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Characterization of Endoxylanase Activity From *Thermomonospora Fusca* BD25

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Abstract: *Thermomonospora fusca* BD25 produces relatively high levels of activity of endoxylanases. Only oat spelt xylan, RBB-xylan and birchwood xylan reacted with endoxylanases with the maximum specific activity of 47.65 U mg^{-1} protein on birchwood xylan. The endoxylanase remained stable up to 70 °C. At 60 °C endoxylanase activity showed stability (92 %) for 16 h, however it showed a half-life of 8 h at 70 °C and 30 min at 80 °C in the absence of substrate at pH 8.0. The relative endoxylanase activity in the pH ranges of 6.5 to 9.5 remained between 68 % and 72 % of the activity at pH 8.0. The apparent K_m value for the crude endoxylanase was 6.66 mg of oat spelt xylan ml^{-1}, while the V_{max} value was 2.5 μmol reducing sugar min^{-1} ml^{-1}. Endoxylanase activity was affected by the addition of xylobiose (1 mM) to the reaction mixture of endoxylanase preparation and resulted in approximately 25 % reduction in activity. Also, the addition of supernatant fluids from cultures grown on xylan (reducing sugar concentration of 1 mg ml^{-1} xylose equivalent) to the reaction mixture resulted in approximately 30 % reduction in activity.

Key Words: Endo-1,4-β-xylanase, lignocellulose degradation, *Thermomonospora fusca,* xylan, actinomycete.

Thermomonospora fusca BD25'ten Elde Edilen Endoksilanaz Enziminin Karakterizasyonu

Özet: Termofilik bir aktinomiset olan *Thermomonospora fusca* BD25, diğer organizmalara oranla daha yüksek seviyede endoksilanaz enzimleri üretmektedir. Endoksilanaz enzimlerinin spesifik aktivitelerini belirlemek amacı ile yapılan çalışmalarda, kullanılan substratlardan sadece yulaf ksileni, huş ağacı ksileni ve RBB-ksilenin reaksiyon verdiği belirlenmiş olup en yüksek spesifik aktivitenin (47,65 U mg^{-1} protein) huş ağacı ksileni ile olduğu saptanmıştır. Endoksilanaz enzimleri için optimum sıcaklık 70 °C olarak tespit edilmiştir. Enzimler (substrat yokluğunda ve pH 8'de) 60 °C'ta 16 saat süre ile stabilitelerini ve maksimum aktivitelerinin % 92'sini korurken yarı ömürleri 70 °C'ta 8 saat, 80 °C'ta ise 30 dak. olarak saptanmıştır. Endoksilanaz enzimleri pH değerleri 6,5-9,5 arasında ise, pH 8'de göstermiş oldukları maksimum aktivitenin % 68-72'sini göstermişlerdir. Substrat olarak yulaf ksileni kullanıldığında görünür K_m değeri 6,66 mg substrat ml^{-1} olarak tespit edilirken V_{max} değeri 2,5 μmol dak^{-1} ml^{-1} olarak tespit edilmiştir. Son ürün inhibitörü olarak ksilobiyozun (1 mM) endoksilanaz enziminin reaksiyon ortamına ilavesi ise enzimin aktivitesinde yaklaşık olarak % 25 azalmaya neden olduğu saptanmıştır. Karbon kaynağı olarak ksilen içeren ortamda yetişen *T. fusca*'nın kültür sıvısının (indirgenmiş olan şekerlerin konsantrasyonu 1 mg ml^{-1} ksiloza eşit) endoksilanaz enziminin reaksiyon ortamına ilavesi ise enzimin aktivitesinde yaklaşık olarak % 30 azalmaya neden olduğu saptanmıştır.

Anahtar Sözcükler: Endoksilanaz, lignosellüloz, *Thermomonospora fusca*, ksilen, aktinomiset.

Abbreviations: TLC, Thin layer chromatography: PAGE, polyacrylamid gel electrophoresis; RBB-xylan, Ramazol Brilliant Blue-xylan; CMC. Carboxymethylcellulose; RS, Reducing sugars; p-NPX, p-nitrophenyl-β-D-xylopyranoside; p-NPA, p-nitrophenyl-α-L-arabinofuranoside; SDS, Sodium dedocyl sulphate; EDTA, ethylenediaminetetraacetic acid.

Introduction

Plant cell walls are the major reservoir of fixed carbon sources in nature. They have three major polymers: cellulose (30-45 % w/w), hemicellulose (30 % w/w) and lignin (15-30 % w/w) (1). As a consequence these structures are commonly referred to as lignocellulose. Hemicellulose is a non-linear heterogeneous polymer composed of three hexoses: glucose, mannose and galactose and two pentoses: xylose and arabinose linked together by a number of β-1,4 glycosidic bonds (2). Xylan is the major hemicellulose component of plant cell walls in most plant species. Depending on the source, the xylose backbones are substituted with mainly acetyl, arabinosyl and glucuronosyl residues. As a result of this molecular complexity, simultaneous action of a range of bioactive enzymes is required to complete the degradation process. Depolymerization is largely achieved through the action of endoxylanases (endo-1,4-β-xylanase or 1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) (3).

As lignocellulose is such an abundant and renewable resource it is being exploited for the generation of numerous products. These include bioconversion of lignocellulose-derived sugars into fuel (4), processing food including bread-making and clarification of beer and juice (5) as well as in the paper and pulp industry (6). In terrestrial habitats (e.g. soils and composts), lignocellulose is degraded by a complex microflora, which includes both eukaryotic and prokaryotic organisms. Actinomycetes are a heterogeneous group of Gram-positive saprophytic eubacteria capable of forming branching hyphae and are prevalent in both terrestrial and aquatic environments (7). *Thermomonospora fusca* is especially significant with respect to lignocellulose degradation because of the high production of lignocellulose degrading enzymes (xylanases, glucanases and peroxidases) reported for this organism (8-13). Lignocellulose degrading enzymes such as xylanases could be employed to enhance the bleachability of kraft pulps (6).

The work described in this study, aimed to characterize some properties of endoxylanase produced by *T. fusca* BD25 when growing on oat spelt xylan as the main carbon and energy sources.

Materials and Methods

Growth and maintenance of *Thermomonospora fusca* BD25

The growth and maintenance conditions of the bacterial strain has been described previously (11,13).

Biomass measurement and protein estimation

Bacterial growth and protein estimation has been described previously (13).

Enzyme assays

Endoxylanase activity was determined by measuring the release of reducing sugars from oat spelt xylan (Sigma). The reaction mixture contained 500 µl of appropriately diluted crude enzyme and 500 µl of 100 mM phosphate buffer (pH 7.0) containing 1 % (w/v) of xylan and incubated at 50 °C for 10 min followed by immediate chilling on ice for 5 min and then

centrifuged at 10,000 *g* for 2 min at 4 °C (14). The amount of reducing sugar levels in the supernatant were determined by the dinitrosalicylic acid (DNS) method of Miller (15). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar (expressed as xylose equivalents) per min at 50 °C and pH 7.0. Enzyme and substrate controls were routinely included, and all enzyme preparations were appropriately diluted for the determination of activity in the presence of a negligible background reducing sugar concentration.

Purification of endoxylanase from *T. fusca* BD25

The initial purification step for endoxylanase was ultra-filtration of the cell-free extracellular liquid cultures from *T. fusca*. This was carried out using Amicon ultra-filtration cells using 10,000 molecular weight cut-off membranes. Then the dialysed crude concentrated preparation was loaded onto the gel filtration column, which was packed with Sepharose 6B column material according to the manufacturer's instructions. The sample was eluted using potassium phosphate buffer (pH 7.0) at a flow rate of 20 ml h^{-1}. Positive fractions for enzyme activity were pooled and used for anion exchange chromatography.

Anion exchange chromatography was carried out using Diethylaminoethyl-Sepharose (DEAE-Sepharose) fast flow column material. The material was packed into a Pharmacia-XK50 column (5.0 id x 5.0 cm) according to the manufacturer's instructions. The pooled active fractions of gel filtration were loaded onto an anion exchange column and eluted with a NaCl gradient (0 to 1 M) in Tris-HCl buffer (50 mM, pH 8.5) (at a flow rate of 0.5 ml min^{-1}). Fractions showing enzyme activity were concentrated by disposable concentration units and the salt content of the sample was removed by washing with sample buffer. Enzyme activities were assayed at each step by the standard assay method.

Temperature studies of endoxylanase

The effect of temperature on the stability of the endoxylanase produced by *T. fusca* BD25 was studied by incubating the enzyme for 15 min in the absence of the substrates over a range of temperatures (50 °C to 90 °C). Activities were measured at 50 °C using the standard enzyme assay methods. Similarly, the effect of time and temperature on the stability of the enzymes was determined by incubating the enzyme in the absence of substrate over a period of 27 h at various temperatures in the range of 50 °C to 80 °C at pH 8.0. The pH was adjusted with 1 M NaOH or HCl at specified reaction temperatures. Again the activity was measured at 50 °C using the standard assay methods. Finally the effect of temperature on the activity was monitored by measuring the activity using the appropriate substrates at specific temperature ranging from 50 °C to 90 °C.

pH studies of endoxylanase

The activities of endoxylanase over a pH range of 3.0-12.0 were investigated using the standard assay method. However, the potassium phosphate buffer was replaced with Britton-Robinson Universal Buffer (16), which contained 6.008 g l^{-1} citric acid; 1.769 g l^{-1} boric acid; 3.839 g l^{-1} KH_2PO_4 and 5.266 g l^{-1} diethylbarbituric acid. The pH was adjusted with 1 M NaOH or HCl. Aliquots (50 μl) of enzyme preparations were mixed with Universal Buffer (950 μl) of

each pH value. These samples were then left to equilibrate for 30 minutes, then the pH was checked. Finally enzyme activities were assayed. Activities were presented as a percentage of the maximum activity.

Kinetic and inhibition studies of endoxylanase

i- Determination of K_m and $V_{max.}$

The kinetics of crude endoxylanase preparation was determined by previously reported methods (17,18). Endoxylanase assays were performed at pH 7.0 and 50 °C and oat spelt xylan concentration varied from 0.5 to 20 mg ml^{-1}.

ii- Inhibition studies of endoxylanase

The effect of end-product inhibition on endoxylanase by xylan-hydrolysis products was determined by addition of D-xylose, D-glucose, L-arabinose, xylobiose and cellobiose (0-30 mM) to the reaction mixture.

In another experiment, supernatant fluids (50-100 µl) taken from the growth culture liquid of *T. fusca* for 48 h in oat spelt xylan containing medium used to identify xylan hydrolysis products by thin layer chromatography (TLC), were added to the enzyme inhibition assays. These supernatant fluids were pre-treated to inactivate any residual enzyme activity by boiling for 5 min. Further studies on end-product inhibition were carried out by serial dilutions of supernatant fluids.

Inhibition by metal ions and reagents, such as citric acid, boric acid, mercury, nickel and ethylenediaminetetraacetic acid (EDTA) was investigated by incorporating appropriate salts (1 mM) in the assay mixture.

Polyacrylamide gel electrophoresis (PAGE)

Concentrated crude supernatant and cell extract of *T. fusca* BD25, grown on oat spelt xylan for 72 h, were separated by non-denaturing discontinuous polyacrylamide gel electrophoresis. Gels were constructed according to the method of Laemmli (19) using 10 % (w/v) acrylamide. Electrophoresis was carried out at 80 V in double strength running buffer (pH 8.8), which contained, 0.303 % (w/v) Tris and 1.44 % (w/v) glycine. Gels were run in an Atto model AE-6450 vertical electrophoresis tank (Atto, Japan). Electrophoresis was stopped when the dye-front marker had run just off the bottom of the gel. Bands of endoxylanase activities were detected by the replica gel technique with Ramazol Brilliant Blue-xylan (RBB-xylan), as described by Biely *et al.*, (20, 21).

Results

Substrate specificity of endoxylanase

The substrate specificity of endoxylanase in partially purified (10.37 fold; 48.96 U mg^{-1}) and crude enzyme preparations was studied using colorimetric assays. Compounds (related to lignocellulose) known to be hydrolyzed by xylan-degrading enzymes from different sources,

such as oat spelt xylan, birchwood xylan, arabinogalactan, cellulose, carboxymethylcellulose (CMC) and synthetic substrate compounds of *p*-nitrophenol- attached to sugar units were used as substrates. Negative reactions were obtained against some of the substrates such as cellulose, arabinogalactan and *p*-nitrophenol- derived monosaccharides by extracellular endoxylanases of *T. fusca* BD25. Only oat spelt xylan, RBB-xylan and birchwood xylan reacted with endoxylanase with the maximum specific activities of 47.65 U mg^{-1} protein on birchwood xylan (see Table 1). Endoxylanases separated by non-denaturing PAGE, developed as zymograms also reacted when RBB-xylan was used as substrate but not with carboxymethylcellulose, *p*-nitrophenyl-β-D-xylopyranoside (*p*-NPX) or *p*-nitrophenyl-α-L-arabinofuranoside (*p*-NPA) (see Figure 1, lanes: 1, 2 and 3, respectively).

Thermostability and the effect of temperature on the activity of endoxylanase

In this study, concentrated crude supernatants of *T. fusca* BD25 were used to assess the effects of temperature and pH on endoxylanase. Thermal inactivation of endoxylanase was studied by pre-incubating the enzyme preparations for a fixed period of time in the absence of substrates at a number of temperatures then cooling and carrying out standard assay procedures described in Materials and Methods. The endoxylanase activity remained stable up to 70 °C (see Figure 2a).

Table 1. The activities of the partly purified endoxylanase from *T. fusca* BD25 against various substrates.

Substrate	Endoxylanase Activities
Oat spelt xylan[1]	+
Birchwood xylan[1]	+
Arabinogalactan[1]	–
RBB-Xylan[1]	+
CMC[1]	–
Cellulose[1]	–
p-NP-β-D-glucopyranoside[2]	–
p-NP-α-D-glucopyranoside[2]	–
p-NP-β-D-mannopyranoside[2]	–
p-NP-α-D-mannopyranoside[2]	–
p-NP-β-D-galactopyranoside[2]	–
p-NP-α-D-galactopyranoside[2]	–
p-NP-α-L-fucopyranoside[2]	–
p-NP-β-D-fucopyranoside[2]	–
p-NP-β-L-fucopyranoside[2]	–
p-NP-α-L-arabinopyranoside[2]	–
p-NP-α-L-arabinofuranoside[2]	–
p-NP-β-D-xylopyranoside[2]	–

[1] 10 mg ml^{-1}

[2] 3 mM.

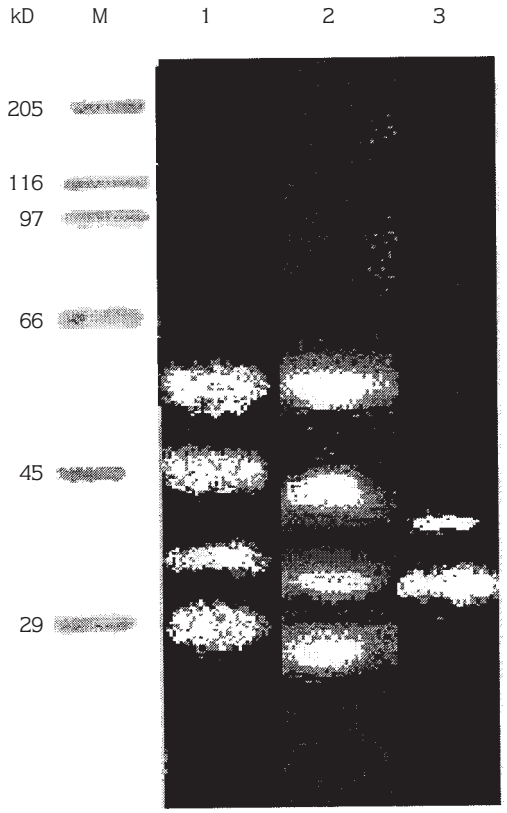

Figure 1. Zymograms of *T. fusca* BD25 endoxylanases separated by PAGE and developed as zymograms. Lines: Crude extracellular (lane 1) and intracellular (lane 2) endoxylanases separated by PAGE; purified endoxylanase (lane 3) separated by SDS-PAGE. M, molecular mass markers.

The effect of temperature on the endoxylanase activity was monitored by measuring the activity using the standard assay methods at specific temperatures ranging from 50 °C to 90°C. The optimum activity occurred at 70 °C for endoxylanase (see Figure 2b). The relative endoxylanase activity in the temperature range of 50 °C to 70 °C remained between 75 and 100 % of 100 % activity at 70 °C, but the relative endoxylanase activity decreased to 40 % at 90 °C.

Finally, the effect of temperature on the stability of the endoxylanase was determined by incubating the enzyme in the absence of substrates over a period of 27 hours within a temperature range of 50 °C to 80 °C. From the results (see Figure 3), it is seen that the endoxylanase was stable for 27 h at 50 °C, whilst at higher temperatures the stability of the endoxylanase was reduced. At 60 °C endoxylanase activity showed stability (92 %) for 16 h, however it showed a half-life of 8 h at 70 °C and 30 min at 80 °C in the absence of substrate at pH 8.0.

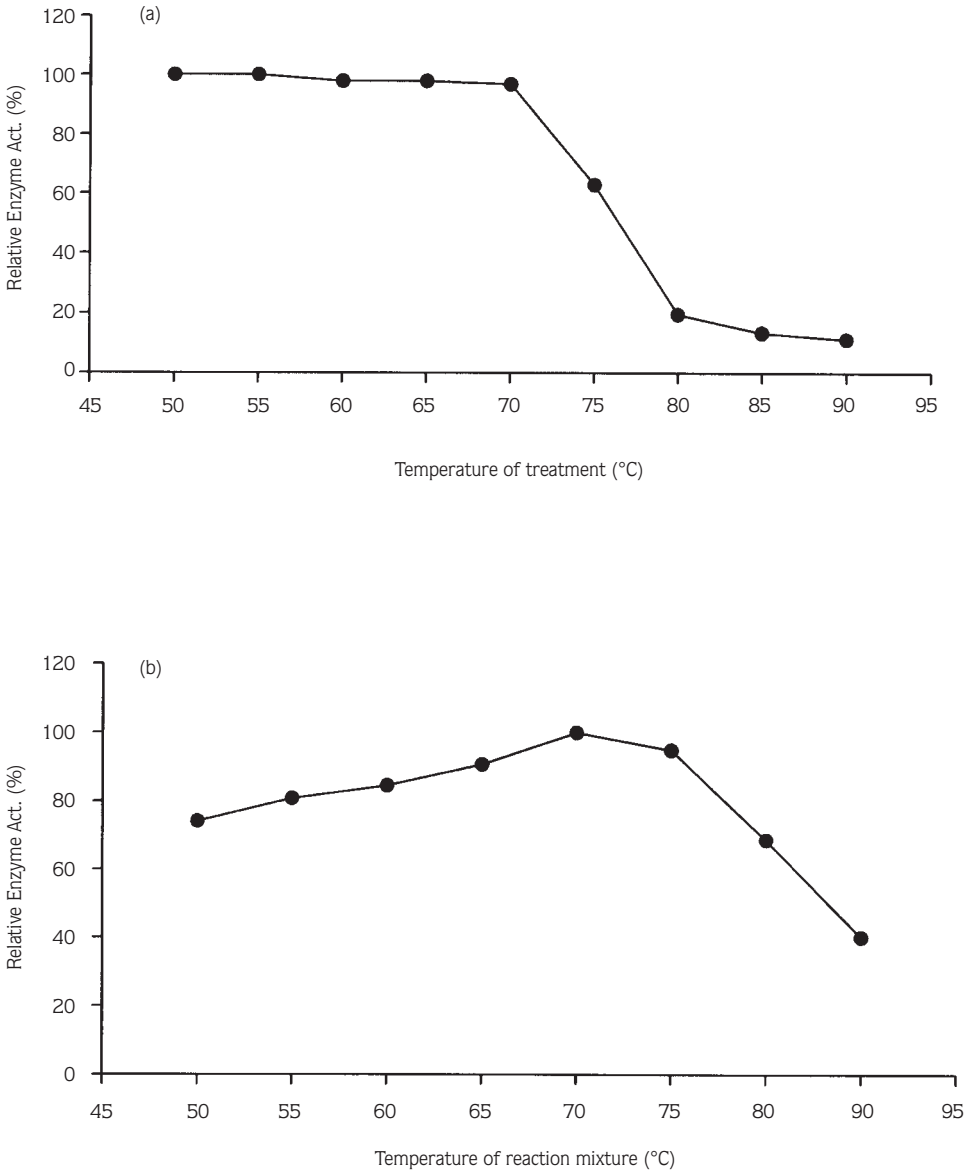

Figure 2. (a) The effect of temperature on the stability of endoxylanase (-●-). The enzyme supernatants were incubated for 15 min at each temperature prior to the enzyme assays using the standard assay method at 50 °C. (b) The effect of temperature on the activity of endoxylanase (-●-). Data are presented as means of three replicates. Standard deviations were ≤ 5 %, in all cases. The enzyme activities are expressed relative to the maximal value (12 U mg^{-1}).

pH studies of endoxylanase

The activity of the endoxylanase over a pH range of 3-12 was investigated using the standard assay methods. The optimum pH for the enzyme activity, as determined under these assay conditions was found to occur between a pH of 6.5 and 9.5 for endoxylanase (see Figure 4). The relative endoxylanase activity in this pH range (6.5 to 9.5) remained between 68 % and 72 % of the activity at pH 8.0 (17.027 U mg^{-1} protein), respectively.

Kinetic studies of endoxylanase

The affinity of *T. fusca* BD25 endoxylanase for the substrate oat spelt xylan was examined using the Lineweaver-Burk plot. The apparent K_m value for the crude endoxylanase preparations was 6.66 mg of oat spelt xylan ml^{-1}, while the V_{max} value was 2.5 µmol reducing sugar min^{-1} ml^{-1} (see Figure 5).

The inhibition effects of D-xylose, D-glucose, L-arabinose, xylobiose and cellobiose on the activity of endoxylanase as hydrolysis end-products of lignocellulose were also determined by

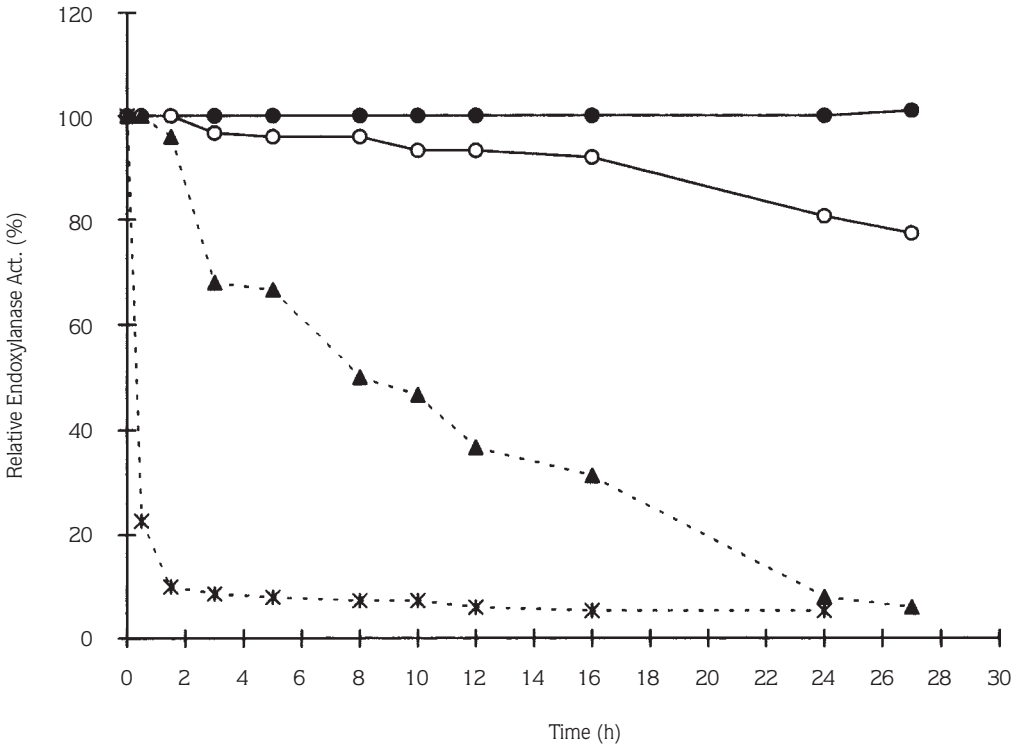

Figure 3. The effect of temperature on the stability of endoxylanase at 50 °C (-●-), 60 °C (-○-), 70 °C (-▲-), and 80 °C (-✶-) at pH 8.0.

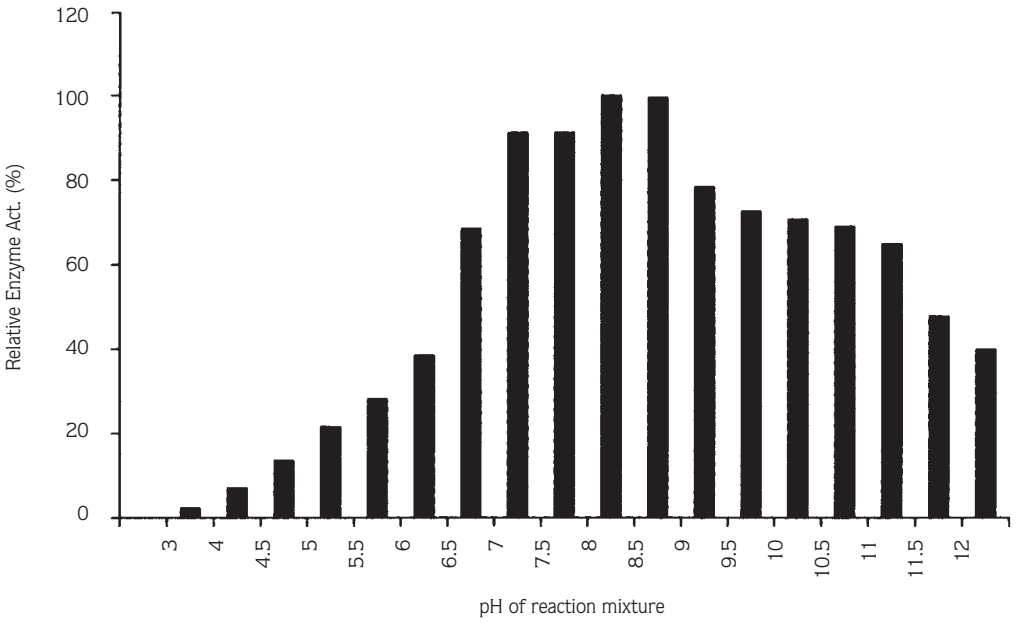

Figure 4. The effect of pH on the activity of endoxylanase. The reactions were monitored at each of the specified pH values using the Universal buffer ranging from 3.0 to 12.0. The enzyme activities are expressed relative to the maximal value (17.07 U mg^{-1}).

adding the appropriate concentration (0-30 mM) of sugars to the reaction mixture. No inhibition of endoxylanase activity appeared when xylose, glucose, arabinose or cellobiose (up to 30 mM) were added to the reaction mixture. In contrast, the addition of xylobiose (1 mM) to the reaction mixture of endoxylanase preparation resulted in approximately 25 % reduction in activity. Also, addition of supernatant fluids from cultures grown on xylan (reducing sugar concentration of 1 mg ml^{-1} xylose equivalent) to the reaction mixture resulted in approximately 30 % reduction in activity.

The effect of different metal ions and chemical agents on the activity of endoxylanase was determined by adding the appropriate salts (1 mM in final reaction mixture) to the reaction mixture. The enzymes retained full activity in the presence of citric acid, boric acid, diethylbarbituric acid, urea, sodium dodecyl sulphate (SDS), EDTA, Zn^{2+} and Mg^{2+}. Little inhibition of endoxylanase activity was caused by Ni^{2+} (5 %) and Li^{+} (7 %). The metals exhibiting a small stimulating effect on the activity of endoxylanase were Co^{2+} (25 %), Fe^{2+} (13 %), Fe^{3+} (18 %), Mn^{++} (11 %) and Cu^{2+} (15 %). Endoxylanase activity was completely inhibited by $HgCl_2$ (1 mM).

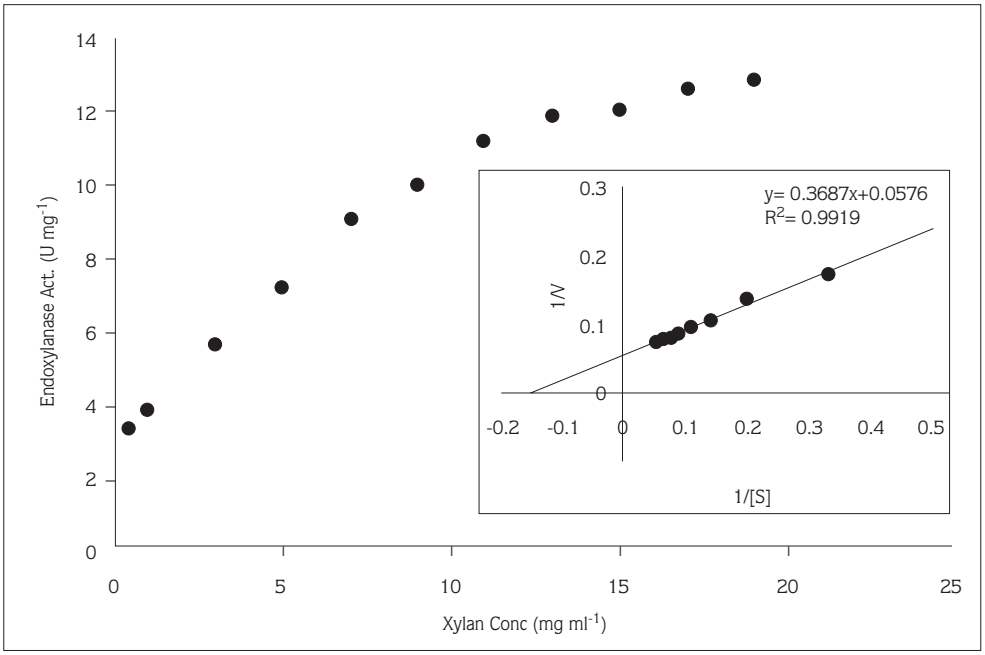

Figure 5. The effect of substrate concentrations on the activity of endoxylanase. The reactions were monitored at 50 °C and at pH 7.0 (100 mM phosphate buffer). Also shown is the Lineweaver-Burk plot. The data are presented as means ± SEM for triplicate measurements.

Discussion

Substrate specificity of endoxylanase

The purpose of this study was to investigate the endoxylanase activity produced by *T. fusca* BD25. A crude extracellular supernatant, such as would be used industrially was used for the determination of substrate specificity, activity conditions, reaction rates, stability and inhibitory studies.

The activity of crude and partially purified endoxylanase was tested on different substrates. Liberation of reducing sugars by endoxylanase was detected only when xylose polysaccharides were used. The highest activity was detected on birchwood xylan and it was approximately two fold higher than the activity obtained on the oat spelt xylan. The high activity on birchwood xylan could be explained by the remarkable solubilization properties of this substrate. The side chains have the positive effects of increasing the solubility of hemicelluloses in aqueous solution, thus facilitating the attacks of main chain-cleaving enzymes, such as endoxylanases. The action of individual endoxylanases is certainly highly dependent on the solubility and the presence and distribution of substituents in their substrate. Puls and Schuseil (22) reported that deacetylated

hemicellulose appears to have an increased molecular weight. The same authors concluded that this phenomenon could be explained by intermolecular aggregation as a result of the reduced degree of substitution. This aggregation renders the polysaccharide less susceptible to endoxylanase action. Therefore, the non-dissolving properties of oat spelt xylan could lead to lower detection of endoxylanase activity on this substrate. No hydrolytic activity was detected on the glucose polymers, such as cellulose and CMC, and other *p*-nitropheneyl-substituted monosaccharides by partially purified endoxylanase preparations (see Table 1).

Endoglucanase activity is unwanted in most biotechnological applications, such as biobleaching of pulp, because it degrades cellulose fibres and destroys pulp properties (23). Some of the endoxylanases attack not only xylan but also cellulose. Such enzymes have been obtained from *Aspergillus niger* (24), *Trichoderma viride* (23) and *Ceratocystis paradoxa* (25). It has been reported that the endoxylanases from *Humicola* sp. (26) and *Streptomyces reseiscleroticus* (*Chainia resea*) (27) not only attack xylan, but also exhibit endoglucanase and debranching arabinofuranosidase activities. The endoxylanases of *T. fusca* BD25 showed no endoglucanase activity when CMC and crystalline cellulose were used as substrate and also were not accompanied by β-xylosidase or α-L-arabinofuranosidase activities against *p*-NPX, *p*-NPA and other substrates (see Table 1).

Thermostability and the effects of temperature and pH on the activity of the endoxylanase from *T. fusca* BD25

The extracellular xylanolytic enzymes from actinomycetes are active at a higher pH range compared with fungal enzymes, and a number of enzymes with thermostable properties have been isolated from cultures of these microorganisms such as *Thermoascus aurantiacus* (28), *Melanocarpus albomyces* (29), a thermotolerant *Streptomyces* sp. (30), *Clostridium stercorarium* (31) and *S. thermoviolaceus* (23). The endoxylanase from *S. thermoviolaceus* was stable in the range of pH 5 to 9 at 50 °C for 30 min.

Endoxylanase activity from *T. fusca* BD25 also exhibited broad pH (6.0-9.5) and temperature (65-70 °C) optima similar to other actinomycetes (32,33), such as *S. roseiscleroticus* (27), *Microtetraspora flexuosa* SIIX (34), and *Bacillus* sp. (3, 35, 36).

Many endoxylanases purified from thermophilic fungi and bacteria are thermostable; the enzymes purified from *M. flexuosa* SIIX (34), *Streptomyces* sp. CH-M-1035 (37), *Bacillus* sp. (36), *Thermoascus aurantiacus* (38), *C. stercorarium* (31), *Bacillus stearothermophilus* and *Humicola lanuginosa* (39) have all been reported to have half-lives of between 10 and 90 min at 80 °C. Similarly, *T. fusca* BD25 endoxylanase demonstrated a half-life of 30 min at 80 °C.

Optimal activity of fungal and most bacterial endoxylanases, such as *Aspergillus nidulans* (40) and *Trichoderma coningii* G-39 (41) occurs at pH 4 to 6. Only a few bacterial enzymes have maximal levels of activity at pH values greater than 7.0. Actinomycete endoxylanases and endoglucanases from *T. fusca, T. curvata, T. chromogenes* and *Sacchoromonospora vridis* were generally similar, in that enzyme activities were optimal in the pH range of 5-8. The pH optima reported here for the endoxylanase activity from *T. fusca* BD25 conform to this general pattern.

Kinetic studies of endoxylanase from *T. fusca* BD25

Crude extracellular enzyme preparations were used throughout, and this can be justified since any process applications are unlikely to be based on purified enzymes. The crude endoxylanase from *T. fusca* BD25 showed affinity for oat spelt xylan and birchwood xylan, with K_m values of 6.66 mg ml^{-1} and 4 mg ml^{-1}, respectively. The specific activities of thermostable endoxylanases, which were purified from thermophilic fungi and bacteria range from 0.28 to 7,6 μmol min^{-1} mg^{-1} of protein, and the K_m values range from 0.15 to 50 mg xylan ml^{-1} (42). The K_m value (6.66 mg of oat spelt xylan ml^{-1} and 4 mg of birchwood xylan ml^{-1}) of endoxylanase from *T. fusca* BD25 is generally lower or equal compared with those of fungal and yeast endoxylanases, which range from 4 to 20 mg of xylan ml^{-1} (43). Also, the specific activity of the partially purified endoxylanase (48.96 μmol $min^{-1}mg^{-1}$ of protein) from *T. fusca* BD25 is greater than most of the other microorganisms. The endoxylanase A and B of *Clostridium acetobutylicum* had K_m values of 6.0 and 6.7 mg of larchwood xylan ml^{-1}, respectively (44). Also, endoxylanases from *Trichoderma longibranchiatum* (45) and *Bacillus* sp. (36) have been reported with K_m values of 10.14 mg xylan ml^{-1} and 9.2 mg xylan ml^{-1}, respectively. However, the endoxylanases from *S. lividans* and *S. sp*. KT-23 showed greater affinity for xylan, with K_m values of 0.78 mg ml^{-1} and 0.2 mg ml^{-1}, respectively (43).

The V_{max} value of the endoxylanase from *T. fusca* BD25 was 16.66 μmol of reducing sugar min^{-1} mg^{-1} which is greater than those reported for endoxylanase (4.02 μmol of reducing sugars min^{-1} mg^{-1} of protein) from *T. longibranchiatum* (45). However, the greater V_{max} values for endoxylanases A and B from *C. acetobutylicum* (44) and *Bacillus* sp. (36) have been reported as 22.4, 22.3 and 1954 μmol of reducing sugars min^{-1} mg of $protein^{-1}$, respectively.

At reducing sugar concentrations >1 mg ml^{-1} xylose equivalents, inhibition of endoxylanase activity occurred (30 %). It is well established that endoxylanases are subject to end-product inhibition. TLC analysis of the supernatant fluid used to inhibit endoxylanase activity revealed that xylose and small xylooligosaccharides were the major products of xylan hydrolysis by *T. fusca* BD25 supernatant enzymes (46). However, endoxylanase activity was not inhibited by the addition of D-xylose, D-glucose and L-arabinose or cellobiose. Actinomycete endoglucanase activity is generally end-product inhibited by cellobiose (47,48) and therefore, by analogy, xylobiose (25 % inhibition at 1 mM) and other small xylooligosaccharides may be responsible for the inhibition of endoxylanase activity described here. Furthermore, the failure of cellobiose to inhibit endoxylanase activity suggests that xylan hydrolysis was not due to the action of endoglucanase with broad substrate specificity (49, 50).

At a concentration of 1 mM, Hg^{2+}, Ni^{2+} and Li^{+} inhibited endoxylanase activity to varying levels (100 %, 5 % and 7 %, respectively). The activity of endoxylanase was unaffected by the presence of boric acid, citric acid, urea, SDS, EDTA, Zn^{2+} and Mg^{2+} (1 mM). Hg^{2+} is known to react with protein sulphydryl groups as well as histidine and tryptophan residues (51). *T. fusca* has been extensively studied as a source of cellulase activity, and comparison of the results presented here with those of other researchers illustrates both similarities and differences between endoglucanases and endoxylanases. Both contain sulphydryl groups in their active sites (52). The addition of EDTA did not affect the activity of endoxylanase, suggesting that no metals are needed for its enzymatic reactions.

From this study, optimum conditions for the activity of endoxylanase from *T. fusca* BD25 can be summarized as:

The extracellular endoxylanase activity from *T. fusca* BD25 remained stable up to 70 °C.

The extracellular endoxylanase activity from *T. fusca* BD25 showed a half life of 8 h at 70 °C and 30 min at 80 °C at pH 8.

The optimum pH and temperature for the activity of extracellular endoxylanase by *T. fusca* BD25 were at pH 8.0 and at 70 °C, respectively.

The apparent K_m value for the crude endoxylanase preparations was 6.66 mg of oat spelt xylan ml^{-1}, while V_{max} value was 2.5 μmol reducing sugar min^{-1} ml^{-1} .

The addition of xylobiose (1 mM) and supernatant fluids from cultures grown on xylan (reducing sugar concentration of 1 mg ml^{-1} xylose equivalent) to the reaction mixture of endoxylanase preparation resulted in approximately 25 % and 30 % reduction in activity, respectively.

The enzymes retained full activity in the presence of urea, SDS, EDTA, Zn^{2+} and Mg^{2+}. Endoxylanase activity was completely inhibited by $HgCl_2$ (1 mM).

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