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Thermostable Xylanase from *Marasmius* sp.: Purification and Characterization

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We have screened 766 strains of fungi from the BIOTEC Culture Collection (BCC) for xylanases working in extreme pH and/or high temperature conditions, the so-called extreme xylanases. From a total number of 32 strains producing extreme xylanases, the strain BCC7928, identified by using the internal transcribed spacer (ITS) sequence of rRNA to be a *Marasmius* sp., was chosen for further characterization because of its high xylanolytic activity at temperature as high as 90ºC. The crude enzyme possessed high thermostability and pH stability. Purification of this xylanase was carried out using an anion exchanger followed by hydrophobic interaction chromatography, yielding the enzyme with >90% homogeneity. The molecular mass of the enzyme was approximately 40 kDa. The purified enzyme retained broad working pH range of 4-8 and optimal temperature of 90ºC. When using xylan from birchwood as substrate, it exhibits K_m and V_{max} values of 2.6 ± 0.6 mg/ml and 428 ± 26 U/mg, respectively. The enzyme rapidly hydrolysed xylans from birchwood, beechwood, and exhibited lower activity on xylan from wheatbran, or celluloses from carboxymethylcellulose and Avicel. The purified enzyme was highly stable at temperature ranges from 50 to 70ºC. It retained 84% of its maximal activity after incubation in standard buffer containing 1% xylan substrate at 70ºC for 3 h. This thermostable xylanase should therefore be useful for several industrial applications, such as agricultural, food and biofuel.

Keywords: Carboxymethyl cellulose, Cellulase, Thermostability, Xylanase, Xylanolytic activity,

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Introduction

Xylans are hemicellulose compounds and are the second most abundant natural polysaccharide behind cellulose (Collins *et al*., 2005). These compounds are present in the cell wall and in the middle lamella of plant cells. Xylanolytic enzymes are a group of enzymes that are involved in the hydrolysis of xylans and arabinoxylan polymers. These enzymes include endo-1,4-β-xylanase, β-xylosidase, a-arabinofuranosidase and acetylxylan esterase (Biely, 1993). Xylanases hydrolyze 1,4-β-D-xylosidic linkages in xylan to produce xylo-oligosaccharide. In natural environment, xylanases are produced mainly by microorganisms, marine algae, protozoans, crustaceans, insects and snails. Among microbial sources, filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase activities are much higher than those found in yeasts and bacteria (Sunna and Antranikian, 1997; Krisana, *et al*., 2005). This property makes fungal xylanases attractive to be used in various industrial applications. For instance, in pulp and paper industry, the xylanases are employed in the prebleaching process to reduce the use of the toxic chlorine chemicals (Bajpai, 1999). In animal feed industry, xylanases are used to increase the body weight gains of the animals (Silversides and Bedford, 1999; Kung *et al*., 2000). In bread and bakery industry, xylanases are used to increase the dough viscosity, bread volume and shelf life (Romanowska *et al*., 2003).

Since Thailand lies near the equator, almost all year round the country experiences hot and humid climate, which are living conditions preferred by most fungi. Consequently, thousands of fungi can grow well and can be found within the border. Here at BIOTEC, we have collected and accumulated thousands of locally isolated fungi (http://mycology.biotec.or.th/current_research/diversity/maindiversity.htm). In this study we screened the fungal strain which possessed high xylanolytic activity, high thermostability and functions well over a wide pH range (pH 3-9). Xylanase present in crude culture medium from fungal strain namely BCC7928 was characterized. The

protein was further purified and characterized to determine its biochemical and physical properties.

Materials and Methods

Microorganisms and plasmids. The fungal strains were collected from several locations in Thailand. All of these strains have been deposited at the BIOTEC Culture Collection. The BCC7928 which exhibited high xylanolytic activity and thermostability was cultured in 5% wheat bran medium supplemented with 1.5% (w/v) xylan to accommodate xylanase production. *Escherichia coli* DH5αF' (F'Δ(*lacZYA-argF*)U169 *deoR endA1 hsdR17 supE44 thi-1 recA1 gyr96 relA1* (*ϕ80dlacZ* Δ*M15*) was grown at 37ºC in LB broth (Sambrook and Russell, 2001) or on agar (1.5% w/v) plates. *E. coli* cells harboring a cloning vector, pGEM®-T Easy vector, were cultured in LB medium supplemented with 100 µg/ml ampicillin.

Identification of the fungal strain. The fungal strain BCC7928 was identified by morphological characteristics as well as comparison of internal transcribed spacer (ITS) sequences of rRNA gene (Buchan *et al.*, 2002). Genomic DNA (gDNA) of this fungus was isolated by CTAB and phenol/chloroform method (Velegraki *et al.*, 1999). Partial DNA fragment of the ITS was amplified by polymerase chain reaction (PCR) using gDNA as template. The ITS-F primer (5'-GCGGAAGGATCATTACTGAG-3') and ITS-R primer (5'-GGGTATCCCTACCTGATCCG-3') were designed from the conserved sequences of ITS among fungi (Yeates *et al.*, 1998). The PCR product, 600 bp in length, was cloned into pGEM®-T Easy vector and the ITS sequences, determined by the dideoxy-nucleotide chain termination method (Sambrook and Russell, 2001), were analyzed.

Gel electrophoresis and zymogram. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% polyacrylamide gels essentially as described by Laemmli (1970). Protein concentration was determined by the Bradford method with bovine serum albumin as a standard (Bradford, 1976).

For detection of xylanase and cellulase activity, either 0.2% (w/v) birchwood xylan or 0.2% (w/v) carboxymethyl cellulose (CMC) was added in the gel. The samples applied to the gel were not boiled and SDS was removed after electrophoresis by gentle shaking of the gel in 20% (v/v) isopropanol at 4ºC for 1 h followed by rinsing 3 times for 10 min each with distilled water. Subsequently, the gel was incubated in 100 mM acetate buffer pH 6 at 60ºC for 1 h to allow the enzyme to digest either xylan or cellulose substrate. The gel was stained with 0.1% (w/v) Congo red for 30 min and washed with 1 M NaCl for 10 min. The gel was then immersed in 0.5% (w/v) acetic acid to stop the reaction.

Purification of endo-1,4-β-xylanase. The BCC7928 strain was cultured for 6 days in 5% (w/v) wheat bran broth in the presence of 1.5% xylan to induce xylanase expression. The culture supernatant containing secreted xylanase, was concentrated in a Minitan system with polysulfone membranes (with 10 kDa cut-off membrane; Millipore). The sample was then purified by using two consecutive columns. First, the unpurified sample (10 ml) was applied to a fast-performance liquid chromatography (FPLC) system equipped with an anionic exchange column (HiTrap™ DEAE Fast Flow), which had been equilibrated with 50 mM MOPS pH 7.0. The protein was eluted with an NaCl gradient from 150-200 mM with flow rate of 1 ml/min. Second, fractions of eluates from the first column eluted protein, containing xylanolytic activity, were then pooled together and applied to a HiTrap™ Phenyl Sepharose Fast Flow column that had been equilibrated with 50 mM MOPS pH 7.0 containing 1 M $(NH_4)_2SO_4$. Then the protein was eluted with gradient from 560 to 170 mM $(NH_4)_2SO_4$ and 50 mM MOPS buffer, pH 7 at a flow rate of 1 ml/min. The fractions containing xylanase activity were then pooled and used for further biochemical characterization.

Quantitative assay for xylanolytic activity. The xylanolytic activity was quantitatively determined by 3,5-dinitrosalicylic acid (DNS) method. The assay was based on enzymatic hydrolysis of birchwood xylan and the reaction of the liberated reducing sugar with DNS (Bailey *et al.*, 1992). Xylose was used as the reference reducing sugar. One unit of xylanase activity was defined as the amount of enzyme that liberated reducing sugar at the rate of 1 µmole/min. The activity was determined carried out at 90ºC for 10 min under otherwise standard conditions. Xylanase activities at different pHs and temperatures were performed with crude and purified enzyme. To determine the optimal pH, pH range from 3 to 10 was used with the following (100 mM) buffers: sodium citrate (pH 3 to 5), acetic acid (pH 5 to 6) sodium phosphate (pH 6 to 8), Tris-HCl (pH 8 to 10) (Ruiz-Arribas *et al.*, 1995). Optimal temperature was determined in the range of 30 to 100ºC in 100 mM acetate buffer (pH 6). For determination of thermal stability, the unpurified or purified enzyme was incubated in acetate buffer (pH 6) with or without 1% xylan for various times at 50, 60 70 and 80ºC. Aliquots were withdrawn at timed intervals and cooled on ice before assaying to determine the residual enzyme activity, using the normal assay procedure. Reducing sugars produced during pre-incubation in the presence of substrate was calculated and specified as base line activity.

For determination of pH stability, the unpurified or purified enzyme was incubated at 40ºC for 4 h in the corresponding buffer and level of activity was determined by using the DNS method.

Enzyme kinetics. Kinetic parameters of the enzyme were determined by measuring the enzymatic activity toward birchwood xylan at various concentrations (2.5 to 20 mg/ml in 100 mM acetate buffer pH 6) (Rogalski *et al.*, 2001). The activity determination of the enzyme was carried out at 90ºC for 10 min by the DNS method. The kinetic parameters were then determined by fitting the initial velocity data to the Michaelis-Menten equation using the Kaleida Graph software (Synergy Software, Reading).

Substrate specificity. The specificity of the purified proteins were determined on the following substrates: 1% birchwood xylan (w/v), 1% beechwood xylan (w/v), 5% wheat bran (w/v), 1% CMC (w/v) and 1% Avicel (w/v). The activities were assayed at 90ºC for 10 min. Levels of reducing sugars produced were measured by the DNS method and calculated from standard curves with known concentrations of xylose or glucose depending on the sugar present in the polymer. The activities were determined as specific activity of enzyme for each substrate.

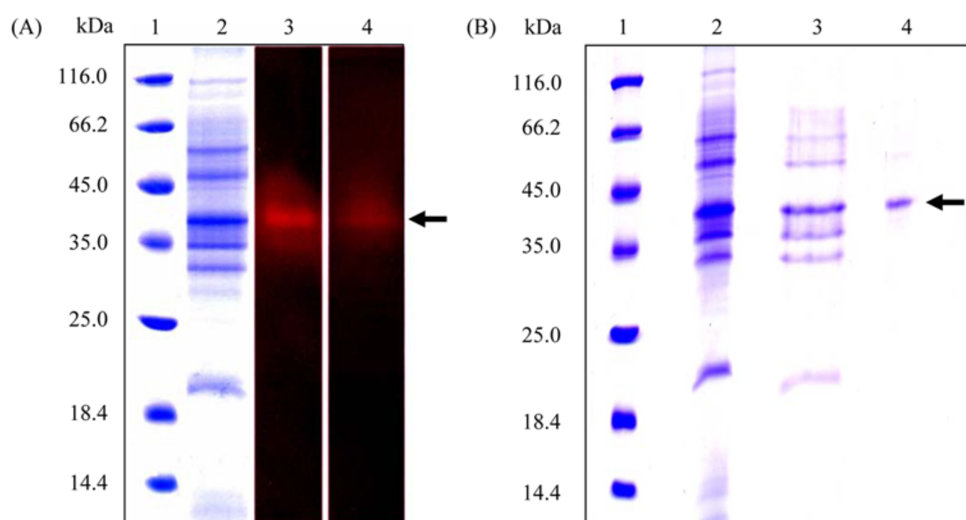

Fig. 1. Electrophoresis analysis of xylanase from *Marasmius* sp. BCC7928 (A) Lane 1, Protein marker; lane 2, Coomassie Blue-stained SDS- polyacrylamide gel of crude culture medium; lane 3, Zymogram analysis of culture medium showing the position of a protein band with xylanolytic activity (arrow); lane 4, Zymogram analysis of crude culture medium showing the position of a protein band with cellulase activity (arrow). (B) SDS-PAGE analysis followed by Coomassie Blue staining of protein components in the unpurified and purified fractions of xylanase from *Marasmius* sp. BCC7928. Lane 1, Protein marker; lane 2, unpurified protein; lane 3, partially purified fraction (eluted fractions from a DEAE Sepharose Fast Flow column); lane 4, purified fraction from a Phenyl Sepharose Fast Flow column. The arrows indicate the position of xylanase from *Marasmius* sp. BCC7928.

Results

Screening and identification of strain producing extreme xylanase. From the screening of the xylanase with the ability of the enzyme to function at broad pH (3-10) range and/or high temperature, the strain BCC7928 exhibited high xylanolytic activity and the highest thermostability. Therefore, this strain was selected for further identification at the molecular level. Its internal transcribed spacer (ITS) sequence of rRNA gene was amplified and analyzed. Alignment of the approximate 600 bp of its ITS showed that it was 95 % identical to ITS sequence of fungus from genus Basidiomycetes; *Marasmius cladophyllus* (AY216475.1). From this result, BCC7928 was identified as *Marasmius* sp.

Crude enzymes show the dual activity of both xylanase and cellulase. The xylanase produced from *Marasmius* sp. BCC7928 was characterized by SDS-PAGE and zymographic analysis. The result in the presence of xylan showed an intense and well-defined xylanase activity at the molecular weight of 40 kDa (Fig. 1). Surprisingly in the SDS-PAGE containing CMC, there was also a protein band showing an intense and well-defined cellulase activity with the molecular weight of 40 kDa, the same position as the band with xylanase activity. This suggested that this enzyme might possess both xylanase and cellulase activity, although the cellulase activity was not as high as the xylanase activity.

Properties of the crude enzyme. The activity of xylanase present in crude culture medium produced by *Marasmius* sp. strain BCC7928 was determined.

First, the effects of pH and temperature on the activity of the xylanase were determined. Optimal conditions for activity were 90ºC and pH 6 (Fig 2A, 2B). The enzyme was highly active in a wide range of pH, showing more than 50% of maximal xylanase activity in the pH range of 3.5 to 9 (Fig. 2B). The enzyme remained highly stable (retained 94% of maximal activity) in acetate buffer (pH 6) at 60ºC and 70ºC for at least 3 h (Fig. 3A). At 80ºC, the enzyme retained approximately 30% of its activity after incubation for 1 h. For pH stability, the test was performed at pH range of 3 to 9. The result showed that at least 80% of activity was maintained from pH 3 to 9 after incubation at 40ºC for 4 h (data not shown).

Properties of the purified xylanase. The xylanase present in the culture medium was purified by FPLC using two consecutive columns as described in materials and methods. The purified xylanase was more than 95% homogeneity with an apparent molecular mass of approximately 40 kDa on SDS-PAGE (Fig. 1B). Optimal temperature and pH for xylanase activity were at 90ºC and pH 6, respectively. These conditions were similar to those of the unpurified enzyme (Fig. 2C, 2D). The purified xylanase was observed to be active over a wide range of pH from 4 to 8 (exhibiting more than 50% of maximal activity) (Fig. 2D). After testing with birchwood xylan, the purified enzyme showed specific activity of 350 ± 32 U/mg protein. When using birchwood xylan at concentrations ranging from 2.5 to 20 mg/ml, the enzymatic reaction was found to follow Michaelis-Menten kinetics with K_m and V_{max} values of 2.6 ± 0.6

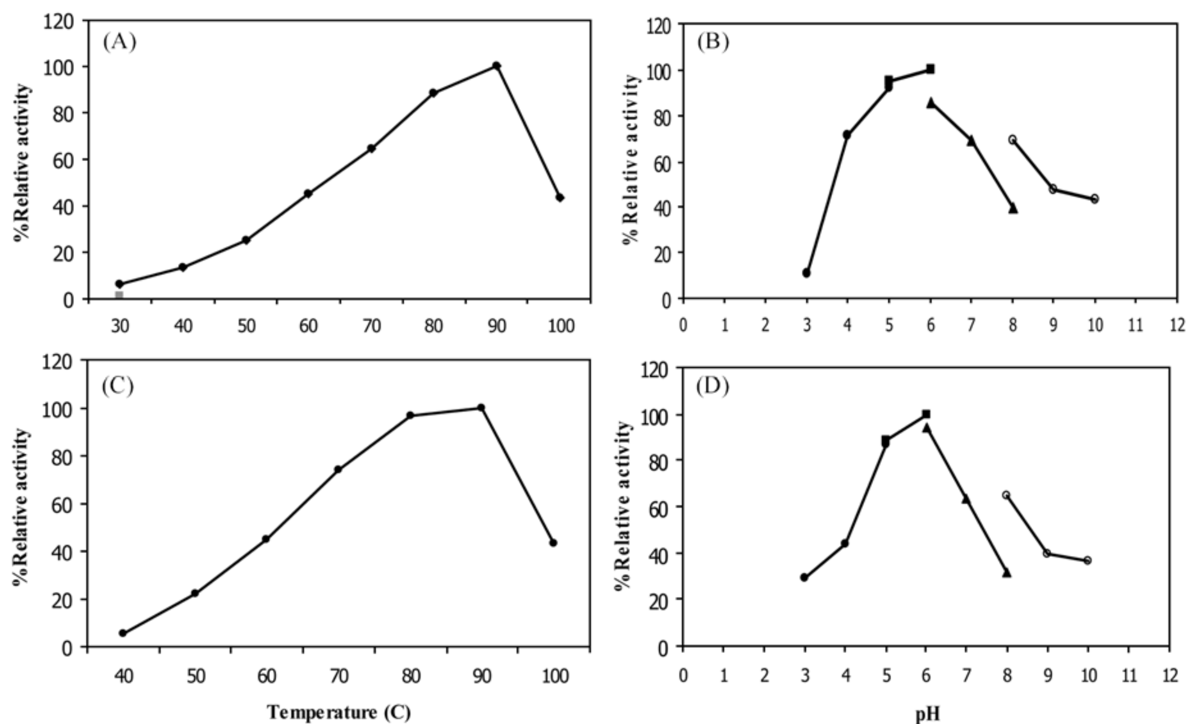

Fig. 2. Activity profiles of the unpurified (A and B) and purified (C and D) xylanase from *Marasmius* sp. BCC7928. Xylanase activities were measured at various temperatures (A and C) or at different pH (B and D) in order to determine optimal temperature and optimal pH for the enzyme. Optimal temperature for activity of the xylanase was determined by carrying out the reaction for 10 min at selected temperatures from 30 to 100ºC: (A), Effects of different temperatures on xylanolytic activity of crude enzyme; (C), Effects of different temperatures on xylanolytic activity of the purified enzyme. The optimal pH was determined by monitoring xylanase activity (90ºC) in the buffer pH range from 3 to 10 with following (100 mM) buffers: sodium citrate (pH 3 to 5) (filled circle), acetatic acid (pH 5 to 6) (filled rectangle), sodium phosphate (pH 6 to 8) (filled triangle) and Tris-HCl (pH 8 to 10) (opened circle). (B); Effect of pH on activity of crude xylanase; (D), effect of pH on activity of purified xylanase. The relative activity is defined as percentage of activity at each temperature or pH compared to a maximal activity.

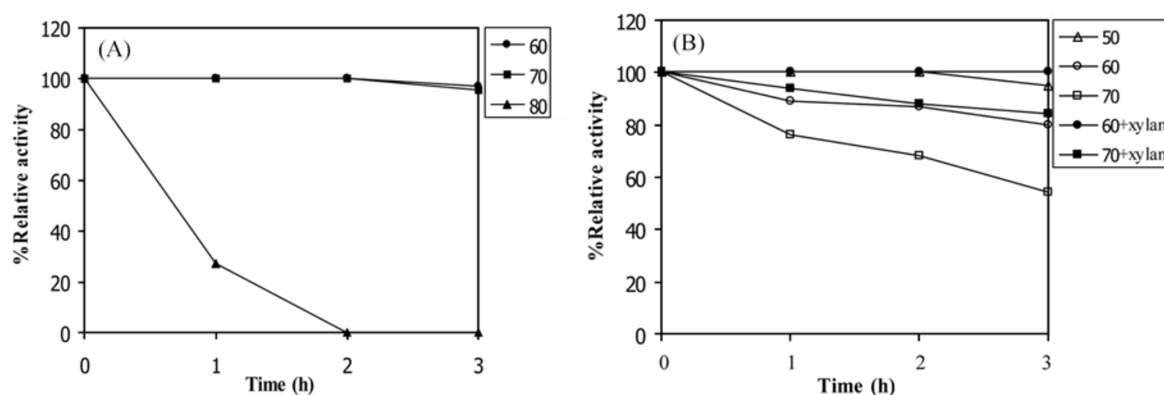

Fig. 3. Thermostability profiles of unpurified and purified xylanase from *Marasmius* sp. BCC7928. For determination of thermal stability, the unpurified or purified enzyme was incubated in acetate buffer (pH 6) with or without 1% xylan for various times at 50, 60 70 and 80ºC. Aliquots were withdrawn at timed intervals and cooled on ice before determining residual enzymatic activity at 90ºC for 10 min by the DNS method. Relative activity was calculated as enzymatic activity at indicated time divided by activity at time zero. (A) Thermostability of unpurified enzyme after incubation at 60 (filled circle), 70 (filled rectangle) and 80 (filled triangle) ºC for indicated times. (B) Thermostability of purified xylanase after incubation at 50, 60 and 70ºC with (filled circle and rectangle) or without (open triangle, circle and rectangle) xylan substrate.

mg/ml and 428 ± 26 U/mg protein, respectively, as determined by Kaleida Graph (n = 3).

Thermostability assays showed that the purified enzyme was highly stable at 50ºC in acetate buffer (pH 6) for at least

Table 1. Activity of purified xylanase on different substrate

Substrate	Activity (U/mg)
Birchwood xylan	350 ± 32
Beechwood xylan	336 ± 22
Wheat bran	66 ± 11
Carboxymethyl cellulose	40 ± 5
Avicel	19 ± 3

The enzyme activities were assayed at 90°C for 10 min and determined the activities as specific activity of the enzyme for each substrate.

3 h (retaining approximately 95% of maximal activity). There was only a slight loss of enzyme activity at 60°C after incubation for 3 h (retaining approximately 80% of maximal activity). The enzyme remained 54% of maximal activity after incubation at 70°C in acetate buffer pH 6 for 3 h (Fig. 3B). However, the enzyme remained 100% and 84% of activity after incubation at 60°C and 70°C, respectively for 3 h in buffer containing xylan substrate (Fig. 3B). Thus, the presence of xylan substrate greatly stabilized the enzyme at high temperature.

For pH stability, the results were similar to those obtained with unpurified enzyme of which at least 80% of enzyme activity remained after incubation at 40°C for 4 h in buffer pH 3 to 9 (data not shown).

The activity of the purified enzyme was tested on several polysaccharides. High hydrolytic activity was displayed toward most xylans obtained from different sources, showing the maximal value toward birchwood xylan followed by beehwood xylan, and the lowest activity was observed on wheat bran polysaccharide. In addition, significant cellulytic activity was detected on carboxy methyl cellulose (CMC) and Avicel (Table 1).

Discussion

Fungi are the most diverse organisms in the world. It is estimated that there are approximately 1.5 million species of fungi (Hawksworth, 2001) and of which approximately 12,000 species have been currently isolated in Thailand (http://mycology.biotec.or.th/Current_Research/Diversity/Main Diversity.htm). This fungi collection is an excellent source of novel natural enzymes. Enzymes with high thermostability and an ability to function at wide pH range are desirable for many industrial applications as many industrial processes take place at very high or low pH and high temperature. Our primary enzyme screening was performed based on those properties desired by industrial processes. The strain BCC7928 was one of the local strains isolated from mushroom, collected from Hala-Bala Wildlife Sanctuary, southern part of Thailand. This strain exhibited high xylanolytic activity and the highest thermostability among those 766 fungal strains that had been screened for extreme xylanases. On the basis of phenotypic characteristics and phylogenetic analysis of ITS data, the BCC7928 strain was identified as *Marasmius* sp.

Currently, thermostable xylanases could be isolated from a number of fungal sources including members of the *Thermoascus aurantiacus* (Kalegoris *et al*., 1998), *Fusarium oxysporum* F3 (Christakopoulos *et al*., 1996), *Penicillium capsulatum* (Ryan *et al*., 2003) and *Trichoderma harzianum* (Tan *et al*., 1985). These strains have been reported to produce xylanases which are active at temperatures between 50 and 80°C. In this study, we obtained xylanase produced by *Marasmius* sp. BCC7928. We found that the unpurified xylanase function well at very high temperature (60 to 90°C) with optimal working temperature of 90°C, which is significantly higher than those xylanases mentioned above. This xylanase had an ability to function at broad pH range (Fig. 2) and is stable over wide range of pH. The optimal conditions and pH stability of the purified enzyme were also similar to crude xylanase. When tested for kinetic properties, it showed similar V_{max} (428 ± 26 U/mg protein) as those xylanases isolated from *Acidobacterium capsulatum* (Inagaki *et al*., 1998) and *Aspergillus fischeri* (Raj and Chandra, 1996). Indicating that this xylanase from *Marasmius* sp. BCC7928 has high potential for being used in a wide range of industrial processes as a result of its high activity and its ability to function in extreme pH or temperature.

Thermostability study was performed and exhibited that the xylanase in crude extract was highly stable when the temperature rose above 60°C (Fig. 3A). These results indicated that there may be some other factors in the crude extract that stabilize the xylanase against thermal shock.

The purified enzyme retained full and about 80% of maximal activity after 3 h incubation at 50°C and 60°C, respectively, in pH 6 buffer (Fig. 3B). Although the purified enzyme lost almost 50% of its maximal activity after incubating at 70°C for 3 h in the absence of xylan, it retained more than 80% of maximal activity after incubating in the presence of substrate. This suggested that xylan substrate could greatly stabilize the enzyme, presumably by protecting conformational change of the enzyme from thermal shock.

Strikingly, xylanase from *Marasmius* sp. BCC7928 possessed endo 1,4 b-D-xylanase and cellulase activity as indicated by activity gel staining and substrate specificity test (Fig. 1, Table1). Most previous reports found that xylanases isolated from various sources were unable to hydrolyse both CMC and Avicel, demonstrating that those enzymes did not possess cellulase activity. However, recent work has identified xylanase KII isolated from *A. niger* (Romanowska *et al*., 2005) that could degrade CMC and Avicel with relative activity of 12.5% and 45 % respectively, compared to xylanase activity. Even though BCC7928 xylanase hydrolyzed both CMC and Avicel with relative activity lower than *A. niger* xylanase KII of 11.5% and 5.6% for CMC and Avicel respectively, compared to xylanase activity, the enzyme still exhibited significant cellulase activity. Thus, this enzyme contains dual specificity toward both xylan and cellulose and

can be effectively used to hydrolyze substrates containing xylan and celluloses at the same time.

Since this enzyme has an ability to hydrolyze both xylan and cellulose and function at high temperature up to 90°C, it can be a great attractive candidate to be utilized in biofuel industry after thermal pretreatment of hemicellulose from corncob, sugarcane bagasse and agricultural wastes to fermentable sugars (e.g., xylose, glucose).

In conclusion, we report the purification and characterization of the thermostable xylanase from fungal strain *Marasmius* sp. BCC7928. Even though various xylanases were obtained from fungal strains, this is the first report of isolation of thermostable xylanase from fungi that belong to basidiomycete family, *Marasmius* sp. In the future, the xylanase gene will be cloned and sequenced in order to study strcucture-function analysis of the enzyme. In addition, over expression of this enzyme in a suitable host will be performed for commercial production of heterologous proteins.

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