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Biochemical property of an endo-glucanase-like enzyme from *Clostridium* sp. Z-7026

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ABSTRACT

Clostridium sp. Z-7026 is a cellulose-degrading anaerobic bacterium, and its genome was recently sequenced. By Blast analysis against the NCBI protein database, one gene encoding a cellulase-like protein was identified. This deduced protein was named Cel_2759, and the Cel_2759 encoding gene was cloned, expressed in *Escherichia coli*, and purified. The purified recombinant protein had an estimated size of 107 kDa. To reveal its hydrolyzing ability, five substrates, namely carboxymethylcellulose (CMC), regenerate amorphous cellulose (RAC), crystalline cellulose Avicel beechwood xylan (BWX), and pretreated rice straw were used for activity assay. Results showed that, with in 15 min, Cel_2759 actively hydrolyzed CMC, yielding a reducing sugar concentration of 689.45 µg/mL. However, activities against RAC, BWX and rice straw required pronged incubation time up to 16 h, yielding reducing sugar concentrations of 73.32 µg/mL, 1393.05 µg/mL, 875.704 µg/mL, respectively. At 24 h, activity against Avicel was not observed, suggesting that Cel_2759 was unable to hydrolyze crystalline cellulose; by contrast, it preferred soluble long chain substrate CMC for hydrolysis. Using CMC as the substrate, Cel_2759 had its own optimal pH and temperature for its biological function at pH 7.0-8.0 and 55 °C. The hydrolysis products from CMC by Cel_2759 were a mixture of cello-oligomers, suggesting that Cel_2759 was an endo-acting enzyme.

Keywords: Cellulose, Cello-oligosaccharide, *Clostridium* species, Endoglucanase

INTRODUCTION

At present, a search for alternative energy source towards sustainable future energy supply for the growing global population is one of the largest challenges that we are facing according to the depletion of fossil resources and the threat of global climate changes (Wu, 2013; Grennan, 2006). Lignocellulosic biomass, such as plant materials, is one of the most promising renewable resources that show potential for meeting a significant portion of the demand for energy (Butterman and Castaldi, 2010).

Lignocellulosic biomass is composed of three main components: cellulose, hemicellulose, and lignin. Cellulose is a polymer consisting of long unbranched chains of β-linked D-glucose units and it represents as the main constituent of plant cell walls. For cellulosic ethanol production, glucose is the most desirable product for ethanol fermenting microorganisms (Srivastava et al., 2014). However, cellulose itself is resistance to enzymatic degradation because of its structural rigidity and insolubility. In addition, a major technical challenge in the cost-effective production of cellulosic biofuel is the need to lower the cost of enzymes, particularly cellulases (Jung et al., 2012).

Endo-glucanases are one of the main cellulases that act randomly on internal amorphous cellulose fibrils to produce free cellulose chain ends and cello-oligosaccharides (Bhalla et al., 2014). This type of cellulolytic enzymes is important because the generation of free chain ends become new sites for exo-glucanases or cellobihydrolases to attack. Therefore, the hydrolysis rate of cellulose is expected to increase when endo-glucanases work in concert with exo-glucanases.

Among glycoside hydrolase families (GH), GH9 family consists mainly of enzymes with endo-glucanase activity. However, some of them shows low substrate specificity towards cellulosic substrates. For example, Thcel9A from *Thermibifida halotolerans*, which is belonged to a classical GH9 group A endoglucanase, showed activity not only on carboxymethyl cellulose (CMC), but also on microcrystalline cellulose and filter paper (Zhang et al., 2011). Moreover, a GH9 enzyme (CenC) from *Clostridium thermocellum* showed a broad activity towards CMC, Whatman filter paper, β-glucan barley, xylan, and laminarin (Haq et al., 2015). Interestingly, one single GH9 is reported to degrade cellulose efficiently (Tolonen et al., 2009). Thus, activity of GH9 enzymes varies and it is worth for further investigation.

Extremophilic cellulolytic bacteria have gained considerable attention due to their great natural diversity, higher growth rate and proficiency for the production of thermotolerant, acid and/or alkali-stable enzymes, (Haq et al., 2015; Zhang et al., 2010 and Essghaier et al., 2018). *Clostridium* spp. is known as a good source of cellulolytic enzymes (Bras et al., 2011). *Clostridium* sp. Z-7026 is an alkaliphilic anaerobic bacterium with an excellent cellulose degrading ability. Recently, the genome of *Clostridium* sp. Z-7026 was sequenced, revealing several cellulase-encoding genes. In the present work, we cloned and expressed one GH9-like enzyme, designated as Cel_2759, and determine the biological functions. Understanding the biochemical property and mode of actions of Cel_7259 could contribute to enzymatic degradation of cellulosic biomass for biofuel production, textile (biopolishing of fabrics) industry, Kraft pulp bleaching process, laundry detergents, and preparation of animal feeds (Liu et al., 2011; Seneesrisakul et al., 2017 and Rungrattanakasin et al., 2018).

MATERIAL AND METHODS

Cloning, expression, and purification

Genomic DNA of *Clostridium* sp. Z-7206 was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany) and used as a gene template for cloning.

PCR amplification of Cel_2759 was performed using the upper primer (5'-AATTAGCTAGCGATCCAGAGTACAACTTTGC-3') and the lower primer (5'- AATTACTCGAGTTAACGCGGCAGTTGTGGAA-3') through the Phusion taq High-Fidelity PCR-System (ThermoFisher Scientific, USA). The PCR product was run on 0.8% (w/v) agarose gel to check the target band and purified using PCR purification kit (Qiagen, USA.).

The purified PCR product and pET28a(+) vectors (Novagen, USA) were doubly digested with restriction enzymes: NheI and XhoI. The digested PCR products and pET28a(+) vectors were then ligated with T4 DNA ligase (New England Biolabs, UK). The ligated plasmids were transformed into NEB 5-alpha *E. coli* cells for sub-cloning.

The positive clones with the target insert was transformed into *E. coli* BL21 (DE3), which was used as a host for protein expression, and the cultivation and protein expression conditions were conducted according to the manufacturer protocols (New England BioLabs). The recombinant Cel_2759 protein was purified from a 400 mL transformed *E. coli* BL21 (DE3) culture with 3 mL Ni-NTA Superflow Agarose columns (Qiagen) and determined its purity on SDS-PAGE.

Assays of enzyme activity

The reaction mixture was prepared in a 1.5-mL microcentrifuge tube. The mixture contained 100 μL of 2% (w/v) substrate, 4.3 μL of 0.25 μM enzyme, and 20 μL of 10x TBS buffer (final concentration of 50 mM, pH 7.4). The reaction mixture was then added with distilled water to a final total volume of 200 μL. The reactions were incubated at 55 °C in a Thermomixer (Eppendorf, Germany) with a shaking speed at 1,000 rpm for 15 min for CMC and 16 h for RAC, Avicel, and pretreated rice straw. The reaction was terminated by incubation on ice bath and then centrifuged at 10,000×*g*, 4°C for 5 min. The amount of reducing sugar released from the substrates was determined by using the 3, 5-dinitrosalicylic acid reagent method (DNS) (Miller, 1959), using glucose as a standard. The absorbance was measured at 540 nm using a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (ThermoFisher Scientific, USA).

Pretreated rice straw (aqueous ammonia treated rice straw) was prepared according to (Phitsuwan et al., 2017). The pretreated rice straw contained cellulose, hemicellulose, and lignin.

Substrate specificity and effect of substrate concentrations on enzyme activity

To find preferred substrates for Cel_2759, activities on different substrates, including CMC, RAC, Avicel, and pretreated rice straw were determined. The enzymatic reactions were carried out in TBS (pH 7.4) and incubated at 55 °C for 10 min for CMC and 16 h for the others.

The effect of substrate concentrations on enzyme activity was studied by preparing the reaction in 1.5-mL microcentrifuge tube using CMC at the concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4% (w/v). The activity against CMC was assayed as described earlier. The reducing sugar concentration was determined by DNS method.

Effects of pH and temperature on enzymatic activity

The effect of pH was determined by measuring the enzyme activity at 55 °C in three buffers (50 mM): Na-citrate (pH 3.0-7.0), Tris-HCl (pH 7.0-9.0) and glycine-NaOH (pH 9.0-11.0). The pH that enzyme is most active, as indicated by the highest reducing sugar release, was considered an optimal pH for the enzyme.

For pH stability, the enzyme (4.3 μL) was pre-incubated in reaction mixture containing 10 μL of 10x buffer, whose pH ranged from 3.0-11.0 at 37 °C for 1 h. After that, the reaction was added with distilled water up to 80 μL and 20 μL of 10x TBS. The reaction tube was placed on Thermomixer with a preset temperature of 55 °C. Then, 100 μL of 2% CMC was immediately introduced to the reaction tube and incubated for 10 min. The reaction was terminated by placing the tube on ice bath and centrifugation at 4 °C. The supernatant was taken and measured for reducing sugar.

To determine the optimum temperature for enzymatic activity of Cel_2759, the reaction temperature for enzymatic assays was carried out in a temperature range of 40-70°C at pH 7.4 (TBS). The temperature that enzyme is most active was considered the optimal temperature for the enzyme.

Time course and TLC analysis

Time course of CMC hydrolysis by Cel_2759 was studied by preparing the reaction mixture containing 1% (w/v) CMC and 4.3 μL of enzyme in TBS. The total volume of the reaction was 200 μL. The samples in individual tubes at specific incubation times were measured for reducing sugar release using DNS method.

The hydrolysis product was analyzed by Thin Layer Chromatography (TLC). The samples were spotted on a silica gel 60 F245 plate and dried. The plate was placed in the TLC chamber containing a mobile phase consisting of n-butanol, acetic acid, and water (2:1:1). After that, the plate was dried and sprayed with visualizing agents (4 g of α-diphenylamine, 4 mL of aniline, 200 mL of acetone, and 30 mL of 80% phosphoric acid) to detect sugars. The sugar spots were developed by heating the plate at 95 °C for 5 min.

RESULTS

Cloning and protein expression

Using a genomic DNA of *Clostridium* sp. Z-7206 as a template and specific primers, a gene encoding Cel_2759 was amplified by PCR. The PCR product with a target size of 2,841 bp was detected after run on an agarose gel and the PCR product was then purified. The purified PCR product and vector were double-digested with NheI and XhoI restriction enzymes, ligated by T4 ligase, and transformed into NEB 5-alpha *E. coli* cells. The identity of recombinant plasmids of positive transformants was checked by colony PCR and DNA sequencing. It was found that the complete nucleotide sequence of Cel_2759 was 2,841 nucleotides in length and encoded a polypeptide of 721 amino acid residues, with a calculated molecular weight of 107 kDa using ProtParam tool (web.expasy.org/protparam/).

The recombinant plasmid containing Cel_2759 was transformed into *E. coli* BL21 (DE3) for protein expression. After cultivation, the *E. coli* cells were harvested and disrupted by sonication. The release proteins were thus purified with Ni-NTA column, as Cel_2759 contained an N-terminal histidine tag in its polypeptide. The purified Cel_2759 was determined for its purity by SDS-PAGE analysis, showing the apparent size of 107 kDa (data not shown). This result was consistent with the calculated molecular weight.

Substrate specificity

The substrate specificity was determined by incubating the enzyme with various substrates at 55 °C in TBS (pH 7.4). Within 15 min-incubation, Cel_2759 was highly active on CMC (689.45 µg/mL). By contrast, activities of other substrates, including RAC, Avicel and pretreated rice straw required prolonged incubation time (16 h). At 16-h incubation, Cel_2759 showed activities of pretreated rice straw at 39.15% (875.704 µg/mL). Activity of Cel_2759 on RAC was inferior and the activity on Avicel at 0.25 µM enzyme load was undetectable (Figure 1). These results indicates that Cel_2759 specifically hydrolyzes β-1,4-glycosidic bonds of soluble cellulose chains, which is a trait similar to several endo-glucanase enzymes (Huang et al., 2012 and Wei et al., 2015). This suggestes the main activity of Cel_2759 is endo-acting. Interestingly, a single Cel_2759 was able to degrade pretreated rice straw, which is a complex substrate containing cellulose, hemicellulose, and lignin. The degradation mechanism on pretreated rice straw by Cel_2759 is still not known. Thus, further study is needed to reveal its biological function.

According to the result of substrate preference, CMC was thus used as substrate to determine the biochemical property of Cel_2759 in the following study.

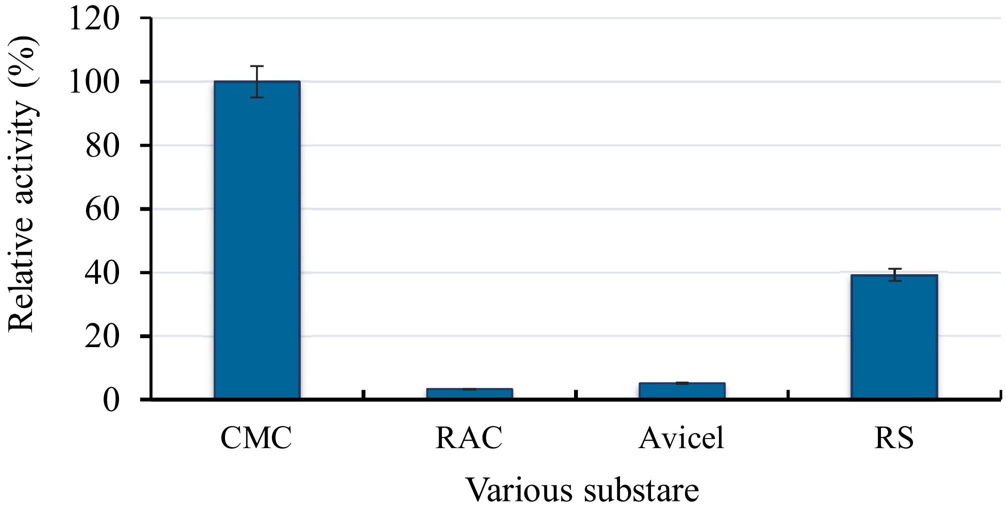


Figure 1. Effect of substrates specificity on activity of Cel_2759 at 16 hours

Effect of pH on activity and stability

Effect of pH on activity of Cel_2759 was examined at different pH range from 3.0-11.0 at 55 °C. Results showed that Cel_2759 had maximum activity around 7.0-8.0 (Tris-HCl buffer) (Table 1). It also exhibited activity in a broad range of pH from 6.0-10.0, and more than 50% of the maximum activity detected at pH from 6.0-8.0. The property of Cel_2759 is similar to the pH profile of alkaline stable GH9 endoglucanase from *T. halotolerans* (optimal pH at 8.0). In general, pH optimum of endo-glucanases is around 6.0-7.0. For example, the Cel9 endo-glucanase from *Clostridium phytofermentan* showed a pH optimum at pH 6.5, (Zhang et al., 2010) and Cel8A endoglucanase from *Serratia proteamaculans* displayed an optimal activity at pH 7.0 and was stable at pH from 4.0 to 8.5 (Cano-Ramirez et al., 2016).

After incubation at specific pHs for 1 h, Cel_2759 was stable across a broad pH range of 6.0-10.0. Cel_2759 showed its high stability at pH 7.0-9.0 (Fig. 2A). It is reported that a endo-β-1,4-glucanase from *T. aurantiacus* showed good stability in a range of pH 2.8–6.8, (Parry et al., 2002), whereas C14 endo-glucanase from *Bacillus* sp. was stable between pH 6.0-12.0, (Aygan and Arikan, 2008). *Thermoascus aurantiacus* endo-glucanase showed pH stability over a broad pH range of 3.0-7.0, (Dave et al., 2015). Therefore, Cel_2759 property is promising to be used as an industrial enzyme having stability at wide range of pHs.

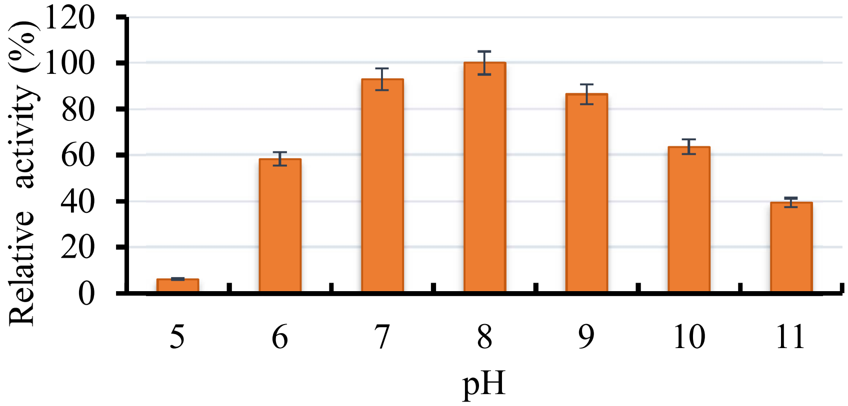
Effect of temperature on activity of Cel_2759

The effect of temperature on Cel_2759 activity was tested over a temperature range of 40-70 °C. The enzyme had the maximal activity at temperature 55 °C (Figure 2B), the enzyme retained nearly 80% of its initial activity at 50 °C. However, the activity decreased rapidly at temperature over 60 °C. Thcel9A endoglucanase from *Thermobifida halotolerans* showed its activity at temperature range from 45 °C to 60 °C with a maximum activity at 55 °C (Zhang et al., 2012). The C14 endoglucanase from *Bacillus* sp. showed activities at temperature from 20-100 ºC, and the optimum activity at 50 ºC (Aygan and Arikan, 2008). Another classical GH9 endoglucanase (Cel9) from *Clostridium phytofermentans* also showed activity at temperature from 40-75 ºC, and it exhibited maximum activity at 65 ºC (Zhang et al., 2010).

Table 1. Effects of pH on recombinant Cel_2759 activity

Buffers	pH	Reducing sugar (µg/mL)
Citric acid	3.0	86.557
	4.0	89.192
	5.0	205.528
	6.0	768.549
	7.0	981.805
Tris-HCl	7.0	1503.269
	8.0	1379.181
	9.0	717.779
Glycine-NaOH	9.0	625.985
	10.0	618.450
	11.0	207.052

(2A)



(2B)

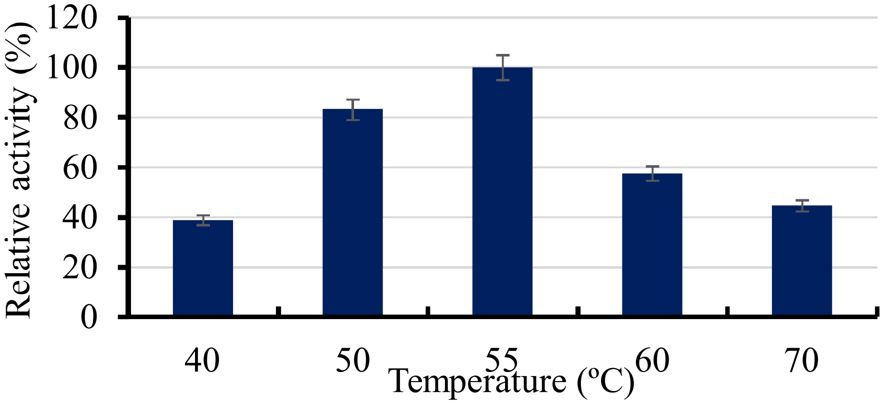


Figure 2. Effects of pH stability (2A), and temperature (2B) on recombinant Cel_2759 activity.

Effect of substrate concentration on enzyme activity and TLC analysis

The effect of substrate concentration on activity of Cel_2759 using CMC as substrate was studied. At a fixed enzyme load of 0.25 µM, activity of Cel_2759 increased as the substrate concentration increased from 0.1-1.0% (w/v) (Figure 3). This indicates the more active sites of the enzyme are used. When the substrate concentration was more than 1% (w/v), there were no significant differences in enzyme activity, indicating that the active sites of the enzymes reach the saturation points. As a consequence, 1% (w/v) load of substrate is an optimal substrate loading under the assayed conditions.

Hydrolysis of 1% (w/v) CMC by Cel_2759 was performed and the hydrolysis product was determined by TLC analysis. After 15-min incubation, a series of oligosaccharides was observed. The oligosaccharides included the cellotetraose (G_4), cellotriose (G_3), and cellobiose (G_2) (Figure 4). It is reported that Cel9 endoglucanase from *Clostridium phytofermentans* produced cellotetraose, cellotriose, cellobiose and glucose as hydrolysis products when regenerated amorphous cellulose was used as the substrate (Zhang et al., 2010).

The cello-oligosaccharides producing ability make Cel_2759 an attractive enzyme for large scale production of cello-oligosaccharides. These cellulose derived oligosaccharides can be used as prebiotics that stimulate growth of beneficial bacterial in the intestine (Chimtong et al., 2016; Jiao et al., 2015 and Sophonputtanaphoca et al., 2018).

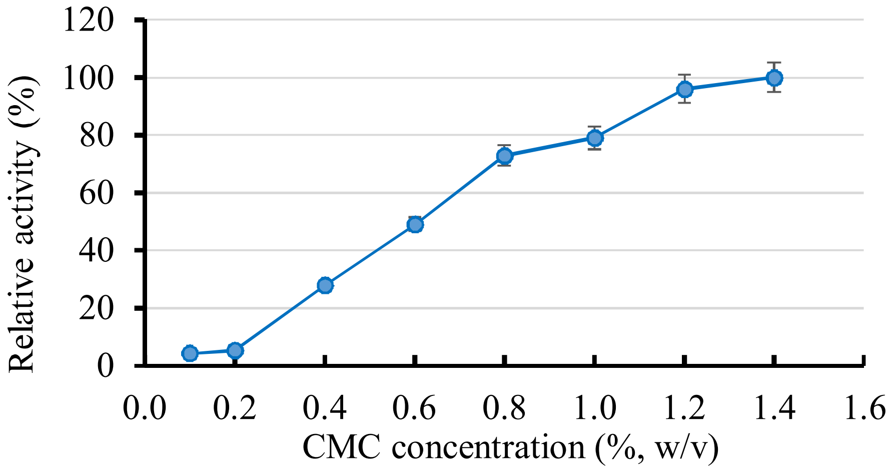


Figure 3. Concentration of CMC on Cel_2759 activity

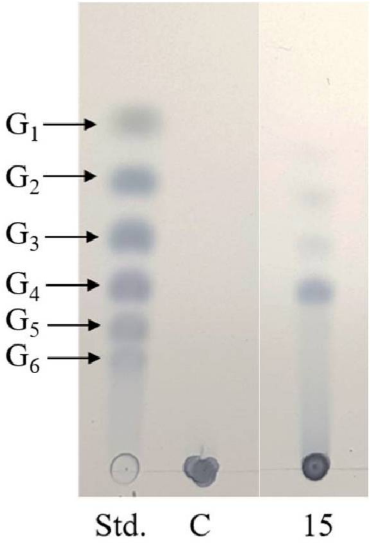


Figure 4. TLC analysis of hydrolyzed of Cel_2759 on CMC.

CONCLUSIONS

Cel_2759 from *Clostridium* sp. Z-7026 was successfully expressed and biochemically characterized. The enzyme showed a good activity on CMC and produced a series of cello-oligosaccharides, which indicated its endo-acting mode. Cel_2759 endoglucanase showed an optimum activity at 55 °C and pH 7.0, with a good stability activity at pH 6.0 – 10.0. This suggests its broad pH working range that is attractive for use in textile industry, biomass conversion, and oligosaccharide production.

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