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Characterization of extracellular cellulose-degrading enzymes from *Bacillus thuringiensis* strains

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Abstract The gram-positive spore-forming bacteria, *Bacillus thuringiensis* (Bt) strains produced novel cellulases which could liberate glucose from soluble cellulose, carboxymethyl cellulose (CMC), and insoluble crystalline cellulose. The maximal cellulase activities were obtained after 60 hrs incubation at 28ºC in a LB broth medium with 1% CMC. Maximum CMCase activities were got at 40ºC and pH 4.0, respectively, and more than 50% of its maximal activity was retained at 40-60ºC for 1 hr, while approximately 40% of its maximal activity was also retained after incubating at 70ºC for 1 hr. Most metal ions and reagents such as Ca^{2+}, Mg^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, EDTA, and SDS inhibited the enzyme activities, but K^{+} and Mn^{2+} activated the activities. The enzymes from *Bacillus thuringiensis* strains could be applied in bioconversion of lignocellulosic biomass into fermentable sugars.

Keywords: *Bacillus thuringiensis*, cellulase, cellulose-degrading, characterization

INTRODUCTION

In the last decade, much concern has been placed on cellulose because of its purpose as a renewable energy alternative for fossil fuel. As the most abundant plant biomass on earth, cellulose is composed of β-1,4 linked D-glucose units which could be hydrolyzed by cellulases, resulting in fermentable sugar for bio-ethanol bioconversion. The widely accepted mechanism of enzymatic cellulose hydrolysis involved the synergistic action of three types of cellulases including endo-β-1,4-glucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-gluosidase (EC 3.2.1.21), which, step by step, nicked the intermolecular β-1,4-glucosidic bonds, cleaved cellulose chain ends to release cellobiose unit, and cut cellobioses and oligosaccharides to produce glucose.

Although many microbes were known for decomposing cellulose, the available application of them was far from satisfactory because the properties and production of their known cellulases could not conform to the specified requirements, such as high catalytic efficiency on insoluble cellulosic substrates, superior stability at elevated temperature and at a certain pH, and strong tolerance to end-product inhibition (Percival Zhang et al. 2006).

One approach to increase enzyme volumetric productivity is to isolate hyper-producers and construct mutants with higher expression (Lin et al. 2009). Till now, cellulose-degrading enzymes have been well studied among *Bacillus agaradhaerens*, *Bacillus amyloliquefaciens*, *Bacillus cellulyticus*, *Bacillus circulans*, *Bacillus pumilus* and *Bacillus subtilis*. Purification as well as enzymologic and biochemical analysis of these enzymes have been performed. Furthermore, more than 20 genes that encode cellulose-degrading enzymes from the four genera above have been cloned and sequenced, including 19 types of endo-β-1,4-glucanase, three types of exoglucanase and one type of β-glucosidase

(Carbohydrate-Active-enZYme: http://www.cazy.org/Glycoside-Hydrolases.html; BRENDA: http://www.brenda-enzymes.info/). Nonetheless, there are few reports concerning cellulose-degrading ability of *Bacillus thuringiensis* species, and this study was done to produce and evaluate cellulases from two kinds of *Bacillus thuringiensis* strains. To our knowledge, this is the first report regarding cellulase from *Bacillus thuringiensis* species, which also provides basic information for the species to be used potentially in industrial cellulases production.

MATERIALS AND METHODS

Bacterial strains

The two different serovar types of *Bacillus thuringiensis* strains, *Bacillus thuringiensis* var. *israelensis* (BtI) and *Bacillus thuringiensis* var. *thompsoni* (BtH) (Itoua-Apoyolo et al. 1995; Lee et al. 2007), were chosen and cultured on CMC plate (0.001% $MgSO_4$, 0.005% KH_2PO_4, 0.001% $CaCl_2$, 0.6% NaCl, 0.2% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.1% yeast extract, 0.5% CMC and 1.5% agar). After growth at 28ºC for 14 hrs, cellulase activity of the colonies were assayed by checking their ability to form halos detected by Congo red staining (Lin et al. 2009).

Production of enzyme

The strains were overnight-cultured in LB-CMC medium (LB broth with 1% CMC) at 28ºC, 200 rpm, and 1% seed culture were progressively transferred and inoculated in fresh LB-CMC medium under the same condition. The culture supernatant at various stages of cell growth (12-84 hrs) was harvested by centrifuging at 4000 rpm for 10 min and was finally used for cellulase activity assay (Shi et al. 2011).

Enzyme assay

The cellulase activities assay was carried out as described by Li with some modifications (Li et al. 2006). The reaction contained 50 µl 0.5% CMC (Sigma) in 100 mM sodium acetate buffer (pH 4.0) and 50 µl enzyme solution. After incubation at 40ºC for 30 min, 100 µl dinitrosalicylic acid reagent (DNS) was added, and the mixture was placed in a boiling-water bath for 5 min and then diluted to 1 ml. Absorbance was measured at 540 nm and one unit of the activity was defined as the quantity of enzyme releasing 1 µmol reducing sugar per min at 40ºC (glucose as a standard). The cellulase activities were also determined by replacing CMC with 1% avicel (Sigma) and 1% filter paper (Whatman) (Ferreira et al. 2009).

Effects of temperature and pH on enzyme activity and stability

The cellulase activities of culture supernatants at different fermentation time (12-84 hrs) were determined using CMC, avicel and filter paper as the substrates, respectively. The profile of enzyme at the optimum fermentation time was evaluated by measuring the cellulase activity toward CMC. The effects of temperature and pH on the enzyme activity were performed at different temperature (20-70ºC) and at different pH values by using 0.2 M HAc-NaAc (pH 3.0-5.0), 0.2 M Na_2HPO_4-NaH_2PO_4 (pH 6.0- pH 8.0) and 0.05 M Glycine-NaOH (pH 9.0-11.0) buffers. Thermal stability studies were carried out by incubating the enzyme at different temperature (30-90ºC) for 1 hr. Then, samples were withdrawn to determine residual enzyme activity (Quiroz-Castañeda et al. 2009).

Effects of different metal ions and reagents on enzyme activity

The effects of different metal ions and chemical reagents on the enzyme activity were determined under optimum assay condition with the reaction mixtures containing 1 mM additional reagents. The activity assayed in the absence of metal ions or reagents was taken as 100%.

Gene prediction and protein modeling

Gene sequence of Bt cellulase predicted in *Bacillus thuringiensis* serovar *israelensis* genome (GenBank accession numbers AAJM00000000; http://www.ncbi.nlm.nih.gov/sites/genome) was

analyzed and the hypothetical conformation of the protein was predicted by Swiss-Model workspace based on the reported crystal structure of endoglucanase from *Thermotoga maritima* (Protein Data Bank code 3 mmw) (Ducros et al. 1995; Arnold et al. 2006).

RESULTS AND DISCUSSION

Identification of cellulase activity

In this study, we explored cellulase-activities of *Bacillus thuringiensis* strains by detecting their ability to form halos in CMC plates, and as a result, a H/C value greater than 2 was obtained (H: hydrolysis halo diameter; C: colony diameter; Figure 1) suggesting that Bt strains could produce cellulases with high activities, which might have the potential ability to liberate glucose from cellulose.

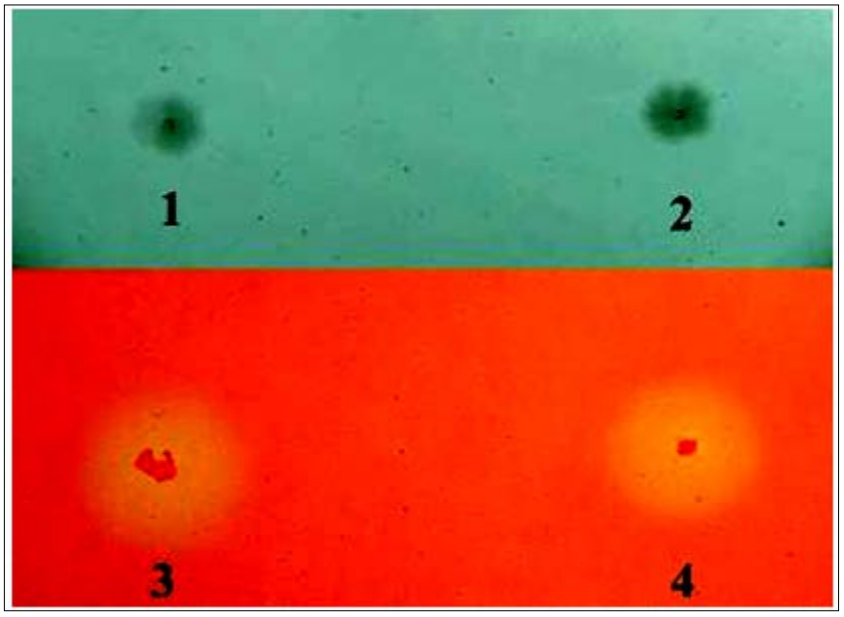

Fig. 1 Congo red staining of *Bacillus thuringiensis* colonies displaying cellulase activities on CMC plates. (1) BtI colony grown on plate without staining; (2) BtH colony grown on plate without staining; (3) BtI colony produced clear hydrolytic zone after staining; (4) BtH produced clear hydrolytic zone after staining. *Bacillus thuringiensis* colonies were grown on CMC plate for 14 hrs at 28ºC and followed by Congo red staining.

Optimum fermentation time

In order to measure the properties of Bt cellulase, the enzymes produced by BtI and BtH, which were cultured in LB with 1% CMC fermentation medium at 28ºC, and showed major cellulose hydrolytic activities towards soluble cellulose CMC, insoluble crystalline cellulose avicel and filter paper. The secreted cellulase of the Bt strains exhibited maximum activities toward all the three substrates in 2.5 days of culture time (Figure 2a). Those of BtI were 0.072 U/ml with CMC, 0.011 U/ml with avicel and 0.009 U/ml with filter paper, at 48, 36 and 60 hrs of culture time, respectively, whereas, the activities of BtH were 0.059 U/ml with CMC, 0.006 U/ml with avicel and 0.008 U/ml with filter paper, at 36, 24 and 36 hrs of culture time, respectively. And the results that maximum cellulase activities occurred at different points in different substrates, were probably due to the different expression of enzymes, which might influence the synergistic action towards the insoluble cellulose. Thus, these strains produced all three required components of the cellulase enzyme complex, which hydrolyzed crystalline cellulose into oligosaccharides first and ultimately into glucose (Qin et al. 2010). Currently, most commercial cellulases are produced by *Trichoderma* species and *Aspergillus* species (Li et al. 2009). Despite of those, cellulases are relatively costly enzymes, and it is significantly important to economize cost of

cellulase used in commerce, such as cotton softening and denim finishing in the textile industry, colour care, cleaning, and anti-deposition in the detergent market; mashing in the food industry; and de-inking, drainage improvement, and fiber modification in the pulp and paper industries (Kirk et al. 2002; Cherry and Fidantsef, 2003). To achieve this aim, strategies toward strain enhancement and process improvement were employed to make the cellulase-production more economical, *e.g.*, particular hyperproducers, cheaper medium for fermentation and alternative inducer system (Knauf and Moniruzzaman, 2004). As shown in Figure 1.1, a rapid increase in cellulase activity occurred after 12 hrs incubation at 28ºC, and the truth that these enzyme complexes were obtained in a very short fermentation time also showed the capability to liberate glucose from soluble cellulose CMC, even from insoluble crystalline cellulose (avicel and filter paper). Thus, these special characteristics might highly benefit industrial applications (Gill and von Hippel, 1989).

Effects of temperature and pH on enzyme activity and stability

Enzymological analysis of Bt cellulases suggest that the enzymes were most active under acidic conditions with optimum pH at 4.0, and presented relatively wider pH-adaptability, showing more than 20% of maximum activity from pH 3.0 to 7.0 (Figure 2b), in comparison with those from other *Bacillus* strains (pH 4.5-7.0) (Mawadza et al. 2000; Lin et al. 2009). The optimal temperature of enzymes was found to be 40ºC at pH 4.0 (Figure 2c), and these cellulases were stable at temperatures below 50ºC and could retain more than 50% of maximum activity even after 1 hr of incubation at 60ºC (Figure 2d), which was consistent with previous reports that cellulases from some *Bacillus* strains were stable at 0-50ºC (Mawadza et al. 2000; Li et al. 2006; Fan et al. 2007).

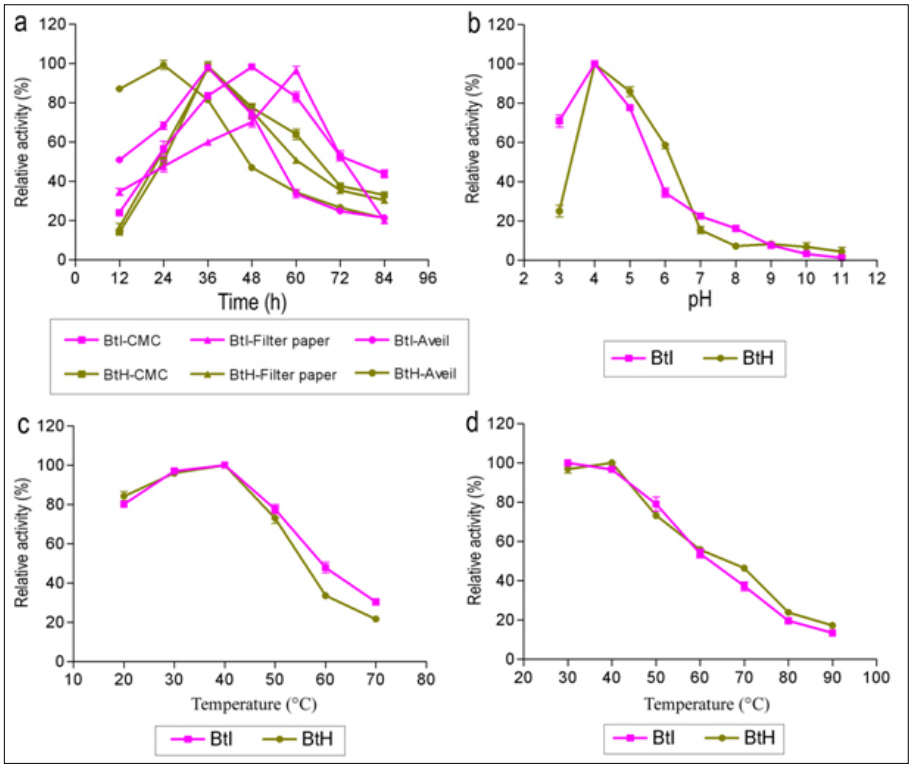

Fig. 2 Characteristics of cellulases secreted by *Bacillus thuringiensis* strains cultured in LB with 1% CMC-Na fermentation medium. (a) Optimum culture time of Bt strains hydrolytic activities toward CMC, avicel and filter paper. Culture time profile was measured under optimum assay condition at varying times (12-84 hrs). (b) Effects of pH on enzyme activity. Enzyme activity was measured under optimum assay condition at the optimum culture time and varying pHs (pH 3.0-11.0). The maximum activity observed was taken as 100%. (c) Effects of temperature on enzyme activity. Enzymes were added to the reaction mixture (100 mM HAc-NaAc pH 5.0, CMC 0.5%) and the reaction was carried out at indicated temperatures. The maximum activity observed was taken as 100%. (d) Effects of temperature on enzyme stability. Enzymes were incubated for 1 hr at indicated temperatures. Then samples were measured under the same conditions of optimum activity assay. The activity without treatment was taken as 100%. Error bars represent the standard deviation of the mean calculated for three replicates.

Effects of different metal Ions and reagents

As shown in Table 1, most metal ions such as Ca^{2+}, Mg^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+} exhibited slight inhibition effect on the enzyme activities, whereas K^{+} and Mn^{2+} enhanced the cellulases to a 10% extent. Among these metals and chemicals, Cu^{2+} could bind the thiol groups and interact with imidazole or carboxyl groups of amino acids, whereas SDS (sodium dodecyl sulfate) could interact with the hydrophobic group of amino acids, resulting in the decreased enzyme activity (Lucas et al. 2001; Yin et al. 2010).

Table 1. Effects of metal ions and chemical reagents on the activity of cellulase from BT.

Metal ions and chemical reagents (1 mmol L^{-1})	Relative activity (%)	
	BtI	**BtH**
None	100	100
K^{+}	115.6 ± 7.1	103.4 ± 4.1
Ca^{2+}	89.3 ± 5.3	82.5 ± 6.7
Mg^{2+}	76.7 ± 5.6	76.5 ± 3.1
Cu^{2+}	69.5 ± 5.5	59.9 ± 4.7
Zn^{2+}	79.5 ± 7.3	70.5 ± 6.4
Mn^{2+}	117.7 ± 7.6	107.6 ± 6.5
Cd^{2+}	89.1 ± 4.9	80.4 ± 5.7
Pb^{2+}	86.2 ± 4.5	71.5 ± 3.7
EDTA	69.5 ± 7.8	77.5 ± 7.4
SDS	57.3 ± 4.3	61.4 ± 3.9

The measurement was carried out at the optimum condition. EDTA, ethylenediaminetetraacetic acid SDS, sodium dodecyl sulphate. Standard deviations were shown behind the specific activities.

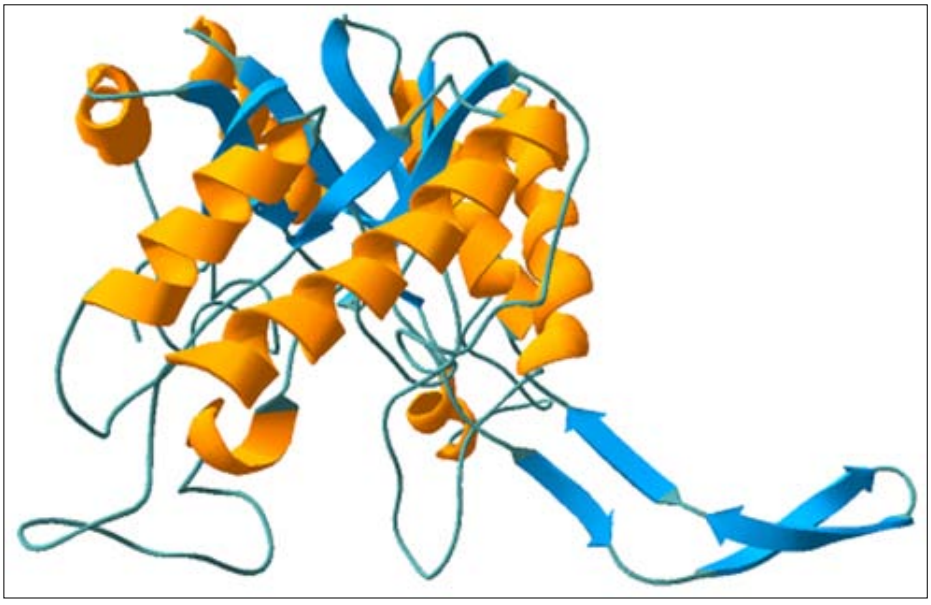

Fig. 3 The hypothetical conformations of the proteins were predicted based on crystal structure of endoglucanase Cel5A from the hyperthermophilic *Thermotoga maritima* (3 mmw) by Swiss-Model workspace and illustrated as ribbon diagrams using Swiss-Pdb viewer. The GH-5 module folded as $(\beta/\alpha)_8$ barrel is rich in α-helix (orange) and β-strand (blue) motifs.

Gene prediction and protein modelling

It should be noted that none of cellulase genes from *Bacillus thuringiensis* had been reported, although CMCase activity of glycoside hydrolase family 8 (GH-8) family chitosanases from Bt species was detected by Lee et al. (2007). Based on the amino acid sequences of predicted cellulase (GenBank accession numbers EAO52015) from *Bacillus thuringiensis* serovar *israelensis* ATCC 35646, crystal structure of the enzyme was predicted and determined as a GH-5 family module using reported crystal structure of endoglucanase Cel5A from the hyperthermophilic *Thermotoga maritima* (Protein Data Bank code 3mmw) as template (Figure 3). And this hypothetical conformation of the protein, consisted of 390 residues, which folded as a typical $(\beta/\alpha)_8$ barrel domain model in GH-5, suggesting this predicted enzyme probably was one of component in Bt cellulase complexes and played a significant role in cellulose hydrolysis. Further studies on Bt cellulase genes cloning and function verification should help to more clearly understand the basis for glycoside hydrolyse function and provide a framework for analysis of synergistic effect essential for cellulose hydrolysis. Actually, these studies were also currently under evaluation.

CONCLUDING REMARKS

The present study was carried out to investigate the catalytic performance of cellulases produced by Bt strains. These enzymes, which had not been reported previously, exhibited hydrolytic activities toward CMC, avicel and filter paper at pH 4.0 and 40ºC, and also showed broad temperature and pH stabilities. The results suggest that BT strains might become a novel and interesting source of lignocellulose-degrading enzymes with important economic advantages, thus might be of potential applications in the industry.

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