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Characterization of the thermostability of xylanase produced by new isolates of *Thermomyces lanuginosus*

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ABSTRACT: Characterization of the thermostability of purified enzymes of low and high thermostable xylanase produced by new isolates of *T. lanuginosus* THKU-9 and THKU-49 were performed. Half-life at 70 °C of the purified xylanases from *T. lanuginosus* THKU-9 and THKU-49, in 50 mM phosphate buffer (pH 6.0) was 178 and 336 min, respectively. These enzymes were unstable at pH 5.0 and completely lost their activity after incubation at 70 °C for 30 min. The xylanase produced by THKU-9 retained 87% and 30% activity in 50 mM sodium phosphate buffer (pH 7.0) after 1080 min incubation at 60 °C and 70 °C, respectively, whereas xylanase produced by THKU-49 retained full activity and 41% activity, respectively. The enzymes were more stable in phosphate buffer than in citrate buffer. When the buffer concentration increased, the half-life of the enzymes decreased significantly. Amino acid sequence analysis of low thermostable *T. lanuginosus* THKU-9 xylanase and high thermostable *T. lanuginosus* THKU-49 xylanase showed that high thermostable xylanase had a single substitution (V96G), which is a small hydrophobic amino acid of β sheet (B5) of the protein located on the outer surface of the enzyme structure.

INTRODUCTION

Endo 1,4-β-D-xylan xylanohydrolases (EC 3.2.1.8) are glycosyl hydrolases and are generally secreted by microorganisms that live by degrading plant biomass and are able to break down xylan from plant cell walls. Xylan-degrading enzymes of microorganisms are potentially important in various industrial processes: food, feedstuffs, and bio-bleaching process[1]. High thermostability of the enzyme is desirable for such applications. Many microorganisms, particularly thermophilic microorganisms, were reported to produce thermostable enzyme. Attempts to improve the thermostability of this enzyme were examined using different methods. Site directed-mutagenesis techniques have also been used for changing amino acids in order to insert a disulphide bridge into the protein structure at positions S110C and N154C, in *Trichoderma reesei* xylanase. Such changes increased the thermal stability, between temperatures 50–80 °C (pH 8.0) by 14 times[2]. An additional aromatic amino acid at position T11Y of *Streptomyces* sp. S38 xylanase improved thermostability at 57 °C (pH 6.0) by a factor of six[3]. Error-prone PCR was also one of the effective methods to improve thermostability of xylanase produced by transformant harbouring *Thermomyces lanuginosus* DSM 5826 xylanase gene. The wild type had a half-life at 70 °C (pH 8.0) for 89 min. By using this technique, single amino acid substitutions (Y58F and D72G) of xylanases of mutant 2B7-10 and mutant 2B11-16 increased the half-life to 215 and 168 min, respectively[4].

Evolution in nature may give rise to strains producing enzymes with different properties including their thermostability. Therefore, intensive isolation of *T. lanuginosus* from soils had been done in order to look for a xylanase with a different thermostability. Among 88 isolated strains of *T. lanuginosus*, 4 strains produced a highly thermal stable xylanase obtained from crude enzymes at 70 °C having half-life in a range of 201–266 min. Another 17 strains produced xylanase with low thermostability having a half-life in the range of 3–20 min[5]. Among 8 selected strains, partially purified xylanase of *T. lanuginosus* THKU-49 showed the longest thermostability whereas xylanase of *T. lanuginosus* THKU-9 yielded the lowest thermostability. To date, *T. lanuginosus* SSBP producing the longest half-life (232 min) at 70 °C of xylanase has been reported[6]. The shortest half-life (40 min) at 70 °C of xylanase was produced by *T. lanuginosus* DSM 10635[7]. The newly isolated *T. lanuginosus* CAU44 from soil in China and *T. lanuginosus* CSB 288.54 from Centraalbureau voor Schimmelculturen, produced thermostable xylanase, which was stable at 65 °C for 30 min[8,9]. We found purified xylanase produced by *T. lanuginosus* THKU-49 having a half-life at 70 °C in 50 mM phosphate buffer (pH 6.0) of 336 min

whereas purified xylanase produced by *T. lanuginosus* THKU-9 was less thermostable with a half-life at 70 °C of 178 min. The main purpose in this study was to clarify thermal stability characteristics of the purified enzymes of low and high thermostable xylanase produced by *T. lanuginosus* THKU-9 and THKU-49. Moreover, the translated amino acid sequences of the xylanase genes of different strains was investigated in order to elucidate their thermostability.

MATERIALS AND METHODS

Strains

This study used *Thermomyces lanuginosus* THKU-9 and THKU-49, which were selected based on the lowest and highest thermostability of partially purified xylanase respectively. Both strains were identified as *T. lanuginosus* according to the manual of fungal taxonomy[10,11] and Internal Transcribed Spacer (ITS) regions sequence[12]. These strains were preserved at the Thailand Institute of Scientific and Technological Research (TISTR) Culture Collection Bangkok MIRCEN.

Cultivation and purification of xylanase

An actively growing 3-day old colony of each *T. lanuginosus* strain (1 or 2 agar blocks) grown on yeast glucose medium was inoculated into synthetic medium (15 ml) consisting of (per litre of water) 2 g of KH_2PO_4, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4$, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and 10 g of oat spelt in 50 ml Erlenmeyer flasks. The flasks were shaken at 120 rpm and incubated at 45 °C for 5 days. The clear supernatant obtained from filtration of cultured broth through Whatman No. 1 filter paper (Whatman International Ltd., USA) was used for purification.

The xylanase produced by *T. lanuginosus* THKU-9 and THKU-49 were purified using the same protocol. Two litres of cultured supernatant were used and subjected to 80% $(NH_4)_2SO_4$ saturation. The precipitated protein was collected by centrifugation at 10,000 rpm for 5 min and dissolved in 50 mM phosphate buffer (pH 6.0). A dialysis bag having a cut-off of 10,000 Dalton was used to remove salt from the sample. The sample from the above step was applied to a DEAE-Sepharose fast-flow column. The bound xylanase was eluted using a 50 mM phosphate buffer (pH 6.0) gradient containing 0–0.4 M NaCl, and the active fractions were pooled. A large volume of the sample from the ion exchange step was loaded to a hydroxylapatite column. The bound xylanase was eluted using 20–500 mM phosphate buffer (pH 6.0) gradient. The active fractions were pooled and concentrated using ammonium sulpfate precipitation. The concentrated sample was applied to a Sephadex G-100 column. The enzyme was eluted using 3 mM phosphate buffer (pH 6.0). The specific activities of combined active fraction of xylanases of *T. lanuginosus* THKU-9 and THKU-49 were 306 and 552 unit/mg protein, respectively. Each purified enzyme showed a single band with molecular weight of 24.9 kDa on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Each purified xylanase was used for determining their thermostability in this study.

Thermal stability of purified xylanases and K_m values

Thermostability of each purified enzyme was determined. Each microtube (1.5 ml) containing 0.5 ml of enzyme (~80 U/ml) in 50 mM phosphate buffer (pH 7.0) was incubated at 50, 60, 70, 80, 90, and 100 °C. The residual xylanase activities were measured at intervals from 0 to 1,080 min.

The effect of buffer concentration on the thermostability of the purified enzymes was determined at 70 °C in 10, 50, 100, 200, and 400 mM of acetate buffer (pH 5.0), citrate buffer (pH 6.0), and sodium phosphate buffer (pH 6.0 and 7.0). The residual xylanase activities were measured at intervals. Half-life at 70 °C of the purified enzymes of each buffer was determined.

To calculate K_m values for the hydrolysis of soluble oat spelt xylan, xylan at different concentrations; 2–10 g/l in 50 mM sodium phosphate

Table 1. Properties of xylanases produced by different strains of *T. lanuginosus*

Strains	MW (kDa)	Optimal pH	Optimal Temp. (°C)	Half-life at 70 °C (min)	K_m (mg/ml)	Isolated from	References
DSM 10635	25.5	6.5	70	40 (pH 6.5)	3.85	Czech Republic	[7]
SSBP	24.7	6.5	70	232 (pH 6.5)	-	South Africa	[6,14]
SSBP	23.6	6.5	70-75	-	3.26	South Africa	[20]
DSM 5826	25.5	7.0	60-70	201 (pH 6.5)	-	Bangladesh	[6,16]
ATCC 46882	25.7	6.0-6.5	75	-	-	Bangladesh	[15]
THKU-49	24.9	6.0	70	336 (pH 6.0)	7.30	Thailand	
THKU-9	24.9	6.0	70	178 (pH 6.0)	10.3	Thailand	

Table 2. Effect of buffer concentration and pH on thermostability of purified xylanase from *T. lanuginosus* THKU-9 and THKU-49

Buffer conc. (mM)	Half-life (min)					
	Citrate buffer (pH6.0)		Phosphate buffer (pH 6.0)		Phosphate buffer (pH 7.0)	
	THKU-9	THKU-49	THKU-9	THKU-49	THKU-9	THKU-49
10	427	484	493	1251	1171	1012
50	83	84	178	336	523	674
100	30	45	144	96	292	455
200	18	33	79	86	173	254
400	10	10	48	69	65	158

buffer (pH 6.0) were incubated with the purified enzyme at 50 °C for 2–20 min. The initial velocities were calculated from the linear regions of product formation curves and plotted against xylan concentrations. The K_m values of both purified xylanases were calculated from Lineweaver-Burk plots.

Determination of xylanase activity

Xylanase was assayed by determination of reducing sugars liberated from oat spelt xylan when incubated with the diluted enzyme solutions at pH 6.0 and 50 °C for 10 min. The reducing sugars were measured using 3,5-dinitrosalisylic acid (DNS) reagent. One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmol of xylose in 1 min.

Sequencing of xylanase gene fragment

Genomic DNA of *T. lanuginosus* THKU-9 and THKU-49 was isolated using the method of Boonlue *et al*[12]. The genes coding for xylanase were amplified by the polymerase chain reaction (PCR) using primers xyn-F (5′-AACATGGATATATAAAGGGC-3′) and xyn-R(5′-ATTGAATACCTCCAACCGGC-3′). PCR product was cloned to pGEM-T easy vector following standard procedures[13]. Quantum Prep Plasmid Miniprep Kit (Bio-Rad, USA) was used for extracting plasmid from transformant. The PCR products were sequenced using a 5500–5 DNA sequencer (Hitachi). Data from the forward and the reverse sequences were translated to amino acids and compared using the Genetyx version 5.0 program. Three-dimension structures of xylanases were predicted from amino acid sequence using Swiss Pdb Viewer program.

RESULTS AND DISCUSSION

Thermal stability and K_m values of pure xylanases

The maximal activities of both pure xylanases of *T. lanuginosus* THKU-9 and *T. lanuginosus* THKU-49 were obtained at a high temperature (70 °C) and pH 6.0 (Table 1). These data were closed to optimal temperature and optimal pH of *T. lanuginosus* xylanases produced by strain DSM 10635, CAU44, CBS 288.54, SSBP, ATCC 46882, DSM 5826, and RT9[7–9, 14–17].

The thermostability of pure xylanases was analysed at 70 °C in 50 mM phosphate buffer (pH 6.0). Pure xylanase of *T. lanuginosus* THKU-9 was less thermostable, having a half-life of 178 min. The thermostability of this enzyme was similar to xylanase produced by *T. lanuginosus* SSBP and DSM 5826 having half-lives of 232 and 201 min, respectively[6]. Although the xylanase of this strain was less stable, it was still more thermostable than xylanase produced by *T. lanuginosus* 10635 having a half-life of 40 min[7]. In contrast, the half-life of pure xylanase from *T. lanuginosus* THKU-49 was 336 min. The xylanase produced by *T. lanuginosus* THKU-49 had half-lives of 1.45, 1.68, and 8.40 times longer than xylanases produced by strains of SSBP, DSM 5826 and DMS 10635, respectively[6,7]. The results implied that xylanase produced by newly isolated *T. lanuginosus* THKU-49 was the most thermostable xylanase (Table 1). This finding is important in view of possible industrial application such as pulps and papers industry or food processes.

Effects of type, concentration and pH of buffers on the thermostability of pure xylanases were investigated. When the buffer concentration increased, the half-lives at 70 °C of xylanases from *T. lanuginosus* THKU-9 and THKU-49 were significantly decreased (Table 2). The thermostability curve steeply decreased at high buffer concentrations. Elevated buffer concentrations caused aggregation of enzymatic molecules, allowing for formation of dimers and trimers of the large molecule, which irreversibly inactivated the xylanase. The synergistic

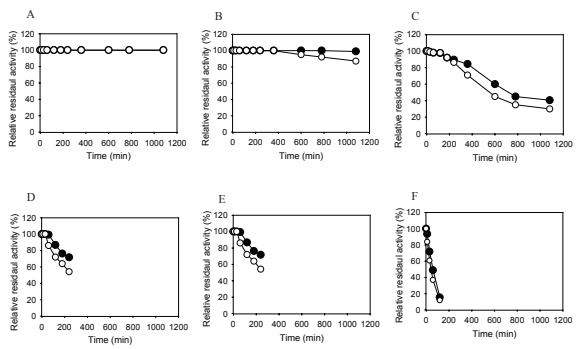

Fig. 1 Relative residual activities of the purified xylanases produced by *T. lanuginosus* THKU-9 (-○-) and *T. lanuginosus* THKU-49 (-●-) in 50 mM sodium phosphate buffer (pH 7.0) at various temperatures; 50 °C (A), 60 °C (B), 70 °C (C), 80 °C (D), 90 °C (E), and 100 °C (F).

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DSM 5826    1:MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTN 60
THKU-49     1:MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTN 60
THKU-9      1:MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTN 60
              ************************************************************

DSM 5826   61:LEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEYYI 120
THKU-49    61:LEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGGYQPNGNSYLAVYGWTRNPLVEYYI 120
THKU-9     61:LEGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEYYI 120
              ************************************.***********************

DSM 5826  121:VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT 180
THKU-49   121:VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT 180
THKU-9    121:VENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGT 180
              ************************************************************

DSM 5826  181:VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG                 225
THKU-49   181:VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG                 225
THKU-9    181:VQTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG                 225
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Fig. 2 Alignment of amino acid sequence of xylanase from strain of *T. lanuginosus* THKU-9, THKU-49, and DSM 5826. The alignment was done using the Genetyx (version 5.0) alignment program. Alignment characters are indicated as follows: (∗) indicates position with a conserved amino acid residue; (·) indicates position with a different amino acid residue.

effect of high temperature and high concentration of buffer solutions may decrease the solubility of the xylanase causing denaturation of enzymes.

The half-lives of the pure xylanases from both strains were longer in phosphate buffer than in citrate buffer, and the longest half-life was obtained when the enzymes were kept in phosphate buffer (pH 7.0) (Table 2). The xylanases from these strains were very unstable at pH 5.0, and their activities were completely lost after incubation at 70 °C for 30 min. The thermostability of pure xylanases produced by *T. lanuginosus* THKU-9 and THKU-49 was greatly dependent on pH, type and buffer concentrations. Because the pI of *T. lanuginosus* xylanase was very low (3.7), differences in the total ionization state of protein between pH 5.0–7.0 may cause the differences in thermostability of the enzymes[18].

The thermostability of each enzyme was determined at various temperatures in 50 mM phosphate buffer (pH 7.0). The enzyme of *T. lanuginosus* THKU-49 was fully stable for 1,080 min up to 60 °C, where as the pure xylanase of *T. lanuginosus* THKU-9 was stable up to 50 °C and retained 87% at 60 °C (Figs. 1A, 1B). At 70 °C, residual activities of xylanase of *T. lanuginosus* THKU-9 and THKU-49 were 30% and 41%, respectively after keeping for 1080 min (Fig. 1C). The results confirmed that xylanase of *T. lanuginosus* THKU-49 was more stable than that of *T. lanuginosus* THKU-9. However, both enzymes were rapidly inactivated starting from 80–100 °C (Figs. 1D, 1E, 1F).The xylanase retained some activity even after incubation at 100 °C, as also observed in other *T. lanuginosus* xylanases[6,7,19].

The K_m values of purified xylanases from *T. lanuginosus* THKU-9 and THKU-49 using soluble xylan as a substrate were 10.3 and 7.30 mg/ml, respectively (Table 1). K_m values of 3.85 and 3.26 mg/ml were found in the xylanase of *T. lanuginosus* strain DSM 10635 and SSBP, respectively[7,20].

Xylanase gene sequencing analysis

The complementary DNA of genes for highly thermostable xylanase produced by *T. lanuginosus* THKU-49 and for xylanase with low thermal stability produced by *T. lanuginosus* THKU-9 were sequenced.

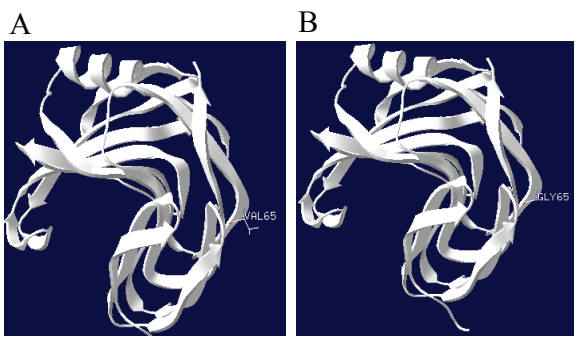

Fig. 3 Three-dimension structures of xylanase produced by THKU-9 (A) and THKU-49 (B) showing hydrophobic amino acid at position 96.

The amino acid sequences derived from the sequence of nucleotides were aligned with the sequence of xylanase from *T. lanuginosus* DSM 5826 which was previously sequenced by Schlacher *et al*.[21] (accession no.U35436) (Fig. 2). The sequences alignment revealed that the active site of xylanase of both strains of *T. lanuginosus* were the position of E117 and E209 (Fig. 2)[22]. The amino acid sequence of xylanase from strain THKU-9 that had a half-life at 70 °C of 178 min was similar to that of *T. lanuginosus* DSM 5826 that had a half-life at 70 °C of 201 min. The sequences of xylanase from THKU-49 that had a half-life at 70 °C for 336 min was different from the other two. There is only one amino acid at position V96G of the β-sheet (A5) on the outer surface of the enzyme structure (Fig. 3). Stephens *et al*.[4] improved the thermostability of the xylanase from *T. lanuginosus* DSM 5826 by directed evolution using error-prone PCR. The amino acid sequence of xylanase from the mutants with enhanced thermostability differed in 3 amino acids for mutant 2B7-6 and had single mutation for mutants 2B11-16 and 2B7-10. Only one amino acid substitution of D72G and Y58F of xylanases from mutant 2B11-16 and mutant 2B7-10 increased the half-lives at 70 °C by 2 and 2.5 times, respectively. Most amino acid substitution for the mutants, except mutant 2B7-10, occurred within the β-sheet of the enzyme which forms the hydrophobic region of the enzyme[4]. The single amino acid substitution of xylanase (V96G) of *T. lanuginosus* THKU-49 and substitution (Y58F) of xylanase in mutant 2B7-10 occurred on the outer surface of the β-sheet, which resulted in an increase of the hydrophilicity of the enzyme increasing the thermostability. In contrast to this finding, glycine substitutions have been reported to unfavourably affect the activity and thermostability of many enzymes[23,24].

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