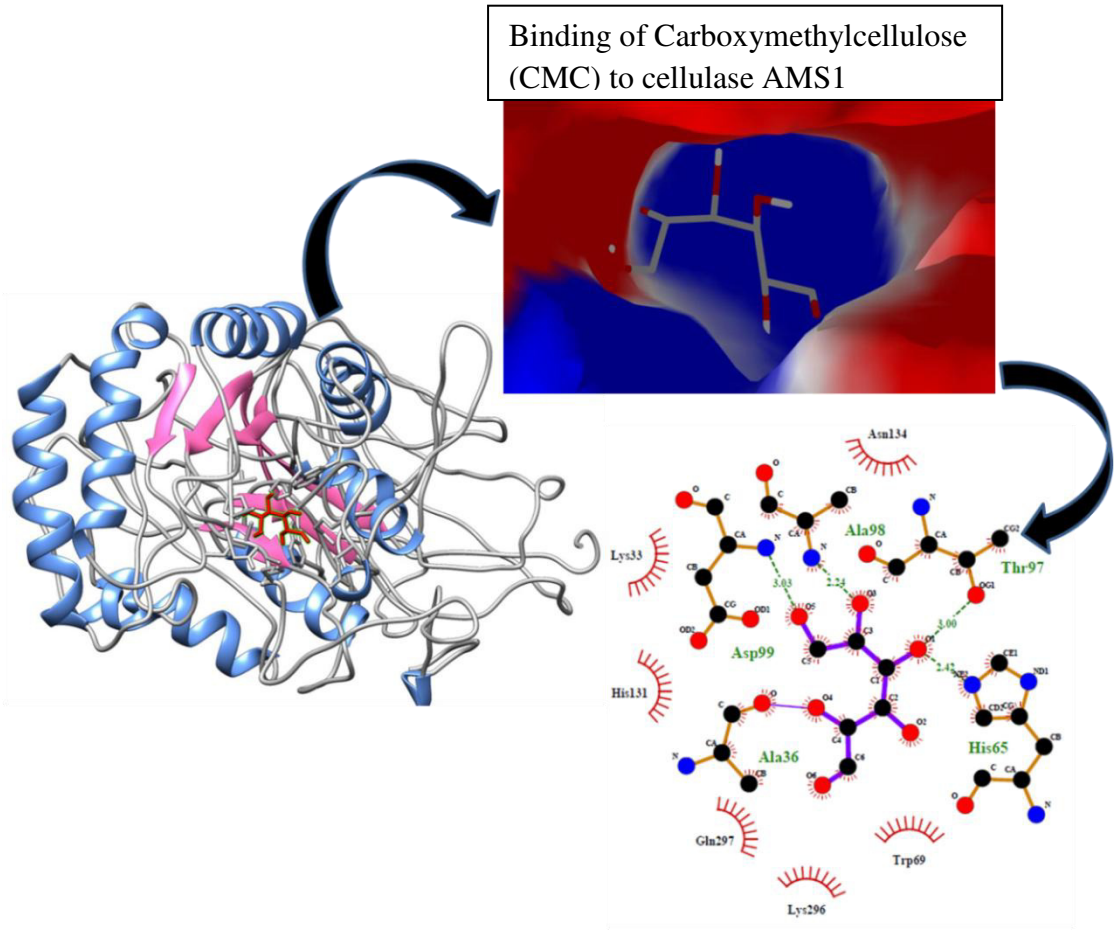


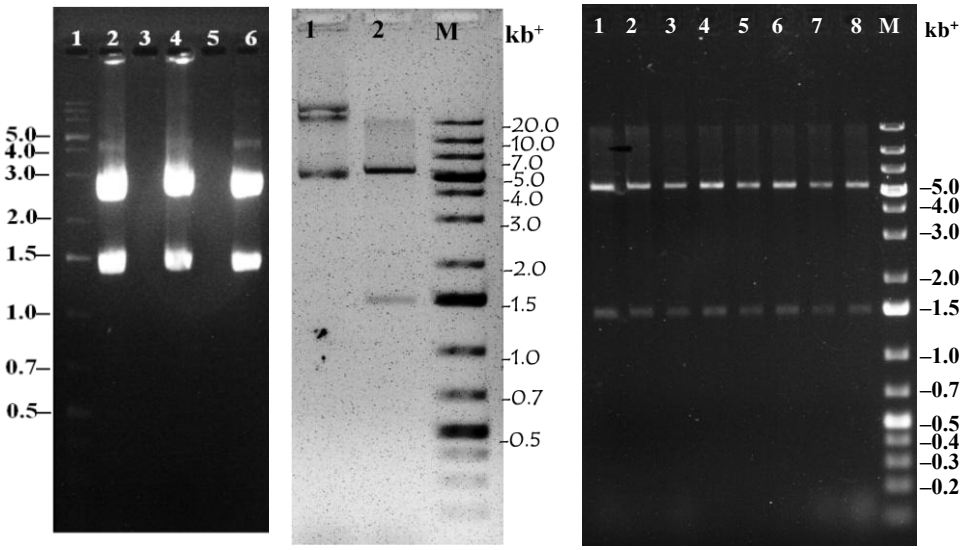
Fig. 6.5 Docking of cellulase AMS1 with carboxy methyl cellulose (CMC). Putative interacting amino acids residues are shown in relation to the CMC substrate.

Docking results showed that CMC binds to cellulase AMS1 with a binding energy of -7.97648 kJ mol^{-1} with MolDock Score and Rerank Score of -36.5071 and -23.4363 respectively in the active site of volume 49.664 $Å^3$ and surface of 139.52 $Å^2$, positioned at (-82.4211, 67.9532, 51.1186). Molecular docking showed the presence of 12 putative residues of cellulase AMS1 that contact with CMC through hydrophobic interaction (Asn134, Lys33, His131, Gln297, Lys296 and Trp69) and hydrogen bonding with Ala98, Asp99, Thr97, Ala36, His65 (Fig. 6.5). The docking studies revealed that interactions of the AMS1 with carbohydrate binding face is mainly by charged and polar residues such as aspartate, lysine, glutamine, threonine, histidine, asparagine and tryptophan. The only aromatic residue interacting with CMC

is Trp69. Our results are in accordance with previous studies reported by Santos et al.[21]. Previous findings also suggest that polar residues were not only responsible for carbohydrate recognition, but could exclusively replace the inter-chain hydrogen bonds which in turn breakdown the complex cellulose structure[22]. The threonine and aspartate residues are reported to protonate the histidine, permitting AMS1 to the classification of glycosyl hydrolases according to this histidine proton donor[21].

6.4.3. Cloning, heterologous expression and purification of recombinant cellulase

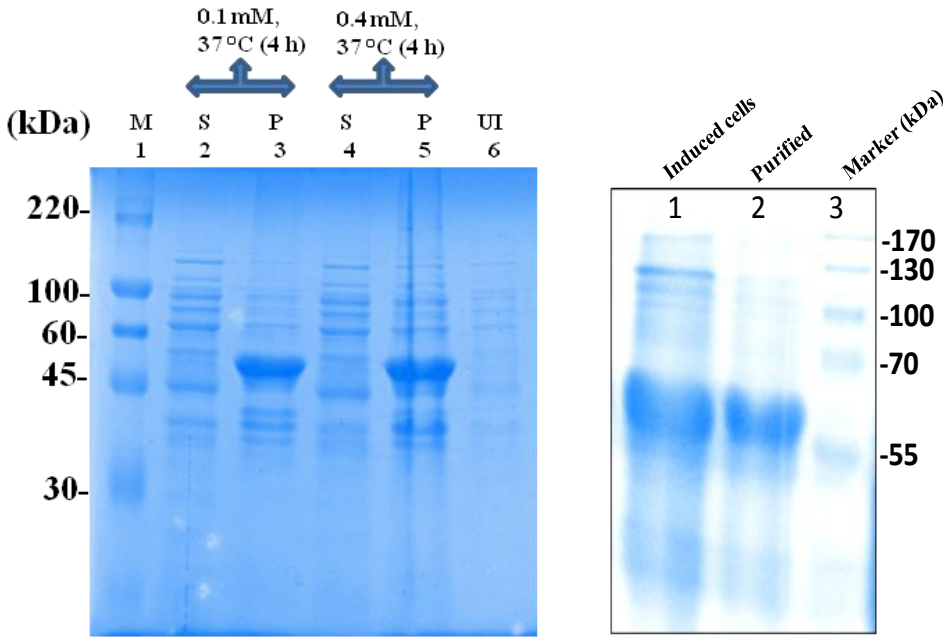
The cellulase gene was firstly cloned into pBluescript KS(+) and then transferred to *E. coli* DH5α. The recombinant pBS-cellulase plasmid was double digested with *Bam*HI and *Xho*I (Fig. 6.6A) and the released cellulase fragment was sub-cloned into expression vector, pET22 b (+) (Fig. 6.6B; Fig. 6.6C).



(A) **(B)** **(C)**

Fig. 6.6 Agarose gel electrophoresis showing the release of DNA fragment corresponding to cellulase gene upon double digestion of pBluescript KS(+) and pET 22b (+) with restriction endonuclease**. (A)** Lane 1: GeneRuler™ 1 Kb Plus DNA ladder (Fermentas); Lanes 2, 4 and 6: positive clones. **(B)** Lane M: GeneRuler™ 1 Kb Plus DNA ladder (Fermentas); Lane 1: undigested recombinant pET22b (+); Lane 2: indicates recombinant pET22b after digestion with restriction enzyme *Bam*HI and *Xho*I. **(C)** Lanes 1 through 8 is positive clones. Lane M: GeneRuler™ 1 Kb Plus DNA ladder (Fermentas).

As shown in Fig. 6.7A, the activity profiles of AMS1 cellulase at different IPTG concentrations showed the cellulase activity induced by 0.1 - 0.4 mM IPTG at 37 °C and the recombinant His tagged cellulase showed a clear band of the induced protein of ~55 kDa (Fig. 6.7B) on SDS-PAGE (12 %) which corroborates with the predicted molecular mass of AMS1 cellulase gene using ExPASy online server. In this study, the major part of cellulase-His was found to be present in the inclusion bodies as confirmed by SDS-PAGE analysis of supernatant and pellet fraction after sonication (Fig. 6.7A). Further, His-tagged cellulase was purified and eluted using Ni-NTA affinity column under denaturing conditions (Fig. 6.7B). The cellulase-His protein was refolded by gradient dialysis method as described in previous chapter (Section 4.3.5.7).

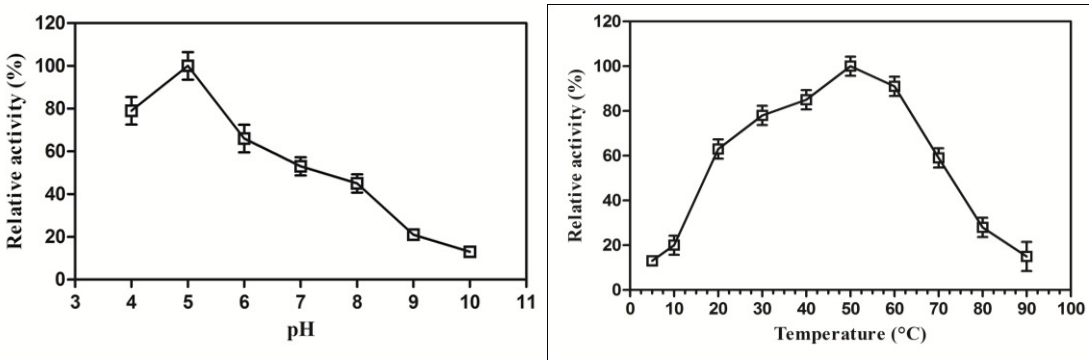


(A) **(B)**

Fig. 6.7 (A) SDS-PAGE (12%) analysis showing the over-expression of BSH in *E. coli* BL21 (DE3) upon induction with IPTG. Lane 1: C1992 SIGMA ColorBurst™ Electrophoresis Marker; lane 2: supernatent; lane 3: pellet fraction; lane 4: supernatent ; lane 5: pellet fraction and lane 6: uninduced cells. (Induction carried out with IPTG at 37 °C for 4h). **(B)** Purification of recombinant cellulase AMS1-His protein by Ni-NTA affinity chromatography. Lane 1: induced cell lysate; Lanes 2: purified eluted fraction; Lane 3: pre-stained protein molecular mass marker (ThermoFisher Cat. 26616).

6.4.4. Characterization of recombinant cellulase AMS1

The purified dialyzed recombinant cellulase protein was characterized and shows maximum activity within a pH range (4.0-7.0) with an optimum at pH 5.0 (0.41 U/ml) as depicted in Fig. 6.8A. The activity was 53–79% within 4–7 pH and the activity was found to be significantly less at higher pH 10.0 (0.06 U/ml). The activity of recombinant cellulase evaluated in this study is similar to those documented from other recombinant endoglucanases from *Bacillus* spp. which showed optimal activity in the acidic to neutral pH (pH 4–7) range[24, 25, 26] . These results indicate that recombinant cellulase AMS1 can perform better in acid pre-treatment process applied on feed for better digestibility and utilization by ruminant animals.



(A) **(B)**

Fig. 6.8 Characteristics of the recombinant cellulase AMS1 from *B. amyloliquefaciens*: **(A)** effect of pH and **(B)** effect of temperature on activity

The purified dialyzed recombinant cellulase showed maximum activity within a broad range of temperature (10 °C - 90 °C) with an optimum at 50 °C (0.43 U/ml). The maximum activity was observed within 40 °C - 60 °C (85-91%), whereas sharp decline in activity was noticed at extreme low temperature conditions (5-10 °C; Fig. 6.8B). The results obtained were in agreement with the previous findings of Li et al. (2008), which reported optimum activity of recombinant cellulase in *Bacillus subtilis* at 50 °C. This result indicates recombinant cellulase AMS1 are thermo active and could be applied for the pretreatment of cellulosic material which involves high temperatures. Since cellulase can be inhibited or activated by various chemical reagents, the effect of metals, ionic and non-ionic reagents on the activity of purified cellulase was also investigated and the results obtained is summarized in Table 6.1.

The cellulase AMS1 activity was significantly enhanced by Mn^{2+} (134 % relative activity). This finding is in accordance with the previous study[27, 28] showing enhanced activity of cellulase produced by *Bacillus* spp. in the presence of Mn^{2+}. However, activity was inhibited to 41% relative activity by EDTA (5mM) suggesting that it was a metalloenzyme. The non-ionic detergents such as Triton X-100, Tween 20 and Tween 80 relatively enhanced the enzyme activity similar to the endoglucanase EG5C from *Paenibacillus* sp. IHB B 3084[29], while the ionic detergent SDS completely inhibited the enzymatic activity like the thermostable β-glucosidase from *Thermotoga thermarum* DSM 5069[30].

Table 6.1 Effect of additives on CMCase activity of cellulase AMS1**.**

Agent	**Concentration**	**Relative activity (%)**
Control[a]	-	100 ± 4.8 [c]
Fe^{+2}	5 mM	83 ± 4.8
Ca^{+2}	5 mM	117 ± 2.4
Mn^{+2}	5 mM	134 ± 4.8
Mg^{+2}	5 mM	112 ± 4.8
Ni^{+2}	5 mM	83 ± 7.3
Zn^{+2}	5 mM	71 ± 7.3
Cu^{+2}	5 mM	73 ± 2.4
EDTA + Mn^{+2}	5 mM	41 ± 4.8
Urea	0.5 M	105 ± 4.8
Tween 80	0.5%	114 ± 4.8
Tween 80	1.0%	126 ± 4.8
Tween 80	2.0%	104 ± 4.8
Triton x 100	0.5%	120 ± 7.3
Triton x 100	1.0%	102 ± 7.3
Triton x 100	2.0%	95 ± 7.3
SDS	0.25%	0.0 ± 0.0
DTT	1mM	105 ± 4.8

[a]The relative activity in the control reaction without additives was defined as 100% and the corresponding absolute value was 0.41 U/ml.

[c] ± Values represent standard error. All of the reactions were performed at pH 5.0 and 50 °C.

6.4.5. Glucose production from enzymatic hydrolysis of maize straw

To assess the applicability of recombinant cellulase AMS1 in biomass saccharification, the acid pretreated maize straw saccharification was studied. The results showed that AMS1 produced maximum 53.33 mg of reducing sugar per liter of the substrate on 3rd day incubation. After 3rd day, the release of reducing sugars started declining and produced upto 50 mg/L on 5th day (Fig. 6.9). These results might be due to the inhibitory compounds such as soluble mono/oligomeric sugars, furans, phenolics, extractives, etc. generated during steam pretreatment process of lignocellulosic maize straw which limit or restrict the performance of recombinant enzyme mediated hydrolysis[31].

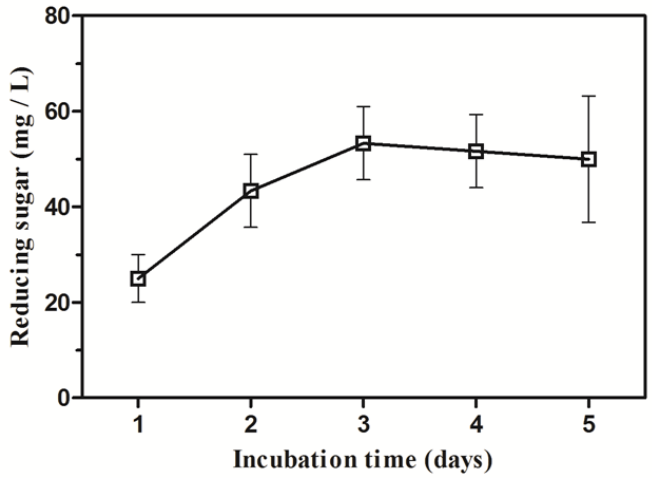


Fig. 6.9 Glucose released after incubating Maize straw with recombinant cellulase

6.5. Conclusion

Animal biotechnology domain continuously demands novel enzymes with enhanced hydrolyzing efficiency and stability under pretreatment conditions. These industrial treatments require cellulolytic activity under a wide range of temperature, pH, and ionic strengths. Considering the pretreatment process of straw and exploitation of cellulosic wastes, cellulase AMS1 mediated saccharification seems to be promising approach for processing of animal feed to ameliorate their utilization and enhance animal productivity. Moreover, the purified cellulase in this study showed activity not only towards CMC but also on natural substrates such as maize

straw and exhibited tolerance to higher temperature and compatibility with ionic and non-ionic detergents. Thus, these interesting attributes exhibited by purified cellulase AMS1 suggest it to be a potential candidate for practical applications in agro-biotechnological processes, including animal feed processing and generation of bioethanol from ligno-cellulosic biomass as a sustainable energy sources.

6.6. References

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