Turk J Biol
33 (2009) 291-300

doi:10.3906/biy-0806-9

Co-operative actions and degradation analysis of purified lignocellulose-degrading enzymes from *Thermomonospora fusca* BD25 on wheat straw

Münir TUNCER[1], Andrew S. BALL[2]

[1]Mersin University, Faculty of Arts and Science, Department of Biology, Çiftlikköy, 33342 Mersin - TURKEY

[2]School of Biological Sciences, Flinders University of South Australia GPO box 2100,
Adelaide, South Australia 5001 – AUSTRALIA

Received: 13.06.2008

Abstract: In industrial hydrolysis processes the lignocellulose-degrading enzymes may have a potential application as an alternative to chemical treatments because they produce high specificity, mild reaction conditions, no toxic chemical hydrolysis inhibitors, and no loss of substrate due to chemical modifications. The amounts of reducing sugars released from the degradation of wheat straw by the action of endoxylanase, peroxidase, β-xylosidase, and α-L-arabinofuranosidase, which were produced by *T. fusca* BD25, were equal to 3.0%, 0.2%, 0.3%, and 0.2% hydrolysis (as xylose equivalents) of the substrate used, respectively, after 10 h of incubation. In the same conditions, the hydrolysis rate of endoglucanase, which was a commercial preparation from *Trichoderma viride* was 4.9%. However, in order, addition of peroxidase, endoglucanase, β-xylosidase, and α-L-arabinofuranosidase preparations to the endoxylanase preparation significantly enhanced the reducing sugar yields (1.33-, 1.52-, 1.61-, and 1.71-fold, respectively) and the hydrolysis of wheat straw (4.3%, 12.3%, 13.5%, and 14.7%, respectively). The concentrations of phenolic-compounds as lignin-equivalents released from wheat straw by the actions of lignocellulose-degrading enzyme preparations either acting alone or in combinations ranged from 2.5 to 129.3 μg mL^{-1} (as p-coumaric acid equivalents). The combination of purified lignocellulose-degrading enzymes significantly increased the degradation of straw 1.33- to 1.71-fold. The results highlight the role of lignocellulose-degrading enzymes in the degradation of wheat straw and suggest that the use of enzyme cocktails may significantly improve the hydrolysis of wheat straw in industrial processes.

Key words: Degradation, endoxylanase, lignocellulose, peroxidase, pulp, wheat straw

Thermomonospora fusca BD25 tarafından üretilen saflaştırılmış lignoselüloz-parçalayıcı enzimlerin buğday samanı üzerindeki kooperatif etkileri ve parçalanma ürünlerinin analizi

Özet: Endüstriyel hidroliz proseslerinde kimyasal muamelelere alternatif olarak, yüksek özgüllükleri, ılımlı reaksiyon koşulları, toksik kimyasal inhibitörlerin oluşmaması ve kimyasal değişimler nedeniyle substrat kayıplarına yol açmamalarından dolayı, lignoselüloz-parçalayıcı enzimlerin bir uygulama potansiyeli bulunabilir. *T. fusca* BD25 tarafından üretilen endoksilanaz, peroksidaz, β-ksilozidaz ve α-L-arabinofuranozidaz enzimlerinin buğday samanı ile 10 saatlik inkübasyonları sonucunda serbest bıraktıkları indirgenmiş şekerlerin (ksiloz eşdeğeri olarak) miktarı, kullanılan

substratın, sırasıyla, % 3,0; % 0,2; % 0,3 ve % 0,2 hidrolizine eşdeğerdir. *Trichoderma viride*'nin ticari enzim preparatı olan endoglukanaz enziminin aynı koşullardaki hidroliz oranı ise % 4,9'dur. Buna rağmen, endoksilanaz preparasyonuna peroksidaz, endoglukanaz, β-ksilozidaz ve α-L-arabinofuranozidaz enzimlerinin ilave edilmesi ise indirgenmiş şekerlerden oluşan ürünü (sırasıyla, 1,33; 1,52; 1,61 ve 1,71 kat) ve buğday samanının hidrolizi (sırasıyla, % 4,3; % 12,3; % 13,5 ve % 14,7) belirgin bir şekilde artırmıştır. Lignoselüloz-ayrıştırıcı enzimlerin tek başlarına veya bir kombinasyon halinde, lignin-eşdeğeri olarak buğday samanından serbest bıraktıkları fenolik-bileşiklerin (p-koumarik asit eşdeğeri olarak) derişimleri ise 2,5 ile 129,3 μg mL^{-1} arasında değişmiştir. Saflaştırılmış olan lignoselüloz-ayrıştırıcı enzimlerin oluşturduğu kombinasyon ise buğday samanının parçalanmasını (ayrıştırılmasını) 1,33 ile 1,71-kat arasında belirgin bir şekilde değiştirmiştir. Bu sonuçlar ise buğday samanının ayrıştırılmasında, lignoselüloz-ayrıştırıcı enzimlerin rolünü ortaya çıkarmakta ve endüstriyel proseslerde enzim-kokteylleri kullanılarak buğday samanı hidrolizinin belirgin bir şekilde artırılabileceğini göstermektedir.

Anahtar sözcükler: Ayrıştırma, endoksilanaz, lignoselüloz, peroksidaz, kağıt hamuru, saman

Introduction

Plant cell walls are the major reservoir of fixed carbon sources in nature. They have 3 major polymers: cellulose (30%-45% w/w), hemicellulose (25%-45% w/w), and lignin (15%-30% w/w) (1). Lignin is composed of phenylpropane monomeric units interconnected by a variety of carbon-carbon and ether linkages. These phenolic compounds may also be involved in cross-linking xylan molecules and in linking xylan to other polysaccharides (2,3). Therefore, lignin is the major factor responsible for limiting the rates of lignocellulose degradation. Nevertheless, in natural environments, lignin can be degraded by means of extracellular enzymes collectively called ligninases. Ligninases include lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13), phenol oxidases, H_2O_2-producing enzymes, laccases, and β-etherases (4-6).

A concerted action of ligninases with cellulose-degrading enzymes and hemicellulose-degrading enzymes systems complete the degradation of lignocellulose. The "lignin barrier" can be disrupted by the activity of lignin peroxidases, rendering the structure more susceptible to hemicellulose and cellulose attack. When xylan was selectively removed from the delignified fiber, the cellulose was more accessible to cellulolytic hydrolysis. However, similar pre-treatments with endoglucanases do not render the fiber more accessible to xylanases (7). Depolymerization of xylan is largely achieved through the action of endoxylanases, but these enzymes are often prevented from cleaving the xylan backbone due to the presence of substituents. Therefore, in many cases these must be removed before extensive degradation of the xylan backbone can occur. The debranching enzymes include acetylesterases, α-L-arabinofuranosidases, and α-glucuronidases.

The enzymes involved in the degradation of lignocellulose, such as endoxylanases, peroxidases, and endoglucanases, may also have applications in biotechnology as alternatives to chemical treatments because of the following advantages: high specificity, mild reaction conditions, no toxic chemical hydrolysis inhibitors (e.g. vanillin and furaldehyde), and no loss of substrate due to chemical modifications (8). The products of such enzymatic hydrolysis may be converted subsequently into liquid fuels, single-cell protein, solvents, and other chemicals by the selective use of specific fermentative microorganisms (9). This can also contribute to the elimination of agricultural wastes. An additional use for xylanolytic enzymes in particular could be for the treatment of cellulosic pulps for the removal or partial hydrolysis of residual xylans (9). Several studies have been undertaken where xylans have been removed by xylanolytic enzymes for the upgrading of dissolving pulps (9-14). The first scientific report of pulp bleaching using xylan-degrading enzymes was published by Viikari et al. (10). There have since been many reports on the efficiency of endoxylanase pre-treatment for bio-bleaching and a number of commercial products are now available. This concept of endoxylanase-aided bleaching has translated into an economically viable and environmentally friendly technology that is presently being used in several industrial mills (11).

It has been well established that extracellular peroxidases are an important part of the lignocellulose-degrading mechanism in fungi. Since

the production of extracellular peroxidases is a common trait amongst lignocellulolytic actinobacteria (formerly actinomycetes), such as *Thermomonospora* (reclassified as *Thermobifida*) (15) and *Streptomyces*, the possible involvement of this enzyme with the xylan-degrading enzymes of *T. fusca* BD25 and endoglucanase in the degradation of lignocellulose was examined.

This paper examines the degradation of ball-milled wheat straw by *T. fusca* BD25 enzymes. The substrate was hydrolyzed using different combinations of purified endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), lignin peroxidase (EC 1.11.1.14), β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37), and α-L-arabinofuranosidase (EC 3.2.1.55) produced by *T. fusca* BD25 and endo-1,4-β-glucanase (1,4-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) (a commercial preparation from *T. viride*). Degradation products from the addition of lignocellulose-degrading enzymes to ball-milled wheat straw were qualitatively characterized by HPLC and TLC.

Materials and methods

Growth and maintenance of *Thermomonospora fusca* BD25

The growth and maintenance conditions of the bacterial strain were performed as described previously (16,17).

Harvesting of culture supernatant fluids

Culture supernatant fluids were harvested as described previously (18).

Biomass measurement, protein estimation, and enzyme assays

Biomass measurement, protein estimation, and enzyme activities were determined as described previously (17,19). Endoxylanase and endoglucanase activities were assayed by the detection of reducing sugars from oat spelt xylan and carboxymethylcellulose, respectively. Reducing sugars were detected by the dinitrosalicylic acid (DNS) method by Miller (20). According to the calibration curve, which was used to calculate the amount of reducing sugars released from the ball-milled straw by lignocellulose-degrading enzymes, the lower detection limit of the DNS method was 0.01 mg of reducing sugars per milliliter. One unit (U) of endoxylanase and endoglucanase activities was defined as the amount of enzyme that released 1 mmol of reducing sugar (expressed as xylose or glucose equivalents, respectively) per minute under assay conditions.

β-xylosidase and α-L-arabinofuranosidase activities were assayed as described by Bachmann and McCarthy (21) and MacKenzie et al. (22), respectively. The substrates used were *p*-nitrophenyl-β-D-xylopyranoside (*p*-NPX, 5 mmol L^{-1}) and *p*-nitrophenyl-α-L-arabinofuranoside (*p*-NPA, 2 mmol L^{-1}) in 100 mmol L^{-1} phosphate buffer, pH 6.5, at 50 °C, respectively. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 mmol of *p*-nitrophenol per minute.

Peroxidase activity was assayed using 2,4-dichlorophenol (2,4-DCP) as the substrate. This method was adapted from the assay described by Ramachandra et al. (23). One unit of enzyme activity was defined as the amount of enzyme required for an increase an absorbance of 1 abs. unit min^{-1}. Enzyme and substrate controls were included in all assays.

Purification of lignocellulose-degrading enzymes from *T. fusca* BD25

The purification procedures for endoxylanase, β-xylosidase and α-L-arabinofuranosidase (18,19), and peroxidase (24) from *T. fusca* BD25 were described previously. Enzyme activities were determined at each step as described previously (17).

Degradation of ball-milled wheat straw by purified enzymes of *T. fusca* BD25

Purified enzyme preparations, i.e. those fractions showing single enzyme activity and single band produced by zymogram and SDS-PAGE analysis (18), were assessed for their contribution to ball-milled wheat straw degradation. The ball-milled wheat straw was washed twice with deionized water and once with 100 mmol L^{-1} phosphate buffer, pH 7.0, to remove the soluble reducing sugars and any freely soluble residual lignin. The wheat straw was then dried at 70 °C overnight. The dried ball-milled wheat straw (10 mg mL^{-1}) was placed in flasks in the presence of sodium azide (0.03%, w/v), H_2O_2 (50 mmol L^{-1}) and enzymes, either solely or in combinations of endoxylanase

(0.175 U mL^{-1}), peroxidase (0.02 U mL^{-1}), β-xylosidase (0.41 U mL^{-1}), α-L-arabinofuranosidase (0.053 U mL^{-1}), and endoglucanase (0.36 U mL^{-1}) in a final volume of 5 mL of phosphate buffer (100 mmol L^{-1}, pH 7.0). The amounts of enzyme used were based upon the amount of the enzyme preparations available. The amount of enzyme used also aimed to reflect the production ratios of lignocellulose-degrading enzymes in *T. fusca*, which are produced naturally. Control samples were also prepared with the same amount of denatured enzymes (achieved through pre-incubation at 100 °C for 10 min). Samples were incubated at 55 °C in an orbital shaker at 150 rev min^{-1} for 10 h. At 2 h intervals, 500 mL samples were removed from each of the flasks and then centrifuged at 12,000 ×g for 10 min prior to being stored at -20 °C.

The sample supernatant fluids were examined for the release of reducing sugars (as xylose equivalents) and lignin-equivalents, using DNS and Folin's phenol reagent (A_{750}) (25), respectively. Reducing sugars and lignin-equivalents were calculated as a lignin component from standard curves of xylose and *p*-coumaric acid, respectively. Wheat straw contains (in w/v) 31.93% glucan, 18.95% xylan, 0.15% mannan, 2.08% arabinan, and 0.56% galactan (26).

Preparation of xylo-oligosaccaharides and TLC of solubilized degradation products

Preparation of xylo-oligosaccaharides and separation of the sugars, released by lignocellulolytic enzymes of *T. fusca* BD25 from ball-milled wheat straw by TLC techniques, were performed as described previously (18).

HPLC of solubilized degradation products

The sugars released by the lignocellulolytic enzymes of *T. fusca* BD25 from ball-milled wheat straw were separated by chromatography in a Beckman System Gold HPLC (Beckman Coulter Ltd, High Wycombe, UK) equipped with a reverse-phase column of Partisil 10 PAC (4.6 mm id × 25 cm; Capital HPLC Ltd, West Lothian, UK) and a 50 µL sampling loop. The mobile phase consisted of acetonitrile:water (80:20 by vol.). Sugar peaks were screened for their refraction spectra using a 156 Refractive Index Detector (Beckman Coulter Ltd, High Wycombe, UK) and their retention times were compared with the standards xylose, glucose, arabinose, xylobiose, xylotriose, and xylotetraose (18).

Results

Degradation of ball-milled wheat straw by lignocellulose-degrading enzymes

Ball-milled wheat straw was incubated with purified endoxylanase, peroxidase, β-xylosidase, and α-L-arabinofuranosidase from *T. fusca* BD25 and endoglucanase from *T. viride*. After a 10 h incubation, which was the end of the linear part of reducing sugar production, endoxylanase, on its own, liberated 0.30 mg of reducing sugar per milliliter, corresponding to approx. 3% hydrolysis (as xylose equivalents) of the total substrate, while incubation with peroxidase, β-xylosidase, and α-L-arabinofuranosidase alone produced lower concentrations of reducing sugar (0.02, 0.03, and 0.02 mg of reducing sugar per milliliter, respectively). However, endoglucanase, on its own, liberated 0.49 mg of reducing sugar per milliliter, corresponding to approx. 4.9% hydrolysis (as xylose equivalents) of the total substrate (Table 1).

Despite the inefficient hydrolysis by peroxidase, β-xylosidase, and α-L-arabinofuranosidase when each enzyme was acting alone, the addition of peroxidase and endoglucanase preparations to endoxylanase significantly enhanced (1.52-fold) the hydrolysis (12.3% hydrolysis, as xylose equivalents) of the substrate used and resulted in a greater liberation of reducing sugars (1.23 mg mL^{-1}). The addition of peroxidase to the endoxylanase preparation also increased the total reducing sugar yield (0.43 mg mL^{-1}) by 1.33-fold (4.3% hydrolysis). Moreover, the addition of β-xylosidase to the endoxylanase-peroxidase-endoglucanase preparation increased the total reducing sugar yield (1.35 mg mL^{-1}) by 1.61-fold (13.5% hydrolysis). Finally, the combination of purified endoxylanase, peroxidase, endoglucanase, β-xylosidase, and α-L-arabinofuranosidase preparation produced the greatest reducing sugar yield (1.47 mg mL^{-1}; 14.7% hydrolysis) and enhanced the total reducing sugar yield by 1.71-fold.

The release of aromatic compounds as lignin-equivalents from ball-milled wheat straw by the lignocellulose-degrading enzymes showed a corresponding increase with the release of reducing

Table 1. Synergistic action of lignocellulose-degrading enzymes from *T. fusca* BD25 on reducing sugar yields and released lignin-equivalents from ball-milled wheat straw. Ball-milled wheat straw (10 mg mL^{-1}) was incubated alone or in combinations of endoxylanase (0.175 U mL^{-1}), peroxidase (0.02 U mL^{-1}), β-xylosidase (0.41 U mL^{-1}), α-L-arabinofuranosidase (0.053 U mL^{-1}) and endoglucanase (0.36 U mL^{-1} from *T. viride*) in the presence of sodium azide (0.03%, w/v) and H_2O_2 (50 mmol L^{-1}) in a final volume of 5 mL of phosphate buffer (100 mmol L^{-1}, pH 7.0) for 10 h at 55 °C at 150 rev min^{-1}.

Enzyme(s) preparations	Reducing sugar yields*		Enhancement fold†	Released Lignin-Equivalents*		Enhancement fold†
	Observed yield	Expected yield		Observed yield	Expected yield	
	(mg of xylose equivalents per mL)			(μg of *p*-coumaric acid equivalents per mL)		
Endoxylanase (X)	0.30 (±0.015)	–	–	23.8 (±0.346)	–	–
Peroxidase (P)	0.02 (±0.010)	–	–	34.7 (±0.520)	–	–
Endoglucanase (C)	0.49 (±0.020)	–	–	20.3 (±0.577)	–	–
β-Xylosidase (B)	0.03 (±0.006)	–	–	2.5 (±0.500)	–	–
α-L-Arabinofuranosidase (A)	0.02 (±0.010)	–	–	8.2 (±1.587)	–	–
X + P	0.43 (±0.026)	0.32 (±0.016)	1.33‡	67.0 (±1.00)	58.5 (±0.541)	1.14**
X + P + C	1.23 (±0.015)	0.81 (±0.018)	1.52§	99.3 (±2.082)	78.8 (±0.901)	1.26§
X + P + C + B	1.35 (±0.020)	0.84 (±0.022)	1.61§	110.5 (±1.803)	81.3 (±1.173)	1.36§
X + P + C + B + A	1.47 (±0.030)	0.86 (±0.019)	1.71§	129.3 (±3.055)	89.5 (±2.050)	1.44§
Control	0.01 (±0.006)	–	–	2.0 (±0.500)	–	–

* Data presented as mean values of 3 measurements with standard deviations in brackets. The lower detection limits of the DNS method and Folin phenol reagent were 0.01 mg of reducing sugars per milliliter and 0.67 μg of *p*-coumaric acid equivalents per milliliter, respectively.
† Increase in the amount of reducing sugar and lignin-equivalents released compared with the amount expected from the action of each enzyme alone
‡ $P < 0.05$ (t-test), § $P \leq 0.001$ (t-test), ** $P < 0.01$ (t-test)

sugars (Table 1). After the 10 h incubation of ball-milled wheat straw with endoxylanase, peroxidase, endoglucanase, β-xylosidase and α-L-arabinofuranosidase with each enzyme acting alone, the release of aromatics was detected at 23.8, 34.7, 20.3, 2.5, and 8.2 mg of lignin-equivalents per milliliter respectively (Table 1). Despite the efficient release of aromatic compounds by the actions of endoxylanase, peroxidase and endoglucanase with each enzyme acting alone, the addition of peroxidase to the endoxylanase preparation significantly enhanced the amount of released aromatic compounds (67 mg mL^{-1}) by 1.14-fold. In contrast, peroxidase did not release a significant amount of reducing sugars (0.02 mg mL^{-1}) from straw; it released 34.7 mg mL^{-1} lignin-equivalents from the substrate when acting alone.

In parallel to the reducing sugar production by lignocellulose-degrading enzyme preparations from straw, the addition of peroxidase to the endoxylanase-endoglucanase preparations also increased the total released aromatics (99.3 mg mL^{-1}) by 1.26-fold. Also the addition of β-xylosidase to the endoxylanase-peroxidase-endoglucanase preparation increased the total released lignin-equivalents (110.5 mg mL^{-1}) by 1.36-fold. Finally, the combination of the purified endoxylanase, peroxidase, endoglucanase, β-xylosidase, and α-L-arabinofuranosidase preparations produced the greatest aromatic compounds yield (129.3 mg mL^{-1}) and enhanced the total released aromatics yield by 1.44-fold. However, the β-xylosidase and α-L-arabinofuranosidase preparations from *T. fusca* BD25 alone did not release a significant amount of reducing sugar and aromatic compounds from the ball-milled wheat straw (Table 1).

Identification of degradation products

Products from the hydrolysis of the ball-milled wheat straw using the same amount of endoxylanase, peroxidase, endoglucanase, β-xylosidase, and α-L-arabinofuranosidase, either alone or in combinations,

were analyzed by HPLC. Samples were also analyzed by TLC to confirm the product patterns; the same results were obtained (Table 2). Xylobiose and substituted-xylotetrose (X_{4+}) were identified as the main saccharification products released using the action of endoxylanase alone on ball-milled wheat straw (Figure 1a and Table 2).

Peroxidase, β-xylosidase, and α-L-arabinofuranosidase liberated neither small oligosaccharides nor monosaccharides from the ball-milled wheat straw. Despite inefficient hydrolysis of ball-milled wheat straw by individual activities of peroxidase, β-xylosidase, and α-L-arabinofuranosidase, endoglucanase was able to release glucose with an oligosaccharide different from the corresponding control, with a retention time identical to xylotriose (Figure 1b).

Hydrolysis of ball-milled wheat straw with the enzyme cocktail preparation of endoxylanase-peroxidase produced xylobiose, xylotriose, and substituted-xylotetrose (Table 2). When the enzyme cocktail contained endoxylanase-peroxidase-endoglucanase the hydrolysis products were glucose, xylobiose, xylotriose and substituted-xylotetrose (Figure 1c). In the presence of 4 purified enzymes, endoxylanase, peroxidase, endoglucanase, and β-xylosidase, the hydrolysis products from straw were glucose, xylobiose, xylotriose and substituted-xylotetrose with an extra peak corresponding to xylose (Figure 1d). Finally, the addition of α-L-arabinofuranosidase to these 4 purified enzyme preparations also changed the pattern of degradation products and resulted in an extra peak corresponding to arabinose (Figure 1e).

Discussion

Degradation of ball-milled wheat straw by lignocellulose-degrading enzymes

The rates of hydrolysis of ball-milled wheat straw by endoxylanase and endoglucanase were 3% and 4.9%, respectively, although the total percentage of available substrate from hydrolysis (assuming a hemicellulose and cellulose content for wheat straw of 30% and 45%, respectively) was similar (10% and 10.9%, respectively). The yield of reducing sugars through the degradation of straw by endoxylanase and endoglucanase activities suggests that these enzymes are capable of efficiently hydrolyzing the substrate. Moreover, the enzyme cocktails of

Table 2. HPLC and TLC analysis of different solubilized sugars released by the synergistic action of lignocellulose-degrading enzymes from *T. fusca* BD25 on ball-milled wheat straw. Ball-milled wheat straw was incubated as described in Table 1 and the Materials and Methods.

	Sugar*	X_1	A	G	X_2	X_3	X_{4+}
	Retention time (min)†	1.95	2.05	2.33	2.6	3.25	4.25
Enzyme(s) preparations	R_f value‡	(100)	(89.5)	(85.6)	(76.4)	(52.4)	(12.5)
Endoxylanase (X)		–	–	–	+	–	+
Peroxidase (P)		–	–	–	–	–	–
Endoglucanase from *T. viride* (C)		–	–	+	–	+	–
β-Xylosidase (B)		–	–	–	–	–	–
α-L-Arabinofuranosidase (A)		–	–	–	–	–	–
X + P		–	–	–	+	+	+
X + P + C		–	–	+	+	+	+
X + P + C + B		+	–	+	+	+	+
X + P + C + B + A		+	+	+	+	+	+
Control		–	–	–	–	–	–

* X_1, xylose; A, arabinose; G, glucose; X_2, xylobiose; X_3, xylotriose and X_{4+}, substituted-xylotetrose

† For HPLC separation conditions see Materials and Methods.

‡ R_f values by TLC were determined relative to D-xylose (100). + Sugar present, – sugar absent.

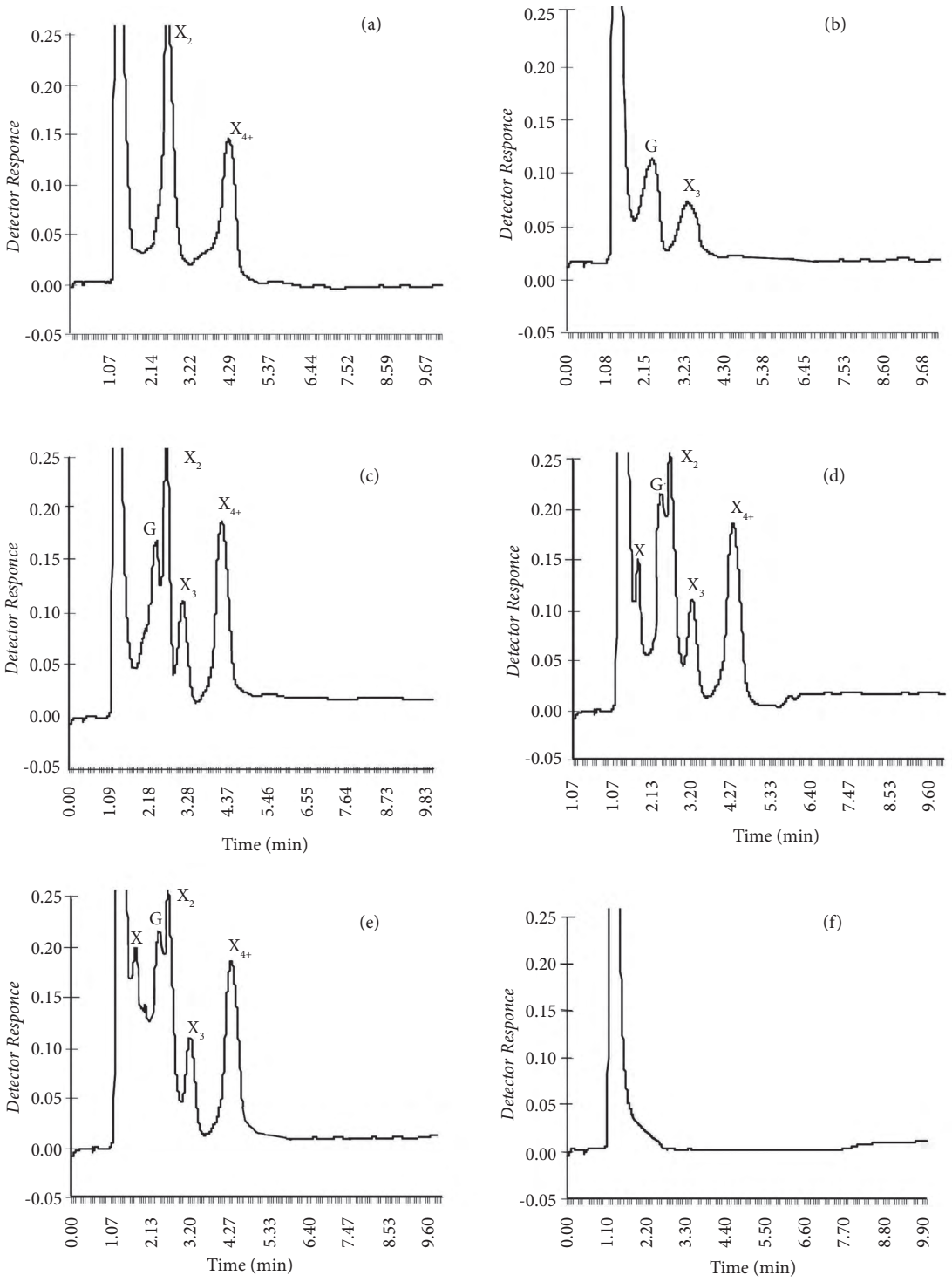

Figure 1. HPLC profiles of ball-milled wheat straw degradation by purified enzymes from *T. fusca* BD25. Samples of ball-milled wheat straw (10 mg mL^{-1}) were incubated in the presence of only endoxylanase (a); endoglucanase (from *T. viride*) (b); endoxylanase, peroxidase, and endoglucanase (c); endoxylanase, peroxidase, endoglucanase, and β-xylosidase (d); endoxylanase, peroxidase, endoglucanase, β-xylosidase, and α-L-arabinofuranosidase (e) and ball-milled wheat straw only; control (f). Peaks were identified as A, arabinose; G, glucose; X, xylose; X_2, xylobiose; X_3, xylotriose; X_{4+}, substituted xylotetrose.

endoxylanase-peroxidase and endoxylanase-peroxidase-endoglucanase preparations significantly enhanced the reducing sugar production. This indicates either that a soluble phenolic substrate must be formed by endoxylanase and endoglucanase before the peroxidase can work, or that the peroxidase may also be capable of releasing phenolic compounds from lignocellulose polymers. The interaction between ferulic acid esterase from *Aspergillus oryzae* and endoxylanases from *Trichoderma reesei* has been reported with increased production of phenolic acids from wheat straw xylooligosaccharides (27). Similarly, ferulic acid esterase from *Penicillilum pinophilum* (28), *Schizophyllium commune* (29), and *Streptomyces olivochromogenes* (30) has been reported with the liberation of a significant amount of ferulic (or coumaric) acid from wheat bran or grass cell walls only in the presence of endoxylanase.

The presence of peroxidase, β-xylosidase, and α-L-arabinofuranosidase in enzyme preparation allows for more efficient hydrolysis of ball-milled wheat straw. An important role for peroxidase and α-L-arabinofuranosidase in plant cell wall degradation can be envisaged, since there is good evidence that the arabinose side groups on the xylan chain are involved in cross-linking between lignin and hemicellulose, through feruloyl and *p*-coumaryl residues of lignin (31). The observation in this study that the addition of purified peroxidase and α-L-arabinofuranosidase from *T. fusca* BD25 to the endoxylanase-endoglucanase preparation enhances the saccharification of ball-milled wheat straw is in agreement with this hypothesis and extends a previous description of synergy between α-L-arabinofuranosidase from *Ruminococcus albus* 8 and other glucanases in the release of sugars from alfalfa cell wall material (32). Furthermore, the synergistic action of α-L-arabinofuranosidase and endoxylanase from *Aspergillus awamori* has been reported to release feruloyl L-arabinose and *p*-coumaryl L-arabinose from oat-spelt xylan and wheat straw, with the α-L-arabinofuranosidase alone having the ability to release a substantial portion (42%) of feruloyl L-arabinose from intact wheat straw arabinoxylan (33). In addition, synergistic interactions between α-L-arabinofuranosidase and endoxylanase in the hydrolysis of arabinoxylan have been reported previously and include those between relevant enzymes from *R. albus* 8 (32), *T. fusca* BD21 (34), and *T. fusca* BD25 (18). In the case of *Penicillium capsulatum,* the amount of arabinose liberated from feruloylxylan by α-L-arabinofuranosidase was enhanced by prior treatment of the substrate with endoxylanase (which did not itself liberate arabinose), and was even further enhanced by prior treatment with endoxylanase and ferulic acid esterase (35). Thus, not only are substituted xylooligomers better substrates than the polymer for this α-L-arabinofuranosidase, the preferred substrates are arabino-xylooligomers in which the arabinose itself is not substituted by ferulic acid.

The enhancement of released aromatic compounds from ball-milled wheat straw by the endoxylanase-peroxidase preparation suggests that these enzymes may hydrolyze some of the bonds between lignin and hemicellulose and enhance the accessibility of the endoxylanase. These observations suggest that the endoxylanase and peroxidase act cooperatively on the lignocellulose polymer and are capable of releasing reducing sugars and phenolic compounds from the substrate. The results indicate that a significant increase in degradation can be achieved with the cooperative actions of lignocellulose-degrading enzymes from *T. fusca*. This enhanced activity using cooperative enzymes confirms a potentially wider role for the enzyme combinations in industrial applications (11,12). These results suggest that more efficient hydrolysis of lignocellulose by *T. fusca* BD25 requires the cooperative actions of lignocellulose-degrading enzymes.

Identification of degradation products

Xylobiose and substituted-xylotetrose were found to be the main products released through the action of endoxylanase alone. The absence of detectable amounts of arabinose and xylose by the endoxylanase activity from ball-milled wheat straw confirms the endo-type mode of action for this enzyme, without any exo-type or debranching arabinofuranosidase activities on the substrate similar to those from *Microtetraspora flexuosa* SIIX (36). Furthermore, no xylose or arabinose was detected until the addition of β-xylosidase and α-L-arabinofuranosidase, indicating

that β-xylosidase hydrolyzed the xylo-oligosaccharides into monomers while α-L-arabinofuranosidase released arabinose from substituted-xylan and xylo-oligosaccharides.

The addition of β-xylosidase to the endoxylanase-peroxidase-endoglucanase cocktail resulted in the release of xylose, glucose, xylobiose, xylotriose, and the substituted-xylotetrose. This suggests that the β-xylosidase enzyme degraded the xylo-oligosaccharides by removing terminal xylose residues, presumably from the non-reducing end of the chain. Therefore, the possible inhibition effects of xylo-oligosaccharides on the endoxylanase activity were relieved by the action of β-xylosidase and resulted in a greater hydrolysis of substrate. The activities of the β-xylosidase were therefore consistent with it being an exoxylanase capable of acting on certain xylo-oligosaccharides. Synergism between endoxylanase and β-xylosidase of *T. curvata* (37), *T. fusca* BD21 (34), and *T. fusca* BD25 (18) in the hydrolysis of insoluble xylan has been reported previously.

Previous studies on the saccharification of wheat straw by actinobacteria have also shown that xylose was the major product (38), and glucose was not detected on TLC plates, although glucose was detected using a glucose oxidase test by Ball and McCarthy (39).

The presence of all 5 purified enzymes resulted in the production of xylose, arabinose, glucose, xylobiose, xylotriose, and substituted-xylotetrose. The proposed action of β-xylosidase and α-L-arabinofuranosidase suggested that these enzymes display synergy with endoxylanase with respect to xylan degradation. Endoxylanases should generate free chain ends upon which the β-xylosidase may act, while the debranching activity of α-L-arabinofuranosidase may remove substitute arabinose that may otherwise block the endoxylanase and peroxidase progress. These findings indicate that the use of enzyme cocktails may significantly improve the hydrolysis of straw in industrial processes.

Corresponding author:

Münir TUNCER

Mersin Üniversitesi,

Fen-Edebiyat Fakültesi,

Biyoloji Bölümü, Çiftlikköy,

33342 Mersin - TURKEY

E-mail: munir.tuncer@gmail.com

References

1. Betts WB, Dart RK, Ball AS et al. Biosynthesis and structure of lignocellulose. In: Betts WB. ed. Biodegradation, natural and synthetic materials. Springer-Verlag, London; 1992: pp 139-156.
2. Ramachandra M, Crawford DL, Pometto III AL. Extracellular enzyme activities during lignocellulose degradation by *Streptomyces* spp.: A comparative study of wild-type and genetically manipulated strains. Applied and Environmental Microbiology 53: 2754-2760, 1987.
3. Joseleau JP, Comtat J, Ruel K. Chemical structure of xylans and their interaction in the plant cell walls. In Visser J, Beldman G, Kusters-van-Someren MA et al. (eds): Xylans and xylanases. Elsevier, Amsterdam, London, New York, Tokyo; 1992: pp 1-15.
4. Kirk TK, Farrell RL. Enzymatic combustion: The microbial degradation of lignin. Annual Review of Microbiology 41: 465-505, 1987.
5. Tien M. Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. CRC Critical Review of Microbiology 15: 141-168, 1987.
6. Eriksson K-EL, Blanchette RA, Ander P. Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, NewYork; 1990: p. 416.
7. Sinner M, Parameswaran N, Yamazaki N et al. Specific enzymatic degradation of polysaccharides in delignified wood cell walls. Applied Polymer Symposium 28: 993-1024, 1976.
8. Yu EKC, Saddler JN. Biomass conversion to butanediol by simultaneous saccharification and fermentation. Trends in Biotechnology 3: 100-104, 1985.
9. Biely P. Microbial xylanolytic systems. Trends in Biotechnology 3: 286-290, 1985.
10. Viikari L, Pauna M, Kantelinen A et al. M. Bleaching with enzymes. In: Proceedings of the third international conference on biotechnology in the pulp and paper industry. Stockholm, 1986: pp. 67-69.
11. Viikari L, Kantelinen A, Sundquist J et al. Xylanases in bleaching: From an idea to the industry. FEMS Microbiology Reviews 13: 335-350, 1994.

12. Nissen AM, Anker L, Munk N et al. Xylanases for the pulp and paper industry. In: Visser J, Beldman G, Kusters-van-Someren MA et al. eds. Xylans and xylanases. Elsevier, Amsterdam, London, New York, Tokyo; 1992: pp 325-337.

13. Senior DJ, Hamilton J, Bernier RL Jr. Use of *Streptomyces lividans* xylanase for bleaching of kraft pulps. In: Visser J, Beldman G, Kusters-van-Someren MA et al. eds. Xylans and xylanases. Elsevier, Amsterdam, London, New York, Tokyo; 1992: pp 555-558.

14. Reid ID, Paice MG. Effects of manganese peroxidase on residual lignin of softwood kraft pulp. Applied and Environmental Microbiology 64: 2273-2274, 1998.

15. Zhang ZS, Wang Y, Ruan JS. Reclassification of *Thermomonospora* and *Microtetraspora*. International Journal of Systematic Bacteriology 48: 411-422, 1998.

16. Tuncer M, Rob A, Ball AS et al. Optimization of production of extracellular non-haem peroxidases by *Thermomonospora fusca* BD25 in aerobic bio-reactor conditions. Biochemical Society Transactions 25: S 65, 1997.

17. Tuncer M, Rob A, Ball AS et al. Optimisation of extracellular lignocellulolytic enzyme production by a thermophilic actinomycete *Thermomonospora fusca* BD25. Enzyme and Microbial Technology 25: 38-47, 1999.

18. Tuncer M, Ball AS. Co-operative actions and degradation analysis of purified xylan-degrading enzymes from *Thermomonospora fusca* BD25 on oat-spelt xylan. Journal of Applied Microbiology 94: 1030-1035, 2003.

19. Tuncer M, Ball AS. Purification and partial characterization of a-L-arabinofuranosidase produced by *Thermomonospora fusca* BD25**.** Folia Microbiologica 48: 168-172, 2003.

20. Miller GL. Use of dinitroslisylic acid reagent for determination of reducing sugar. Analytical Chemistry 31: 426-428, 1959.

21. Bachmann SL, McCarthy AJ. Prufication and characterization of a thermostable b-Xylosidase from *Thermomonospora fusca*. Journal of General Microbiology 135: 293-299, 1989.

22. MacKenzie CR, Bilous D, Schnieder H et al. Induction of cellulolytic and xylanolytic enzyme systems in *Streptomyces spp.* Applied and Environmental Microbiology 53: 2835-2839, 1987.

23. Ramachandra M, Crawford DL, Hertel G. Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete *Streptomyces viridosporus*. Applied and Environmental Microbiology 54: 3057-3063, 1988.

24. Rob A, Ball AS, Tuncer M et al. Thermostable novel non-haem extracellular glycosylated peroxidase from *Thermomonospora fusca* BD25. Biotechnology and Applied Biochemistry. 24: 161-170, 1996.

25. Lowry OH, Rosebrough NJ, Farr AL et al. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-275, 1951.

26. Puls J, Schuseil J. Chemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis. In: Coughlan MP, Hazlewood GP eds. Hemicellulose and hemicellulases. Portland Press, London; 1993: pp. 1-27.

27. Tenkanen M, Schuseil J, Puls J et al. Production, purification and characterization of an esterase liberating phenolic acids from lignocellulosics. Journal of Biotechnology 18: 69-84, 1991.

28. Castanares A, McCrea SI, Wood TM. Purification and properties of a feruloyl/*p*-coumaroyl esterase from the fungus *Penicillium pinophilum*. Enzyme and Microbial Technology 14: 875-884, 1992.

29. MacKenzie CR, Bilous D. Ferulic acid esterase activity from *Schizophillum commune*. Applied and Environmental Microbiology 54: 1170-1173, 1988.

30. Faulds CB, Williamson G. The purification and characterization of 4-hydroxy-3-methoxycinnamic (ferulic) acid esterase from *Streptomyces olivochromogenes*. Journal of General Microbiology 137: 2339-2345, 1991.

31. Mueller-Harvey I, Hartley RD, Harris PJ et al. Linkage of *p*-coumaryl and feruloyl groups to cell wall polysaccharides of barley straw. Carbohydrate Research 148: 71-85, 1986.

32. Greve LC, Labavitch JM, Hungate RE. a-L-arabinofuranosidases from *Ruminococcus albus* 8: purification and possible role in hydrolysis of alfalfa cell wall. Applied and Environmental Microbiology 47: 1135-1140, 1984.

33. Wood TM, McCrae SI. Arabinoxylan-degrading enzyme-system of the fungus *Aspergillus awamori*: purification and properties of an α-L-arabinofuranosidase. Applied Microbiology and Biotechnology 45: 538-545, 1996.

34. Bachmann SL, McCarthy AJ. Purification and co-operative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. Applied and Environmental Microbiology 57: 2121-2130, 1991.

35. Filho EXF, Puls J, Coughlan MP. Purification and characterisation of two arabinofuranosidases from solid-state cultures of the fungus *Penicillium capsulatum*. Applied and Environmental Microbiology 62: 168-173, 1996.

36. Berens S, Kaspari H, Klemme JH. Purification and characterization of 2 different xylanases from the thermophilic actinomycete *Microtetraspora flexuosa* SIIX. Antonie Van Leeuwenhoek, International Journal of Gen. Mol. Microbiol. 69: 235-241, 1996.

37. Stutzenberger FJ, Bodine AB. Thermostable b-xylosidase from *Thermomonospora curvata*. Journal of Industrial Microbiology and Biotechnology 20: 55-60, 1998.

38. Ball AS, McCarthy AJ. Production and properties of xylanases from actinomycetes. Journal of Applied Bacteriology 66: 439-444, 1989.

39. Ball AS, McCarthy AJ. Saccharification of straw by actinomycete enzymes. Journal of General Microbiology 134: 2139-2147, 1988.