Fusion Endoglucanase Cel12B from *Thermotoga maritima* with Cellulose Binding Domain

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Four fusion enzymes were expressed in *Escherichia coli* BL21 (DE3), and their properties against sodium carboxymethyl cellulose (CMC-Na) and microcrystalline cellulose were characterized. When endoglucanase *cel12B* genes fused with the cellulose binding domain (CBD) of the N-terminal of xylanases from *Thermotoga maritima* and *Thermotoga thermarum*, CBD1, CBD2, CBD3, and CBD4 fusion proteins were obtained. The four fusion proteins exhibited a certain adsorption of microcrystalline cellulose, and CBD4 showed the best performance. In addition, the optimum pH and temperature of fusion proteins were all somewhat decreased, and they became more sensitive to cations. The CBD1, CBD2, CBD3, and CBD4 displayed some enzyme activity towards microcrystalline cellulose; however, the CMC-Na enzyme activity was remarkably reduced.

INTRODUCTION

In recent years, research on cellulases derived from extreme microorganisms is becoming more extensive. Extremely thermophilic cellulases are mainly obtained from some thermophilic bacteria and archaea, such as the Thermotogales family, which comprises four kinds of bacteria (*Thermotoga maritima*, *T. petrophila*, *T. lettingae*, *T. napthophila, Thermosiphi* melanesiensis), *Fervidobacterium nodosum*, and *Sulfolobus islandicus* (Hreggvidsson *et al.* 1996; Reno *et al.* 2009; Zhaxybayeva *et al.* 2009; Wang *et al.* 2010; Lian *et al.* 2016; Zhang *et al.* 2017). Endoglucanase from the extremely thermophilic anaerobic bacteria *F. nodosum* possesses thermostability above 80 °C. This endoglucanase that was incubated at pH 5.0 to 5.5, 80 °C for 48 h still retained 50% of the enzyme activity and exhibited a strong thermal stability (Wang *et al.* 2010). Endoglucanase *EglA* from *Pyrococcus furiosus*, in which the optimum temperature was 100 °C, could maintain more than 50% of the residual enzyme remaining activity at 95 °C for 40 h, while all of them become inactivated at 112 °C after the required 40 h (Bauer *et al.* 1999). Cellulase genes from strict anaerobes *Rhodothermus marinus* that are expressed in *E. coli* showed 80% activity after incubation at 90 °C for 16 h (Hreggvidsson *et al*. 1996). Biochemical characterization of a new cellulase from an

Icelandic hot spring isolate revealed that it is a glycoside hydrolase with optimal activity at 70 °C and pH 5.0 that shows high heat-tolerance (Zarafeta *et al*. 2016). In the past few years, many more genes from extreme thermophilic bacteria have been sequenced; therefore, studies of thermostable cellulases derived from these thermophiles and their applications have received more attention. Some research has been conducted in high temperature hydrolysis of cellulosic materials; however, these cellulases mainly obtained from extreme thermophilic bacteria could not act directly on crystalline cellulose. Therefore, it is necessary to conduct molecular transformation of these extremely heat-resistant cellulases, making them efficient and stable for the degradation of natural cellulose in the future.

In view of the above problems, research of cellulose binding domain (CBD) creates a channel for further degradation of cellulose using fusion enzymes. After the extremely heat-resistant cellulase gene being fused with its matched CBD, the acquired fusion enzymes are expected to possess novel properties that may be more fitting for the hydrolysis of natural cellulosic material. Through fusing of the endoglucanase *cel74* gene from *T. maritima* with the CBD gene of *Pyrococcus furiosus* chitinase, compared to none-CBD Cel74, the fusion enzyme gained the ability to hydrolyze the microcrystalline cellulose (Avicel), releasing 0.18 μmol of reducing sugar per min (Chhabra and Kelly 2002).

However, the intervention of exogenous CBD is bound to change the structure of the fusion enzyme and then affect the thermal stability of the fusion enzyme. By studying *Bacillus subtilis* JA18 endoglucanse Egl499 and analyzing its amino acid sequence, there was a CBD domain in the protein N-terminal (Wang *et al.* 2009). After removal of the CBD domain, the endoglucanase Egl330 (without CBD) and Egl499 were both expressed and characterized. Compared to Egl499, Egl330 showed higher activity and thermostability. When incubating at 80 °C for 10 min, Egl330 kept 60% of its initial activity, while Egl499 kept only 12%. This indicates CBD's role in maintaining the enzyme's thermostability. Therefore, it is quite necessary to discover a certain CBD with higher activity and thermostability for further research.

Thermostable cellullases serve as potential advantages in industrial processes, due to their excellent specific activity for reducing supplementation of enzymes, higher thermostability, low risk of contamination, and improved pH flexibility (Zhang *et al*. 2017). Thus, this study aims to obtain fusion enzymes with desired properties for cellulose biodegradation at high temperature by fusing the expected *cbd* gene with the *T. maritima cel12B* gene. It is envisioned that this recombinant fusion enzyme may have an efficient effect on crystalline cellulose, and could be a candidate for application to lignocellulose enzymatic saccharification at high temperatures.

EXPERIMENTAL

Materials

Primers were synthesized by Sangon Biotech (Shanghai, China). The TaqDNA polymerase, restriction endonucleases, and DNA ligase were purchased from Takara Bio. Inc. (Dalian, China). Avicel (microcrystalline cellulose) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Others were analytical reagents from Sango Biotech (Shanghai, China).

Bacterial strains and plasmid

The *E. coli* Top10 and *E. coli* BL21(DE3) strains (Novagen, Madison, WI, USA) were respectively used for DNA molecule manipulation and recombinant protein expression. Plasmid pET-20b (Novagen, Madison, WI, USA) was used for cloning and the construction of expression vectors.

Extraction and amplification of genomic DNA

Genomic DNA of recombinant *T. maritima* was used as a template, and the following primers (Table 1) were used for the CBD fusion expression studies. In addition, the *cbd* gene from *T. thermarum* was synthesized by Generay Biotech (Shanghai, China). The PCR amplification conditions were as follows: pre-denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s; then extension at 72 °C for 10 min. In this study, primers 1 through 8 were utilized to fuse one *cbd* gene derived from N-terminus of *T. maritima* xylanase A with the *cel12B* coding gene (catalytic domain gene), primers 9 and 10 were used for the fusion of *T. thermarum* xylanase A 1 or 3 *cbd* genes and *cel12B*, as shown in Fig. 1.

Table 1. Nucleotide Sequences of Used Primers

Primers	Nucleotide Sequences
Primer 1	5'-GGAATTC**CATATG**AGTTTTGAAGGAACAACAGAAGGTG-3'
Primer 2	5'-CGC**GGATCC**CTTTGGGCCCGATTCTTTTGGAG-3'
Primer 3	5'- CGC**GGATCC**ATGACGAGCGTTGGTGCAACGG-3'
Primer 4	5'- CGC**GGATCC**TTTTACAACTTCGACAGAGAAGTCC-3'
Primer 5	5'-GGAATTC**CATATG**ACGAGCGTTGGTGCAACGGACA-3'
Primer 6	5'-CGC**GGATCC**TTTTACAACTTCGACAGAGAAGTCC-3'
Primer 7	5'-CGC**GGATCC**AGTTTTGAAGGAACAACAGAAGGTG-3'
Primer 8	5'-CCG**CTCGAG**CTTTGGGCCCGATTCTTTTGGAG-3'
Primer 9	5'-CGC**GGATCC**GATGACGAGCGTTGGTGCAACGG-3'
Primer 10	5'- CGC**GGATCC**TTTTACAACTTCGACAGAGAAGTCC-3'
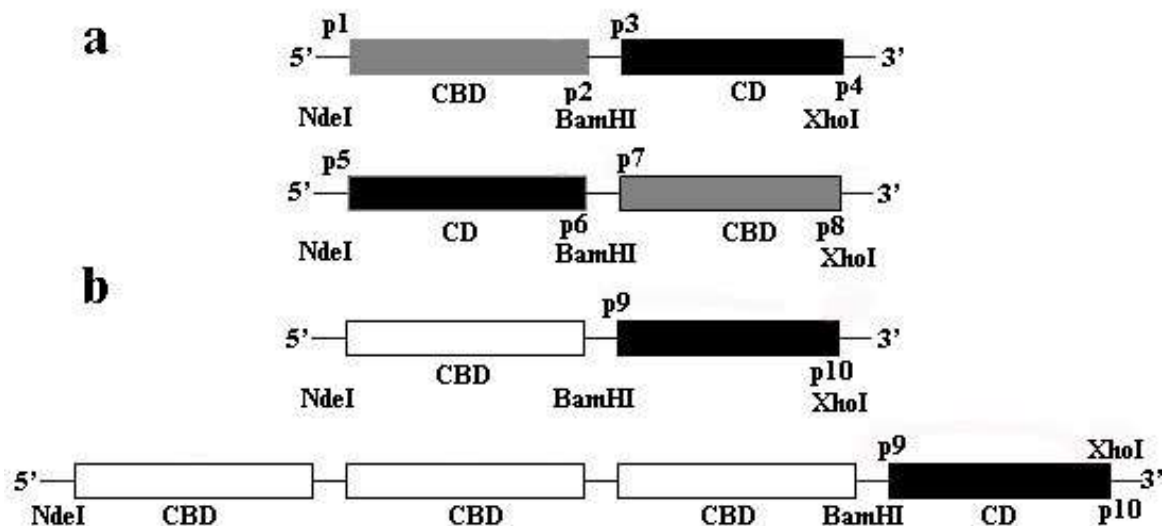
Fig. 1. Construction of fused Cel12B-CBD: a) CBD obtained from *T. maritima* and b) CBD obtained from *T. thermarum*

Construction and sequencing of expression vectors

The polymerase chain reaction (PCR) products were purified by a Biomiga PCR Purification Kit (Shanghai, China), followed by double-enzyme digestion of the purified products and pET-20b vector with restriction endonucleases NdeI, and BamHI or XhoI (Fig. 1). Next, they were ligated and transformed into *E. coli* Top10 competent cells. The positive transformants were screened by colony PCR, followed by double digestion verification. Afterwards, the positive colonies were sent to Sango Biotech (Shanghai, China) for sequencing, ensuring the correct recombinant vectors were obtained.

Expression and purification of fusion proteins

The four recombinant plasmids, which contained pET-20b-*cel12B*-CBD, were transformed into *E. coli* BL21(DE3) cells. The obtained single colony was inoculated into 5 mL of Luria-Bertani (LB) medium at 37 °C and 200 rpm overnight, then 2 mL of the above inocula were added into 200 mL of LB medium with appropriate ampicillin (100 μg mL^{-1}), and were incubated at 37 °C and 200 rpm. When OD_{600} reached 0.6 to 0.8, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM and inducted at the same condition for another 5 h. Cells were harvested by centrifugation at 4 °C, 10000 rpm for 5 min, washed twice with 20 mM Tris-HCl buffer (pH 7.9), followed by resuspending cell debris in 5 mL of 20 mM Tris-HCl containing 5 mM imidazole and 0.5 M NaCl. Cells after sonication (10% output, 40% duty cycle 5 s, 10 min) were centrifuged by 5000 g, at 4 °C for 20 min. Next, the supernatant was purified using affinity chromatography by loading samples onto a Ni^{2+} column (5 cm, Novagen, Madison, WI, USA), then eluted with different concentrations of imidazole buffer (50 mM to 1000 mM). Purified fractions were concentrated by an Amicon Ultra-4 10K centrifugal filter, and then kept in 20% glycerol at -20 °C for further use.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The analysis of yield and purity of proteins was carried out with a 12% SDS-PAGE medium (Lammirato *et al.* 2011) using a wide range of molecular weight markers (MBI Fermentas, Foster, USA) as standards. Concentration of protein was measured by the Bradford method using bovine serum albumin (BSA) as a standard. The electrophoretic results were then analyzed by a gel imaging system (Bio-Rad, Foster, USA).

Enzyme assay

The enzyme activity was assayed at pH 6.0 and 85 °C in a reaction mixture (200 μL) containing 90 μL of 0.1 mol L^{-1} pH 6.0 imidazole/potassium-phthalate buffer, 100 μL of CMC-Na substrate, and 10 μL of diluted enzyme solution. After incubation of the mixture for 10 min, 300 mL of terminator DNS was added to stop the reaction by boiling for 5 min. The reducing sugar was measured at 520 nm. One unit of enzyme activity was defined as the amount of enzyme necessary to liberate 1 μmol of glucose per min.

Methods

Determination of properties of fusion proteins

The optimum pH of fusion enzymes was determined by incubation at 75 °C for 10 min in 50 mM imidazole/potassium-phthalate buffer adjusted to various pH values in the

range of 3.0 to 8.5. For optimum temperature, the enzymes were reacted with substrate in 50 mM Tris-HCl buffer (pH 6.5) for 10 min at temperatures ranging from 65 °C to 100 °C (5 °C intervals). The highest activity of each enzyme was defined as 100%, and the relative activity was calculated.

Metal ions and chemical reagents on enzyme activities were also investigated. In the reaction system, the final concentration of Mg^{2+}, Zn^{2+}, Ba^{2+}, Ni^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, or Al^{3+} was 1 mM, and the final concentrations of the chemical reagents Tween 60, Tris, or SDS were respectively 0.05%, 0.05%, and 0.1%, respectively. Before the reaction, a variety of fusion protein or unfused protein and metal ions or reagents were added into 50 mM imidazole imidazole/potassium-phthalate buffer, incubation at 85 °C for 1 h, then 1% CMC-Na was added to start the reaction. Under the same conditions, the enzyme activity without metal ions or chemical was defined as 100%.

Hydrolysis experiment of fusion protein on natural cellulose

A total of 100 µL of diluted enzyme solution was added into 900 µL of 0.1 mol l^{-1} imidazole/potassium-phthalate buffer (pH 6.0) and 1 mL of 10% microcrystalline cellulose; then the mixture was incubated at 80 °C for 30 min, followed by centrifugation at 12000 rpm for 20 min. Then, 200 µL of the reaction solution was removed and immediately mixed with 300 µL DNS reagents. The mixture was boiled for 5 min. The enzyme activity was determined using the same conditions as above, and using reactions without the enzyme as a control.

Fusion protein adsorption experiment

Each fusion protein and cellulose were mixed into the buffer solution as shown above. The control group was treated with the same fusion protein without microcrystalline cellulose. After incubation in a water bath at 60 °C for 1 h, the absorption of microcrystalline cellulose was confirmed by SDS-PAGE gel.

RESULTS AND DISCUSSION

Microbes that produce cellulases are widely distributed in fungi, bacteria, and actinomycetes. In the past several decades, numerous cellulase-producing strains have been reported due to their ability of hydrolysis against cellulosic fibers. For a long time, the study of cellulases mainly originated from *Trichoderma reesei*, *Trichoderma viride*, and *Trichoderma koningii*. Because these cellulases possess both CMC-Na enzyme activity and filter paper (FP) enzyme activity, these strains have been generally explored for industrial applications. Among these industrial strains, *T. reesei* are usually widely used in cellulose-degradation related research. Currently, thousands of cellulase genes have been cloned and expressed in *E. coli* or yeast, including endoglucanase, beta-glucosidase and exoglucanase, *e.g.*, endoglucanase and exoglucanase from *T. reesei*, endoglucanase from *Clostridium cellulovorans* (Ghose and Sahai 1979; Sacco *et al.* 1984; Liebl *et al.* 1996; Kovacs *et al.* 2008). So far, cellulase cloning, expression, and production technology have tended to be quite mature, but their enzymatic properties do not satisfy all aspects of current demand; therefore, it becomes more urgent to seek enzymes that can hydrolyze cellulose in some specific conditions. Perhaps thermophilic enzymes are possible to be good candidates for applications in cellulose biodegradation.

T. maritima and *Fibrobacter succinogenes* are thermophilic bacteria. They were reported to have cellulases with specific properties that may meet the need for specific applications (Huang *et al.* 1990; Salleh *et al.* 2006; Wang *et al.* 2010). In general, cellulases do not bind CBD domains, and therefore they tend to exhibit low degradation properties when exposed to insoluble cellulose. Studies have shown that CBD has an important role in certain capacities in cellulose adsorption and degradation (Linder and Teeri 1997; Srisodsuk *et al.* 1997; Chhabra and Kelly 2002). Thus, it is advantageous for degradation of crystalline cellulose by fusing cellulase with CBD.

Identification of Recombinant Fusion Proteins

Confirmed by restriction enzyme digestion and sequencing, the recombinant plasmids obtained above and plasmids without target genes were then transferred into *E. coli* BL21 (DE3). Purification was carried out using the purified procedure above. A total of four fusion proteins were obtained and identified as CBD1, CBD2, CBD3, and CBD4, as shown in Fig. 2. The amino acids of Cel12B (no CBD linked), fusion protein CBD1, CBD2, CBD3, and CBD4 were 253, 410, 410, 410, and 730 base pairs, respectively, and their theoretical molecular weights were 28 kDa, 45 kDa, 45 kDa, 45 kDa, and 80 kDa, respectively. These were consistent with the SDS-PAGE results.
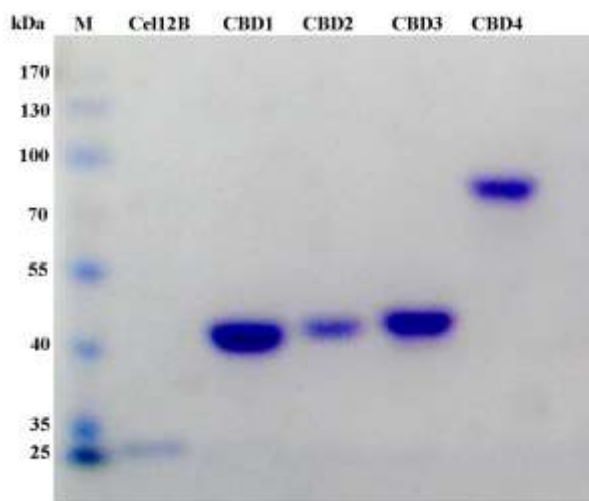

Fig. 2. SDS-PAGE of fusion enzymes

Enzymatic Properties of Fusion Proteins

In the present study, Cel12B derived from *T. maritima* was fused with CBD originated from *T. maritima* and *T. thermarum* xylanase A, respectively. The optimum temperature of every fusion enzyme was measured at a variety of temperatures ranging from 65 °C to 100 °C. The results showed that the optimum temperature of CBD1 and CBD4 decreased from 90 °C to 85 °C, with no obvious change towards CBD2 and CBD3. Compared to the original Cel12B, the enzyme activity of CBD3 and CBD4 were dramatically increased 70% in the temperature range of 70 °C to 85 °C. However, almost all of the fusion enzymes exhibited a noticeable decrease of enzyme activity over 90 °C (Fig. 3). The results showed that the optimum temperature of four fusion enzymes were noticeably lower than that of native Cel12B. This result was same as for *Bacillus subtilis* JA18 truncated endoglucanase by removal of CBD (Wang *et al.* 2009).
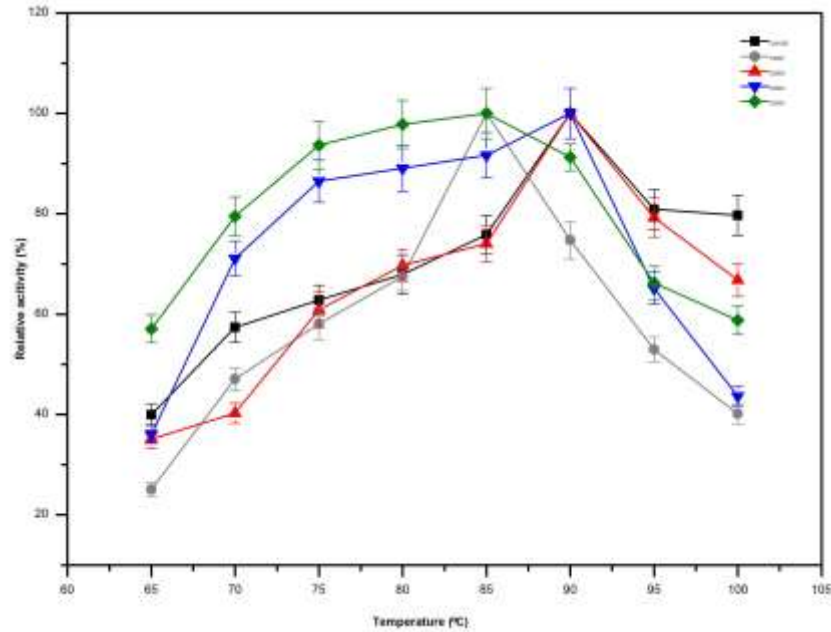
Fig. 3. Effect of temperature on fusion enzymes activities

To determine the optimum pH of fusion enzymes, a series of different pH buffers (pH 4.0 to 8.5) were assayed. As shown in Fig. 4, the optimal pH of all of the fusion enzymes decreased 5 °C or 10 °C. Among them, CBD2, CBD3, and CBD4 dropped down to pH 5.0, while CBD1 attained pH 4.5. The optimum pH of fusion enzymes was also decreased to some extent, which suggested that this may have been caused by the interaction of certain acidic amino acids present on the CBD or the structure change at active region.
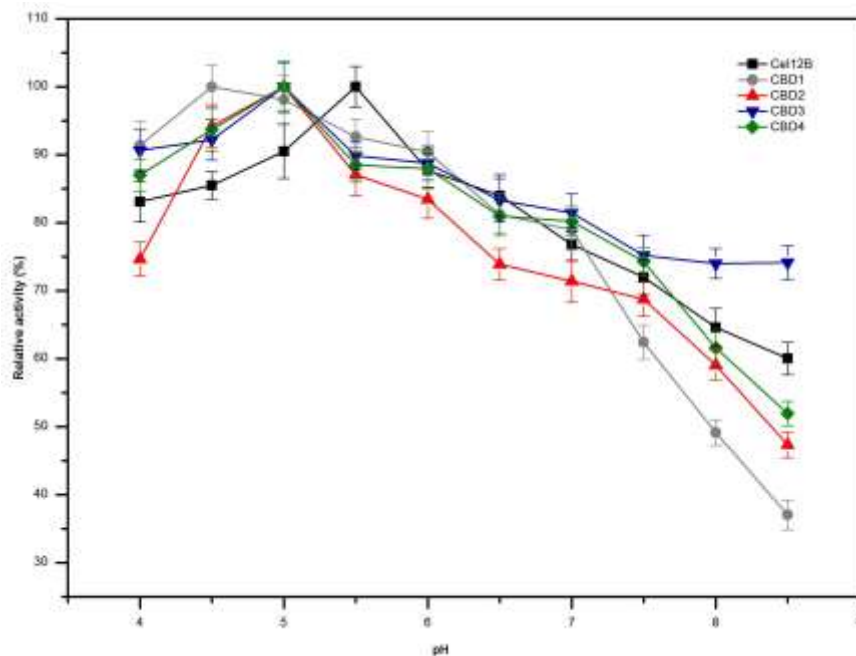
Fig. 4. Effect of pH on fusion enzymes activities

When testing the effects of metal ions on fusion enzymes, 0.1 μg enzyme, a variety of ions (1 mM), or chemical reagents (shown in methods) in corresponding buffers were incubated for 10 min before the CMC-Na was present. Figure 5 shows that the sensitivity of the fusion protein to metal ions was enhanced compared with the control. In particular, the sensitivity towards Al^{3+} and Cu^{2+} was reduced by 60%. Although most of the metal ions inhibited the enzyme activity of fusion enzymes, Mn^{2+} and Co^{2+} only showed a slight effect. Among reagents, SDS displayed a greater impact on fusion enzyme activity, while Tris and Tween exhibited little influence. In addition to Mn^{2+} and Co^{2+}, all other metal ions remarkably weaken the enzyme activity, indicating that Mn^{2+} and Co^{2+} as metal cations play a vital role in promoting the enzymatic activity of many cellulosic hydrolases, *e.g.*, xylanases and xylosidase from *T. thermarum* (Shi *et al.* 2013a, 2013b), and β-glucosidase from *T. thermarum* (Pei *et al.* 2017). Based on analyzing the catalytic domain of these enzymes, it was found that there were some binding sites to Mn^{2+} and Co^{2+}. Therefore, it is proposed that Mn^{2+} or Co^{2+} can stabilize the catalytic center of the enzyme, and then they can promote the enzyme activity. When Cu^{2+} or Al^{3+} are present, the enzyme catalytic center is squeezed, thereby inhibiting the enzyme hydrolysis.
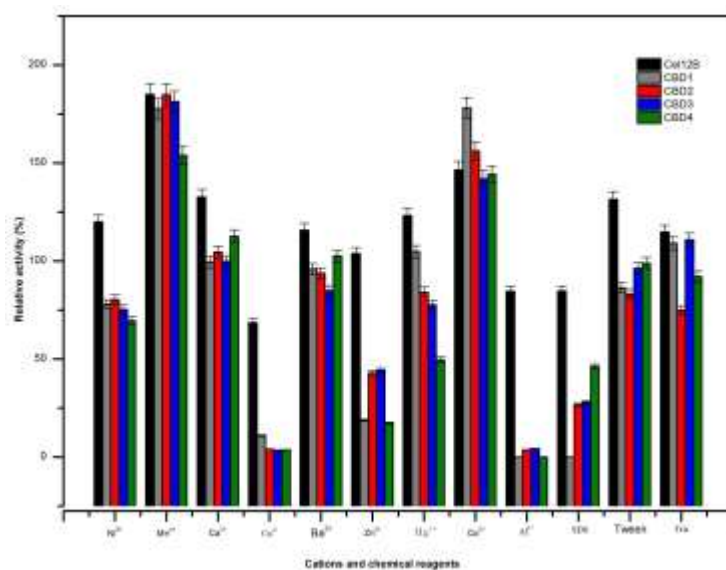

Fig. 5. Effect of cations and chemical reagents on fusion enzyme activities

Fusion Enzymes on Adsorption of Crystalline Cellulose

The four purified fusion proteins were mixed with the microcrystalline cellulose, while the control group was each fusion protein without adding microcrystalline cellulose. After being in a water bath shaker (100 rpm) at 60 °C for 1 h, Fig. 6 shows that Cel12B did not had the ability to adsorb microcrystalline cellulose, while CBD1, CBD2, and CBD3 showed a certain adsorption capacity. Surprisingly, CBD4 was completely adsorbed with microcrystalline cellulose, and after centrifugation, there was no soluble protein in the supernatant.

The fusion enzymes of this research showed a certain ability to bind microcrystalline cellulose, especially CBD4 that can be completely bound to the substrate. Such behavior is closely related to the species properties of the CBD, and the structure formed by the catalytic domain.
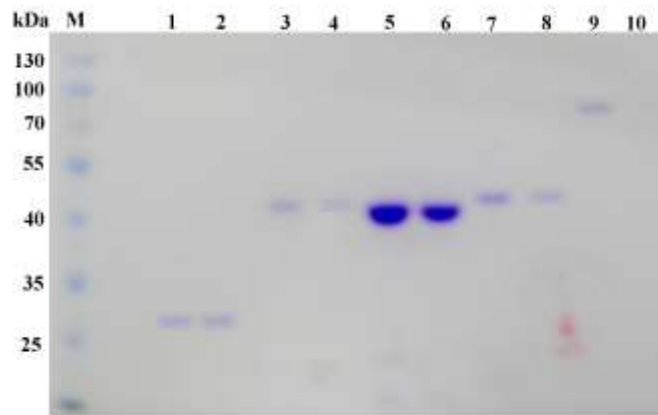
Fig. 6. Adsorption of fusion enzymes and Cel12B to avicel. Lanes 1, 3, 5, 7, 9: Ce12B, CBD1, CBD2, CBD3, and CBD4 without addition of microcrystalline cellulose, respectively; Lanes 2, 4, 6, 8, 10: Ce12B, CBD1, CBD2, CBD3, and CBD4 with addition of microcrystalline cellulose

Specific Activity of Fusion Enzymes

When using CMC-Na and microcrystalline cellulose as substrates, the recombinant enzyme Cel12B and fusion enzymes hydrolyzed the substrates at the appropriate temperature and pH conditions as listed above. The ability of fusion enzymes degrading CMC-Na dropped remarkably, but they promoted the degradation of microcrystalline cellulose (Table 2). CBD1, CBD2, and CBD3 showed a certain degradation to the microcrystalline cellulose, however, compared to soluble CMC-Na, the fusion enzyme on the insoluble crystalline cellulose degradation capacity was still poor.

In this study, the adsorption of microcrystalline cellulose by fusion enzymes achieved good results; however, the degradation of natural cellulose was still not satisfactory, which is consistent with Chhabra's results (Chhabra and Kelly 2002). When a foreign gene is chosen to be fused with, a linker peptide is also critical to fuse an enzyme's fold between two domains. Therefore, linker peptides and varieties of CBDs will be explored in the authors' further investigations.

Table 2. CMC-Na and Avicel Activity of Recombinant Cel12B and Fusion Enzymes

Recombinant Protein	Enzyme Activity ($\mu mol\ min^{-1}\ mg^{-1}$)	
	CMC-Na	Avicel
Cel12B	99.64 ± 4.2	0.03 ± 0.01
CBD1	53.12 ± 2.6	0.76 ± 0.05
CBD2	23.55 ± 1.1	0.16 ± 0.02
CBD3	36.33 ± 1.8	0.05 ± 9.01
CBD4	31.42 ± 2.1	0.01 ± 0

CONCLUSIONS

1. Four thermophilic fusion endoglucanases, CBD1, CBD2, CBD3, and CBD4, were obtained with a few specific properties.

2. The results revealed that the four fusion proteins were able to adsorb a certain amount of microcrystalline cellulose, and CBD4 showed the best performance. In addition, the optimum pH and temperature of fusion proteins were all somewhat decreased, and they became more sensitive to cations. The CBD1, CBD2, CBD3, and CBD4 displayed some enzyme activity towards microcrystalline cellulose; however, the CMC-Na enzyme activity was remarkably reduced.

3. Maybe caused by the inflexibility of Cel12B itself, desirable recombinant enzymes were still lacking.

ACKNOWLEDGMENTS

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