0273-2289/92/3435—0247$02.60

Purification and Characterization of a Xylanase from the Thermophilic Ascomycete *Thielavia terrestris* 255B

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ABSTRACT

Thielavia terrestris 255B, a thermophilic ascomycete, produced two major forms of xylanase with pIs of 4.6 (xylanase I) and 6.1 (xylanase II). The latter enzyme could be purified to > 99% homogeneity using anion-exchange chromatography and gel filtration. Xylanase II had a mol wt of 25.7 kDa (SDS-PAGE) and a pH and a temperature optimum of 3.6–4.0 and 60–65°C, respectively. The ratio of the enzyme's activity against xylan and carboxymethylcellulose was 500–1000 to 1, indicating a possible application of this enzyme in biobleaching processes. The amino acid sequence of this protein is being determined, and initial data suggest that the enzyme belongs to a group of low-mol wt xylanases that have been isolated from both bacteria and fungi.

Index Entries: *Thielavia terrestris;* xylanase; thermophile.

INTRODUCTION

Lignocellulosics are the most abundant renewable resource available. The efficient exploitation of this resource requires the use of all of its three major components, i.e., cellulose, hemicellulose, and lignin. Xylans are

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the major hemicellulose in angiosperms where they account for 20–30% of the dry wt of woody tissue (*1*). Xylans are also present in gymnosperms, but are less abundant, contributing about 8% of the tissue dry wt (*1*). Xylans are heterogeneous polysaccharides consisting mainly of linear chains of D-xylosyl residues. Depending on the source, substituents, such as arabinofuranosyl, 4-*O*-methylglucuronosyl, and acetyl groups, can be present at various frequencies.

Hydrolysis of xylans involves two major types of enzymes: endo β-1,4 xylanases and β-xylosidases. Endoxylanases cleave β-1,4 linkages between xylosyl residues inside xylan chains. β-xylosidases release xylosyl residues from the nonreducing ends of xylooligosaccharides. Although xylanases can act on substituted xylans, complete hydrolysis probably also requires the action of esterases and arabinosidases to remove the substituents that might hinder the access of some bonds along the xylan chains to xylanases.

The potential industrial application of xylanolytic systems include the hydrolysis of xylans to fermentable products in bioconversion processes, and the selective removal of hemicelluloses and, consequently, lignin in biobleaching pulp treatments (*2*). The latter application requires the use of enzymes that are very specific for the hydrolysis of hemicelluloses in order to preserve the cellulose structure during the biopulping process. Thermostability is also a desirable property of the enzymes used in industrial processes. The advantages of thermophilic systems are numerous, and include rapid kinetics, reduced risk of contamination, and an increased opportunity for enzyme recycling (*3*). *Thielavia terrestris* was previously shown to produce large amounts of cellulases and xylanases (*4,5*), which are more thermostable than the enzymes produced by mesophilic organisms, such as *Trichoderma* spp. Our previous work (*4*) showed that *T. terrestris* produced two major forms of xylanase (designated xylanase I and II). In this work, we present the purification and characterization of xylanase II, which was the predominant form of xylanase produced when *T. terrestris* was grown on oat-spelts xylan.

MATERIAL AND METHODS

Organism and Culture Conditions

T. terrestris 255B was obtained from the Forintek Canada Corp. culture collection. It was grown on modified Durand medium as described earlier (*6*). The characterization of the profile of enzymes produced after growth on various substrates was carried out using 200-mL vol in 1-L shake flasks. The following substrates were used at a 1% (w/v) concentration: glucose, Solka Floc BW300, and oat-spelts xylan. Xylanase II was obtained after *T. terrestris* 255B was grown in a New Brunswick Sc. Co. Inc. (Edison, NJ, USA) 16L Model SF116 fermentor operated with a total

vol of 12 L. Oat-spelts xylan at a concentration of 0.5% was used as the substrate. After 48 h, the culture was harvested and filtered through two glass fiber filters (Whatman 934-AH). Sodium azide (0.02% final concentration) was added as a preservative to the culture filtrate.

Purification of Xylanase II

The culture filtrate was concentrated using a Pellicon ultrafiltration unit (Millipore, Bedford, USA) fitted with a 10,000 mol wt cutoff membrane. The concentrated material was applied to a Q-Sepharose column equilibrated at pH 7.2 (20 m*M* Bis-Tris). The column was washed with a column volume (200 mL) of this equilibration buffer. The bound material was eluted with a linear gradient of 0–0.5*M* NaCl (in equilibration buffer). When the conditions described above were used, most of the xylanase activity did not bind to the column and was eluted with the equilibration buffer. The unbound material was applied to a Superose 12 column and eluted using 50 m*M* sodium acetate (pH 4.8). The peak that eluted last contained xylanase II purified to at least 99% homogeneity.

Electrophoresis

Isoelectrofocusing (IEF) was performed using Phastgel IEF 3-9 run in the automated Phastsystem (Pharmacia, Uppsala, Sweden). The separation was performed at a constant current of 2.5 mA for 500 accumulated V h. The gels were developed by silver staining using the "Phastgel Silver Kit" according to the manufacturer's manual (Pharmacia). For the detection of bands with xylanase activity, an unstained gel was layered on 2% agarose gel containing 0.1% oat-spelts xylan (*4*). The gels were incubated at 65°C for 5–10 min and stained for 60 min in 0.1% Congo Red. The gels were destained with 1*M* NaCl until clearing zones were visible. To increase the contrast, the gels were placed in 5% (v/v) acetic acid, which turns the background from red to dark blue.

SDS-PAGE was run according to Laemmli (*7*). A 14% gel was cast and run at 100 V for 40 min using the Mini-Protean II slab cell (Bio-Rad Lab., Richmond, CA, USA). The mol-wt markers were the Pharmacia low-mol-wt calibration kit.

Protein and Enzymatic Assays

Protein was determined by the bicinchoninic acid method using the #BCA-1 kit for total protein determination (Sigma Chem. Co., St. Louis, MO, USA). The assay was carried out in a microtiter plate (*8*) and read at 550 nm using a microplate autoreader EL-310 (Biotek Instr. Inc., Winooski, USA).

The xylanase assays were performed in 50-m*M* sodium acetate buffer (pH 4.8) using 0.5% oat-spelts xylan (#X 0376, Sigma Chem. Co) as the substrate. The standard assay for xylanase determination was carried out at 65°C for 30 min, whereas chromatography fractions were analyzed using a 10-min assay. Reducing sugars released during incubation were quantified by the 3,5-dinitrosalicylic acid (DNS) method of Miller (*9*).

Characterization of Xylanase II

The substrate specificity of xylanase II was studied with the following substrates: 0.5% oat-spelts xylan (Sigma #X-0376), 0.5% larchwood xylan (Sigma #X-3875), 0.5% carboxymethylcellulose (Sigma #C-4888), 0.5% acid-swollen cellulose (4), 0.5% salicin (Sigma #S-0625), 5 m*M* *p*-nitrophenyl β-D-xylopyranoside (Sigma #N2132), and Whatman #1 filter paper (70 mg in a final vol of 2 mL). All the substrates, other than filter paper, were incubated with the enzyme for 30 min at 65°C. The filter paper assay was carried out for 60 min at 65°C. Between 50–100 ng of xylanase II were added to each of the xylan-rich substrates. The cellulosic substrates had either 50 or 2500 ng of xylanase II added to them to determine if hydrolysis could occur over a wide range of enzyme concentrations. Reducing sugars were assayed using the DNS method (9). Release of *p*-nitrophenol from *p*-nitrophenyl xylopyranoside was determined by absorbance measurement at 410 nm following the addition of 1 mL of 1*M* Na_2CO_3 to the 1.1-mL assay vol.

The pH optimum of xylanase II was determined by the standard xylanase assay using either 50 m*M* sodium acetate or 50 m*M* sodium citrate at various pHs. The optimum temperature of the xylanase was determined by incubating 0.5% oat-spelts xylan (50 mM Na acetate, pH 4.8) for 30 min at temperatures ranging from 45 to 80°C. Temperature stability was determined by incubating xylanase II (0.2 mg/mL) at temperatures ranging from 50 to 65°C for 10 h. Aliquots were taken at various times and assayed using the standard xylanase assay.

Amino Acid Analysis and *N*-Terminal Sequencing

For amino acid analysis, xylanase II was hydrolyzed with 6*M* HCl for 24 h at 110°C and analyzed on a Durrum D-500 analyzer. *N*-terminal sequencing was done by automated Edman degradation of native xylanase using an Applied Biosystems 475A protein sequencing system incorporating a model 470A gas phase sequencer equipped with an on-line model 120A PTH analyzer under the control of a model 900A control/data analysis module.

RESULTS AND DISCUSSION

Initially, we used IEF to separate the extracellular protein secreted after growth of *T. terrestris* 255B on various substrates (Fig. 1). *T. terrestris* 255B produced two major forms of xylanase, which we designated xylanase I (pI = 4.6) and xylanase II (pI = 6.1). Only xylanase II was detected after growth on glucose, which apparently failed to repress the production of this form of xylanase completely. Xylanase II predominated after growth

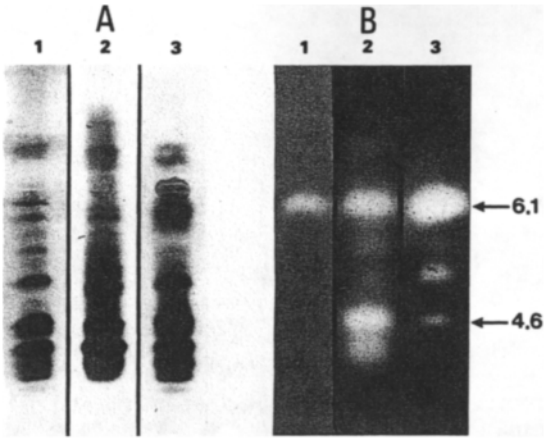

Fig. 1. Separation by IEF of the protein obtained after growth of *Thielavia terrestris* 255B on glucose (lane 1), Solka Floc BW300 (lane 2), and oat-spelts xylan (lane 3). Panel A: Silver staining of the gel. Panel B: Overlay with gel containing oat-spelts xylan to detect bands with xylanase activity. The arrows indicate xylanase I (pI 4.6) and xylanase II (pI 6.1).

on oat-spelts xylan. Xylanase I and another minor active band with a pI of 5.2 were also detected. The higher levels of activity found after growth on oat-spelts xylan (*4*) were largely the result of the induction of xylanase II.

Both xylanase I and II were detected with comparable clearing zone intensities after growth on cellulose (Solka Floc BW300). A third clearing zone was also detected at a pI of approx 4.3, but the fuzzy definition of the band suggested that it might have been caused by more than one protein. Solka Floc BW300 contains contaminating xylan, which is probably responsible for the induction of relatively high xylanase activity after *T. terrestris* 255B is grown on this substrate (*4,10*). However, the production of xylanase I is probably also influenced by the presence of cellulose, because the intensity of the clearing zone suggests that more is produced after growth on Solka Floc BW300 than after growth on oat-spelts xylan. Both the cellulolytic and xylanolytic systems comprise many components, and it is possible that the close association of their substrates in nature leads to enzyme regulation systems that interrelate.

Purification of Xylanase II

Previously, we reported higher production of xylanase activity after *T. terrestris* 255B was grown for 4–6 d on oat-spelts xylan (*4*). However, proteases were also detected in the culture filtrate after 3–6 d of growth. This was probably a consequence of autolysis, since the cell biomass decreased over the same period of time. We therefore decided to use a 2-d-old culture of *T. terrestris* 255B grown on oat-spelts xylan as the source for the purification of xylanase II. The crude material was applied to an

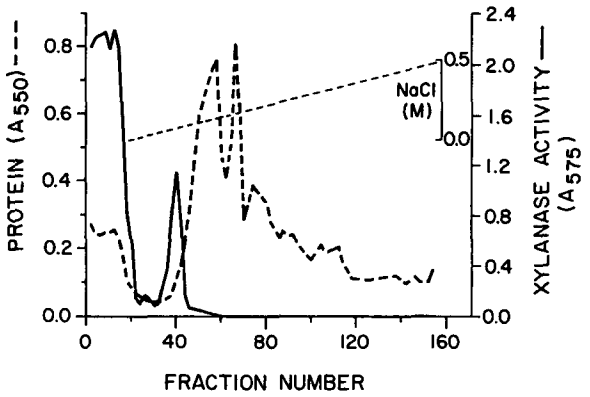

Fig. 2. Anion exchange on a Q-Sepharose column of the Pellicon retentate. A linear gradient of 0–0.5*M* NaCl (in 20 m*M* Bis-Tris, pH 7.2) was applied from fraction 21 to 160.

Table 1
Steps Involved in the Purification of Xylanase II
from Culture Filtrates of *Thielavia terrestris* 225B

Purification step	Protein, mg	Activity, U	Specific activity, U/mg	Yield, %
Culture filtrate	2806	128600	46	100
Pellicon retentate	445	78400	176	61
Anion exchange	140	64500	462	50
Gel filtration	2.8	6032	2154	4.7

anion-exchange column (Fig. 2) under conditions (20 m*M* Bis-Tris, pH 7.2) that should normally have bound a protein such as xylanase II, with a pI of 6.1. However, most of the xylanase activity was eluted with the equilibration buffer. A small xylanase peak containing < 5% of the applied activity was eluted early after the application of the salt gradient (0–0.5 NaCl). This fraction was not further characterized. Although the net charge of xylanase II was probably negative under the ion-exchange conditions, the negative groups could have been prevented from interacting with the anion exchanger. This effect might be the result of the structure of the enzyme itself or of interactions with other molecules present in the crude material, such as polysaccharides or other proteins. Nevertheless, the anion-exchange step achieved purification with an increase in specific activity from 176 U/mg for the Pellicon retentate to 462 U/mg for the material that did not bind to the anion exchanger (Table 1).

This latter fraction was subjected to gel filtration (Fig. 3) using a Superose 12 column. Two xylanase peaks were observed. The peak that eluted first contained 25–30% of the recovered activity and was not further characterized. The peak that eluted last contained xylanase II. Compari-

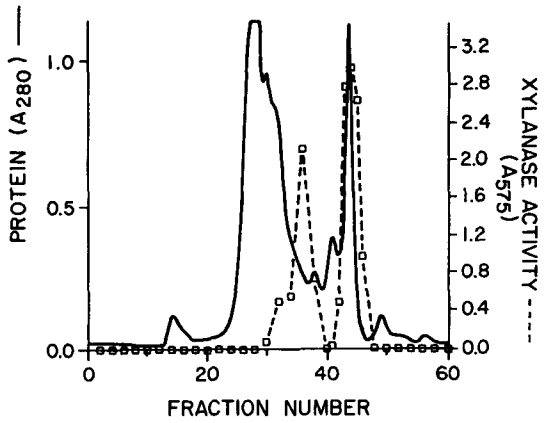

Fig. 3. Gel filtration on a Superose 12 column of the material that did not bind to the anion exchanger. Sodium acetate (50 m*M*) (pH 4.8) was used as the elution buffer.

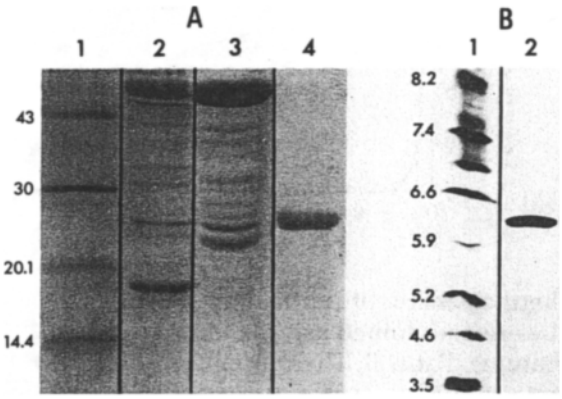

Fig. 4. Analysis of purified xylanase II. Panel A: SDS-PAGE on 14% acrylamide gel; lane 1: mol-wt standards in kilodaltons; lane 2: crude xylanase; lane 3: material that did not bind to the anion exchanger; lane 4: xylanase II after gel filtration. Panel B: IEF gel; lane 1: pI standards; lane 2: xylanase II after gel filtration.

son with elution volumes of standard proteins showed that xylanase II must have interacted with the gel matrix, because the apparent mol wt of 500–1000 daltons calculated from the late elution was not representative of its true mol wt. Unusually high gel filtration elution volumes have also been reported during the purification of xylanases from other microorganisms (*11–13*). It is apparent that this interaction is not limited to polysaccharides, because it has been reported for both glucan-based (*11,12*) and acrylamide-based (*13*) matrices. It has been suggested (*11*) that the high tyrosine content of these xylanases could be responsible for the interaction with the gel filtration matrix (*11*).

Analysis of the preparation obtained after gel filtration by SDS-PAGE and IEF (Fig. 4) showed that xylanase II was purified to at least 99% homo-

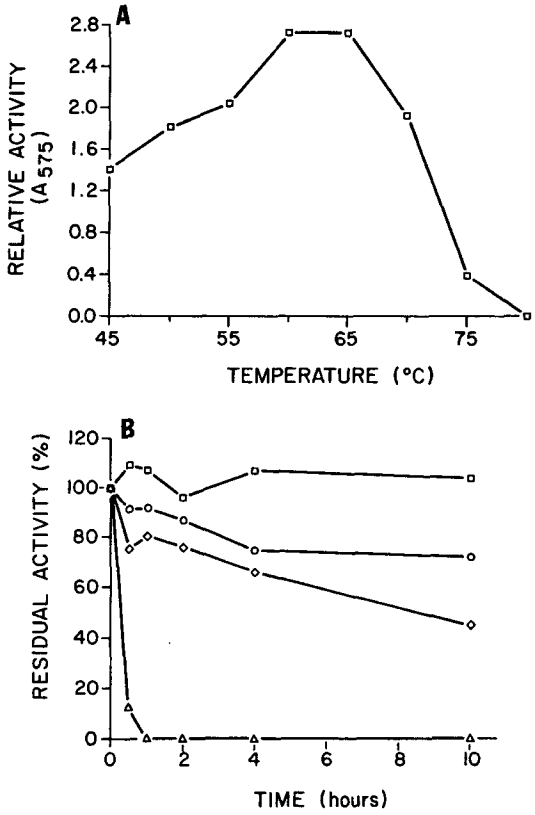

Fig. 5. Thermostability of purified xylanase II. Panel A: Relative activities of xylanase II were determined using 30-min assays with 0.5 oat-spelts xylan at various temperatures. Panel B: Thermostability of xylanase II incubated in the absence of substrate at 50°C (□), 55°C (○), 60°C (◇), and 65°C (△). After various durations of incubation, aliquots were assayed using the 30-min xylanase assay at 65°C.

geneity. Although the interaction with the gel filtration matrix was not fully determined, it acted as an efficient final purification step. The interaction is known to be influenced by the elution buffer composition (*11*) and could possibly be optimized in commercial scale processes to obtain large amounts of xylanases.

Characterization of Xylanase II

Xylanase II was shown to have a mol wt of 25.7 kDa and a pI of 6.1 as determined using SDS-PAGE and IEF, respectively (Fig. 4). The optimal temperature for hydrolysis over a 30-min assay was 60–65°C (Fig. 5A). However, the purified xylanase was found to be very unstable when incubated in the absence of substrate at temperatures above 60°C (Fig. 5B). The optimal pH of xylanase II was determined in different buffers. Citrate

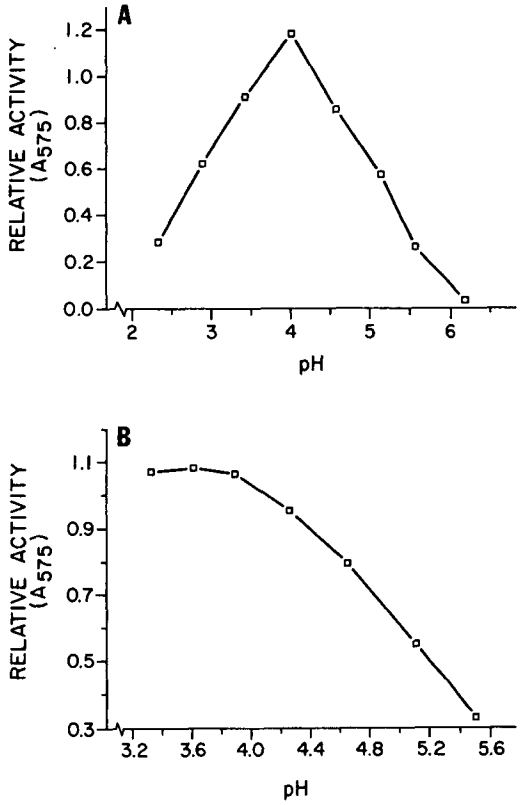

Fig. 6. Optimum pH values of xylanase II determined using 50 m*M* of citrate buffer (A) and 50 m*M* of acetate buffer (B).

buffer gave a symmetrical curve with an optimum pH of 4.0 (Fig. 6A). Acetate buffer gave a slightly more acidic pH optimum of 3.6, although the decline in activity on the acidic side of the curve was less dramatic (Fig. 6B).

The effect of various ions on xylanase activity was tested using 2 m*M* of Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, and Pb^{2+}. Only Cu^{2+} and Pb^{2+} affected xylanase II and reduced the original activity by 50 and 40%, respectively.

Substrate Specificity

Xylanase II was shown to be highly specific in only cleaving the β-1,4 linkages between xylosyl residues in xylans (Table 2). Although xylanase II showed very high activity towards oat-spelts xylan (2154 U/mg) and larchwood xylan (1206 U/mg), it did not cleave *p*-nitrophenyl xylopyranoside and, therefore, did not exhibit xylosidase activity. The enzyme showed no activity on filter paper or acid-swollen cellulose, and very low activity on carboxymethylcellulose (<2 U/mg). The very high ratio of xylanase/cellulase activity makes this enzyme a potential candidate for applications, such as biobleaching of pulps.

Table 2
Substrate Specificities of Xylanse II

Substrate	Activity, U/mg enzyme
Oat-spelts xylan	2154
Larchwood xylan	1206
p-Nitrophenyl xylopyranoside	0
Filter paper	0
Acid-swollen cellulose	0
Carboxymethylcellulose	<2
Salicin	0

Table 3
Amino Acid Composition of Low-Mol-Wt Xylanases from *Thielavia terrestris* 255B, *Bacillus subtilis*, *Bacillus pumilus*, and *Trichoderma viride*

Amino acid	*Thielavia terrestris*	*Trichoderma viride* (*19*)	*Bacillus subtilis* (*15*)	*Bacillus pumilus* (*18*)
Asp	(Asp+Asn)	(Asp+Asn)	7	6
Asn	20	23	18	18
Glu	(Glu+Gln)	(Glu+Gln)	2	7
Gln	15	10	5	7
Thr	31	17	24	19
Ser	25	24	19	20
Cys	?	0	0	1
Pro	5	6	6	5
Gly	24	28	25	23
Ala	17	7	9	11
Val	15	13	14	8
Met	3	1	2	6
Ile	6	10	6	10
Leu	6	5	4	8
Tyr	17	18	15	16
Phe	9	7	4	9
Trp	?	6	11	6
His	2	4	2	4
Lys	2	4	5	9
Arg	4	6	7	8

Amino Acid Composition and *N*-Terminal Sequencing

Amino acid analysis revealed a high threonine and tyrosine content (Table 3). These data, along with the enzyme's low-mol-wt, suggest that xylanase II is related to other low-mol wt xylanases that have been isolated from both mesophilic bacteria and fungi (*11,14–19*). Determination

Table 4
N-Terminal Sequence
of Xylanase II Determined
by Automated Edman Degradation

Cycle	Identity
1	Ala
2	Ala
3	Gly
4	Ile
5	Asn
6	Tyr
7	Val
8	Gln
9	Asn
10	Tyr
11	Asn
12	Gly
13	Asn
14	Leu
15	Gly
16	Tyr
17	Phe
18	Thr
19	Tyr
20	Asn
21	Glu
22	Gly
23	Ala
24	Gly
25	Gln
26	Phe
27	Ser
28	Met
29	Tyr
30	Thr

of a single *N*-terminal sequence by automated Edman degradation (Table 4) confirmed the purity of the isolated enzyme. When the homology of this sequence was compared with *N*-terminal sequences of other low-mol-wt xylanases, it was found to be marginal. Leathers (*17*) previously proposed an *N*-terminal consensus sequence between some low-mol-wt xylanases. However, the comparison of the sequences of the xylanases from two closely related bacteria (*Bacillus subtilis* and *Bacillus pumilus*) suggests that higher homology is found in the carboxy-terminal two-thirds of the related low-mol-wt xylanases (*15*). We are currently continuing the

sequencing of xylanase II to determine the extent of homology with other low-mol-wt xylanases.

CONCLUSION

The major xylanase from the thermophilic fungus *Thielavia terrestris* 255B was shown to be highly specific for the hydrolysis of xylans, and did not attack either crystalline or amorphous cellulose. Although xylanase II was produced by a thermophilic fungus, it was found to be only moderately more thermostable than xylanases from mesophilic sources. The amino acid composition suggests that this xylanase is related to other low-mol-wt xylanases from bacterial and fungal origins. We are currently determining the mode of action of xylanase II and comparing it with the activity of xylanases from mesophilic sources.

ACKNOWLEDGMENTS

We would like to thank Forintek Canada Corp. for allowing us to carry out this work in its laboratory. We are grateful to David Watson (NRCC) for his technical expertise in determining the amino acid sequence. We also thank K. Wong for his comments and suggestions.

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