Purification and Characterization of Xylanase from Spent Mushroom Compost and its Application in Saccharification of Biomass Wastes

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The activities of xylanase extracted from spent mushroom composts (SMCs) of *Coprinus comatus*, *Auricularia auricular*, *Pleurotus ostreatus*, *Pleurotus citrinopileatus*, *Agrocybe cylindracea*, *Hericium erinaceus*, *Hypsizygus marmoreus*, and *Tremella fuciformis* were investigated. The crude extract from *T. fuciformis* SMC showed high xylanase activity with a value of 255.2 U/mg. Furthermore, this xylanase was purified using a combination of ammonium sulfate precipitation, diethylaminoethyl-cellulose (DEAE-cellulose), and gel filtration column chromatography. The enzyme was purified 20.7-fold with a yield of 43.1% and activity of 5293.8 U/mg. The purified xylanase showed maximum activity at 50 °C and pH 6, retained 80% activity after 1 h incubation at 50 °C, and sustained stability over a wide range of pH values (2 to 10). Under the optimal conditions, the enzyme exhibited a K_m value of 2.5 mg/mL towards birchwood xylan. The activity of xylanase was enhanced in the presence of Mg^{2+}, Ca^{2+}, Ba^{2+}, NH_4^+, and Tween 80, while some metal ions, particularly Fe^{3+}, inhibited its activity. The saccharification of several biomass wastes using the crude xylanase enzyme was studied. The results showed the potential for saccharification of alkaline-pretreated wheat bran solution where 75% saccharification was achieved.

Keywords: Tremella fuciformis; Spent mushroom compost; Xylanase; Enzyme purification; Saccharification

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INTRODUCTION

The mushroom industry has grown at a rapid rate since the late 1990s. Global mushroom production increased more than 25-fold during the last 35 years, from about one million tons in 1978 to 27 million tons in 2012 (Li *et al*. 2012; Zhang *et al*. 2014). China has a long history of mushroom cultivation, and it has become the largest mushroom producer, consumer, and exporter. More than 30 million tons of mushrooms were produced in 2013, which resulted in an accumulation of a large amount of spent mushroom compost (SMC) production. Approximately 1 kg of mushrooms generates 5 kg of SMC (Lau *et al*. 2003; Phan and Sabaratnam 2012). Therefore, the waste management of SMC is a major problem confronted by mushroom producers. The disposal strategies of SMC include burning, spreading on land, burying, composting with animal manure, or landfilling (Singh *et al*. 2003; Phan and Sabaratnam 2012). Recently, some studies used SMC as a raw material to produce value-added products such as biogas, bulk enzymes, and organic fertilizer by bioconversion and as animal feed supplements (Bisaria *et al*. 1990; Soechtig

and Grabbe 1995; Kumaran *et al*. 1997; Lim *et al*. 2013). New applications for SMC utilization would be economical and beneficial for human health and environmental protection.

SMC is mainly composed of fungal mycelia, extracellular enzymes, and relatively decay-resistant components of lignocellulosic substrates. The residual lignocellulosic substrates can be reused by some microorganisms (Wu *et al*. 2014) or hydrolyzed into the fermentable sugars (Balan *et al*. 2008; Wu *et al*. 2013). Extracellular enzymes from SMC include cellulases, xylanases, and ligninases (Isikhuemhen and Mikiashvilli 2009; Mayolo-Deloisa *et al*. 2009; Yang *et al*. 2015), the activities and amounts of which depend on the mushroom species and its cultivation conditions. These extracellular enzymes have shown wide applications in the brewing, baking, leather, textile, and saccharification industries (Xin and He 2013; Goncalves *et al*. 2015; Kocabas *et al*. 2015). The extracellular enzymes extracted from these SMCs have broad potential applications.

In this study, the activities of xylanases extracted from spent mushroom composts (SMCs) of eight mushroom species were determined. The xylanase from *T. fuciformis* SMC showed higher activity than the other seven SMC samples. When the xylanase obtained from SMC of *T. fuciformis* was purified and characterized, the purified enzyme showed potential in saccharification of agro-residues into sugars.

EXPERIMENTAL

Materials

Post-harvest media from bag-cultivations of *Coprinus comatus*, *Auricularia auricular*, *Pleurotus ostreatus*, *P. citrinopileatus*, *Agrocybe cylindracea*, *Hericium erinaceus*, *Hypsizygus marmoreus*, and *Tremella fuciformis* were collected from eight farms (Fujian province, China). The SMC was obtained immediately after harvest of mushrooms and was stored at 4 °C.

Methods

Extraction of extracellular enzymes from SMCs

Approximately 20 g of each sample from the collected eight SMCs was suspended in 100 mL of tap water. The SMC-water mixtures were incubated in a shaker at 150 rpm and 25 °C for 3 h. Each sample was filtered through multi-layer gauze and centrifuged at 10,000 × g at 4 °C for 20 min. The supernatant, which constituted the crude enzyme extract, was assayed for xylanase activity.

Purification of xylanase from the SMC extract of T. fuciformis

The xylanase from the SMC extract of *T. fuciformis* was purified by a four-step process. Approximately 100 mL of the crude enzyme extract was prepared by the above extraction method. Solid ammonium sulfate (24.3 g) was added slowly to the crude extract to roughly 40% saturation. This mixture was incubated in a shaker at 150 rpm and 25 °C for 3 h and centrifuged at 10,000 × g at 4 °C for 20 min. The residual pellet was discarded, and the supernatant was adjusted to a final concentration of 80% saturation by the addition of solid ammonium sulfate. The mixture was again incubated and centrifuged under the same conditions. The precipitated proteins at 80% saturation were redissolved in 25 mL of distilled water and transferred into a dialysis bag at 4 °C overnight. The crude enzyme after dialysis was freeze-dried and re-suspended in 10 mL of 50 mM Tris buffer (pH 8.5). The

re-suspended sample was loaded into a column (2 × 50 cm) of DEAE-cellulose (DE52) pre-equilibrated with 50 mM Tris buffer (pH 8.5). The enzyme was eluted with a linear gradient of zero to 0.5 M NaCl in the same buffer at a flow rate of 0.5 mL/min. The eluted fractions (1 mL/tube) were collected using an automatic collector. The xylanase activity assay was performed for the obtained fractions. The fractions with xylanase activity were then pooled together, assayed, and concentrated by freeze-drying. The pooled enzyme was loaded on a Sephadex G-100 column (2 × 50 cm) pre-equilibrated with five column volumes of 20 mM potassium phosphate buffer (pH 6). The enzyme was eluted with the same buffer at a flow rate of 0.5 mL/min and concentrated by freeze-drying. The obtained enzyme solution was assayed for protein content and enzyme activity.

Enzyme assay

Xylanase activity was determined using birchwood xylan as a substrate (Bailey *et al*. 1992; Xin and He 2013). The reaction mixture containing 0.5 mL of diluted enzyme and 1 mL of birchwood xylan solution (1% w/v in 50 mM potassium phosphate buffer, pH 6) was incubated at 50 °C for 30 min, and then 1.5 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added to stop the reaction. The amount of reducing sugar released from the reaction was determined by the DNS method. One unit of xylanase activity was defined as the amount of enzyme required to release 1 μmol xylose from xylan in 1 min. The protein concentrations of all samples were determined using the Bradford method, and bovine serum albumin served as the standard protein (Lowry *et al*. 1951).

Enzyme characterization

The effect of pH on the activity of pure xylanase was measured in the range of pH 2 to 10 using 20 mM glycine-HCl (pH 2 to 3), sodium acetate (pH 4 to 5), potassium phosphate (pH 6 to 8), and glycine-NaOH buffers (pH 9 to 10) under standard assay conditions. To test its stability, the purified xylanase was pre-incubated at different pH levels (pH 2 to 10) at 25 °C for 1 h prior to the assay. The remaining enzyme activity was determined using the standard conditions. The effect of temperature on the purified xylanase activity was determined at 20 °C to 80 °C. The thermal stability of xylanase was determined by incubating appropriate enzyme in 20 mM potassium phosphate buffer (pH 6) at 20, 25, 30, 40, 50, 60, 70, and 80 °C for 1 h. The residual enzymatic activity was measured using the standard assay conditions. The effect of metal ions and reagents on xylanase activity were also evaluated with purified xylanase in the presence of metal salts (1 mM $FeCl_2$, $MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$, $BaCl_2$, $CuSO_4$, NH_4Cl, $FeCl_3$, and KCl) or common enzyme inhibitors/activators (50 mM EDTA, urea, β-mercaptoethanol, SDS, and Tween 80). The enzyme activity without metal ions and inhibitors/activators was used as the control.

To determine the kinetic constants, all parameters were kept constant under optimal assay conditions (50 °C, pH 6, and 30 min), and only the substrate (birchwood xylan) concentration was changed from one to 10 mg/mL. The Michaelis-Menten equation was used to determine the kinetic parameters (Boucherba *et al*. 2014).

Saccharification of biomass wastes

Biomass wastes such as wheat bran, sugarcane bagasse, cottonseed hull, waste paper, and waste carton were dried and ground. A total of 1.5 g of each sample was pre-treated with 10% NaOH at 121 °C for 1 h followed by centrifugation at 10,000 × g for 30 min. The pH of the supernatant was adjusted to neutrality by adding distilled water, and

the sample was oven-dried at 80 °C. Each biomass waste was suspended in 15 mL of 20 mM potassium phosphate buffer (pH 6) and supplemented with the concentrated crude xylanase enzyme (1000 U). The saccharification reaction was carried out at 50 °C for 2 h. The quantification of reducing sugar in the reaction mixture was performed by DNS method using xylose as standard. The saccharification percentage was evaluated by Eq. 1 (Baig *et al*. 2004),

$$\text{Saccharification (\%)} = \text{Xylose (mg/mL)} / \text{substrate (mg/mL)} \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Xylanase Activity in SMC Extracts of Various Mushroom Species

The enzyme activities in SMC extracts of *C. comatus*, *A. auricular*, *P. ostreatus*, *P. citrinopileatus*, *A. cylindracea*, *H. erinaceus*, *H. marmoreus*, and *T. fuciformis* under standard assay conditions are shown in Fig. 1. The SMC extract of *T. fuciformis* showed the highest xylanase activity (39.3 U/mL) of with 1.1 U/mL of cellulase activity and 0.1 U/mL of laccase activity among the eight SMCs. The result represented 196.5 U/g *T. fuciformis* SMC for the xylanase activity, which was obviously higher than the xylanase activities of SMC extracts from the other reported mushroom species (Singh *et al*. 2003; Lim *et al*. 2013). The highest xylanase activity of 91.6 U/g *Pleurotus* spp. SMC was achieved among previous studies (Lim *et al*. 2013). Therefore, the SMC of *T. fuciformis* was selected for further experiments.

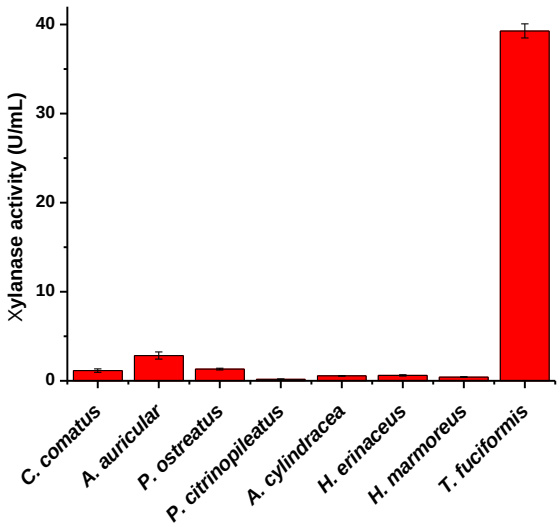

Fig. 1. Comparison of xylanase activities in SMC extracts from eight mushroom species

Purification of Xylanase from the SMC Extract of *T. fuciformis*

The extraction of crude enzyme was carried out using an SMC and deionized water ratio of 1:5 (w/v). The extract acquired after centrifugation was used to find the optimal ammonium sulfate saturation level for precipitation. The results showed that more than 94% of xylanase activity could be retained in the extract when the saturation level was below 40%. When the saturation was at 80%, about 96% of xylanase activity was achieved.

Ammonium sulfate was added to the crude enzyme extract to 40% saturation and was precipitated. The resulting precipitate was discarded, and the supernatant was collected. Again the obtained supernatant was added with 80% saturation of ammonium sulfate. The precipitated enzyme was collected by centrifugation and was subjected to dialysis overnight. As shown in Table 1, the crude enzyme extract showed a xylanase specific activity of 255.2 U/mg. The salt precipitation resulted in 1.3-fold purification with an 85.1% enzyme recovery. The dialysate was used in a DEAE-cellulose column pre-equilibrated with 50 mM Tris buffer (pH 8.5), and the enzyme was eluted with a linear gradient of zero to 0.5 M NaCl in the same buffer at a flow rate of 0.5 mL/min. The eluted solution was collected as 1 mL/tube using an automatic collector. The elution process was demonstrated in Fig. 2A. As shown in Fig. 2A, the first peak observed between 200 (fraction 100) and 230 min (fraction 115) represented the xylanase enzyme according to the results of activity assay. However, some xylanase activity overlapped another peak after about 224 min (fraction 112), implying xylanase in these fractions (112-115) was mixed with other proteins. To obtain the relatively pure protein for further purification, the fractions from 104 to 111 exhibiting the xylanase activity were pooled. The results showed that an enzyme purity of 16.4-fold with 61.9% of enzyme recovery was obtained by this step, which led to 4194.8 U/mg of the xylanase specific activity (Table 1). The pooled active fractions were used to perform further purification by Sephadex G-100 column chromatography. Figure 2B shows the elution profile of Sephadex G-100 column. The fractions 23 to 39, which showed high enzyme activity, were combined and concentrated. Finally, the xylanase specific activity of 5293.8 U/mg was obtained with 20.7-fold purification and 43.1% enzyme recovery. After a series of purified steps, 56.9% xylanase loss was observed, though high xylanase specific activity and purification fold were achieved during the purification process. The activity loss was from the process of ammonium sulfate precipitation (14.9 %), DEAE-cellulose step (23.2 %) and Sephadex G-100 step (18.8 %), respectively. It suggested that further improvement during the purification process was necessary for enhancing xylanase recovery. Subsequently, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzyme was carried out for checking its purity and molecular weight. As shown in Fig. 3, the pure enzyme was observed as a single band on SDS-PAGE, indicating that the relatively pure enzyme was obtained. According to the protein marker bands, the molecular weight of the purified xylanase was approximately 32 kDa.

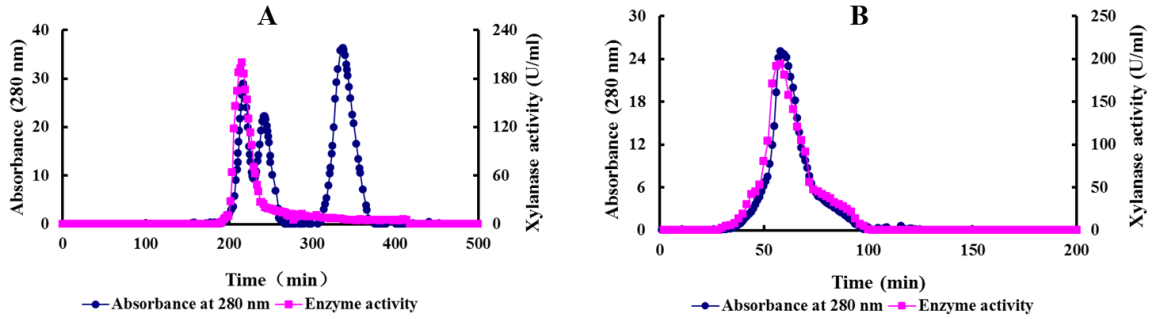

Fig. 2. The elution profiles of xylanase from DEAE-cellulose column chromatography (A) and Sephadex G-100 column chromatography (B): (A) The enzyme was eluted with a linear gradient of zero to 0.5 M NaCl in 50 mM Tris buffer (pH 8.5) and the fractions (1 mL/tube) were collected with a flow rate of 0.5 mL/min; (B) The enzyme was eluted with 20 mM potassium phosphate buffer (pH 6.0) and the fractions (1 mL/tube) were collected with a flow rate of 0.5 mL/min

Table 1. Purification Profile of Xylanase from SMC of *T. fuciformis*

Purification Steps	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	3930.1	15.4	255.2	100	1
$(NH_4)_2SO_4$ precipitation	3343.6	9.7	344.7	85.1	1.3
DEAE-cellulose column	2433.0	0.58	4194.8	61.9	16.4
Sephadex G-100 column	1694.0	0.32	5293.8	43.1	20.7

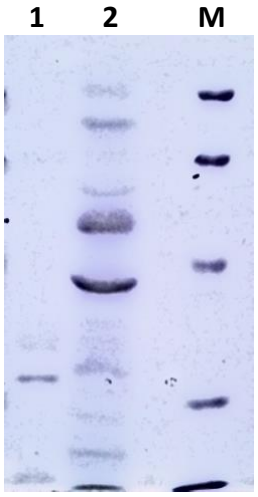

Fig. 3. SDS-PAGE analysis of purified xylanase: Lane M - protein markers (97.2, 66.4, 44.3, 29.0, 20.1 kDa); Lane 1 - purified xylanase protein; and Lane 2 - the precipitated enzyme from extract of *T. fuciformis* SMC were subjected to electrophoresis on a 10% polyacrylamide gel under denaturing conditions

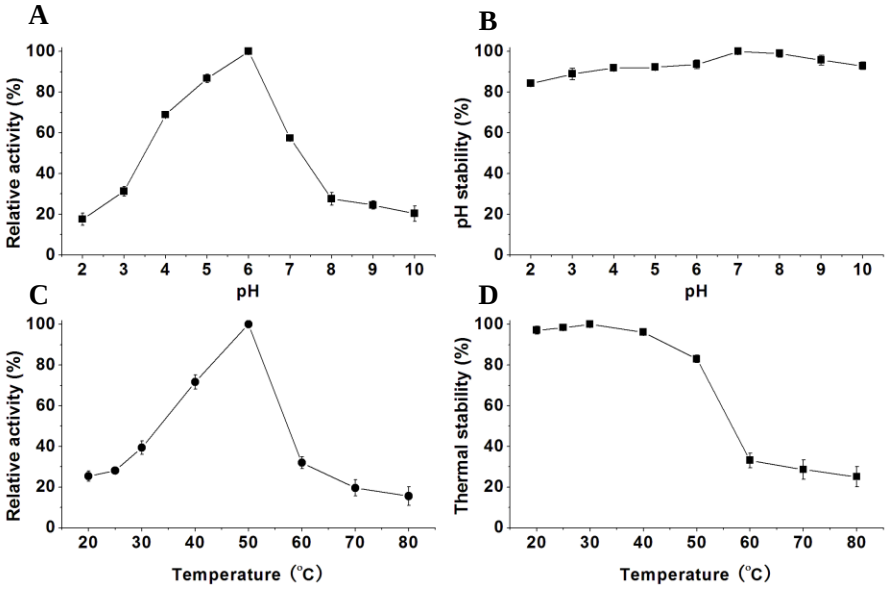

Fig. 4. Effects of pH and temperature on the purified xylanase activity: (A) The optimum pH; (B) the pH stability; (C) the optimum temperature; and (D) the thermal stability of the purified enzyme

Effects of pH and Temperature on the Purified Xylanase Activity

The effects of pH on the purified xylanase activity were investigated, as shown in Fig. 4A. The optimum pH required for birchwood xylan as substrate was 6. The pH value over 7 resulted in a rapid decrease of enzyme activity. The pH stability of the enzyme was studied in the range of pH 2 to 10 by measuring its activities after incubation at each pH value for 1 h at 25 °C (Fig. 4B). More than 84% of xylanase enzyme activity was retained in the pH range of 2 to 10, implying that the purified xylanase from the *T. fuciformis* SMC had good pH stability. The temperature effects were determined in the range of 20 °C to 80 °C at pH 6. Maximum xylanase activity was observed at 50 °C (Fig. 4C). The enzyme was very stable below 40 °C, and more than 95% of its activity could be retained (Fig. 4D). An obvious decrease in the stability of the enzyme was observed over 50 °C probably due to enzyme denaturation.

Effects of Metal Ions and Reagents on the Purified Xylanase Activity

The effect of various metal ions and reagents on the enzyme activity was studied (Table 2). The xylanase activity was enhanced when it was incubated with Mg^{2+} (10%), Ca^{2+} (19%), Ba^{2+} (27%), and NH_4^+ (6%) at a final concentration of 1 mM (Table 2). Other ions inhibited its activity, particularly Fe^{3+}, which exhibited strong inhibition to 26% of maximum activity. For common enzyme reagents, Tween 80 (10 mM) increased the enzyme activity by 10%, but SDS (10 mM) showed complete inhibition of the enzyme with the residual activity of only 1%.

Table 2. Effects of Various Ions and Reagents on the Purified Xylanase Activity

Ions/Reagents	Relative Activity (%)
Control	100
Fe^{2+}	64 ± 4
Mg^{2+}	110 ± 2
Ca^{2+}	119 ± 1
Mn^{2+}	71 ± 2
Zn^{2+}	80 ± 3
Ba^{2+}	127 ± 1
Cu^{2+}	68 ± 3
NH_4^+	106 ± 4
Fe^{3+}	26 ± 2
K^+	99 ± 4
EDTA	37 ± 4
Urea	97 ± 2
β-Mercaptoethanol	93 ± 3
SDS	1 ± 1
Tween 80	110 ±2

Kinetic Analysis

The kinetic parameters of the purified xylanase were determined using birchwood xylan as substrate (1 to 10 mg/mL) under optimal assay conditions (50 °C, pH 6, and 30 min). The K_m and V_{max} values of the purified enzyme towards birchwood xylan were 2.5 mg/mL and 6748 U/mg, which were obtained by using linear regression from double-

reciprocal Lineweaver-Burk plots. The lower K_m of 2.5 mg/mL indicated that the purified xylanase from SMC extract of *T. fuciformis* had a high affinity for xylan.

Saccharification of Biomass Wastes

The saccharification of various biomass wastes including wheat bran, sugarcane bagasse, cottonseed hull, waste paper, and waste carton using concentrated crude xylanase enzyme from *T. fuciformis* SMC extract was studied. Biomass wastes were pre-treated with 10% NaOH at 121 °C for 1 h, washed until they reached neutral pH, and then treated with the xylanase preparation. The saccharification of the biomass wastes is presented in Fig. 5. Among these biomass wastes, the xylan of wheat bran was the optimal substrate for the xylanase from SMC extract of *T. fuciformis*. 95.17 mg of xylose with 75 % saccharification percentage could be achieved from 126.22 mg xylan of wheat bran after 2 h. By comparison, low saccharification rates of xylan from sugarcane bagasse (37 %), cottonseed hull (25 %), waste paper (17 %), and waste carton (41 %) were observed by the xylanase from SMC extract of *T. fuciformis*. These results implied that the xylanase from SMC extract of *T. fuciformis* has potential in saccharification of wheat bran into sugars.

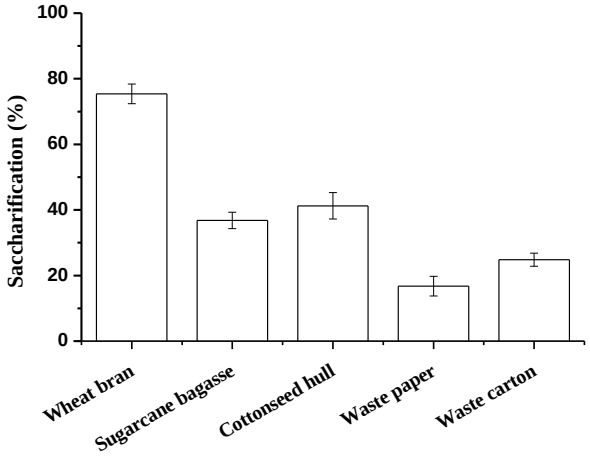

Fig. 5. Saccharification of waste biomass by the crude xylanase enzyme from SMC extract of *T. fuciformis*

This study focused on the xylanase from the SMCs. The xylanase activities of various SMC extracts from eight edible fungi after harvesting of mushroom were evaluated, and the SMC extract from *T. fuciformis* exhibited higher xylanase activity of 39.3 U/mL (196.5 U/g *T. fuciformis* SMC) with weak cellulase and laccase activity, whereas the other seven SMCs extracts showed lower xylanase activity. In previous studies, the highest xylanase activity is 91.6 U/g SMC from *Pleurotus* spp. (Lim *et al*. 2013). A possible explanation is that the concentration of xylanase in *T. fuciformis* SMC is higher or the xylanase from *T. fuciformis* SMC possesses high specific activity compared with that of other SMCs. Thus, *T. fuciformis* SMC has potential for xylanase production since a large amount of *T. fuciformis* SMC as waste source was produced in China each year. To evaluate enzyme properties, the xylanase from SMC of *T. fuciformis* was purified using ammonium sulfate precipitation, DEAE-cellulose, and gel filtration column chromatography. The purified xylanase showed high catalytic activity towards birchwood xylan with 5294 U/mg of specific activity under the optimal assay conditions (50 °C and

pH 6). In addition, the enzyme showed high pH stability from pH 2 to pH 10 and temperature stability below 40 °C with less activity loss. These features are favourable for its application. Kinetic analysis revealed the K_m value and V_{max} of the enzyme were 2.5 mg/mL and 6748 U/mg, respectively. Previous studies showed that the xylanase from *Termitomyces clypeatus* among mushroom species exhibited the highest V_{max} of 6910 U/mg similar to that of the purified xylanase from SMC of *T. fuciformis* (Soren *et al*. 2009). However, the xylanase K_m value (10.4 mg/mL) from *Termitomyces clypeatus* was significantly lower than that (2.5 mg/mL) of the purified xylanase from SMC of *T. fuciformis*, which suggested that the purified xylanase from SMC of *T. fuciformis* had higher catalytic efficiency for xylan. Fungal xylanases vary greatly in their affinities towards xylan, and K_m values have been reported in a wide range (0.5 to 12.5 mg/mL) (Teunissen *et al*. 1993; Chen *et al*. 1997). The purified xylanase from SMC of *T. fuciformis* with a lower K_m suggested that it has a high-affinity xylanase to xylan. Some ions could activate the enzyme activity such as Mg^{2+} (10%), Ca^{2+} (19%), Ba^{2+} (27%), and NH_4^+ (6%) at a final concentration of 1 mM. Finally, the concentrated crude enzyme was used to carry out the saccharification of several biomass wastes. The results showed that the crude enzyme exhibited obviously different saccharification rates for several biomass wastes. Similar results were also found in previous studies (Saha and Ghosh 2014; Kocabas *et al*. 2015). A reasonable explanation is that the xylanases from different organisms have different substrate specificities (Saha and Ghosh 2014). In this work, a relatively high yield (75%) of alkaline pretreated wheat bran demonstrated its potential in saccharification of second generation feed stocks into sugars.

CONCLUSIONS

1. The extract from *T. fuciformis* SMC exhibited high xylanase specific activity.
2. The purified xylanase from *T. fuciformis* SMC extract showed high activity and thermal resistance with a wide range of pH values.
3. The crude xylanase from *T. fuciformis* SMC extract exhibited potential for the saccharification of alkaline pretreated wheat bran.

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