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Preliminary Characterization of a New Processive Endoglucanase from *Clostridium alkalicellulosi* DSM17461 †

Techly San [1] and Paripok Phitsuwan [1,2,*]

[1] Division of Biochemical Technology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkuntien, Bangkok 10150, Thailand
[2] LigniTech-Lignin Technology Research Group, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkuntien, Bangkok 10150, Thailand
* Correspondence: paripok.phi@kmutt.ac.th; Tel.: +662-470-7751
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Abstract: The *Clostridium alkalicellulosi* DSM17461^T genome contains several glucoside hydrolase encoding genes essential for cellulose degradation. Herein, the family 9 glycoside hydrolase enzyme (*Cal*GH9_2089) was cloned and expressed. The enzyme contains one GH9 catalytic module, a family 3 carbohydrate-binding module, and one Type I dockerin at its C-termini. The optimal pH and temperature for *Cal*GH9_2089 to hydrolyze CMC were 55 °C and pH 6.0, with the remaining activity of more than 60% at pH 10.0. *Cal*GH9_2089 produced a series of cello-oligomers (G2-G6) from CMC, suggesting that the enzyme has an endo-acting capability. When regenerated amorphous cellulose was hydrolyzed with *Cal*GH9_2089, the ratio of reducing ends in the soluble fraction to that in the insoluble pellets was 4.8, suggesting that this enzyme acts processively on RAC. This work extends our knowledge of the behavior of the GH9 endoglucanase from the microorganism living in an alkaline environment.

Keywords: family 9 glycoside hydrolase; endoglucanase; processivity; carbohydrate-binding module; dockerin

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1. Introduction

Cellulose is an unbranched glucose polymer that is linked by the β-1,4-glycosidic bond. Individual cellulose chains are closely associated through intra- and inter-hydrogen bonds, forming a tightly packed structure with a few amorphous regions (semi-crystalline). The crystalline structure makes cellulose insoluble, thus posing difficulty in hydrolysis [1,2]. To break down cellulose, three main enzymes, i.e., endo-1,4-β-glucanases, exo-glucanases/cellobiohydrolases, and β-glucosidases, are required to work in concert to degrade crystalline cellulose to simple sugar successfully. The endo-1,4-β-glucanases are important enzymes. They attack the β-1,4-glycosidic bonds at random sites along the cellulose chain, freeing sections of the chain from the crystal surface and creating free ends upon which exo-glucanases can act, thereby generating cellobioses, which are a substrate for β-glucosidases for producing glucose molecules [1].

According to the carbohydrate-active enzyme (CAZy) database, glycosidases are classified on the basis of similarities in the amino acid sequence and three-dimensional structure. Cellulases belong to glycoside hydrolase families (GH) 5-10, 12, 26, 44, 45, 48, 51, 61, 74, etc. [3]. Most endo-1,4-β-glucanases belong to GH9. GH9 endoglucases catalyze the hydrolysis of β-1,4 linkages with the inversion of anomeric carbon configuration. Several GH9 endoglucanases are tightly associated with a carbohydrate-binding module (CBM) classified in family 3 [4].

In bacterial cellulase systems, GH9 endoglucanases are distinctive from other cellulase families. GH9 endoglucanases have shown either non-processive or processive activity.

The processivity is the ability to remain in adsorbing the substrate in between subsequent hydrolytic reactions. This ability is important for the degradation of crystalline cellulose [5,6]. Therefore, the processive glucanases, behaving similarly to exoglucanases, are thought to be a major cellulose-degrading factor in addition to the exoglucanase component [5,6]. A recent study showed that a single GH9 endoglucanase is essential for cellulose degradation by *Clostridium phytofermentans*, despite its production of several other cellulases [7].

Carbohydrate-binding modules (CBMs) are non-catalytic modules found in some carbohydrate-active enzymes. They play an important role in degrading insoluble substrates [8,9]. Based on the structure of the binding site and functional similarity, CBMs are grouped into three types: Type A CBMs that bind the planar surfaces of crystalline polysaccharides; Type B CBMs bind internally on the glycan chains (endo-type); Type C CBMs bind the termini of glycan (exo-type). CBMs have been classified into 81 families based on their amino acid similarities (http://www.cazy.org, accessed on 12 August 2021) [10], and a family 3 CBM has been shown to bind cellulose effectively. There are numerous examples of fusing CBMs to catalytic modules, subsequently resulting in 2- to 10-fold increase in enzyme activities on insoluble substrates but not on soluble polysaccharides [11,12].

Clostridium spp. have received considerable attention as a source of cellulolytic and enzymes [13]. A number of endo-1,4-β-glucanases from several *Clostridial* species have been studied in recent years, some of which show promising applications in biofuel and biochemical production due to their high activity and high thermostability [13]. The *Clostridium alkalicellulosi* DSM17461^T genome contains several glycoside hydrolase encoding genes important for cellulose degradation. Herein, the family 9 glycoside hydrolase enzyme (*Cal*GH9_2089) was cloned and expressed. The recombinant enzyme's biochemical properties (i.e., pH and temperature optima, mode of action, and processivity) were determined.

2. Materials and Methods

2.1. Cloning and Protein Expression

Genomic DNA of *C. alkalicellulosi* DSM17461^T was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany).

PCR amplification of the putative endoglucanase gene (*Cal*GH9_2089) was performed using the upper primer (5′-AATTAGCTAGCTCAACTTTTAACTACGGAGA-3′), and the lower primer (5′-AATTACTCGAGTTACTGGTTTACTGGGAAAA-3′) using the genomic DNA as the template and the Phusion Taq High-Fidelity PCR-System (ThermoFisher Scientific, Waltham, MA, USA). The PCR-amplified material was cloned in pET28a(+) (Novagen, Madison, WI, USA) by digestion with restriction enzymes NheI and XhoI.

The purified PCR products and pET28a(+) vectors were doubly digested with NheI and XhoI. The digested PCR products and pET28a(+) vectors were ligated with T4 DNA ligase (New England Biolabs, Hitchin, UK). The ligated plasmids were transformed into NEB 5-alpha *E. coli* cells.

The positive clones with the target insert were transformed into *E. coli* BL21 (DE3) for protein expression following the manufacturer protocols (New England BioLabs). The recombinant *Cal*GH9_2089 protein was purified from a 400 mL transformed *E. coli* BL21 (DE3) culture.

2.2. Enzyme Activity Assay

The enzyme assay was performed in a total reaction volume of 200 µL. The reaction mixture consisted of 1% (*w*/*v*) substrate (i.e., CMC and RAC) and 0.25 µM of *Cal*GH9_2089 in 50 mM citrate buffer pH 6.0. The reaction mixture was incubated at 55 °C for 15 min (and 3 h for RAC). The reaction was terminated by immersion in ice water and centrifuged at 13,000× *g* at 4 °C for 10 min to separate the substrate from the soluble fraction. One hundred microliters of the soluble fraction was taken to a new 1.5 mL microcentrifuge tube, and 150 µL of dinitrosalicylic acid (DNS) [14] was added. The reaction mixture was boiled for 10 min and centrifuged at high speed. A sample was taken and measured at an absorbance of 540 nm. The amount of reducing sugar released in the mixture was estimated

using a glucose standard curve. One unit of the enzyme was defined as the amount of enzyme that produces 1 µMol of glucose equivalent in 1 min under the assayed conditions.

2.3. Effect of pH and Temperature

The influence of pH on enzyme activity was determined. The activity at different pH values from pH 3.0 to 11.0 was measured at 55 °C for 15 min. Citrate–sodium citrate buffer for pH 3.0–7.0, Tris-HCl buffer for pH 7.0–9.0, and glycine-NaOH for pH 9.0–11.0 with a concentration of 50 mM were used as a reaction buffer. The maximum activity at the corresponding pH was taken as 100%. A pH stability assay was performed by incubating 5.5 µL of the 11.5 µM enzyme in 10 mM of the buffers mentioned above at 30 °C for 60 min. After that, the enzyme solution was diluted, and the residual activity was measured under optimal assay conditions.

The effect of temperature on enzyme activity was tested by incubating 0.25 µM enzyme in a 200 µL reaction mixture containing 1% (*w*/*v*) substrate at a temperature range of 40–70 °C, pH 6.0 (citrate buffer), for 10 min. The maximum activity at the corresponding temperature was taken as 100%. The thermal stability of the enzyme was determined by incubating 5.5 µL of the 11.5 µM enzyme at temperatures of 37, 55, 60, and 70 °C for 0–24 h. The enzyme solution was diluted, and the residual activity was determined under optimal assay conditions.

2.4. Analysis of the Hydrolysis

The reaction mixture (total volume of 200 µL) containing 0.25 µM enzyme and 1% (*w*/*v*) substrate (i.e., CMC) was assayed under optimal conditions with varying times from 15 min to 16 h. The reaction was stopped at specific time intervals by boiling for 5 min and centrifuging at 13,000× *g* at 4 °C for 10 min to remove the undigested substrate. The soluble fraction was transferred to a new tube, and 4 µL of the soluble fraction was mixed with 4 µL of absolute ethanol. The mixed sample (8 µL) was spotted on TLC plates, and the plates were immersed in a TLC chamber containing n-butanol:acetic acid:water at a ratio of 2:1:1 as the mobile phase and heated at 90 °C in a hot air oven for visualization [15].

2.5. Processivity Assay

The content of reducing ends in the soluble and insoluble fractions was determined using RAC as a substrate [16]. Different concentrations of enzyme were added to a 1.5 mL microcentrifuge tube containing 1% (*w*/*v*) RAC in citrate buffer (pH 6.0) (final volume of 200 mL). The reaction mixture was incubated at 55 °C for different incubation times. After incubation, the reaction tube was centrifuged at 13,000× *g* at 4 °C for 10 min. The supernatant was removed, of which 100 µL was taken to measure reducing ends (soluble fraction) by the DNS method. The pellet was washed three times with 500 µL citrate buffer (pH 6.0) by centrifugation at 13,000× *g* at 4 °C for 5 min. After washing, the pellet was resuspended in 100 µL citrate buffer (pH 6.0) and taken to measure reducing ends (insoluble fraction) by the DNS method.

3. Results and Discussion

3.1. Bioinformatic Analysis

The draft genome sequence of *C. alkalicellulosi* DSM17461 was sequenced, revealing several putative endoglucanase encoding genes essential for cellulose hydrolysis in its genome. One ORF possessing 2899 bp was designated as *Cal*GH9_2089. This gene was predicted to encode a putative glycoside hydrolase family 9 endoglucanase connected with a family 3 CBM and a type I dockerin according to BlastP analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 6 July 2021). This gene was predicted to have 748 amino acid residues with a theoretical molecular weight of 82.86 kDa and pI of 5.1 (https://web.expasy.org/cgi-bin/protparam/protparam/, accessed on 6 July 2021) (Figure 1). *Cal*GH9_2089 was successfully expressed in *E. coli* BL21 (DE3), and it was determined for

its molecular weight and purity, showing a single band with a molecular weight of about 83 kDa.

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        10         20         30         40         50         60
MKKIISVVIV VAVLVGVLTV TPSPAEASTF NYGEALQKSI MFYEFQMSGK LPDNLRTNWR

        70         80         90        100        110        120
GDSCLDDGSD VGLDLTGGWF DAGDHIKFNL PMSYTSTMLA WAVYEYKDAL ERSGQLPYIK

       130        140        150        160        170        180
QQIRWATEYF IKCHPEKYVY YYQVGDGITD HRWWVPAEVI HLQSVRKSHK VTLDSPGSAV

       190        200        210        220        230        240
VAGTAAALAS AAIVFQDSDP AYAALCLKHA KDLFDFADRT QSDAGYTAAN NYYDSWSGFW

       250        260        270        280        290        300
DELSWAGVWI YMASGEKAFL DKAESYVANW NREERTNLLA YKWGHCWDDV MYGASLLLAK

       310        320        330        340        350        360
ATNKSIYKEH VERHLDYWSV GYNGERITYT PKGLAHLFVW GVLRHATTTA FLASVYSDWS

       370        380        390        400        410        420
ECPPAKAKTY MDFAKQQVDY ALGSSGRSYV VGFGVNPPQH PHHRTAHSSW IDTMEEPSYH

       430        440        450        460        470        480
RHVLYGALVG GPNQSDAYVD DIGDYITNEV ACDYNAGFVG ALAKMYDVYG GDPIPGFNAI

       490        500        510        520        530        540
EEVPYPEIYV TASLSSRTTA TEVKAFLINK SGWPARVKDT LSFKYFVDLT DFINAGHSPN

       550        560        570        580        590        600
EITSSIIYSA APTAKITGPI AYDTSKNIYY FELDLKGTAI FPGSRMDHQK EVQFHIVPPN

       610        620        630        640        650        660
GAPWNIPTDP SYPGTLSADE PVPQIPVYDN GVLLFGLEPD GSTPQPTTPP TTTPPTTTPP

       670        680        690        700        710        720
TTTTPTPQPA IMAGDINGDG LINSTDYILL RRYILEVTPS LPTTDVSGNP YRGDLAADLN

       730        740
GDGLIDSIDV ILMRRYILEI ITVFPVNQ
```

Figure 1. A translated amino acid sequence of *Cal*GH9_2089.

3.2. Effects of pH and Temperature

The effect of pH on *Cal*GH9_2089 activity was tested using CMC as a substrate. *Cal*GH9_2089 exhibited an activity from pH 4.0 to 11.0, with maximal activity at pH 6.0–7.0. The enzyme showed more than 50% activity from pH 5.0 to 10.0 (Figure 2A). For pH stability, the enzyme was pre-incubated in different buffers at 55 °C for 60 min and was assayed at the optimal conditions. It was found that the enzyme showed good stability from pH 5.0 to 10.0 with residual activity of more than 60% (Figure 2B). The pH profile suggested that *Cal*GH9_2089 is active in a broad pH range and tolerates alkaline pHs.

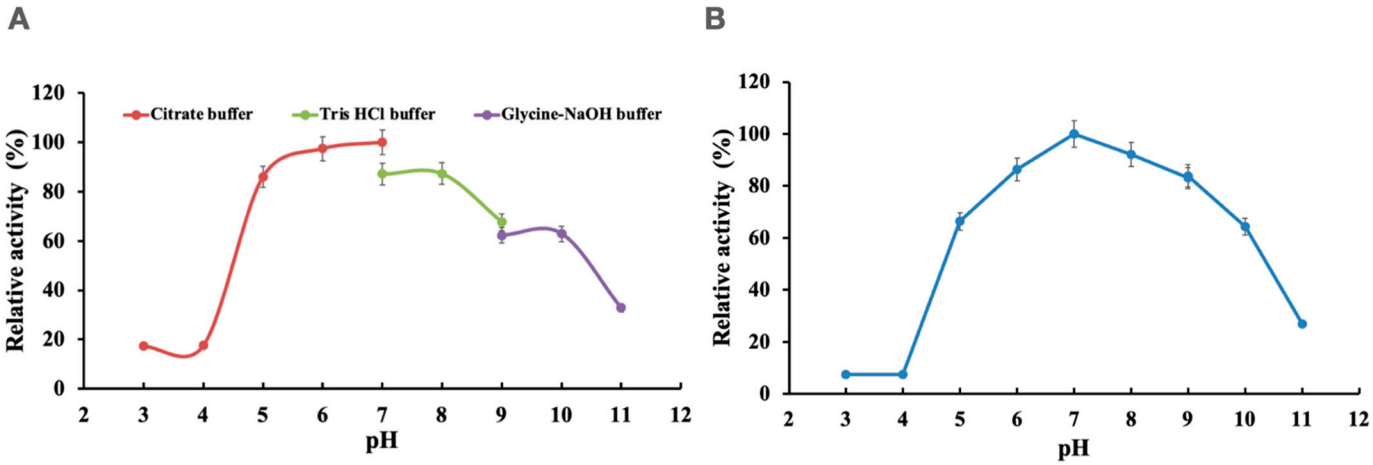

Figure 2. Effect of pH on *Cal*GH9_2089 activity towards CMC (**A**) and its pH stability (**B**).

The effect of temperature on the activity of *Cal*GH9_2089 was examined with the CMC. *Cal*GH9_2089 exhibited the highest activity of 1804.98 µmol product/min/µmol protein on CMC. The optimal temperature of *Cal*GH9_2089 toward CMC was 55-60 °C (Figure 3A). At 80 °C, the enzyme was almost completely inactivated. The thermal stability analysis of CalGH9_2089 was investigated at pH 6.0 with different temperatures (37, 55, 60, and 70 °C) (Figure 3B). The result showed that *Cal*GH9_2089 was stable at 37 °C, retaining more than 80% activity for 24 h. At 55 °C, the residual activity remained about 60% when the enzyme was incubated for 30 min. The enzyme activity remained 40% of its maximal activity from 6 to 24 h incubation. At 60 °C and 70 °C, the activity of *Cal*GH9_2089 lost rapidly, retaining 20% and 10% of its maximal activity, respectively, when it was incubated for 30 min. This result likely indicates that *Cal*GH9_2089 is a mesophilic enzyme and tolerates a moderate temperature of around 50 °C.

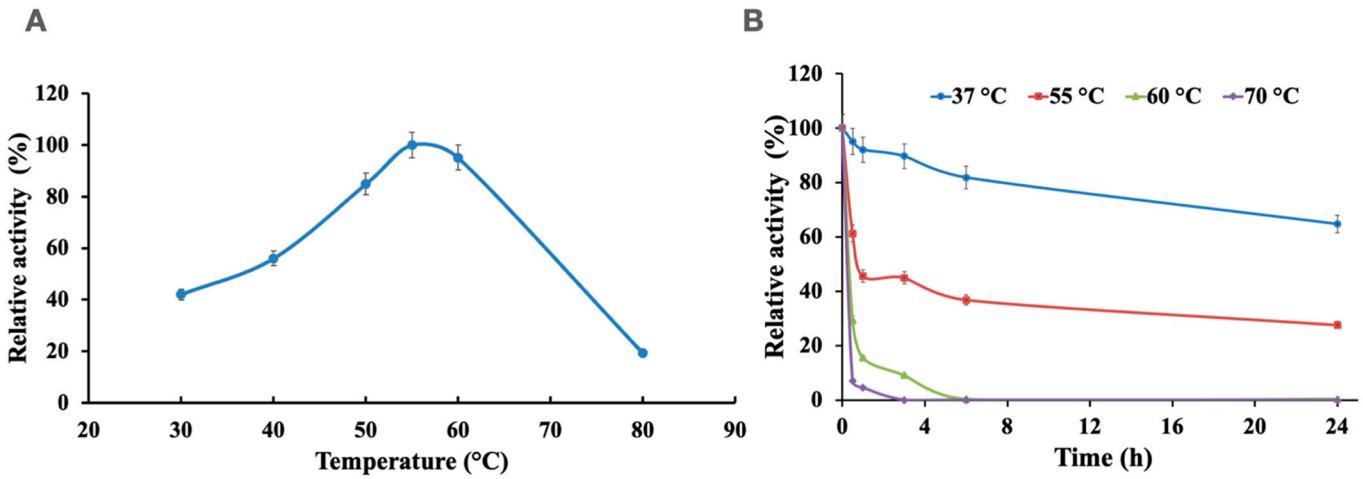

Figure 3. (**A**) Effect of temperature on *Cal*GH9_2089 activity towards CMC and (**B**) its thermal stability.

3.3. Mode of Action

To investigate the mode of action of *Cal*GH9_2089, the hydrolysis of CMC was tested at pH 6.0 and 55 °C, and the hydrolysis products at different incubation times were determined by TLC (Figure 4). It was found that *Cal*GH9_2089 was able to hydrolyze CMC. At 1 min, small amounts of oligosaccharides were present around cellotriose (G3) and cellotetraose (G4) compared to the standard. From 3 to 10 min, more oligosaccharides, particularly cellobiose (G2), G3, and G4, were accumulated, and they were clearly observed on the TLC plate. After 15 min to 16 h, the amounts of G2, G3, and G4 greatly increased, together with the accumulation of glucose (G1). This hydrolysis pattern likely indicates the endo-acting mode of *Cal*GH9_2089.

3.4. Processivity

Some GH9 endoglucanases have been shown to have processivity similar to exoglucanases [17]. The processivity of endoglucanase is usually determined by the ratio of the generated soluble reducing ends to insoluble reducing ends [16]. Here, the distribution of reducing sugars generated by *Cal*GH9_2089 on RAC was studied. It was found that the ratio of reducing ends in the soluble fraction to that in the insoluble fraction increased from 1.6 to 4.8 as incubation time was prolonged from 30 to 180 min (Figure 5). This result indicates that *Cal*GH9_2089 is a processive endoglucanase, differing from the common endoglucanases, for which their actions randomly cut and produce more reducing ends in the insoluble fraction than in the soluble fraction.

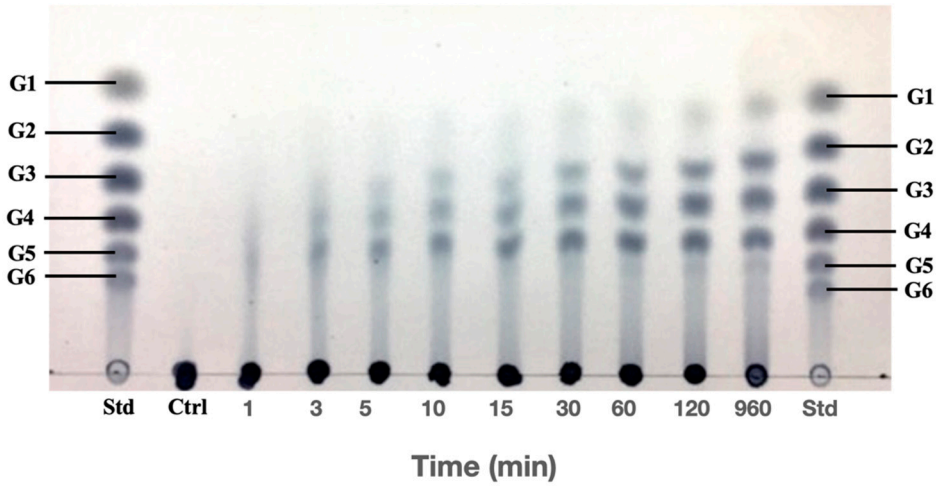

Figure 4. TLC analysis of hydrolysis product from CMC by *Cal*GH9_2089.

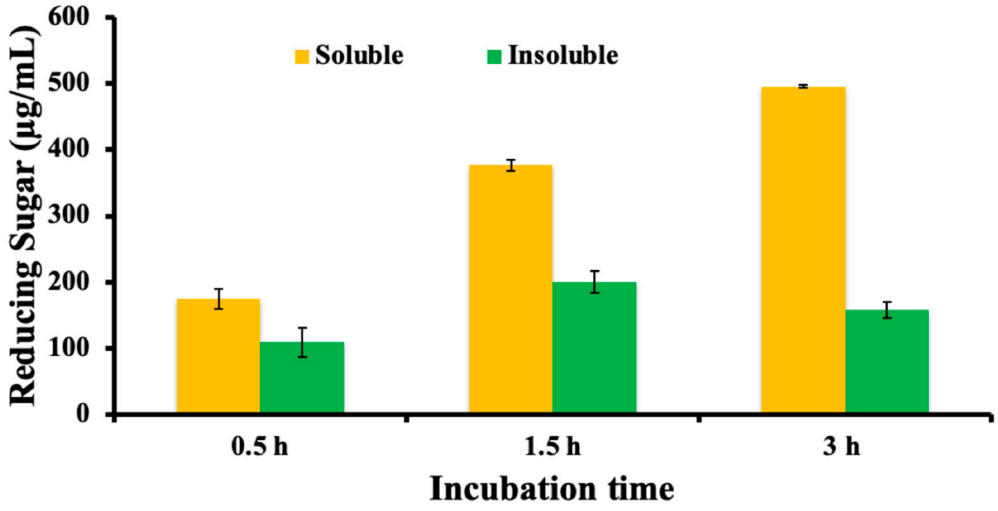

Figure 5. The processivity of *Cal*GH9_2089 towards RAC.

4. Conclusions

Herein, we successfully expressed a putative GH9 endoglucanase possessing a modular structure containing a GH9 catalytic module, a family 3 CBM, and one Type I dockerin (*Cal*GH9_2089) from *C. alkalicellulosi* genome. The enzyme was functionally active from pH 5.0 to 10.0 with an optimal pH around 6.0–7.0. The optimal temperature for this enzyme is 55 °C; however, its half-life was around 1 h. The production of a series of oligosaccharides from CMC indicates an endo-acting mode of *Cal*GH9_2089. Moreover, the ratio of reducing ends in the soluble fraction to that in the insoluble fraction using RAC as a substrate was 4.8 (3 h incubation), suggesting this *Cal*GH9_2089 endoglucanase possesses processive activity.

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