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A Single Mutation in the Carbohydrate-Binding Module Enhances Cellulase Activity in *Bacillus Amyloliquefaciens* Mutant

Nitipol POLSA[1], Chomphunuch SONGSIRIRITTHIGUL[2,3], Wasana SUYOTHA[4], Sugunya SUEBSAN[5,6], Somboon ANUNTALABHOCHAI[5,6] and Kanta SANGWIJIT[6,*]

[1]*Applied Science, School of Science, University of Phayao, Phayao 56000, Thailand*
[2]*Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima 30000, Thailand*
[3]*Center for Biomolecular Structure, Function and Application, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand*
[4]*Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Songkla 90112, Thailand*
[5]*Department of Biology, School of Science, University of Phayao, Phayao 56000, Thailand*
[6]*Plasma Bioengineering Unit, School of Science, University of Phayao, Phayao 56000, Thailand*

([*]Corresponding author's e-mail: sangwijit.k@gmail.com)

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Abstract

From our earlier work, we modified the carbohydrate-binding module (CBM) of *Bacillus amyloliquefaciens* to increase cellulase activity using cold plasma technology. The cellulase gene (*BglC-M*) from the mutant was expressed in *Escherichia coli* BL21(DE3) under the T7 promoter. The hydrolysis activity of the cellulase mutant (BglC-M) was approximately 2.5-fold higher than the control (BglC-W) over a wide range of pH and temperature conditions. The amino acid sequence of the mutant BglC-M contained 471 residues that were almost identical to the control BglC-W. Only a single amino acid, lysine, was replaced by glutamic acid at position 370 (K370E) within the carbohydrate-binding module (CBM). Structure prediction and substrate docking of BglC-M indicated that the single mutation (K370E) might involve cellulose binding of the β-sandwich facilitated by hydrogen bonding. The docking study of cellopentaose with the model structure of BglC-M indicated that the replacement of lysine-370 led to the formation of a hydrogen bond with 436Y, which has a shorter distance (2.6 Å) compared with the control (5.4 Å). As a result, the structure becomes more compact and stable, resulting in increased catalytic efficiency. Finally, the biomass hydrolysis ability of cellulase was investigated on lignocellulosic wastes such as pineapple peel, corncob, and durian peel. The BglC-M enzyme showed a more significant amount of reducing sugar released from all lignocellulosic wastes than the control. This was the first evidence that altering the base composition of the cellulose binding module enhanced the catalytic activity.

Keywords: Agricultural wastes, *Bacillus amyloliquefaciens*, Carbohydrate-binding module (CBM), Cellulase, Mutation

Introduction

Because the majority of agricultural waste in Thailand is burned in open fields, it contributes significantly to environmental degradation. In addition, agricultural waste was an inexpensive and renewable resource that could be processed into animal feed [1]. The problems of lignocellulosic materials fed directly to livestock usually include low protein content, high crude fiber, low digestibility

coefficients, and the presence of some anti-nutrient factors such as tannins and alkaloids [2]. To increase the digestibility of these agricultural residues, it is important to hydrolyze the cellulose chains that interact with each other through hydrogen bonds and form microfibrils [3]. Microorganisms such as fungi, bacteria, and algae have specific enzymes that break down cellulose and convert it into sugars that the organism can readily use as food [4,5]. These enzymes were known as cellulases and were the enzymes that hydrolyze β-1,4 bonds in cellulose chains. Cellulases have been classified into three major types: endoglucanase (EG, EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [6,7]. Basically, cellulase sequences are composed of catalytic domains (CDs) and carbohydrate-binding modules (CBMs). These were connected by a variety of linkers, differing in length, and sequence [8]. The catalytic module of cellulases is responsible for cleaving β-1,4- linkages in cellulose polymer in the active site. Moreover, CBMs did not have catalytic activity but functioned as substrate binding modules. The binding property of a CBM improved the enzyme's catalytic function by increasing substrate-enzyme proximity [9,10].*B. amyloliquefaciens* was an attractive strain for several industrial applications. This strain secretes cellulase and has probiotic attributes, which could apply to many types of animal feed [11]. The production of microbial cellulases in cells is generally regulated by genetic and biochemical approaches involving induction and end-product inhibition [12-14]. These approaches are effective under cellulase production conditions, resulting in limited yields of enzymatic components or their catalysis activity. However, random mutagenesis has been successfully used in *Penicillium oxalicum* to improve cellulase production [15]. Also, an endoglucanase from *Thermotoga maritime* Cel5A was subjected to site-directed mutagenesis and CBM engineering to achieve hyperthermostability [16]. Furthermore, a single mutation in a non-catalysis domain of xylanase produced from *Bacillus subtilis* was performed to increase its hydrolysis activity [17]. This evidence indicated that a single missense mutation caused by a random mutation technique could enhance the catalysis activity of enzymes. More attention has been paid to finding cellulases with higher specific activity, better stability, as well as high affinity for substrate [18,19]. To understand these, computer simulations, or *in silico* techniques, were launched to characterize the properties and structure of proteins. The study was thought to potentially speed up understanding of protein-protein interaction or protein-ligand docking, a method that predicts 1 molecule's preferred orientation in a second when bound to each other to form a stable complex [20].

Cold plasma immersion implantation (P- III) technology has been shown as a novel and highly effective mutagenesis tool. Experimental evidence has shown that energetic ion beam bombardment is an environmentally friendly technique capable of inducing mutations in various living organisms, including crops, fungi, and bacteria [21,22].

In our previous work, *B. amyloiquefaciens* was mutated by cold plasma immersion (P- III) to obtain higher cellulase activity [23]. In this work, the enzyme activity of BglC-M was characterized, and its structure was analyzed. In addition, the model of the 3-dimensional structure of BglC-M was constructed to show the contribution of K370E to increasing the cellulase activity. This was the first demonstration that a single mutation in the non-catalytic domain (carbohydrate-binding module) was involved in the increase of cellulase enzyme activity [23,24].

Materials and methods

Bacterial strains and vectors

The cellulase producing bacterium, *B. amyloliquefaciens* (BglC-W), was mutated and a mutant (BglC-M) was obtained as described in previous work [23]. *E. coli* strain DH5α and BL21 (DE3) were used as hosts for transferring plasmids in this work. All bacterial strains were grown at 37 °C in Luria-Bertani (LB) broth and shaken at 120 rpm. Cloning and expression vectors, pTZ57R/T and pETDuet-1, were purchased from Thermo Fisher Scientific (USA) and Novagen (USA).

Cellulase gene (*BglC*) cloning and vector construction

Chromosomal DNA of the wild-type strain and the mutant was extracted and used as a DNA template for cellulase gene cloning. The known sequences of the cellulase gene from *B. amyloliquefaciens*'s registered in GenBank (accession numbers: KY797654.1, MF134665.1 and EU022559.1) were chosen as templates to design primers for amplifying the full-length sequence. Both primers, forward primer 5' ccggcacatatggcagggacaaaaacgccag g 3' with *Nde*I restriction site, and reverse primer 5' ctaatgctcgaggcctaaagcttaactaatt 3' with *Xho*I restriction site were designed. Then, the entire cellulase gene sequences of *BglC-W* and *BglC-M* were amplified by PCR. The 1,416 bp of both PCR products were obtained and subcloned into the pTZ57R/T cloning vector. DNA sequencing was performed using an autosequencer (ABI) by Macrogen, Korea, and nucleotide sequences were determined online with NCBI blast (www.ebi.ac.uk).

Both *BglC-W* and *BglC-M* were digested with *Nde*I and *Xho*I and ligated into the corresponding sites of the pETDuet-1 expression vector. The recombinant plasmids of the mutant and control were transformed into *E. coli* BL21(DE3), respectively. The transformed cells were cultured on LB plates with 100 μg/ml ampicillin and incubated overnight at 37 °C.

Expression of recombinant *BglC-W* and *BglC-M* in *E. coli* BL21 (DE3)

The transformants harboring recombinant *BglC-W* and *BglC-M* were cultured in LB-ampicillin (100 μg/ml) medium at 28 °C and shaken at 200 rpm until the OD_{600} reached 0.6 [17]. The expression of the recombinant cellulase was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG, 1mM) for 12 h at 28 °C. The bacterial cells were harvested by centrifugation followed by ultrasonication. Protein concentration was measured using the Bradford method with bovine serum albumin as standard.

The effect of temperature and pH on BglC-W and BglC-M hydrolysis activity

The hydrolysis activity of BglC-W and BglC-M was assayed using CMC as a substrate. The effect of pH was determined by incubating the mixture containing 1 % (w/v) of CMC with each enzyme at 50 °C for 30 min at pH 2.0 - 11.0. Four different buffers were used: citrate buffer (pH 2.0 - 5.0), potassium phosphate buffer (pH 6.0 - 7.0), Tris-HCl buffer (pH 8.0 - 9.0) and CAPS buffer (pH 9.0 - 11.0). The optimal temperature of each enzyme activity was determined by incubating 1 % (w/v) substrate with the enzymes in 50 mM citrate buffer (pH 4.0) for 30 min at various temperatures ranging from 10 - 100 °C. The amount of reducing sugar was determined by the DNS method [25], and the activity at each pH was calculated. All experiments were carried out in triplicate, and the data were expressed as the mean ± standard deviation. The statistical analysis was analyzed by Duncan's multiple range test, and the values were considered significant at $p < .05$.

Effect of Ca^{2+}, $Fe^{2+}$and EDTA on cellulase activity

The BglC-W and BglC-M in 50 mM citrate buffer (pH 4.0) with Ca^{2+}, Fe^{2+} and EDTA were incubated at room temperature for 30 min. The final concentrations of metals and EDTA were 1, 5 and 10 mM. After incubation, the cellulase activities were measured. All experiments were carried out in triplicate, and the data were expressed as the mean ± standard deviation. The statistical analysis was analyzed by Duncan's multiple range test, and the values were considered significant at $p < .05$.

Structure prediction and molecular docking of BglC

ClustalW (1.83) multiple sequence alignment software [26] was chosen for amino-acid sequence alignment. After that, ESPript [27] was linked to illustrate the secondary structure of the templates selected. The catalytic core of BglC was aligned with that obtained from *B. subtilis* cellulase 5A (PDB code 3PZT). The carbohydrate-binding module of mutant K370E BglC and *B. subtilis* cellulase 5A (PDB code 2L8A) were aligned.

The structural models of BglC were created using SWISS-MODEL [28]. The model of the catalytic domain of BglC was built by using the X-ray structure PDB code 3PZT, and the carbohydrate-binding module of BglC-M was built using PDB code 2L8A, which corresponded to a family of 5 catalytic domains and a family of 3 carbohydrate-binding modules (CBM3), respectively.

The coordinates of divalent cations (Ca^{2+} and Fe^{2+}) were simulated within the catalytic core of the model structure. In addition, the coordinates of cellopentaose were docked into the carbohydrate-binding module of the predicted structure of BglC-M. Docking studies in both catalytic and carbohydrate-binding domains were performed by AutoDock Vina [29]. Finally, all structures were displayed with PyMOL [30].

Analysis of the hydrolysis of cellulases against lignocellulosic substrates

The hydrolysis activities of BglC-W and BglC-M were determined using lignocellulosic substrates including pineapple peel, corncob, and durian peel. The hydrolysis activities were carried out by incubating the enzyme in 50 mM citrate buffer with 5 % (w/v) substrates for 7 days at 50 °C. Then, the reaction mixtures were collected every 24 h. The reducing sugar released from the substrates was measured by the DNS method. All experiments were carried out in triplicate, and the data were expressed as the mean ± standard deviation. The statistical analysis was analyzed by Duncan's multiple range test, and the values were considered significant at $p < 0.05$.

Results and discussion

Cloning and sequence analysis of *BglC*

B. amyloliquefaciens was induced mutation by P-III in our previous work, and the mutant with greater cellulase activity was described [23]. Sequence comparison between cellulase genes of the wild type (*BglC-W*) and the mutant (*BglC-M*) revealed that *BglC-W* and *BglC-M* consisted of 1,416 nucleotides, encoding a protein of 471 amino acids. The amino acid sequence alignment of BglC-W and BglC-M was almost identical. However, a single amino acid, lysine, located at position 370, was substituted with glutamic acid (K370E). Analysis of the conserved domain further confirmed that our cellulase gene was comprised of the catalytic domain (from 1M to 301I), which belongs to family 5 of glycoside hydrolases (GH5) and a family 3 cellulose binding module (CBM3) (from 326I to 471H). A catalytic domain (CD) and a family 3 carbohydrate-binding module (CBM3) were connected by a short linker sequence (from 302L to 325G). Based on sequence alignment and domain analysis, the mutant residue (K370E) was situated in CBM3, not in the catalytic site.

Characterization of cellulase enzymes

Our previous study [31] *B. amyloliquefaciens* was selected as a microorganism source for cellulase production and applied for animal feed. However, the more stable and higher catalytic activity of enzymes under a broad range of pH and temperature is required for a feed fermentation process. Both cellulase genes (*BglC-W* and *BglC-M*) were subcloned and introduced into host cells, then incubated with 1 % CMC in pH ranging from 2 to 11. The reducing sugar was liberated from the reaction and measured by using the DNS method, and the activity of the enzyme was also calculated. As shown in **Figure 1,** cellulase activity of BglC-W and BglC-M attained the highest values at pH 4.0. The effect of temperature on cellulase activities was measured, and the result is shown in **Figure 2**. he highest activity of BglC-M was observed at 50 °C, and its cellulase activity was approximately 2.5 times higher over a wide range of pH and temperature, retaining approximately 80 % of the activity at 90 °C after 30 minutes of incubation (**Figure 2**). During the feed fermentation process, the temperature within the fermented chamber

increased gradually, up to 42 °C [32]. Therefore, our mutant had higher catalytic activity between 40 - 60 °C, which was suitable for the fermented process.

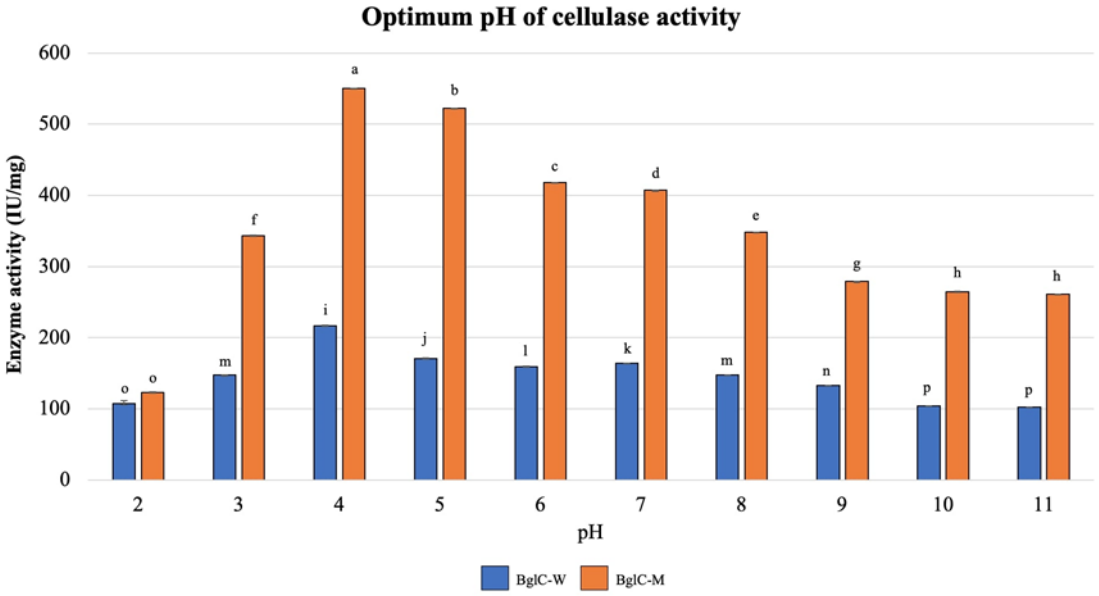

Figure 1 Cellulase activities of BglC-W and BglC-M at different pH ranging from pH 2 - 11 at 50 °C. The letters indicate a significant difference ($p < 0.05$) as determined by Duncan's multiple range test.

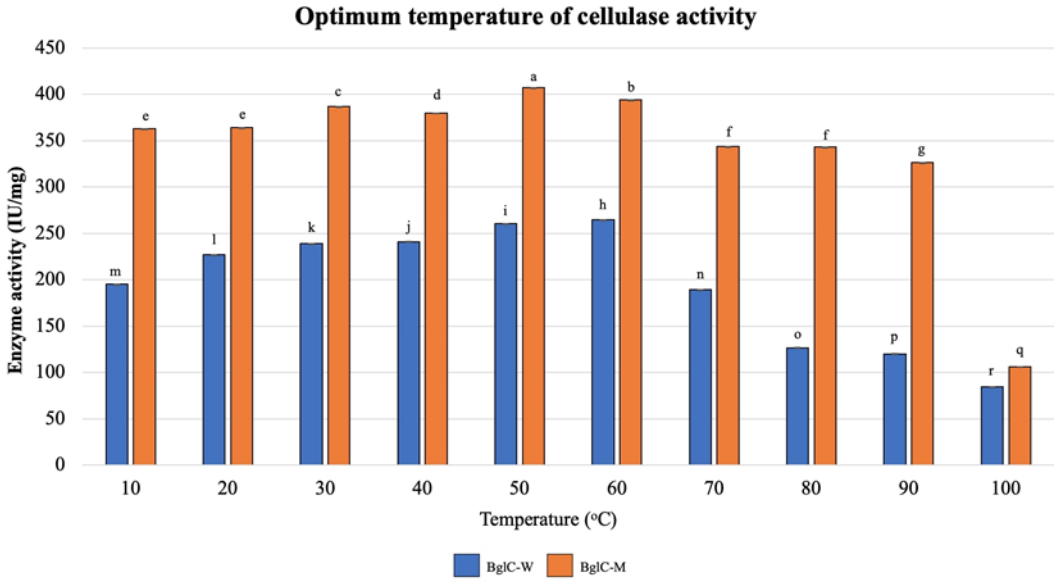

Figure 2 Cellulase activities of BglC-W and BglC-M at different temperature ranging from 10 - 100 °C. The letters indicate a significant difference ($p < 0.05$) as determined by Duncan's multiple range test.

The effects of Ca^{2+}, Fe^{2+} and chelating agents

As shown in **Table 1**, the effect of Ca^{2+} and Fe^{2+} on cellulase activity of BglC-W and BglC-M was reported. The effects of these ions proved to be dependent on their concentration. Under the lowest concentration of 1 mM, Ca^{2+} and Fe^{2+} were able to increase cellulolytic activity slightly. The activity of both enzymes slightly increased in the presence of Ca^{2+} and Fe^{2+} at low concentrations, but it decreased when ion concentrations increased. In contrast, increasing EDTA concentration provided only a slight decrease in enzyme activity under given conditions.

Presumably, the docked metal atoms (Ca^{2+} and Fe^{2+}) were coordinated by the side chains of polar serine and asparagine residues, carbonyl oxygen, and the amide nitrogen. The metal binding motif with the possible hepta coordination may contribute to its stability, thus further enhancing cellulase activity. Such a metal binding site had been previously reported for the crystal structure of the catalytic core of *B. subtilis* cellulase 5A [33]. Yaniv *et al.* [34] also confirmed that the role of metal ions was to facilitate holding the loop together, but not for protein reconformation. And EDTA was a strong chelating agent and effectively removed divalent cation concentrations from the catalytic domain, thus eliminating the metal-binding motif and suggesting lower stability. Therefore, the range of 1 to 10 mM of EDTA reduced cations, resulting in decreased stability and activity of the enzyme.

Table 1 Effect of ions and chelating agent on cellulase activity

	Relative cellulase activity		**BglC-W**		**BglC-M**			
	0 mM	**1 mM**	**5 mM**	**10 mM**	**0 mM**	**1 mM**	**5 mM**	**10 mM**
Ca^{2+}	100.00±0.11^{c}	103.57±0.36^{b}	94.14±0.11^{d}	81.44±0.21^{g}	100.00±0.25^{c}	115.97±0.47^{a}	92.57±0.21^{e}	88.66±0.39^{f}
Fe^{2+}	100.00±0.28^{b}	110.27±0.05^{a}	53.14±0.45^{e}	45.97±0.01^{f}	100.00±0.47^{b}	100.10±0.07^{b}	80.52±0.24^{c}	66.30±0.12^{d}
EDTA	100.00±0.34^{a}	73.34±0.15^{f}	74.20±0.35^{e}	64.19±0.22^{g}	100.00±0.30^{a}	83.29±0.31^{c}	86.60±0.30^{b}	80.54±0.23^{d}

The letters indicate a significant difference ($p < 0.05$) as determined by Duncan's multiple range test.

Structural comparison between BglC-W and BglC-M

Sequence analysis of the cellulase enzyme

The sequence's alignment between the control (BglC-W) and mutant (BglC-M) *B. amyloliquefaciens* cellulases (BaCel5) was shown in **Figure 3**. Analysis of the conserved domain (**Figure 3**) confirmed that our *BglC* gene belonged to a cellulase superfamily, consisting of a GH5 family catalytic domain (CD ~301 residues) and a family 3 cellulose-binding module (CBM3 ~144 residues), according to CAZy classification (http://www.cazy.org). The 2 domains were joined by a short linker sequence (302LGSKDSTKERPETPAQDNPAQENG327), which was rich in hydrophobic residues. Based on sequence alignment and domain analysis, the mutant residue (K370E) was suggested to be located in CBM3, not the catalytic motif. Typically, carbohydrate-binding modules (CBMs) are amino acid sequences that play an important role in carbohydrate-binding [35]. The alignment result showed that our CBM belongs to family 3 CBM (CBM3), which forms a planar surface for cellulose recognition [35]. The aromatic residues that contribute to its planar carbohydrate-binding site, assumingly conduct the substrate in a proper conformation to the active site, thus being considered in our molecular docking studies of cellopentaose. This would be further discussed in detailed information.

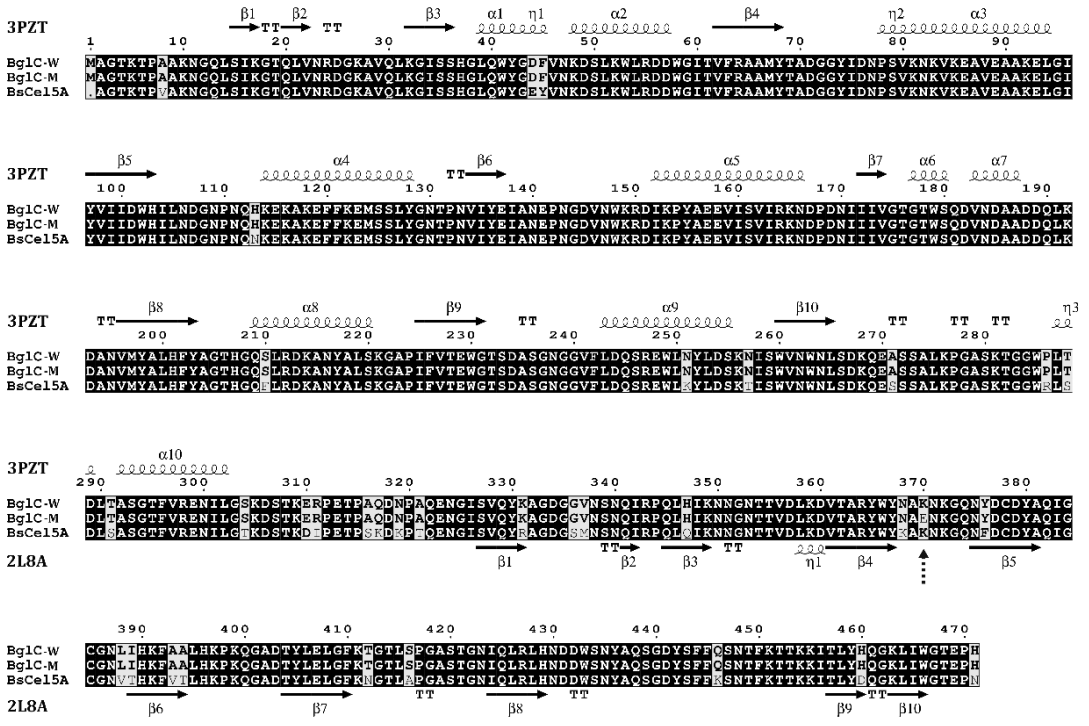

Figure 3 Amino acid sequence alignment of wild type and mutant cellulase from *B. amyloliquefaciens* (BglC-W and BglC-M, respectively) and cellulase from *B. subtilis* 168 (BsCel5A). Secondary structures were predicted using the X-ray structures of the catalytic domain and carbohydrate-binding module of BsCel5A as templates (PDB entries 3PZT and 2L8A, respectively). The catalytic domain is shown above the sequences, but the carbohydrate-binding module is below the sequences. The mutant residue (K370E) was indicated by a dashed arrow. α-helices and β-strands are displayed as squiggles and arrows, respectively. TT indicates turns. Fully conserved residues are printed as white characters on a black background. Similar residues are written in black bold characters and boxed.

Analysis of BglC catalytic core

The catalytic core of BsCel5A (PDB code 3PZT) was used as a template to compare with that of BglC. The alignment result showed that our cellulase shared 96 % amino acid identity with the catalytic core of the structural template. In the previous report, the BsCel5A catalytic core structure was composed of a typical (β/α)8-TIM-barrel domain with the catalytic residues located on β-strands [33]. Structural analysis revealed that the model structure of the catalytic core of our cellulase displayed a common TIM barrel fold. Ca^{2+} and Fe^{2+} were docked in the model structure of the catalytic core, thus having the same binding free energy (in kcal/mol) of -1.4. Docking studies presume that the coordination of metal ions by serine and asparagine may stabilize enzyme structure and lead to an increase in its cellulolytic activity. Fe^{2+} and Co^{2+} have been previously reported to increase the enzyme activity of endocellulase Cel-5A under the low concentration of 1 mM [36]. Moreover, the Co^{2+} ion in the endoglucanase CelCM3 was able to enhance the enzyme activity [37]. Metal ions have been proposed to guide the enzyme into its optimized and stabilized conformation [33,38]. The contribution of metal ions in our study may facilitate the enzyme structure becoming more stable, thus further increasing its activity.

Analysis of BglC carbohydrate-binding module (CBM3)

BglC-W and BglC-M CBM3 amino acid sequences shared 90 and 89 percent sequence identity, respectively, with BsCel5A (PDB code 2L8A) as a template model. The predicted structure revealed that CBM3 folded into a β-sandwich, a dominant fold among CBMs. It comprised 2 β-sheets which consisted of 4 and 5 antiparallel β-strands, being similar to the selected template (**Figure 4**). There is also one short -helix similar to BsCel5A at the C-terminus of the predicted structure (**Figure 4**). The structure of cellopentaose docked in the structure of BglC-M had a binding free energy of -7.5 kcal/mol.

From the docking study, 376Y is close to the reducing end terminal residue of the substrate (~8 Å apart), which may facilitate the occupation of the substrate. 376Y is proposed to lie along with the hydrophobic platform since it may form a hydrophobic stack against the pyranose rings of the incoming cellulose chain (**Figure 4**). From the docking study of cellopentaose with the model structure of BglC-M, the residues which were proposed to form hydrogen bonds with cellopentaose were 370E of BglC-M and 398K of BsCel5A CBM3 (homologous to 370K of BglC-W) (**Figure 4**). The oxygen atom of the sugar residue at the non-reducing end (subsite -3) recognizes the side chain oxygen of 370E and the amide side chain of 398K with the predicted hydrogen bond distances of 3.8 and 3.6 Å, respectively (**Figure 4**). 398K of BsCel5A CBM3 (homologous to 370K of BglC-W) was located on a surface loop, and its amide side chain was far from the hydroxyl side chain of 464Y (BsCel5A CBM3) with the distance of 5.4 Å, thus being predicted in BglC-W similarly. The predicted hydrogen bonding with the distance of 2.6 Å between 370E (BglC-M) and 436Y, which linked the flexible loops together, thus making the structure more compact. This might increase the stability of the enzyme and collectively contribute to the accommodation of the cellulose chain. Our biochemical assays indicated that the K370E mutant exhibited approximately 2.5 times the activity improvement when compared to the wild type. Docking studies of BglC-M CBM3 with cellopentaose reported that the carbohydrate-binding site is involved in a hydrophobic stack against the pyranose rings and hydrogen bonds formed between the hydroxyl groups of cellopentaose and the model protein.

The backbone carbonyl of 370E of BglC-M was structurally homologous to 398K of BsCel5A and was proposed to form a hydrogen bond within the sugar ring at subsite -2, with a distance of 3.0 Å (**Figure 4**). Similarly, the backbone carbonyl of 373G formed a hydrogen bond with the oxygen atom of the sugar residue (subsite -1) docked at a predicted distance of 3.9 Å (**Figure 4**). The glucosyl substrate at subsite -2 is proposed to be stacked against the indole ring of 433W (**Figure 4**).

Two aromatic residues on this face were 433W and 436Y, whereas 376Y was buried in the β-sandwich core, making hydrophobic contacts. The findings indicated that the interactions of BglC CBM3 with cellulose were contributed by aromatic residues and polar side chains. Yaniv *et al*. [34] found that base substitution by site mutagenesis from asparagine to tryptophan (N126W) in the carbohydrate binding domain enhanced the binding ability of cellulase from *Clostidium thermocellum*, thus proposing its crystal structure. However, the activity of the cellulase was not demonstrated.

Thermostability and pH stability generated from the mutants are also the keys to the improvement of enzyme properties and activities [39]. Moreover, CBM played an important role in substrate binding, which further enhanced the catalytic activity [40] and thermostability [41]. Our study showed that a mutant, which involved in the alteration of a single charged residue in the CBM3 domain by P-III enhanced its cellulase activity.

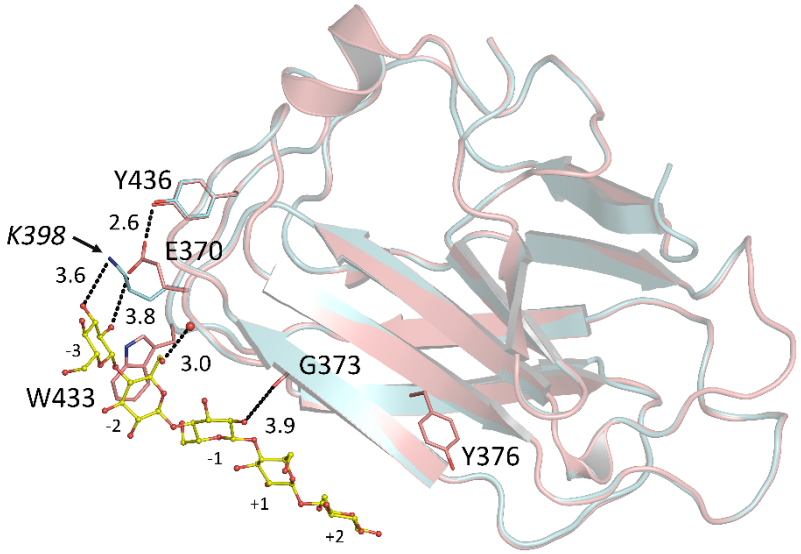

Figure 4 The superposed structures of the cellulose binding domain. The structure model of BglC-M from *B. amyloliquefaciens* was shown in salmon. The structure of BsCel5A CBM3 from *B. subtilis* 168 (PDB code 2L8A) was shown in cyan. The residues involved in carbohydrate recognition in the structure model of BglC-M K370E were shown as a stick model with carbon in salmon, nitrogen in blue, and oxygen in red. 398K, labeled in italic, was from the structure of BsCel5A CBM3 shown as a stick model with carbon in cyan. The docked cellopentaose was shown as a ball-and-stick model with carbon in yellow. Sugar-binding subsites were labeled. The black dashed line represented a predicted hydrogen bond formed in the structure model of BglC-M between the surrounding residues and the cellopentaose molecule; their distances are given in Å.

Hydrolysis activities on lignocellulosic substrates

The hydrolysis activity of cellulases on pineapple peel, corncob, and durian peel was tested. The results showed an increase in hydrolysis activity by measuring the amount of reducing sugar liberated from substrates since the first day of incubation. As shown in **Figure 5**, the results indicated that BglC-M exhibited the highest hydrolysis activity against all substrates. The most suitable substrate for this experiment was the corncob since it released the highest amount of reducing sugar on the 7th day.

According to the composition of lignocellulosic biomass, which contains 45 - 55 % of cellulose, the reducing sugar released during our experiment could be calculated as 20 - 40 % of total cellulose in each lignocellulosic substrate. This result indicated that the mutant could be used to hydrolyze these lignocellulosic wastes for animal feed. In the future, this cellulase mutant would be applied to hydrolyze lignocellulosic wastes for fermented animal feed, particularly corncob and durian peel. This could provide low-cost animal feed and reduce air pollution by avoiding the burning of lignocellulosic waste.

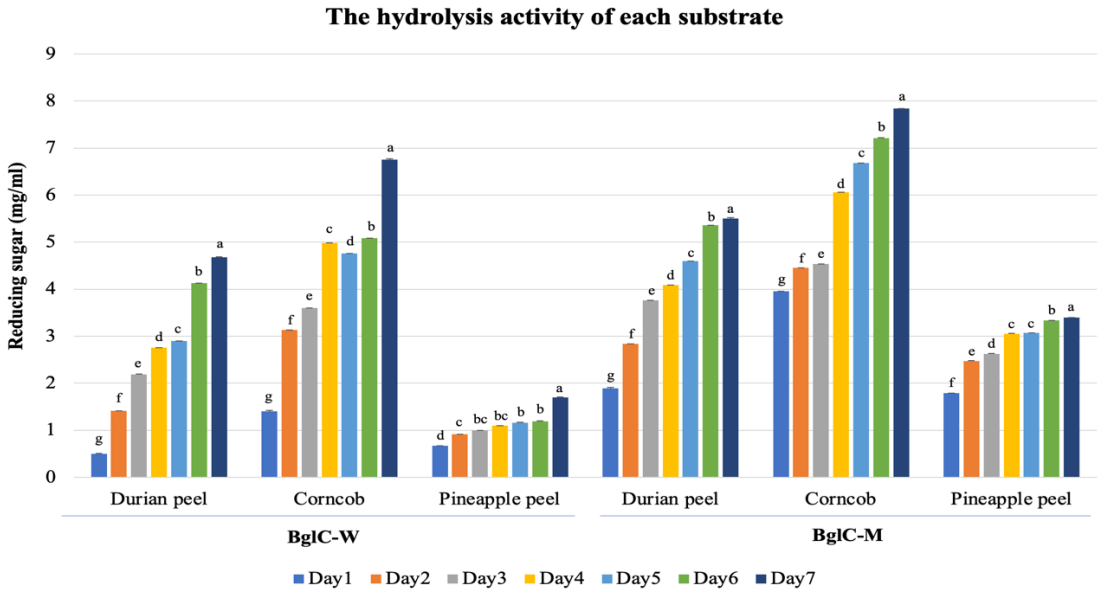

Figure 5 Hydrolysis activity of BglC-W and BglC-M on durian peel, corncob, and pineapple peel. The letters indicate a significant difference ($p < .05$) as determined by Duncan's multiple range test.

Conclusions

By using cold plasma implantation, *B. amyloliquefaciens* was mutated to increase its cellulase activity. Under a wide variety of temperature and pH conditions, the cellulase mutant (BglC-M) demonstrated about 2.5-fold greater catalytic activity than the control. The molecular docking study proposed the carbohydrate-aromatic and hydrogen bonding interactions between cellopentaose and BglC. Moreover, the residue which recognized the incoming substrate was predicted. Sequence analysis and structure prediction revealed that a single amino acid residue substituted from lysine to glutamine at position 370 (K370E) in the cellulose binding module, while not in the catalytic domain. The predicted structure of BglC-M with bound substrate revealed the role of mutant K370E, thus facilitating the attachment of the 2 loops. This amino acid (K370E) involved a charge residue change on the CBM3, resulting in increased structural stability and enzymatic activity. In addition, the hydrolysis ability of cellulase was investigated on agricultural wastes, including pineapple peel, corncob, and durian peel. A greater release of reducing sugar was detected in the corncob. Although an amino acid mutation in CBM (N126W) increasing the binding activity of *C. thermocellum* cellulase has been reported [34], its enzyme activity was not demonstrated. This was the first evidence shown that a single amino acid substitution in CBM, not in the catalytic domain, increased its enzyme activity.

Acknowledgements

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