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Research paper

Fusion of beta glucosidase (BglA) to endoglucanase (Cel5L), enhanced their activities and thermostability

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Abstract

Beta-glucosidase gene (BglA) from *Thermotoga maritima* was fused to C-terminus of the catalytic domain of Cel5L from *Clsotridium thermocellum* with a linker sequence between the two domains. Fusion construct Cel5L-BglA was expressed in *E. coli.* The cellulase and the beta glucosidase activities in the fusion construct increased 2.0 and 1.8-fold, respectively as compared to their activities in free states. The component enzyme activities in the fusion were stable at 80 ºC for 2 h. The optimum temperatures for endoglucanase and beta glucosidase activities of the fusion construct were found to be 70 and 90 ºC, respectively under the assay conditions used. Optimum pH for endoglucanase activity was found to be 6.0, while for β-glucosidase activity optimum pH was 5.0. The HPLC analysis of the hydrolysates showed that the bifunctional enzyme Cel5L-BglA can efficiently hydrolyze CMC, producing glucose as the major end product.

Key words: Fusion constructs, *T. maritima, C. thermocellum,* Thermostability

Introduction

Cellulose is an organic compound with hundreds to thousands of linear chains of D-glucose units linked by β-(1→4) linkage. Cellulose exists in two main forms; amorphous cellulose which is water soluble and crystalline cellulose which is insoluble in water [1]. Due to its large availability, lignocellulosic plant material is the target for industrial production of ethanol. However, the production of bioethanol from plant biomass is still a challenge due to the high cost of the enzymes required for its breakdown to sugars. Efficient and cost effective production of bioethanol requires cellulases with high catalytic efficiency and thermostability [2]. Breakdown of cellulose requires the synergistic action of three enzymes; endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and beta glucosidases (EC 3.2.1.21) [3]. Endoglucanases and exoglucanases produce cellodextrins or cellobiose, which serve as the substrate for β-glucosidases to produce glucose [4,5]. Considering that hydrolysis of plant biomass requires more than one enzyme, fusion of two or more polypeptides to build a multifunctional enzyme can prove beneficial for industrial applications. Several natural enzymes with two or more functional domains on a single polypeptide chain have been reported previously [6,7]. Studies also reported multifunctional enzymes that are produced in vitro by the means of gene fusion [8,9]. Most of the chimera proteins synthesized by gene fusion strategy maintain the activities of all the fused polypeptides usually providing a significant advantage [10].

Synergistic degradation of cellulose by different cellulase enzymes results in the accumulation of a soluble disaccharide, cellobiose. The end product, cellobiose, acts as a inhibitor of exocellulases; therefore, removal of inhibitory cellobiose from the system is essential for constant degradation of lignocellulosic substrates. Previous studies have shown that the addition of β-glucosidase, the cellobiose-degrading enzyme, can increase the rate of plant

biomass hydrolysis by cellulases [11,12]. Therefore, fusion of endoglucanase and β-glucosidase can prove to be promising for efficient glucose synthesis which finally produces ethanol. Cel5L from *C. thermocellum* is a thermostable endoglucanase with optimum temperature in the range of 70-80°C and high activity on different β-1,4 linked polysaccharides including carboxymethyl cellulose, β-glucan, and cellooligosaccharides [13]. β-glucosidase from *T. maritima* is a 56 kDa protein with optimum temperature of 80-90 °C with significant activity on cellobiose [14]. In this study a β-glucosidase A from *T. maritima* was fused to the C- terminus of the catalytic domain of Cel5L from *C. thermocellum.* Two domains were joined with a linker sequence present after the catalytic domain of Cel5L. The bifunctional enzyme was analyzed for its properties and activities against different substrates.

Material and Methods

Bacterial strain and growth conditions

Genomic DNA of *T. maritima* (DSM-3109) and *C. thermocellum* (ATCC 27405D) purchased from DSMZ, Braunschweig, Germany were used to amplify BglA and Cel5L gene sequences, respectively. *E. coli* DH*5α* cells were used for the propagation of recombinant plasmids while *E. coli* BL21 (DE3) cells were used for the expression analysis. Both the strains were grown in LB medium.

Cloning, expression and purification

Nucleotide sequence of Cel5L and BglA were obtained from NCBI with Genebank accession# ABN51643 and CAA52276, respectively. Primers for Cel5L and BglA were designed using NEBcutter [15] Primer3 [16], and OligCalc [17]. Gene sequence of Cel5L and BglA were amplified using taq DNA polymerase followed by cloning in pET28a (+) vector. Recombinant plasmids Cel5L-pET 28a(+) and BglA-pET 28a(+) were digested with restriction endonucleases and then ligated so that BglA was fused to C-terminus of Cel5L. Recombinant plasmids Cel5L, BglA and Cel5L-BglA were used to transform competent cells of DH5-*α* and were confirmed through restriction analysis and colony PCR. Combinations of *NdeI* and *HindIII, NheI and XhoI,* and *NdeI* and *XhoI* were used for restriction confirmation of Cel5L, BglA and Cel5L-BglA, respectively.

Competent cells of *E. coli* BL21 (DE3) were then transformed with the recombinant plasmids. Transformed cells were incubated in LB medium at 37°C until OD_{600} reached to 0.7. The cells were induced using 0.5mM IPTG as an inducer and were grown additionally at 37 °C for 4 hr. For expression analysis of Cel5L, BglA, and Cel5L-BglA, 12% SDS-PAGE was used. Induced cells were harvested by centrifugation and resuspended in 50Mm tris-Cl (pH 8.0). Sonication was then performed using UP400S ultraschall processor (Germany) for 40-45 minutes. The sonicated samples were centrifuged at 14,000 rpm for 5 minutes at 4°C to separate soluble and insoluble fractions which were analyzed on 12% SDS-PAGE. Cel5L, BglA and Cel5L-BglA were purified by heat treatment at 70°C for 30 min.

Enzyme Activities

Enzyme activities were performed using the heat treated purified supernatant. Endoglucanase activities of Cel5L and Cel5L-BglA were determined using 2% CMC as a substrate, while β-glucosidase activities of BglA and Cel5L-BglA were determined using 2% Salicin. For endoglucanase activity, 0.5 mL of the diluted enzyme solution was incubated with 0.5 mL of 2% CMC prepared in 0.05M phosphate buffer (pH 6.0) at 70°C for 10 minutes. For β-glucosidase activity, 0.5 mL of the diluted enzyme solution was

incubated with 0.5 mL of 2% salicin prepared in 0.05M phosphate buffer (pH 6.0) at 90°C for 10 minutes. The amount of released reducing sugars was determined by using DNS method [18]. One unit of activity is defined as the amount of enzyme that releases1µmole of glucose per minute under the assay conditions [19].

Enzyme Characterization

Optimal pH of Cel5L-BglA was determined by measuring activities against 2% CMC and 2% salicin prepared in buffers of pH range 4.0-9.0. Optimal temperature was determined by measuring enzyme assay at different temperatures in the range 50-90°C for 10 minutes using 2% CMC or 2% salicin as substrate. Thermal stability was determined by incubating the enzyme at different temperatures ranging from 50-90 °C for different time periods upto 2 h and then measuring the residual activity.

HPLC analysis

Processivity of fusion construct was determined by analyzing the Cel5L-BglA digested hydrolysates through HPLC. 2% CMC and 2% salicin were treated with Cel5L-BglA as described above. The supernatant obtained after the reaction was passed through 3.0x78 mm column, HPX-42A, (Bio-Rad Laboratories, Inc.) with deionized water as mobile phase. Temperatures of column and the detector were maintained at 85 and 45°C, respectively. Peaks were detected through a refractive index detector (S3580; SyKam GmbH, Germany). Glucose, Cellobiose, cellotriose and cellotetrose were used as standards [20].

Results

Cloning, Expression and Purification

Cel5L, BglA and Cel5L-BglA were successfully cloned in pET 28a(+) vector and were confirmed through restriction digestion and colony PCR. Recombinant plasmids were expressed in *E. coli* BL21 (DE3) cells. All the recombinant plasmids were successfully expressed as is evident from SDS-PAGE analysis. Cel5L and BglA and fusion construct were expressed as partially soluble. Expression level was 30, 35 and 12% for Cel5L, BglA and Cel5L-BglA, respectively (Figure 1).

Enzyme Activities

Endoglucanase activity of Cel5L and Cel5L-BglA on 2% CMC was 170.5 and 343 U/µmol, respectively. Fusion construct showed 2.0-fold increase in endoglucanase activity compared to the Cel5L alone. β -glucosidase activity of BglA and Cel5L-BglA on 2% salicin was 11,907 and 21,756 U/µmol, respectively. β -glucosidase activity of the fusion construct was found to be 1.8 fold higher than the free BglA (Table 1).

Table 1: Specific activities of Cel5L, BglA and Cel5L-BglA on 2% CMC and 2% salicin.

Enzymes	Size (kDa)	Substrate	Specific Activity (U/µmol)	Fold increase
Cel5L	46	CMC	170.5	-
BglA	52	Salicin	11,907	-
Cel5L-BglA	98	CMC	343	2.0
Cel5L-BglA	98	Salicin	21,756	1.8

Enzyme Characterization

For endoglucanase activity of Cel5L-BglA, optimum temperature was 80ºC and optimum pH was 6.0, whereas, for β-glucosidase activity, optimum temperature was 90 ºC and optimum pH was 5.0 (Figure 2, 3). The results of exposure of the fusion construct to varying temperatures for different time period showed that Cel5L domain retained almost 80% of endoglucanase activity when incubated at 80ºC for 2h. BglA domain retained 70% of β- glucosidase activity when incubated at 80ºC for 2 h. Endoglucanase and β-glucosidase activity was reduced to 70% and 50% respectively, when the fusion construct was incubated at 90 ºC for 2 h (Figure 4).

HPLC Analysis

HPLC analysis of the hydrolysates produced by Cel5L-BglA showed that about 7% of glucose is produced by the bifunctional enzyme. These results show that Cel5L domain is hydrolyzing CMC to cellobiose or cellotetorse and as no traces of both of these products appeared on the graph which showed that these products were further taken up by the BglA domain of the fusion protein to produce glucose (Figure 5).

Discussion

Efficient hydrolysis of plant biomass to produce bioethanol requires enzyme engineering in order to produce highly efficient, cost-effective enzymes that are stable under industrial conditions. Since, for complete hydrolysis of plant biomass a consortium of enzyme is required, production of bifunctional enzymes would significantly reduce the cost for the production of biomass-based biofuels. In this study, we constructed a bifunctional enzyme by fusion of β -glucosidase (BglA) domain from *T. maritima* with endoglucanase domain (Cel5L) from *C. thermocellum*, and analyzed the enzymatic potential of the fusion construct. Cel5L, BglA, and Cel5L-BglA were expressed in E. coli BL21 (DE3) cells with expression level of 30, 35, and 12% respectively. The lower expression level of fusion construct is due to the large size of enzyme as we reported in previous study [21, 22, 23].

Activity analysis showed that the fusion construct, Cel5L-BglA, showed 2.0 and 1.8 fold increase in cellulase and β-glucosidase activity, respectively. One of the possible explanations for increase cellulase activity could be the substrate channeling i.e. cellobiose and cellotetroses that are produced by cellulase domain of the bifunctional enzyme are immediately taken up as substrates by β-glucosidase domain of the enzyme. This was confirmed through HPLC analysis of Cel5L-BglA digested hydrolysates, which showed that cellulose was efficiently hydrolyzed to glucose with only the traces of cellobiose if any. Similar results have been reported previously, where the fusion of β -glucosidase BglA to endoglucanase tCel5A1 resulted in 1.3 and 2.6 fold increased endoglucanase and β-glucosidase activity, respectively [22]. Additionally, in another study, specific fusion of β-glucosidase to cellulase showed significant increase in both the endoglucanase and β-glucosidase activity. Fusion construct Cel5L-BglA was stable at 80 °C for 2 h as the enzyme retained both of the activities. Cel5L alone was less thermostable than the fusion construct as reported previously [24]. The increased thermo-stability could be the result of protective effect caused by thermophilic BglA. This may give the hybrid enzyme a benefit to work under higher temperature for converting cellobiose to glucose [25].

In conclusion, the present study demonstrates a single polypeptide formed by the fusion of cellulase and β-glucosidase domain can efficiently hydrolyze the cellulose to glucose without the accumulation of end product cellobiose.

This makes bifunctional enzyme a suitable candidate for industrial production of bioethanol.

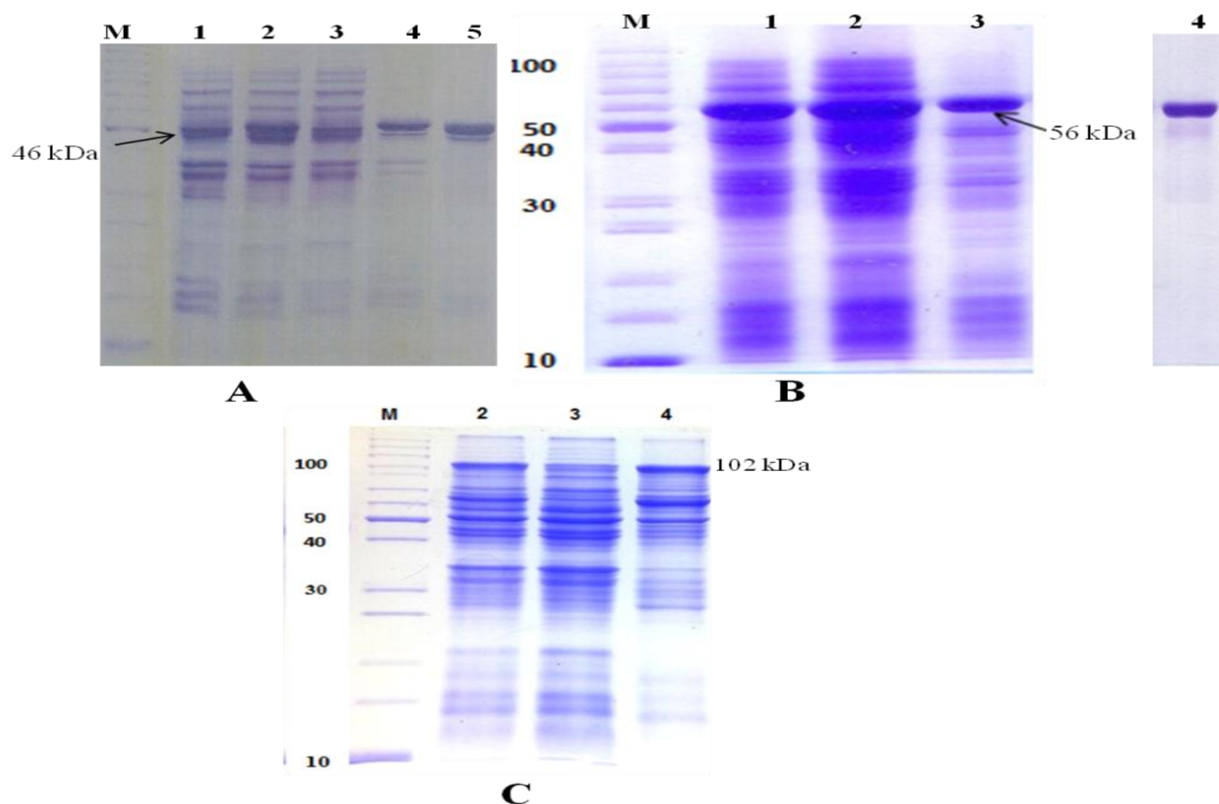

Figure 1: 12% SDS-PAGE showing the expression of Cel5L (A) BglA (B), and Cel5L-BglA (C) **A:** Lane M; protein size marker, lane 1: Uninduced cells, lane 2: TCP showing the expression of Cel5L, lane 3: supernatant, lane 4: pellet and lane 5: purified Cel5L. **B:** Lane M: protein size marker, lane 2: TCP showing expression of BglA, lane 2: supernatant, lane 3: pellet, lane 4: purified BglA. **C:** Lane M: protein size marker, lane 2: TCP showing the expression of Cel5L-BglA, lane 3: supernatant, lane 4: pellet.

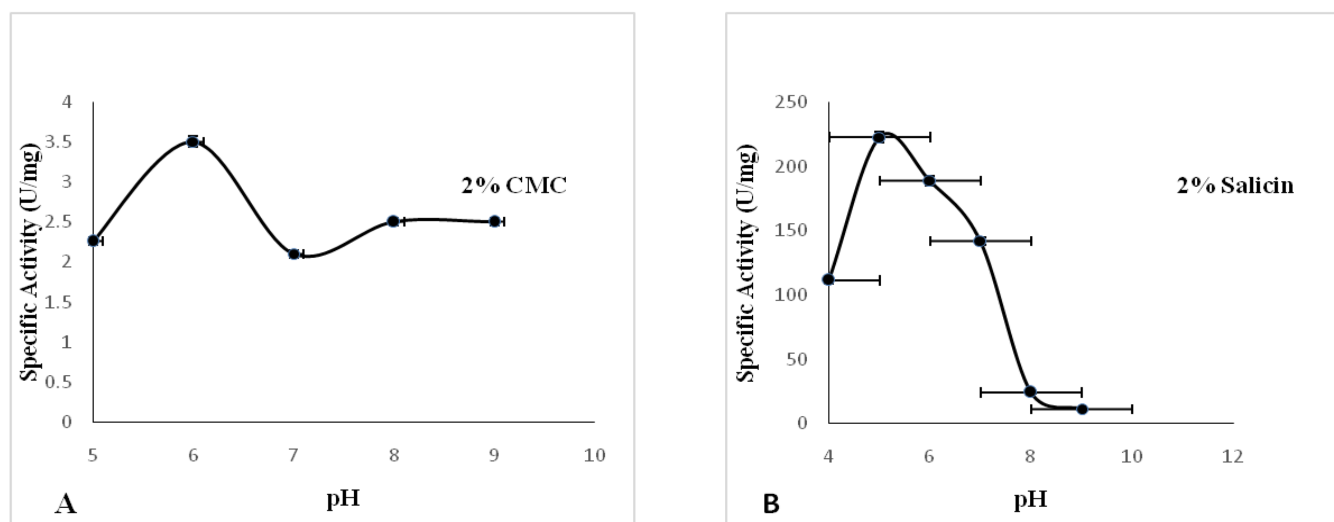

Figure 2: Effect of temperature on endoglucanase (A) and β-glucosidase activity (B) of fusion construct Cel5L-BglA.

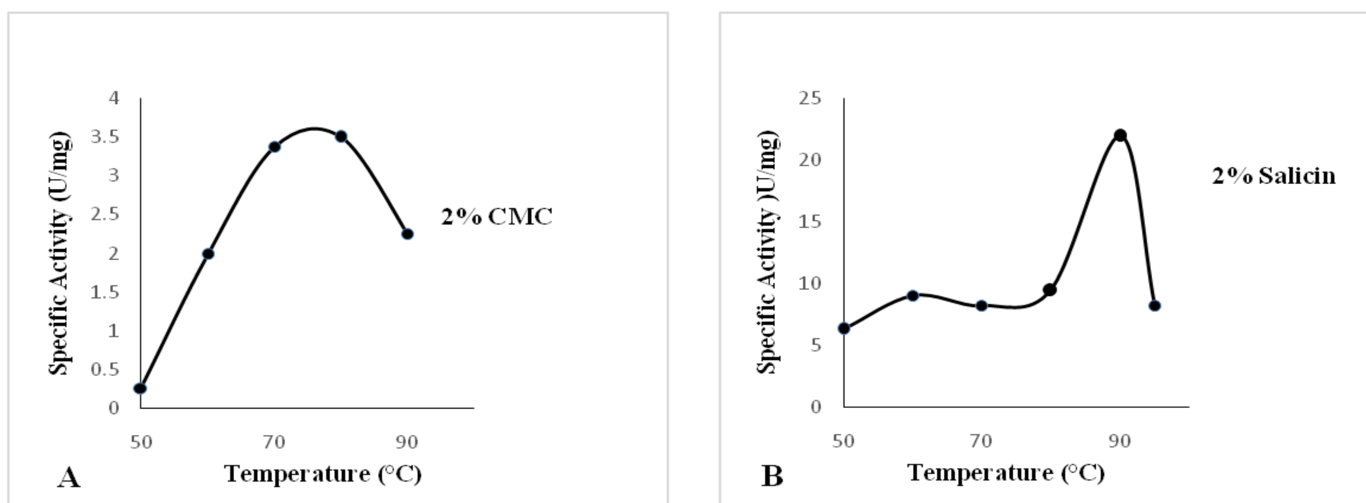

Figure 3: Effect of pH on endoglucanase **(A)** and β- glucosidase (B) activity of fusion construct Cel5L-BglA.

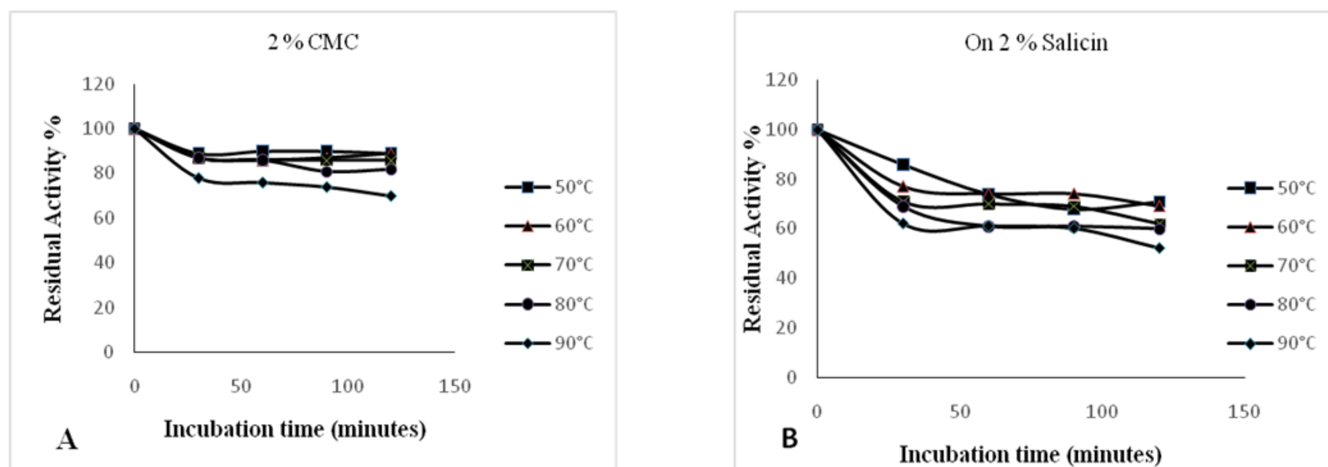

Figure 4: Effect of thermostability on endoglucanase (**A**) and β- glucosidase activity (**B**) of fusion construct Cel5L-BglA.

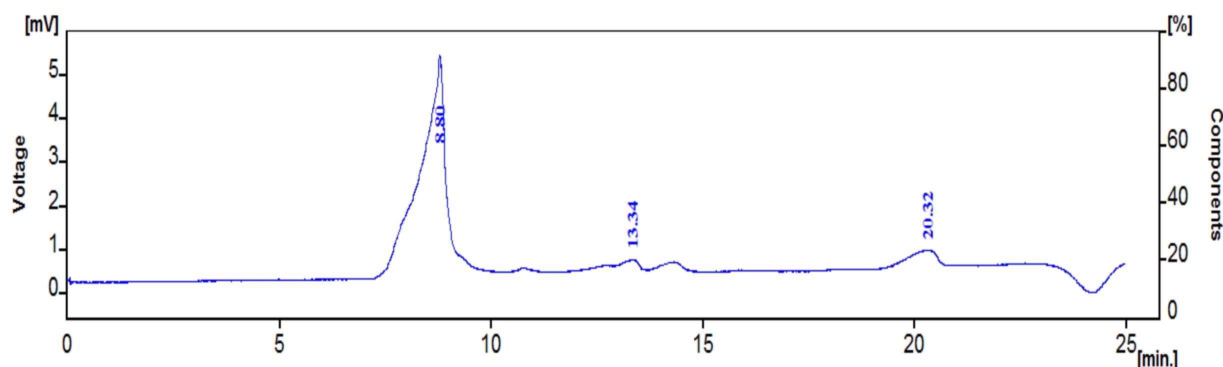

Figure 5: HPLC analysis of hydrolysates of Cel5L-BglA with 2% CMC.

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