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Purification, Biochemical Characterization of a *Macrotermes gilvus* Cellulase and Zymogram Analysis

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ABSTRACT

Cellulase plays an important role in cellulose degradation. The enzyme catalyzes the cleavage of b-1,4 glycosidic bond between glucose residues. The *Macrotermes gilvus* cellulase was purified by using ammonium sulfate precipitation and anion exchange column with 1.38% recovery and 22-fold purification. The SDS-PAGE coupled with zymogram analysis revealed the molecular weights approximately of 54 kDa. The biochemical properties of the enzyme exhibited the optimum temperature and optimum pH of 45°C and 5.2. Interestingly, the enzyme was active over a wide range of temperatures (7-70°C) and a broad range of pH values (4.5-8). At the indicated temperatures and pH values, the enzyme exhibited more than 84 and 50% of its activity. The thermal stability and pH stability of the enzyme were also investigated. The result showed that the enzyme retained nearly 40% of its original activity after incubation in mild acidic (pH 5.2), neutral (pH 7.0) and basic (pH 10.0) conditions for 5 h. The enzyme retained its activity more than 70% of initial activity at both 37 and 45°C after incubation for 3 h. Moreover, the activity of the enzyme was strongly inhibited by Cu^{2+} and slightly affected by Fe^{2+} and EDTA, whereas the presence of Ca^{2+} and Mg^{2+} slightly increased the enzyme activity. Due to the wide temperature and pH range of enzyme activity, the *Macrotermes gilvus* cellulase might be potential enzyme for industrial or agricultural application.

Key words: Cellulase, glucosidase, cellulolytic enzyme, *Macrotermes gilvus*, termite

INTRODUCTION

Cellulose is the most abundant carbohydrate on earth mainly produced by terrestrial plants. It is a linear polymer of glucose residues, which are linked to each other by b-1,4 glycosidic bonds. The number of glucose unit inside the cellulose molecule varies and the degree of polymerization ranges from 250 to over 10,000 (Klemm *et al*., 2005). As its conversion to glucose sugar, cellulose is considered as the most important source of raw materials for production of renewable energy such as bio-fuel. In addition, there are many industrials and agricultural cellulosic wastes are being accumulated. The utilization of cellulolytic enzyme for cellulose degradation is interest.

Cellulases or cellulolytic enzymes are the enzymes catalyze the hydrolysis of b-glycosidic linkage in cellulose molecule. The complete hydrolysis of cellulose requires three types of enzyme which work synergistically. All of them are endoglucanases, cellobiohydrolases and b-glucosidases. Endoglucanases (EC 3.2.1.4) randomly cleave the internal b-1,4-glycosidic bonds to minimize the length of cellulose chain. Cellobiohydrolases (EC 3.2.1.91) or exoglucanases hydrolyze the external

b-1,4-glycosidic bonds at both reducing and non-reducing ends of cellulose to release either cellobiose or glucose. β-glucosidases (EC 3.2.1.21) generally function to break down cellobiose into glucose and also help to reduce the cellobiose accumulation, which inhibits the activities of both exo-and endoglucanases (Harhangi *et al*., 2002).

Cellulolytic enzymes have revealed their biotechnological potential in various industries (Kuhad *et al*., 2011) including pulp and paper (Bajpai, 1999; Mai *et al*., 2004; Pere *et al*., 2001), food, wine, animal feed (Galante *et al*., 1993, 1998b; Dhiman *et al*., 2002), textile (Hebeish and Ibrahim, 2007; Galante *et al*., 1998a), wine and brewery (Bamforth, 2009), food processing (De Carvalho *et al*., 2008), agricultural (Agosin and Aguilera, 2005) and bio-ethanol production (Ghosh and Singh, 1993; Gupta *et al*., 2011; Sukumaran *et al*., 2009). The cost of production and low yield of the enzyme are the major problems for industrial application (Thongekkaew *et al*., 2008).

Cellulase enzymes produced from bacteria and fungi have been characterized. Some bacterial cellulases are produced in relative low amounts and no exoglucanase activities is detected (Gilbert *et al*., 1987), whereas fungal cellulases are produced in large amount in the presence of all enzyme components including exoglucanases, endoglucanases and b-glucosidases (Ogawa *et al*., 1991; Konstantin *et al*., 1999; Coughlan *et al*., 1985; Coughlan, 1985). However, the produced enzymes tightly associate in the multienzyme complexes called cellulosomes which are difficult to isolate without loss of total activity (Bayer *et al*., 1998a, b; Doi *et al*., 1998). Besides fungal and bacterial cellulases, the insect enzymes such as beetle and termite cellulases also have been investigated (Lee *et al*., 2004; Ueda *et al*., 2010).

Termites utilize cellulosic materials as their food source and thus they are regarded as harmful insects because of their ability to destroy all materials containing cellulose. Termites are classified into four groups based on their feeding habit comprising wood-feeding, leaf and litter-feeding, soil-feeding and fungus-growing termites (Creffield, 1991). The cellulolytic enzymes from wood-feeding termites are well characterized (Potts and Hewitt, 1974; Tokuda *et al*., 1997; Zhang *et al*., 2009, 2011; Inoue *et al*., 2005; Zhou *et al*., 2007; Ni *et al*., 2010), but the information of cellulase enzyme produced from fungus-growing termites is limited.

Macrotermes gilvus (Hagen), the fungus-growing termite widely distributed in South-eastern Asia (Ahmad, 1965; Roonwal, 1970) was used for this study. In the present study, we aimed to purify and characterize the biochemical properties of cellulase enzyme extracted from *Macrotermes gilvus* in order to understand more catalytic function of cellulase enzyme and use as the local enzyme source for further agricultural and industrial applications, as well as for development of cellulase-specific inhibitor to eliminate and kill the termite.

MATERIALS AND METHODS

Materials: The Q Sepharose Fast Flow resin was purchased from GE healthcare, Uppsala, Sweden. Quick Start™ Bradford Protein Assay was purchased from Bio-rad, CA, USA. Sodium carboxymethyl cellulose was procured from Gentaur, CA, USA. Unstained protein molecular weight marker was purchased from Thermo Fisher Scientific, MA, USA.

Sample collection and preparation: *Macrotermes gilvus* termites including worker and soldier termites (253 g) were taken directly from their nest at the agricultural farm, Faculty of Agriculture, Khon Kaen University, Khon Kaen province, Thailand. The sample was cleaned with distilled water and then grinded on ice with mortar and pestle. Proteins were extracted with ice-cold

0.05 M sodium acetate buffer, pH 5.2 with the wet weight per buffer volume of 1:2 ratio. Centrifugation at 36,000 g for 30 min was performed to separate protein sample from cell debris. Protein concentration was determined based on Bradford method (Bradford, 1976) by using Quick Start™ Bradford Protein Assay supplied with bovine serum albumin as the standard. The absorbance at 595 nm was monitored after 7 min of sample and dye incubation.

Protein precipitation with ammonium sulfate: Initially, protein sample was precipitated with 20% saturation of ammonium sulfate. The precipitated proteins were separated by using centrifugation at 36,000 g for 30 min, resuspended with 0.1 M sodium acetate buffer, pH 5.2 followed by dialysis in ten sample volumes of the same buffer for removal of the remaining salt. The non-precipitated proteins in supernatant fraction were subsequently precipitated with 40 and 60% saturation, respectively. The precipitate was recovered and dialyzed as described above. All fractions of precipitated and non-precipitated proteins were subjected to cellulase activity assay and enzyme zymography. Fractions containing cellulase were pooled and used for purification with column chromatography.

Purification of cellulase using anion-exchange column: The purification of *M. gilvus* cellulase was achieved by using Q Sepharose Fast Flow column. All purification steps were done at 4°C. The pooled protein sample was dialyzed against twenty sample volumes of equilibration buffer (0.1 M Tris-HCl, pH 8.0 containing 0.02 M NaCl and 5% glycerol) and then loaded onto the column, which pre-equilibrated with ten column volumes of equilibration buffer. Bound proteins were eluted with stepwise gradient of 0.2-1.0 M NaCl. Eluted fractions were aliquoted and subjected to cellulase activity assay. The active fractions were pooled and dialyzed against ten sample volumes of equilibration buffer and loaded onto the Q Sepharose column again. At the second purification, the proteins bound to the column were eluted with a stepwise gradient of 0.05-0.2 M NaCl. The fractions showed cellulase activity were verified with native-PAGE and zymographic analysis. The purity of enzyme was analyzed by SDS-PAGE. The cellulolytic activity toward sodium carboxymethyl cellulose of native form and refolded state of the enzyme were monitored by native-PAGE and SDS-PAGE coupled with zymogram analysis.

Cellulase activity assay: The activity of cellulase was determined by using DNS (3, 5-dinitrosalicylic acid) method (Miller, 1959) with some modification. The reaction tested for cellulase activity composed of 400 μL of 0.1 M sodium acetate buffer, pH 5.2, 500 μL of 0.5% sodium carboxymethyl cellulose and 100 μL of enzyme samples. The mixture was incubated at 45°C for 20 min and 500 μL of DNS solution was added to stop reaction and in order to determine the amount of the produced glucose. The reaction was boiled for 10 min prior to measure the absorbance at 540 nm. Two control reactions were also set. The first control contained substrate and buffer without enzyme solution and used as a blank. The second one contained all components as described for assayed reaction, but the DNS solution was added to substrate and buffer solution before adding enzyme solution. The resulting absorbance of the second control was subtracted from those of the tested samples. The amount of produced glucose was calculated from the glucose standard curve. One unit of cellulase activity was defined as the amount of enzyme that could hydrolyze sodium carboxymethyl cellulose and release 1 mmol of glucose per minute. The specific activity was presented in mmol glucose min^{-1} mg^{-1} protein.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): The SDS-PAGE was performed according to Laemmli method (Laemmli, 1970) with some modification by using 5 and 12% stacking and separating gels, respectively. The protein samples were mixed with sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 0.01% bromophenol blue, 2% SDS, 10% b-mercaptoethanol and then heated for 3 min before loading to the gel. The electrophoresis was carried out in running buffer (0.25 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3) and the gel was then stained by a solution of 0.15% Coomassie Brilliant Blue (CBB) R-250 in 50% ethanol and 10% glacial acetic acid. The gel was destained with destaining solution containing 40% ethanol and 10% glacial acetic acid.

SDS-PAGE and zymogram analysis: The protein samples were mixed with the sample buffer without b-mercaptoethanol or any reducing agent. The sample without heating was subsequently loaded on 5% stacking gel. Sodium carboxymethyl cellulose with a final concentration of 0.1% (w/v) was incorporated into 12% separating gel during gel preparation. After electrophoresis, SDS gel was soaked in 0.1 M sodium acetate buffer, pH 5.2 containing 0.1% Triton X-100 for 30 min to remove SDS from the gel. To allow the proteins to renature, the gel was incubated in the same buffer without Triton X-100 for 1 h. The gel was then stained in 0.2% Congo red for 1 h and destained with 2 M NaCl until the clear zone against red background was observed. Another duplicated SDS gel containing the same loading pattern was stained with Coomassie brilliant blue R-250 and destained with the destaining solution. The positions of the cellulase enzyme on the both SDS gels were determined.

Native-PAGE and zymogram analysis: For the native-PAGE, the protein samples were mixed with native sample buffer containing the same compositions as described for those of SDS-PAGE except no SDS and reducing agent were presented. The samples without heating were loaded onto 4% stacking gel and 8% separating gel containing 0.1% (w/v) sodium carboxymethyl cellulose. The electrophoresis was performed at 4°C. The native gel was then soaked in 0.1 M sodium acetate buffer, pH 5.2 for 1 h prior to staining with 0.2% Congo red. Gel was destained with 2 M NaCl until the clear zone was observed. Another duplicated native gel was stained with Coomassie brilliant blue R-250 and destained as described previously. The positions of the cellulase enzyme on the both native polyacrylamide gels were monitored.

Determination of optimum pH and optimum temperature for cellulase activity: To determine the optimum pH for *M. gilvus* cellulase, 100 μL of purified enzyme was added to the reaction mixture, which composed of 500 μL of 0.5% sodium carboxymethyl cellulose and 400 μL of buffers. The investigated buffers included 0.1 M of sodium acetate buffer, pH 4.0-5.2, sodium phosphate buffer, pH 6.0-7.5, Tris-HCl buffer, pH 8.0, glycine-NaOH buffer, pH 9.0-10.0. The reactions were incubated at 45°C for 20 min. Five hundred microlitters of DNS solution was added and then boiled for 10 min prior to measure absorbance at 540 nm. Glucose produced from activity of cellulase was calculated from glucose standard curve.

Determination of optimum temperature for *M. gilvus* cellulase was examined by incubating the purified enzyme and sodium carboxymethyl cellulose in 0.1 M sodium acetate buffer, pH 5.2 at different temperatures ranging from 7-70°C. The amount of glucose released from sodium carboxymethyl cellulose during the reaction was measured by DNS method as described earlier. All assays were duplicated.

Effects of temperature and pH on activity and stability of enzyme: The effects of temperatures on enzyme activity were evaluated at 37 and 45°C. The purified *M. gilvus* cellulase was incubated at both temperatures for 300 min. During incubation, 100 μL of enzyme was aliquoted and determined for the amount of glucose produced from the enzyme reaction by using cellulase activity assay. The residual activities at different times of incubation were calculated.

The effects of pH on enzyme activity were determined at pH 5.2, 7.0 and 10.0. The purified cellulase was incubated in the tested buffers at room temperature (30°C) for 300 min. The remaining or residual activity of *M. gilvus* cellulase was determined by monitoring the hydrolysis of sodium carboxymethyl cellulose at 45°C for 20 min and the produced glucose was quantitated by DNS method. The activity of the enzyme before incubation was considered as 100%. The residual or remaining activities were calculated in the percentage for the different times of incubation.

Effect of chemical reagent and metal ions on enzyme activity: Metal ions ($CaCl_2$, $MgCl_2$, $CuSO_4$ and $FeSO_4$) and chelating agent (EDTA) were included in this experiment. The reactions containing 500 μL of 0.5% sodium carboxymethyl cellulose, 430 μL of 0.1 M sodium acetate buffer, pH 5.2 and 50 μL of purified cellulase were incubated at 45°C for 10 min in the presence of 5 mM of EDTA or metal ions. The amount of glucose produced from the reaction was determined as described above and the residual activity was then analyzed. The activity assay in the absence of any chemical reagent or metal ions were recorded as 100%.

RESULTS AND DISCUSSION

Purification of *Macrotermes gilvus* cellulase: Ammonium sulfate precipitation was initially used to partially separate some proteins from cellulase enzyme. The result indicated that the cellulase enzyme was precipitated with 20-60% saturation, which verified by enzyme activity assay and zymogram analysis. Purification with the first Q Sepharose column displayed the cellulase enzyme in flow-through fractions (fraction No. 1-18) and fractions eluted with 0.2 M NaCl (No. 20-35) corresponding to the zymogram analysis which showed clear bands revealing cellulolytic activity in gel (Fig. 1a). Whereas fractions no. 47-49 exhibited very low enzyme activity and were not seen by zymogram. The second Q Sepharose column displayed two activity peaks eluted with 0.05 and 0.1 M NaCl. However, the fractions eluted with 0.1 M NaCl did not show cellulolytic activity on the zymographic gel (Fig. 1b). The cellulase was finally purified 22-fold with a recovery of 1.38% and a specific activity of 105.93 U mg^{-1} protein as summarized in Table 1. The specific activity of *M. gilvus* cellulase was higher than those of cellulases produced from *Salinivibrio* sp., *Clostridium thermocellum* and *Reticulitermes speratus* (Wang *et al*., 2009; Ng and Zeikus, 1981; Watanabe *et al*., 1997). The SDS-PAGE revealed a few protein bands after purification (Fig. 2).

Isoform determination and molecular weight estimation using native PAGE and SDS-PAGE: Figure 3 showed native gels stained with Coomassie Brilliant Blue (Fig. 3a) and Congo red (Fig. 3b). The zymogram in Fig. 3b exhibited a single significant activity band of cellulase suggesting that the enzyme was able to hydrolyze the sodium carboxymethyl cellulose and had only one isoform. The denatured cellulase separated by using SDS-PAGE revealed the cellulolytic activity with the molecular weight around 54 kDa (Fig. 4b) suggesting that the enzyme was properly refolded after SDS-gel electrophoresis and the following incubation step. The molecular weight of the enzyme estimated from SDS-PAGE was similar to the molecular weight of the

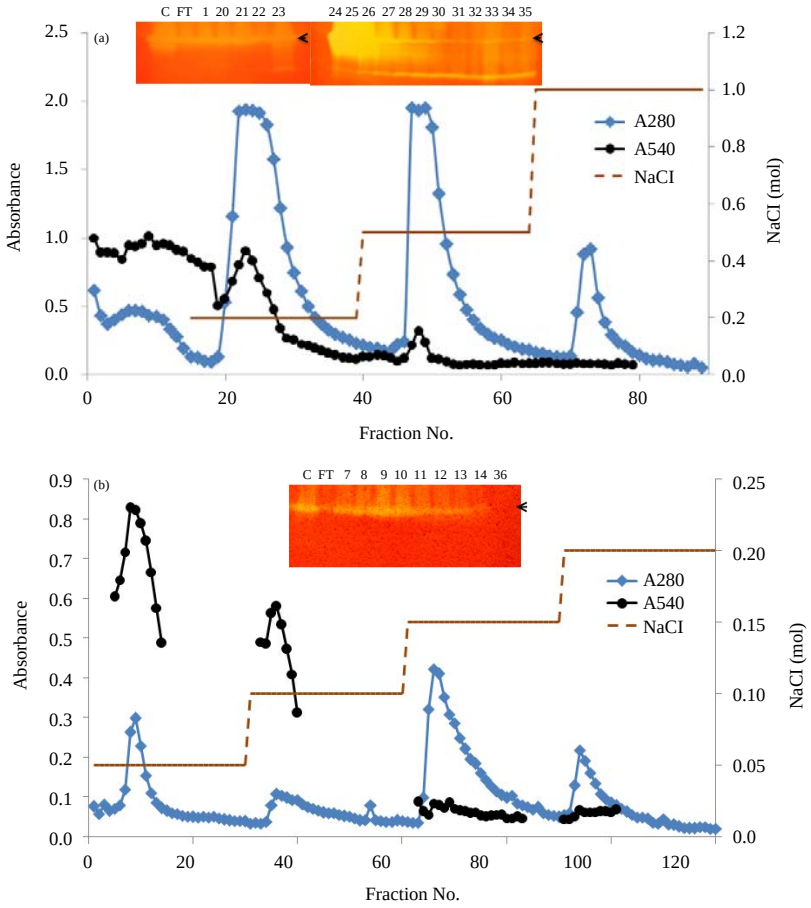

Fig. 1(a-b): Purification profiles of cellulase from first and second Q-Sepharose columns. At the first column (a), the proteins were eluted with stepwise gradient of 0.2-1 M NaCl. The cellulase activity was determined in all fractions and the fractions showed enzyme activity were also verified by zymography. The fractions containing cellulase were pooled and next purified by second column. At the second column (b), proteins bound to the column were eluted with stepwise gradient of 0.05-0.2 M NaCl. The resulting fractions were subjected to cellulase activity assay and zymogram analysis. The protein content in each fraction was determined by measuring the absorbance at 280 nm as indicated in the blue ( ) and the cellulase activity measured at 540 nm were presented as a dark line ( ). The NaCl concentration was represented as a dash line in brown color ( ). For zymogram analysis, the clear zones under red background indicate the presence of cellulase enzyme. Arrows indicate the position of cellulase enzyme

Table 1: Purification table of *Macrotermes gilvus* cellulase

Steps	Total protein (mg)	Total activity (unit)	Specific activity (unit mg^{-1} protein)	Yield (%)	Fold
Crude enzyme extract	786.60	3687.52	4.69	100.00	1.00
Q-Sepharose	0.48	50.72	105.93	1.38	22.60

cellulases produced from *Geobacillus* sp. T1 and *Bacillus amyoliquefaciens* DL-3 (Assareh *et al*., 2012; Lee *et al*., 2008), but different from the molecular weights of enzymes produced from some

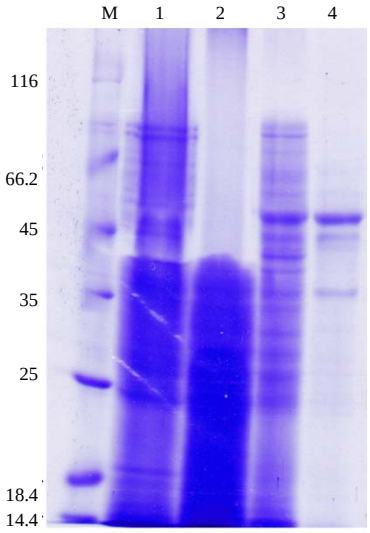

Fig. 2: SDS-PAGE revealing the protein patterns of enzyme samples. Lane M: Unstained protein molecular weight marker, Lane 1: Crude enzyme sample, Lane 2: Proteins precipitated at 20-60% saturation of ammonium sulfate, Lanes 3 and 4: Purified enzymes after first and second Q Sepharose columns, respectively

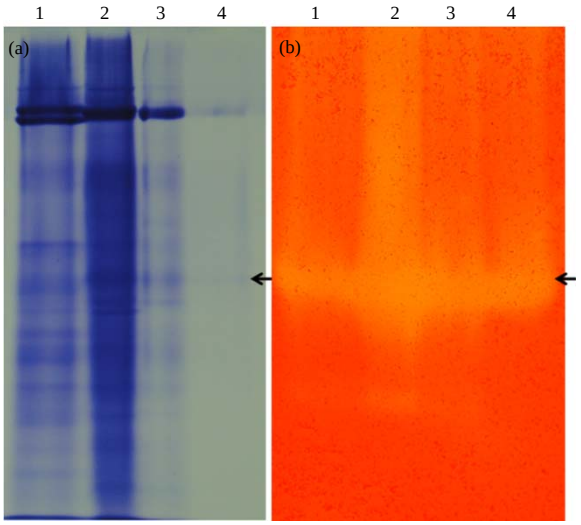

Fig. 3(a-b): Native PAGE and zymogram analysis of cellulase enzyme from *M. gilvus*. (a) Stained gel with Coomassie brilliant blue R-250 and (b) in-gel activity staining or zymography. Lane 1: Crude sample, Lane 2: Proteins precipitated at 20-60% saturation of ammonium sulfate, Lanes 3 and 4: Purified enzyme from first and second Q Sepharose columns. Black arrows indicate the position of cellulase enzyme

bacteria, yeast, beetle and termites, which are in the range of 29-52 kDa (Inoue *et al*., 2005; Harshvardhan *et al*., 2013; Mawadza *et al*., 2000; Annamalai *et al*., 2013; Wang *et al*., 2009; Thongekkaew *et al*., 2008; Lee *et al*., 2004; Tokuda *et al*., 1997; Rouland *et al*., 1988).

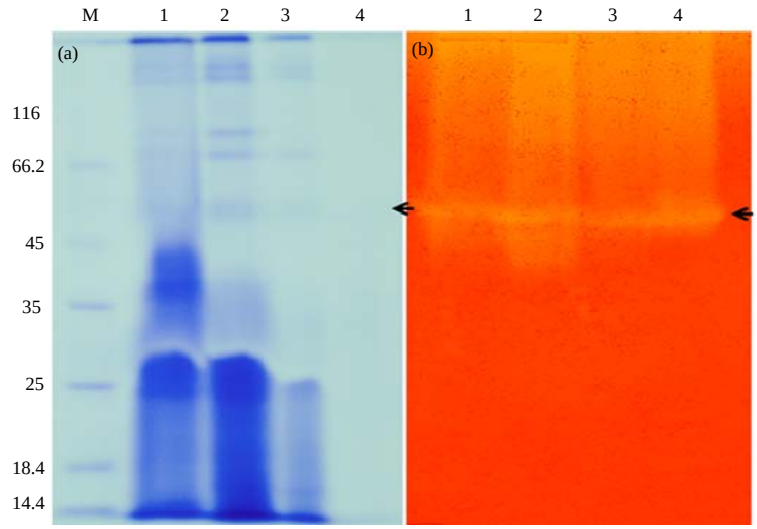

Fig. 4(a-b): SDS-PAGE and zymogram analysis of cellulase enzyme from *M. gilvus* (a) Stained gel with Coomassie brilliant blue R-250 and (b) In-gel activity staining or zymography. Lane M: Unstained protein molecular weight marker, Lane 1: Crude sample, Lane 2: Proteins precipitated at 20-60% saturation of ammonium sulfate, Lanes 3 and 4: Purified enzymes from first and second Q Sepharose columns. Black arrows indicate the position of cellulase enzyme. Sizes of standard proteins are shown on the left

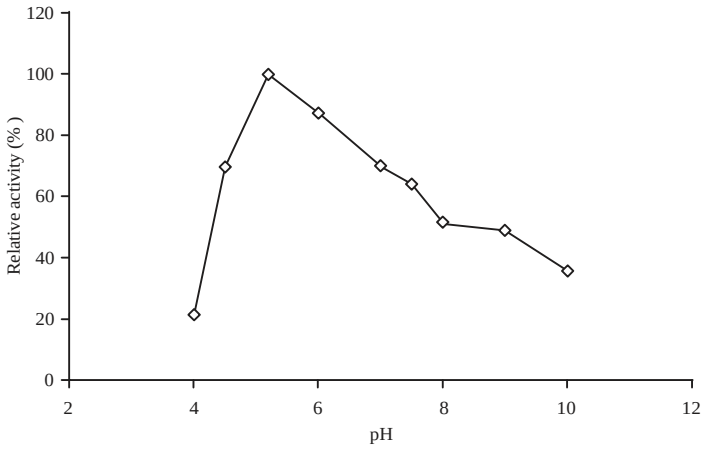

Fig. 5: Optimum pH profile of cellulase enzyme from *M. gilvus*

Optimum condition for cellulase activity: As shown in Fig. 5, the cellulase enzyme had a maximum activity at pH 5.2 in the sodium acetate buffer and the enzyme was also active over a wide range of pH. More than 50% of its maximum activity was observed from pH 4.5 to 8. The optimum pH for *M. gilvus* cellulase was similar to those of *Fervidobacterium nodosum* (Wang *et al*., 2010), *Catharanthus roseus* (Smriti and Sanwal, 1999), *Coptotermes formasanus*, (Zhang *et al*., 2011) and *Nasutitermes takasagoensis* (Tokuda *et al*., 1997), whose optimum pH values are between 5.0 and 5.8. In contrast, the optimum pH of those enzymes from *Geobacillus* sp. T1 (Assareh *et al*.,

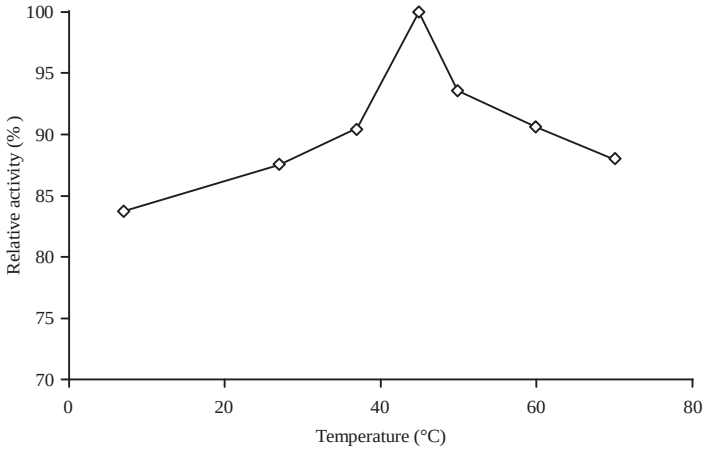

Fig. 6: Optimum temperature profile of cellulase enzyme from *M. gilvus*

2012), *Bacillus amyoliquefaciens* DL-3 (Lee *et al*., 2008), *Bacillus* sp.H1666 (Harshvardhan *et al*., 2013) and *Bacillus halodurans* CAS 1 (Annamalai *et al*., 2013) are in the range of 6.5 to 9. Cellulase sensitive to acidic condition has also been reported for endoglucanase from *Bacillus* sp. HR68. The enzyme is completely inactive at pH 4, while the CH43 enzyme displays over 40% activity (Mawadza *et al*., 2000). Based on that pH, the *M. gilvus* cellulase revealed about 20% of enzyme activity.

For optimum temperature, the *M. gilvus* cellulase revealed the highest activity at 45°C which is similar to the data reported for *Coptotermes formasanus* cellulase expressed in *E. coli* (Zhang *et al*., 2011). Interestingly, the *M. gilvus* cellulase was able to catalyze the reaction with a wide range of temperature. More than 84% of its maximum activity was detected throughout the tested temperatures (7-70°C) as seen in Fig. 6. Based on this property, the enzyme may be applied for industrial process. For the previous studies, cellulase enzymes from many organisms revealed different optimum temperatures. The optimum temperatures of endoglucanases from *Bacillus* sp. CH43 and HR68 were 70 and 65°C, which are quite high compared to other cellulase enzymes. Nevertheless, the activities of those enzymes are dramatically declined to 40 and 0% at 75 and 80°C, respectively (Mawadza *et al*., 2000). In *Bacillus* sp. H1666 and *Bacillus subtilis*, cellulase activities are very sensitive to the temperature. Their activities immediately decrease when the temperature increased over 50°C (Harshvardhan *et al*., 2013; Li *et al*., 2008).

Effects of pH and temperature on enzyme stability: The enzyme exhibiting high activity and revealing pH and thermal stabilities is required for biotechnological and industrial applications. The stability of *M. gilvus* cellulase was studied at all acidic, neutral and basic conditions in order to understand more catalytic function of the enzyme. As shown in Fig. 7, the initial enzyme activity at pH 5.2 was higher than those at neutral (pH 7.0) and basic (pH 10.0) conditions. After 1-3 h of incubation time, the enzyme at pH 7.0 was more stable than those at pH 5.2 and 10.0. Only 13% of the original enzyme activity was loose at pH 7.0, while 25 and 38% of those at pH 5.2 and 10.0 were disappeared. Nevertheless, the enzyme activities at all pH tended to decline and showed similar stability at the end of incubation (5 h). About 40, 37 and 39% of the initial enzyme activities were retained at pH 5.2, 7.0 and 10.0, respectively. This result indicated that the enzyme was stable at a wide range of pH and the pH did not influence the enzyme stability after prolonged incubation.

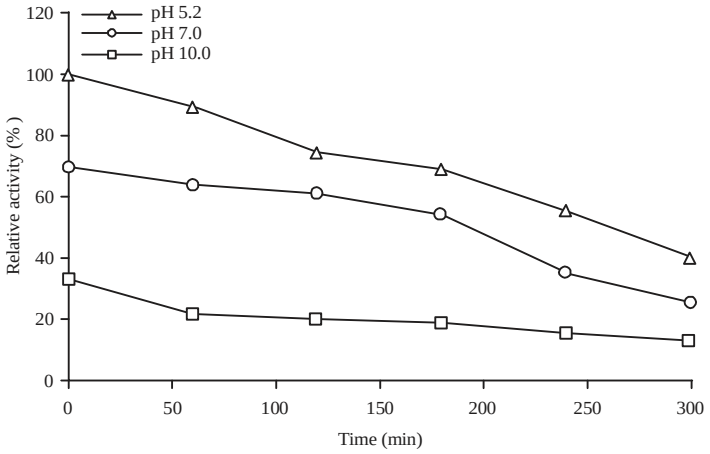

Fig. 7: Effect of pH on the cellulase activity of *M. gilvus*. The 100% enzyme activity was the maximum activity in 0.1 M sodium acetate buffer, pH 5.2

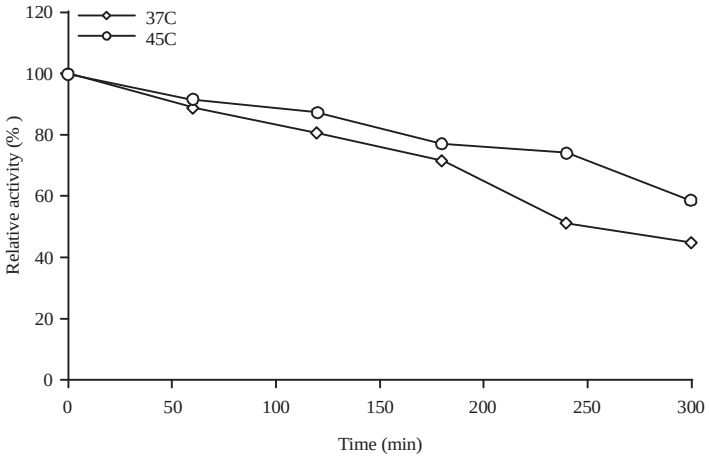

Fig. 8: Effect of temperature on cellulase activity of *M. gilvus*. The activity of the enzyme before incubation was considered as 100%

The thermal stability was examined by incubating the purified cellulase at 37 and 45°C for 5 h and the residual activity was assayed under standard condition at 45°C for 20 min in 0.1 M sodium acetate buffer, pH 5.2. As indicated in Fig. 8, the stabilities of the enzymes at both temperatures were not significantly different over the first three hour of incubation and their activities were remained more than 70%. Nevertheless, the enzyme activity at 37°C was rapidly dropped and showed 45% of remaining activity at 5 h of incubation, whereas the enzyme at 45°C displayed more stability with 59% remaining at indicated time. For the stability assay, the enzyme was present in the same buffer at pH 5.2, therefore increasing the temperature from 37-45°C increases the pH stability of the *M. gilvus* cellulase. The thermal stability of *Bacillus amyloliquefaciens* DL-3 cellulase has also been reported. The enzyme maintained its activity about 60% at the temperature ranging from 40-80°C (Lee *et al*., 2008).

Table 2: Effects of metal ions and chemical reagent on cellulase activity

Metal ions/chemical reagent	Relative activity (%)
No	100
EDTA	82.09±2.49
$CaCl_2$	105.36±0.29
$MgCl_2$	105.29±0.58
$CuSO_4$	18.72±0.38
$FeSO_4$	97.69±0.77

Effects of metal ions and chemical reagent on cellulase activity: The effects of 5 mM of metal ions and chemical reagent on enzyme activity were determined by incubating the individual ions or reagent with enzyme reaction. The results showed that the enzyme activity was strongly inhibited by Cu^{2+} and was moderate affected by EDTA, whereas Fe^{2+} slightly influenced the enzyme activity. Therefore, the rate of inhibition of the *M. gilvus* cellulase activity was in the order of Cu^{2+}>EDTA>Fe^{2+}. The inhibition by the same divalent cations and EDTA was also reported in cellulase enzymes from *Catharanthus roseus* (Smriti and Sanwal, 1999), *Bacillus amyoliquefaciens* DL-3 (Lee *et al*., 2008) and *Bacillus flexus* (Trivedi *et al*., 2011). On the other hand, the enzyme activity was increased for 5% in the presence of Ca^{2+} and Mg^{2+} ions. The effects of Ca^{2+} and Mg^{2+} ions on *M. gilvus* cellulase activities were identical to the effects of those ions on cellulase generated from *Bacillus subtilis* YJ1 (Yin *et al*., 2010b) and also similar to those of haloalkaline cellulase produced from *Bacillus halodurans* CAS 1 (Annamalai *et al*., 2013). The presence of Ca^{2+} in *Clostridium thermocellum* endoglucanase D has been reported to stabilize the enzyme against thermal denaturation and increase substrate binding affinity (Chauvaux *et al*., 1990). The strong inhibitory effect of Cu^{2+} on cellulase activity might due to the binding of ion to the thiol groups of amino acids in the active site of enzyme results in the decrease of enzyme activity (Lin *et al*., 2012; Yin *et al*., 2010a; Lucas *et al*., 2001) (Table 2).

CONCLUSION

This study conclude that the enzyme retained nearly 40% of its original activity after incubation in mild acidic (pH 5.2), neutral (pH 7.0) and basic (pH 10.0) conditions for 5 h. The enzyme retained its activity more than 70% of initial activity at both 37 and 45°C after incubation for 3 h. Moreover, the activity of the enzyme was strongly inhibited by Cu^{2+} and slightly affected by Fe^{2+} and EDTA, whereas the presence of Ca^{2+} and Mg^{2+} slightly increased the enzyme activity. Due to the wide temperature and pH range of enzyme activity, the *Macrotermes gilvus* cellulase might be potential enzyme for industrial or agricultural application.

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