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Contributed Paper

Comparison and Characterization of Purified Cellulase and Xylanase from *Bacillus amyloliquefaciens* CX1 and *Bacillus subtilis* B4

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ABSTRACT

Cellulase and xylanase producing bacteria, *Bacillus amyloliquefaciens* CX1 and *Bacillus subtilis* B4, were isolated from horse feces and cow's rumen fluid. Both enzymes were purified, characterized, and determined their molecular weight. The molecular weight of purified cellulase and xylanase of *B. amyloliquefaciens* CX1 was found to be 53 kDa and 26 kDa when cellulase and xylanase produced from *B. subtilis* B4 was estimated to be 46 and 23 kDa, respectively. Both cellulases exhibited their thermostability in wide range of 20-80°C and were stable over a broad range of pH 5-10. While both xylanases showed thermostability ranging 20-50°C, and were stable over a broad pH range of 4.0-9.0. Cellulase and xylanase activities were stimulated by Fe^{3+}, in contrary both enzymes' activities were strongly inhibited by 10 mM Mn^{2+} and Cu^{2+}. Xylanase from *B. amyloliquefaciens* CX1 showed the highest hydrolysis activity on various pretreated lignocellulosic substrates including rice straw, corn stover, paragrass and napier grass to total reducing sugar by 2.23, 3.52, 2.72 and 2.39 mg/ml, respectively. Furthermore, cellulase produced from *B. subtilis* B4 showed maximum release of reducing sugar from napier grass (0.72 mg/ml), followed by paragrass (0.58mg/ml), and rice straw (0.45mg/ml). Cellulase and xylanase produced from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 could also be considerable biotechnological interest particularly in fermented food process for cattle feed since the enzymes showed a dramatically stability over broad pH and temperature range.

Keywords: cellulase, xylanase, biomass degradation, ruminants, *Bacillus*

1. INTRODUCTION

Cellulase and xylanase have been produced by wide range of microorganisms such as fungi, bacteria, actinomycetes and yeast, but the most common industrial producer is bacteria [1]. Ruminal microbes, especially bacteria, present multiple functions in the process of dietary organic matter digested. Rumen bacteria represent an abundant source of novel enzymes and produce several potential enzymes such as cellulase, xylanase, glucanase, pectinase, amylase and protease [2]. Microbial cellulases and xylanases have shown their potential application in various industries including bleaching of pulp and paper, biofuel production, food industry, brewing, improving the digestion of cereal-based feeds for poultry and improving the nutritional quality and digestibility of ruminant feed [3]. Generally most of agriculture waste was eliminated by burning after harvest, causing serious air pollution and greenhouse gas emissions. In addition, shortage and low quality of feed stuff have become a problem for animal livestock production. At present, agricultural wastes such as corn stover, rice straw, paragrass, and durian peel which mainly compose of cellulose and hemicellulose are an alternative solution for animal feed using bacterial enzymes. The principle of agricultural waste materials includes cellulose, hemicellulose and lignin, the major structures can be hydrolysed β (1-4) glycosidic linkages by cellulases and xylanases, respectively. Several researches have shown that the products from plant structure hydrolysis by the enzymes contain not only homodisaccharides but also heteropolysaccharide form and finally turn small units into single glucose and xylose [4]. For lignocelluloses degradation to glucose, microorganisms capable of degrading both cellulose and xylan are considerable of importance for biomass utilization [5].

We have screened microorganisms producing cellulase and xylanase from various ruminants for the utilization of lignocellulosic biomass. In this report, we describe the purification and characterization of cellulase and xylanase produced from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 isolated from horse feces and cow's rumen which is remarkably efficient microbial breakdown of lignocellulosic materials.

2. MATERIALS AND METHODS

2.1 Isolation and Screening of Bacteria

Samples of cow's rumen fluid were obtained from the Faculty of Agriculture, Chiang Mai University, and samples of horse feces were obtained from the Chiang Mai Zoo (Thailand). Suitable dilutions were spread onto agar medium containing 0.05% yeast extract, 0.05% KH_2PO_4, 0.05% K_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.1% $(NH_4)\ SO_4$, 1.5% agar, 0.5% CMC, and 0.5% birchwood xylan as carbon sources. The organisms were incubated at 37°C for 24 h. Bacterial colonies were purified by repeated streaking. For screening of cellulase production, bacterial isolates were spotted on agar medium containing 1% CMC.

For screening of xylanase production, bacterial isolates were spotted on agar medium containing 1% birchwood xylan. Both samples were incubated at 37°C for 24 h. To visualize the hydrolysis zone, the plates were flooded with 1% (w/v) congo red for 15 min, and then destained with 1 M NaCl [6]. The isolates exhibiting both enzyme activities were selected for further investigation.

2.2 Bacterial Identification by 16S rDNA Sequencing

Genomic DNA of the selected isolates

were extracted [7] and used for 16S rDNA amplification, using universal primers: 16SF: 5'-AGT TTG ATC CTG GCT C-3' and 16SR: 5'-GGC TAC CTT GTT ACG A-3'. The PCR was performed using the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, extension at 72°C for 90 s, and extra elongation at 72°C for 10 min. The resulting 1.5 kbp PCR product was purified using Viogene's DNA extraction kit, and the nucleotide sequence was analyzed using NCBI blast (www.ebi.ac.uk).

2.3 Enzymatic Assay and Protein Determination

The activity of each enzyme was determined with each substrate, CMC or birchwood xylan. For cellulase activity, the reaction mixture contained 1% CMC in 50 mM citrate buffer (pH 5.0) and appropriate amounts of enzyme. After incubation at 50°C for 30 min, the reaction was stopped by immersing it in boiling water for 10 min. Xylanase activity was measured with the same process as cellulase activity using birchwood xylan as a substrate instead of CMC. The released reducing sugar was determined by the dinitrosalicylic acid (DNS) method [8]. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar per minute. Protein concentration was determined by the method of Bradford [9] by using the Bio-Rad protein assay with bovine serum albumin (BSA) as the protein standard.

2.4 Enzyme Production and Purification

For cellulase production, CMC was used as a carbon source while birchwood xylan was used as a carbon source for xylanase production. The cultures were grown at 37°C on a shaker (100 strokes/min) in 2-L Sakaguchi flasks containing 500 mL of media. After 48 h of cultivation, cells were removed from the culture broth by centrifugation at 10,000 × g for 10 min at 4°C and the supernatants were collected and stored at 4°C. Cellulase and xylanase were partially purified by ammonium sulfate precipitation.

To purify cellulase produced from *B. subtilis*, ammonium sulfate was added to the supernatant prepared from CMC-medium to 90% saturation and stored overnight at 4°C. Enzyme was collected by centrifugation at 10,000 × g for 15 min, and the pellet was suspended in 30 mL of 10 mM Tris-HCl buffer (pH 9.0). The enzyme solution was dialyzed against the same buffer. The dialyzed solution was applied to a DEAE-650M column (2 × 2 cm) equilibrated with 10 mM Tris-HCl (pH 9.0). After it was washed with the same buffer, the column was developed with buffer containing 50 mM NaCl, which eluted the enzyme. The active fractions were dialyzed against 10 mM Tris-HCl (pH 9.0). Ammonium sulfate was added at 30% saturation to the active fraction and then applied to a Butyl-650M column (2 × 2 cm) equilibrated with Tris-HCl (pH 9.0) containing 30% ammonium sulfate. After washing with the buffer containing 30% ammonium sulfate, the column was developed with buffers containing 20%, 15% and 10% ammonium sulfate. Cellulase was eluted with the buffer containing 15% ammonium sulfate. The active fractions were dialyzed against 10 mM Tris-HCl (pH 8.0) and then applied to a SuperQ-650M column (2×2 cm). The column was washed with the buffer. Cellulase was found in the unabsorbed fractions.

Cellulase produced from *B. amyloliquefaciens* was purified by adding ammonium sulfate to 70% saturation for the supernatant from CMC-medium and

stored overnight at 4°C. Enzyme was collected by centrifugation at 10,000 × g for 15 min, and the pellet was re-suspended in 30 mL of 10 mM Tris-HCl buffer (pH 8.0). The enzyme solution was dialyzed against the same buffer. The dialyzed solutions were placed onto a DEAE-Toyopearl 650M (2 × 20 cm) column equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The elution was performed with a linear gradient from 0 to 0.5 M NaCl at flow rate 0.5 mL/min. Fractions were collected and analyzed for enzyme activities and protein concentration. The active fractions were collected and dialyzed overnight against 10 mM Tris-HCl buffer (pH 8.0).

To purify xylanase produced from *B. subtilis* and *B. amyloliquefaciens*, ammonium sulfate was added to the supernatant prepared from xylan-medium to 30% saturation and stored overnight at 4°C. Enzyme produced from *B. subtilis* was collected by centrifugation at 10,000 × g for 15 min, and the pellet was suspended in 30 mL of 10 mM Tris-HCl buffer (pH 9.0) then applied to a DEAE-650M column (2 × 2 cm) equilibrated with Tris-HCl (pH 9.0) and the column was washed with this buffer. Xylanase was found in the unabsorbed fractions. The precipitate collected from *B. amyloliquefaciens* was suspended in 30 mL of 10 mM Tris-HCl buffer (pH 8.0) then enzyme solution was dialyzed against the same buffer. The dialyzed solution was placed onto a DEAE-Toyopearl 650M column (2 × 20 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The elution was performed with a linear gradient from 0 to 0.5 M NaCl at flow rate 0.5 mL/min. Fractions were collected and analyzed for enzyme activities and protein concentration.

2.5 Characterization of Purified Cellulase and Xylanase

2.5.1 Molecular weight determination

The molecular mass of purified cellulase and xylanase was determined using 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) [10]. Pre-stained Protein Markers (Broad Range) were used as molecular weight markers including myosin (200 kDa), β-Galactosidase (120 kDa), Bovine Serum Albumin (91 kDa), Glutamine Dehydrogenase (62 kDa), Ovalbumin (46 kDa), Carbonic Anhydrase (38 kDa), Myoglobin (26 kDa), Lysozyme (19 kDa) and Aprotinin (9 kDa).

2.5.2 Effect of temperature on activity and stability of cellulase and xylanase

The optimal temperature of each enzyme activity was determined by incubating 1% (w/v) substrate (CMC and birchwood xylan) with the enzymes in 50 mM citrate buffer (pH 5.0) for 30 min at various temperatures ranging from 20-100°C. To study the thermal stability, the enzymes were pre-incubated at temperatures ranging from 20-100°C for 30 min. The residual activity was measured according to Miller [8].

2.5.3 Effect of pH on activity and stability of cellulase and xylanase

The optimal pH of each enzyme activity was determined by incubating the mixture containing 1% (w/v) substrate with each enzyme at 37°C at pH 3.0-11.0. Four different buffers were used: citrate buffer (pH 3.0-5.0), potassium phosphate buffer (pH 6.0-7.0), Tris-HCl buffer (pH 8.0-9.0) and CAPS buffer (pH 9.0-11.0). To determine the pH stability, the enzymes were incubated in 50mM buffer at pH 3.0-11.0 for 24 h. The residual activity at each pH was calculated.

2.5.4 Effect of metal ions

The purified enzymes in 50 mM citrate buffer (pH 5.0) with various metals (Na^{+}, K^{+}, Li^{+}, NH^{4+} , NH^{4+} , Ca^{2+} ,MN^{2+} , Mg^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , Zn^{+}) were incubated at room temperature for 30 min. The final concentrations of metals were 5 and 10 mM. After incubation, the residual activity was measured according to Miller [8].

2.5.5 Hydrolysis activities against lignocellulosic substrates

The hydrolysis activity of cellulase and xylanase was tested on non-treated and pretreated rice straw, corn stover, paragrass and napier grass. The lignocellulosic substrates were pretreated with steam at 121°C for 2 hours and followed by 2 %w/v of sodium hydroxide solution. The appropriate amounts of purified enzymes were incubated with 5% (w/v) non-treated and treated substrates in 50 mM citrate buffer (pH 5.0) at 50°C for 24 h. The reducing sugar released from the substrates was measured by the DNS method.

2.6 Cloning of cellulase (*BglC*) and xylanase (*XynA*)

According to published sequences of *B. subtilis* and *B. amyloliquefaciens* endoglucanase gene in the GenBank, the complete reading frame (ORF) of endoglucanase gene was amplified using designed primer. All primers used for the cloning in this part are listed in Table 1. PCR reaction mixture was performed in a total volume of 20 µl then amplified using the following cycling profile: 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1.50 min, and followed by a final extension at 72°C for 10 min. The resulting 1.5 kbp PCR product was purified using Viogene's DNA extraction kit, and the nucleotide sequence was analyzed using NCBI blast (www.ebi.ac.uk). According to the known sequences of xylanase (*XynA*) genes in the GenBank database, two pairs of primers were designed and synthesized to amplify the entire gene from chromosomal DNA by PCR reaction. The PCR reaction mixture was performed under the same condition as the cellulase gene amplification. The PCR started with a 5 min denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C and 30 s at 72°C, followed by a final extension at 72°C for 5 min. The amplified PCR product was purified using Viogene's DNA extraction kit and the nucleotide sequence was analyzed using NCBI blast (www.ebi.ac.uk).

Table 1. List of primers used for genes cloning.

Primer name	5' to 3' Sequence
CelCX1F	ccggcacatatggcagggacaaaaacgccag
CelCX1R	ctaatgctcgaggcctaaagcttaactaatt
XyCX1F	aggaggggatccatgtttaagtttaaaaag
XyCX1R	aaagataagcttttaccacactgttacatt
CelB4F	catatggcagggacaaaaacgccagtagcc
CelB4R	aaactcgagctaatttggttctgttcccca
XyB4F	ggatccgctggcacagattactggcaaaat
XyB4R	aaagataagcttttaccacactgttacatt

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Microorganisms

Samples from horse feces and cow's rumen fluid were collected in order to find microorganisms that can use xylan and cellulose. The cultures were grown on medium containing both birchwood xylan and CMC. After growing bacteria on agar plates with 48 h of incubation time at 37°C, 32 isolates of distinguished morphology were selected to test cellulase and xylanase activities. As a consequence, two isolates, namely CX1 and B4, one collected from horse feces, the other obtained from cow's rumen fluid, produced both cellulase and xylanase activities. A preliminary test of cellulase activity of both isolates was performed; cellulase produced from isolate CX1 showed higher activity against CMC substrate than isolate B4. On the other hand, xylanase activity of B4 showed higher enzyme activity than CX1. Consequently, both isolates were selected for further investigation. Analysis of a 16S rDNA sequence of isolate CX1 showed the highest homology (99%) to *Bacillus amyloliquefaciens* (accession no. NR_075005.1), and isolate B4 displayed the highest match of 99% similarity with *Bacillus subtilis* (accession no. NR_112116.1). Based on the evolution distance resulting from 16S rDNA sequence and the neighbor-joining method [11], the bacteria were designated as *B. amyloliquefaciens* CX1 and *B. subtilis* B4 (Figure 1).

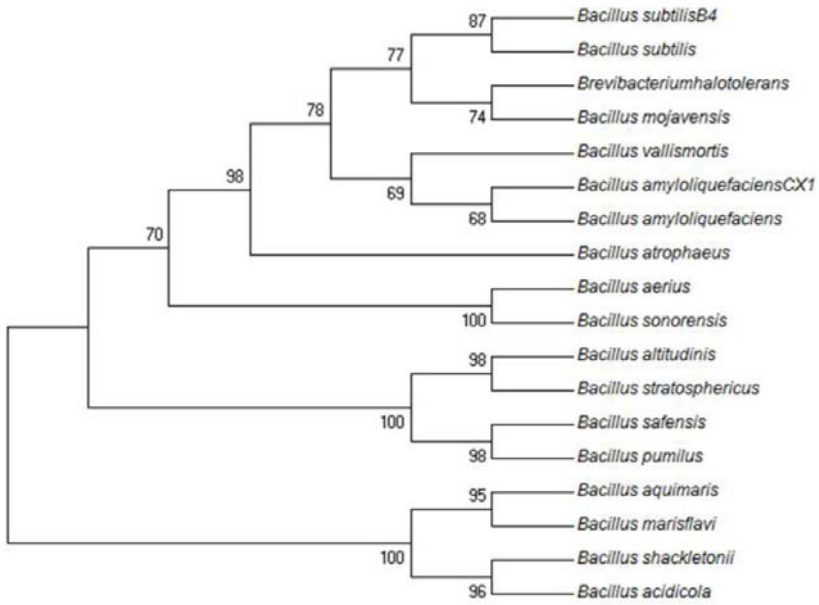

Figure 1. Neighbor-joining tree [11] based on 16S rDNA sequences of *Bacillus* sp. Numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled dataset.

3.2 Purification of Cellulase and Xylanase

The cellulase produced from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 were purified as previously described. The cellulase secreted by *B. amyloliquefaciens* CX1 was purified to homogeneity with 27.7-fold purification and a yield of 35.9% (Table 2a). Molecular mass of the purified cellulase was estimated to be 53 kDa (Figure 2) which was similar to *Bacillus* sp. C1AC5507 (55 kDa) [12]

and *Bacillus licheniformis* ATCC 14580 (53kDa) [13]. The molecular mass was larger than those of cellulases from *B. licheniformis* [14] and *B. circulans* [15] (37-43 kDa). On the contrary, the molecular mass of cellulase from *B. amyloliquefaciens* CX1 was smaller than those of the cellulases from *Bacillus* sp. AC-1 [16] and *Bacillus* sp. KSM-522 [17] (61-78 kDa). The cellulase produced from *B. subtilis* B4 showed a single band in SDS-PAGE gel with 46 kDa, indicating homogeneity. Purified cellulase had a yield of 9.50% with 67.20-fold purification (Table. 3a). The molecular mass of purified cellulase from *B. subtilis* B4 was larger than some *Bacillus* spp., *Chalara paradoxa* and *Aspergillus niger* sized around 35-40 kDa [18-19].

Table 2a. Summary of purification of the cellulase produced by *B. amyloliquefaciens* CX1.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	8463.28	595.00	14.22	1.00	100
$(NH_4)_2SO_4$ precipitation	3311.49	21.00	158.21	11.12	39.12
DEAE-Toyopearl 650M	3037.65	7.70	394.50	27.74	35.90

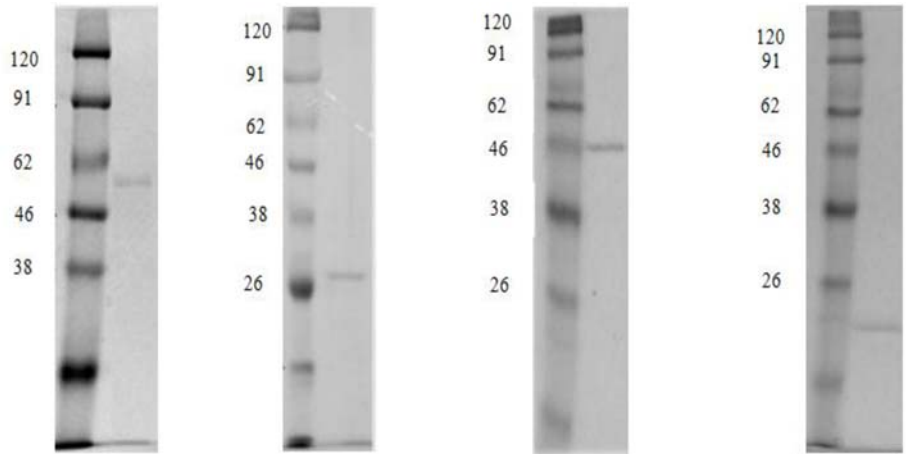

Figure 2. SDS–PAGE analysis of the purified cellulase (a) and xylanase (b) produced by *B. amyloliquefaciens* CX1, cellulase (c) and xylanase (b) produced by *B. subtilis* B4. Electrophoresis of cellulase and xylanase were performed using 12% and 10% polyacrylamide gel, respectively. The gels were stained with coomassie brilliant blue R-250. Size markers (kDa) described in the section of "Materials and Methods" were used.

Table 2b. Summary of purification of the xylanase produced by *B. amyloliquefaciens* CX1.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	10058.86	778.58	12.919	1.00	100
$(NH_4)_2SO_4$ precipitation	4451.42	41.00	108.57	8.40	44.25
DEAE-Toyopearl 650M	3370.62	11.00	306.42	23.72	33.5

The xylanases produced from both isolates were also purified. The purified enzyme secreted from *B. amyloliquefaciens* CX1 was purified homogenously with 23.7-fold purification and a yield of 33.5% (Table 2b). Moreover, the *B. subtilis* B4 xylanase was purified with one step using a DEAE-650M column with a purification fold of 6.67 and recovery yield of 33.37% (Table. 3b). The molecular mass of purified xylanases from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 were estimated to be 26 kDa and 23 kDa, respectively (Figure 2). This molecular mass was larger than 22 kDa of xylanase from *B. licheniformis* [20], yet it was smaller than 83-99 kDa of xylanases from *Anoxybacillus pushcinoensis* A8 and *Bacillus* sp. SPS-0 [21, 22].

Table 3a. Summary of purification of the cellulase produced by from *B. subtilis* B4.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	141.30	1994.00	0.07	1.00	100.0
$(NH_4)_2SO_4$ precipitation	48.20	214.80	0.22	3.20	34.20
DEAE-Toyopearl 650M	17.20	32.60	0.53	7.50	12.20
Butyl-Toyopearl 650M	14.00	15.40	0.90	12.90	9.90
SuperQ-650	13.60	2.89	4.70	67.20	9.50

Table 3b. Summary of purification of the xylanase produced by *B. subtilis* B4.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	53.51	944.00	0.06	1.00	53.51
$(NH_4)_2SO_4$ precipitation	39.26	170.00	0.23	4.03	39.26
DEAE-Toyopearl 650M	33.37	75.00	0.56	6.67	33.37

3.3 Characterization of Cellulase and Xylanase

3.3.1 Effect of temperature on activity and stability of cellulase and xylanase

The optimal temperature of cellulase activity from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 were 50 and 60°C, respectively (Figure 3a). The enzymes were stable over a broad range of temperatures from 20-80°C, while cellulase activity of both species retained about 50% of the activity at 90°C (Figure 3c).

The cellulase from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 showed higher optimal temperature than that of *B. amyloliquefaciens* DL-3 (50°C) [23] and *Mucor circinelloides* (50°C) [24] and presented higher thermal stability than *B. subtilis* TD6 (0-50°C) [25]. Due to their activity and stability at optimal

temperatures. Xylanase from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 exhibited optimal activity at 50°C (Figure 3b) and thermostability was between 20-50°C, respectively (Figure 3d). The xylanases from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 were much more thermostable than xylanase from *B. amyloliquefaciens* CH51 which exhibited the optimum temperature for xylanase activity at 25°C and retained 25% less of the activity after incubation at 50°C for 10 min [26]. Both enzymes show great potential for several applications, particularly, feed fermentation and ensilage. Moreover, increasing of temperature during feed fermentation is another reason for wide range thermostable enzymes are required.

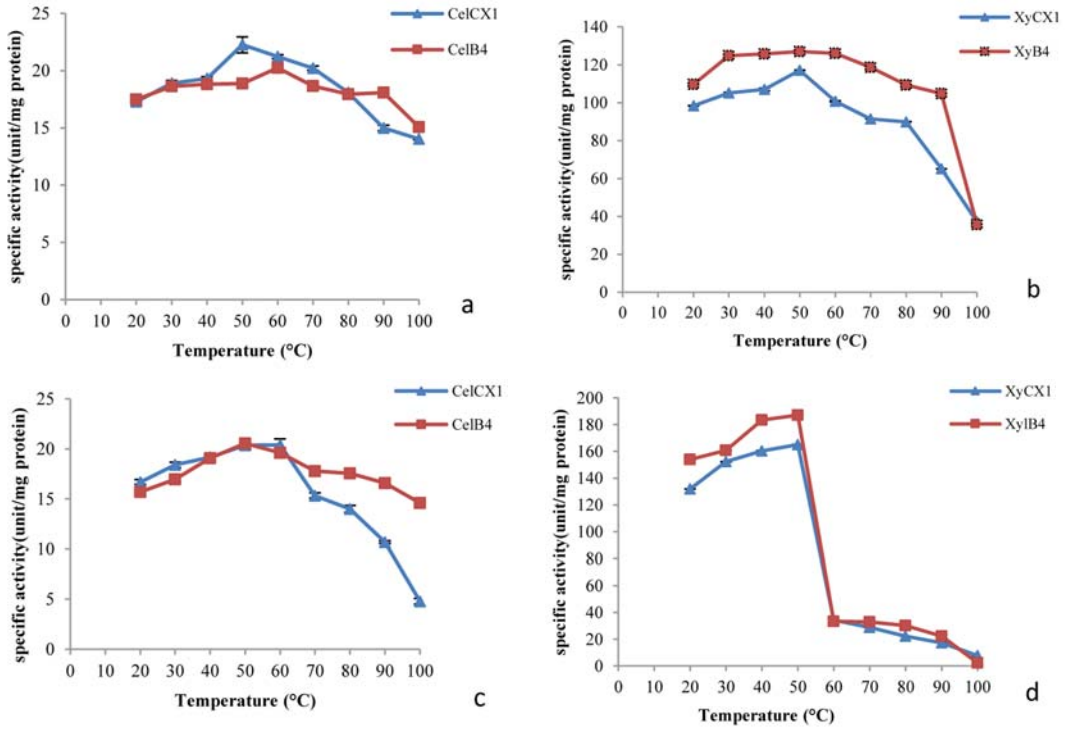

Figure 3. Effect of temperature on purified cellulase and xylanase. (a) Optimum temperature of cellulases. (b) Optimum temperature of xylanases. (c) Temperature stability of cellulases. (d) Temperature stability of xylanases.

3.3.2 Effect of pH on activity and stability of cellulase and xylanase

Optimal pH for cellulase activity from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 were pH 5.0 and pH 8.0, respectively (Figure 4a). The optimal pH of cellulase activity produced by *B. subtilis* B4 measured higher than the other *Bacillus* strains (pH 5.0-6.5) [17], *Bacillus vallismortis* RG-07 (pH 7.0) [12] and *A. niger* (pH 6.0-7.0) but lower than *Bacillus sp.* HSH-810 (pH 10) [27]. Both cellulases were stable over a wide range of pH 5-10 (Figure 4c), and exhibited rather high pH stability than *M. circinelloides* (pH 4.0-7.0) [28]. Xylanases from both species showed optimum pH at 6.0 and were stable over a broad pH range of 4.0-9.0 (Figure 4b, 3d). The xylanase from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 demonstrated a much more stable pH than xylanase from *B. amyloliquefaciens* CH51 (pH 6.0-7.0) [26]. Hydrolysis of agricultural waste with

cellulase and xylanse may help reducing crude fiber content of plant which is one of limiting factor to improve feed quality during fermentation process [29].

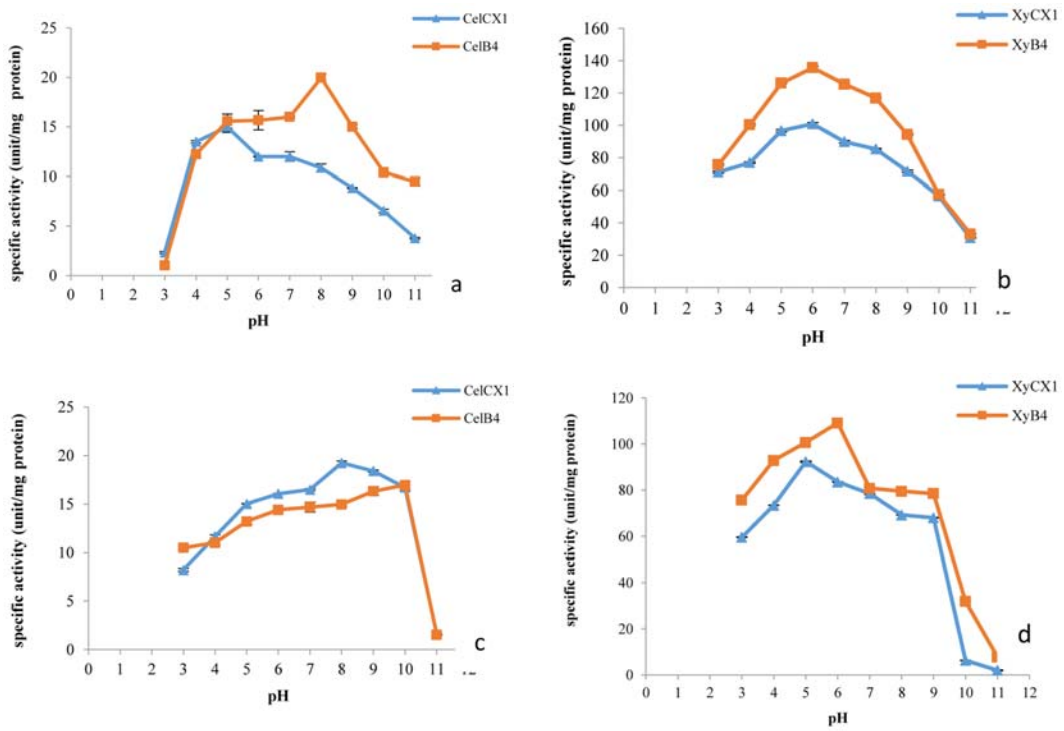

Figure 4. Effect of pH on purified cellulase and xylanase. **(**a**)** Optimum pH of cellulases. **(**b**)** Optimum pH of xylanases. **(**c**)** pH stability of cellulases. **(**d**)** pH stability of xylanases.

3.3.3 Effect of metal ions

Effects of various metal ions on the cellulase and xylanase activities were investigated under the standard assay condition in the presence of metal ions at 5 mM and 10 mM concentration. As shown in Table 4, most metal ions slightly affect the activity of cellulases and xylanases. Mn^{2+} strongly inhibited both of cellulases and xylanases activity about 20-60%, while Fe^{3+} activated the enzymes activity. This result is similar to *Bacillus* sp. DSNC 101 [30]. The addition of Mn^{2+} and Mg^{2+} resulted in about 50% loss of cellulase and xylanase activity.

3.3.4 Hydrolysis activities against lignocellulosic substrates

The hydrolysis activity of cellulases and xylanases on several non-treated and pretreated lignocellulosic substates were tested. The results from Table 5 show high ability of xylanase produced from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 to hydrolyze pretreated rice straw, paragrass, corn stover and napier grass by liberate reducing sugar. Furthermore, non-pretreated substrates can be hydrolyzed by the xylanase and cellulase from both of species. The enzymes produced from *B. amyoliquefaciens* CX1 and *B. subtilis* B4 could be useful in the hydrolysis of lignocellulosic biomass which will apply for animal feed.

Table 4. Effect of various additives (metal ions) on cellulase and xylanase activities.

Metal ions	Relative activity							
	5 mM				10 mM			
	CelCX1	CelB4	XyCX1	XyB4	CelCX1	CelB4	XyCX1	XyB4
none	100	100	100	100	100	100	100	100
Na^{+}	86	105	102	77	101	99	87	81
K^{+}	97	87	106	99	104	102	93	87
Li^{+}	108	107	102	111	95	97	94	93
NH^{4+}	92	94	109	99	76	100	96	92
Ag^{+}	99	99	103	102	111	113	82	100
Ca^{2+}	95	86	119	106	96	86	92	103
Mn^{2+}	52	77	69	68	47	65	59	70
Mg^{2+}	76	87	93	96	98	89	85	104
Cu^{2+}	84	97	120	115	38	66	51	55
Fe^{2+}	92	91	100	98	96	88	89	81
Fe^{3+}	112	118	122	124	152	140	118	125
Zn+	81	106	93	94	83	106	66	77

* Data are expressed as means of triplicate experiments.
* CelCX1 is purified cellulase from *B. amyloliquefaciens.*
* CelB4 is purified cellulase from *B. subtilis* B4.
* XyCX1 is purified xylanase from *B. amyloliquefaciens* CX1.
* XyB4 is purified xylanase from *B. subtilis* B4.

Table 5. Comparison of enzymatic hydrolysis of different lingocellulosic substrates by cellulases and xylanases.

Substrate	Reducing sugar (mg/ml)			
	CelCX1	CelB4	XyCX1	XyB4
non-treated rice straw	0.25	0.17	0.06	0.04
pretreated rice straw	0.17	0.45	2.33	2.28
non-treated corn stover	0.05	0.08	0.14	0.08
pretreated corn stover	0.16	0.2	3.52	3.24
non-treated paragrass	0.26	0.34	0.08	0.18
pretreated paragrass	0.38	0.58	2.68	2.72
non-treated napier grass	0.21	0.54	0.11	0.11
pretreated napier grass	0.66	0.72	2.39	2.14

3.4 Cloning of Cellulase (*BglC*) and Xylanase (*XynA*)

The molecular weight of purified cellulase from *B. amyoliquefaciens* CX1 and *B. subtilis* B4 were approximately 53 kDa and 46 kDa, respectively, corresponding to the predicted molecular weight of the endoglucanase gene of *B. amyloliquefaciens* and *B. subtilis*. The endoglucanase gene of *B. amyloliquefaciens* CX1 and *B. subtilis* B4 were amplified using primers based on sequences available in GenBank. A full length gene of β-1,

4-endonuclease of *B. amyloliquefaciens* CX1 consisting of 1,497 nucleotides was identified through sequence analysis of the amplified product. The ORF encoded for a protein of 499 amino acids with a predicted molecular weight of 53 kDa. Moreover, this amino acid sequence showed a similarity to sequences reported for *B. amyoliquefaciens* and *B. subtilis* cellulase genes (accession no. ZP_16170636.1, ZP_17180290.1, ABS70711. 1, and YP_001421404.1) with 99% identity. The sequence result of *B. subtilis* B4 showed that the cloned sequence of *BglC* contained ORF consisted of 1,499 nucleotides encoding a protein of 499 amino acids. Furthermore, the *BglC* gene presented similarities to sequences reported for other *B. subtilis* cellulase genes (accession nos. HM470252.1, AF355629.1, HQ000093.1, JX567739.1) above 98%. These results suggested that cloned fragments were endo-1,4-β-glucanase.

Likewise, *XynA* fragments of *B. amyoliquefaciens* CX1 and *B. subtilis* B4 were cloned using primers based on sequences already available in GenBank, and both fragments consisted of 642 nucleotides encoding a protein of 214 amino acids with a predicted molecular mass of 26 kDa and 23 kDa, respectively. *XynA* sequence of *B. amyoliquefaciens* CX1 showed a similarity to xylanase from *B. amyoliquefaciens*, *B. subtilis* and *B. circulans* (accession nos. YP_001422939.1, AEG2103 9.1, and AAM08360.1) with 98% identity. Moreover, the *XynA* of *B. subtilis* B4 was similar to the *B. subtilis* xylanase genes (accession nos. HM060310.1, U51675.1, CP0 06952.1, CP004065.1) with 98% identity. Further sequence analysis showed that the amino acid sequence deduced from both the nucleotide sequences were a xylanase of glycosyl hydrolase family 11 with a signal peptide at N terminus. The results suggested that these *XynA* fragments were endo-1, 4-β-xylanase.

4. CONCLUSION

We have isolated *B. amyloliquefaciens* CX1 and *B. subtilis* B4, both of which produce cellulase and xylanase. Purified cellulases produced from *B. amyloliquefaciens* CX1 and *B. subtilis* B4 were both thermostable and characteristically similar. Nevertheless, cellulase produced from *B. subtilis* B4 exhibited good thermostability between 20-80°C, higher than cellulase from *B. amyloliquefaciens* CX1. Moreover, the optimal temperature and pH for cellulase activity of *B. subtilis* B4 were higher than that of cellulase from *B. amyloliquefaciens*. In contrast, the cellulase activity of *B. amyloliquefaciens* CX1 at 50°C was higher than *B. subtilis* B4's cellulase activity. Due to the wide stability range of temperature, the cellulases demonstrated high potential to be used in many applications which require several enzymes working in the same conditions. These enzymes could contribute to efficient hydrolysis of lignocellulosic waste to reducing sugar. In addition, the high temperature and pH stability character of cellulase produced from *B. subtilis* B4 suggested its potential use in hydrolyzing cellulosic materials in high temperature and alkaline pH. Likewise, the xylanase produced from *B. subtilis* B4 presented higher xylanase activity and stability than *B. amyloliquefaciens* CX1 at pH range of 4.0-9.0. The hydrolysis activity of cellulase from *B. subtilis* B4 to hydrolyze lignocellulosic biomass was also higher than the xylanase from *B. amyloliquefaciens* CX1. Since the enzymes produced from both strains demonstrated high temperature stability, stability over broad pH range and high hydrolysis activity, these enzymes could be consider to biotechnology application especially for cattle and poultry dietary feed. Since feed fermentation process is carried

out under close system and during this process there are not only increasing of temperature but pH also changed. Therefore, the enzymes with high stable to wide range of temperature and pH would be useful.

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REFERENCES

[1] Perez J., Munoz-Dorado R.T. and Martinez J., *Int. Microbiol.*, 2002; **5**: 53-63. DOI 10.1007/s10123-002-0062-3.

[2] Cheng K.J., Lee S.S., Bae H.D and Ha J.K., *J. Anim. Sci.*, 1999; **12**: 84-92. DOI 10.5713/ajas.1999.84.

[3] Barry V.M. and Paraic M.G., *Appl. Biochem. Biotechnol.*, 2015; **177**: 1152-1163. DOI 10.1007/s12010-015-1803-z.

[4] Brandon C.K., Majid H.M., Michael F.C., Lloyd F.M., Andreas W.G., Mats S., Stephen G.W., Jerry S. and Gregg T.B., *J. Am. Chem. Soc.*, 2014; **136(1)**: 321-329. DOI 10.1021/ja410291u.

[5] Sakamoto T., Hasunuma T., Hori Y., Yamada R. and Kondo A., *J. Biotechnol.*, 2012; **158**: 203-210. DOI 10.1016/j.jbiotec.2011.06.025.

[6] Ronald M.T. and Peter J.W., *Appl. Environ. Microbiol.*, 1982; **34(4)**: 777-780.

[7] Ifaku S., *Biomacromolecules*, 2005; **8(6)**: 1973-1978. DOI 10.1021/bm070113b.

[8] Miller G.C., *Anal. Chem.*, 1959; **31**: 426-428. DOI 10.1021/ac60147a030.

[9] Bradford M.M., *Anal. Biochem.*, 1976; **72**: 248-254. DOI 10.1016/0003-2697(76)90527-3.

[10] Laemmli U.K., *Nature*, 1970; **227**: 680-685. DOI 10.1038/227680a0.

[11] Saitous N. and Nei M., *Mol. Biol. Evol.*, 1987; **4**: 406-425.

[12] Gaur R. and Tiwari S., *BMC Biotechnol.*, 2015; **15**: 19. DOI 10.1186/s12896-015-0129-9.

[13] Zahoor S., Javed M.M., Aftab M.N. and Haq I.U., *Biologia*, 2011; **66/2**: 213-220. DOI 10.2478/s11756-011-0020-7.

[14] Bischoff K.M., Liu S. and Hughes S.R., *Process Biochem.*, 2007; **42**: 1150-1154. DOI 10.1016/j.procbio.2007.05.001.

[15] Hakamada Y., Endo K., Takizawa S., Kobayashi T., Shirai T., Yamane T. and Ito S., *Biochem. et. Biophys. Acta General Subjects*, 2002; **1570**: 174-180. DOI 10.1016/S0304-4165(02)00194-0.

[16] Li Y.H., Ding M., Wang J., Xu G.J. and Zhao F., *Appl. Microbiol. Biot.*, 2006; **70**: 430-436. DOI 10.1007/s00253-005-0075-x.

[17] Okoshi H., Ozaki K., Shikata S., Oshino K., Kawai S. and Ito S., *Agric. Biol. Chem. Tokyo*, 1990; **54**: 83-89.

[18] Mawadza C., Rahni H., Zvauya R. and Bo M., *J. Biotechnol.*, 2000; **83**: 177-187. DOI 10.1016/S0168-1656(00)00305-9.

[19] Akiba S., Kimura Y., Yamamoto K. and Kumagai H., *J. Ferment. Bioeng.*, 1995; **79(2)**: 125-130. DOI 10.1016/0922-338X(95)94078-6.

[20] Lee C.C., Kibblewhite-Accinelli R.E., Smith M.R., Wagschal K., Orts W.J. and Wong D.W., *Curr. Microbiol.*, 2008; **57**: 301-305. DOI 10.1007/s00284-008-9193-x.

[21] Kacagan M., Canakci S., Sandalli C., Inan K., Colak D.N. and Belduz A.O., *Biologia*, 2008; **63**: 599-606. DOI 10.1007/s13369-016-2110-x.

[22] Bataillon M., NunesCardinali A.P., Castillon N. and Duchiron F., *Enz. Microb. Technol.*, 2000; **26(2-4)**: 187-192. DOI 10.1007/s11274-007-9516-2.

[23] Lee Y.J., Kim B.K., Lee B.H., Jo K.I., Lee N.K., Chung C.H., Lee Y.C. and Lee J.W., *Bioresour. Technol.*, 2008; **99(2)**: 378-86. DOI 10.1016/j.biortech.2006.12.013.

[24] Saha B.C., *Process Biochem.*, 2004; **39(12)**: 1871-1876. DOI 10.10 I6/j.procbio.2003.09.013.

[25] Dian A. and Don H., *Proceeding of the 2nd International Seminar on Chemistry*, Jatinangor, Korea, 24-25 November 2001; 281-287.

[26] Baek C.U., Lee S.G., Chung Y.R., Cho I. and Kim J.H., *Indian J. Microbiol.*, 2005; **52**: 695-700. DOI 10.1007/s12088-012-0260-4.

[27] Kim J.Y., Hur S.H. and Hong J.H., *Biol. Lett.*, 2005; **27**: 313-316. DOI 10.1007/s10529-005-0685-5.

[28] Lucas R., Robles A., Garcia M.T., Alvarez C.G and Galvez A., *J. Agric. Food Chem.*, 2001; **49(1)**: 79-85. DOI 10.1021/jf000916p.

[29] Nuraini A.D and Maria E.M., *Int. J. Poult. Sci.*, 2015; **14(6)**: 354- 358. DOI 10.3923/igps.2015.354.358.

[30] Nam-Chul C. and Suk B., *J. Microbiol. Biotechnol.*, 1997; **7**: 386-39. DOI 10.1016/1381-1177(95)00003-8