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A new xylanase from a *Trichoderma harzianum* strain

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A new xylanase (XYL2) was purified from solid-state cultures of *Trichoderma harzianum* strain C by ultrafiltration and gel filtration. SDS-PAGE of the xylanase showed an apparent homogeneity and molecular weight of 18 kDa. It had the highest activity at pH 5.0 and 45°C and was stable at 50°C and pH 5.0 up to 4 h xylanase. XYL2 had a low K_m with insoluble oat spelt xylan as substrate. Compared to the amino acid composition of xylanases from *Trichoderma* spp, xylanase XYL2 presented a high content of glutamate/glutamine, phenylalanine and cysteine, and a low content of serine. Xylanase XYL2 improved the delignification and selectivity of unbleached hardwood kraft pulp.

Keywords: *Trichoderma*; xylan; xylanase; characterization

Xylans are major components of renewable plant biomass. A wide variety of fungi, including *Trichoderma* spp, produce xylan-degrading enzyme systems [1,13,25,29]. One of the most promising approaches in pulp prebleaching is the use of these enzymes in order to decrease the liberation of chlorinated phenolic compounds and their subsequent environmental problems [11]. In this paper, we report the purification, characterization and application in pulp prebleaching of a xylanase (XYL2) isolated from solid-state cultures of *Trichoderma harzianum* strain C.

T. harzianum strain C was kindly provided by CJ Ulhoa (University of Goias, Brazil). The fungus was maintained at 4°C after growth for 7 days in MYG medium (0.2% malt extract, 0.2% yeast extract, 2% glucose and 2% agar) at 28°C. For production of xylanase activity, the fungus was cultured at 28°C for 7 days in a solid-state medium containing wheat bran as the carbon source [21,26]. The crude extract obtained from extraction procedure [21] was filtered and used for purification of xylanase XYL2.

Xylanase activity was determined by mixing 100 μl of enzyme solution with 200 μl of oat spelt xylan (1%, w/v) in 100 mM sodium acetate buffer, pH 5.0 at 50°C for 30 min. The enzyme activity was expressed as μmol product formed min^{-1} ml^{-1} enzyme solution, ie, as IU ml^{-1} and IU mg^{-1} protein. β-Glucanase, carboxymethyl-cellulase, β-mannanase, β-xylosidase, α-arabinofuranosidase, β-glucosidase, β-mannosidase and filter paper degrading enzyme (FPase) activities were determined as reported elsewhere [21]. The reducing sugar released was measured using the dinitrosalicylic reagent method [17]. The activity of the purified xylanase was also performed in the presence of reducing agents (β-mercaptoethanol and dithioerythrito), glycerol and amino acids (l-cysteine and l-tryptophan). The reaction mixtures contained individual reagents at a final concentration of 10 mM. For the kinetic experiments, the substrates were used at concentration ranges of 1.05–50 mg ml^{-1} (soluble oat spelt and birchwood xylans), 0.50–10 mg ml^{-1} (insoluble oat spelt xylan) and 6.0–60 mg ml^{-1} (insoluble birchwood xylan). They were prepared as described elsewhere [9]. The assay conditions were as previously described. Values of K_m were estimated from the Michaelis–Menten equation with a non-linear regression data analysis program [15]. All values represent the mean of three separate determinations. The determination of optimum temperature was carried out from 30–65°C in 100 mM sodium acetate buffer, pH 5.0. To determine the optimum pH at 50°C, the range was 3.0–9.0. McIlvaine type buffer systems were adjusted to the same ionic strength with KCl [6]. The thermostability of xylanase was determined by preincubating samples of the enzyme at 50°C and pH 5.0. After various times, aliquots were withdrawn and the residual activity was measured under standard conditions. Protein concentration was measured by the method of Petterson [18], using bovine serum albumin as standard.

All purification steps were carried out at 4°C unless otherwise specified. The crude extract was concentrated by ultrafiltration using an Amicon system with a 10 kDa cut-off membrane (PM 10). Aliquots of permeate were fractionated by gel filtration on Sephacryl S-100 (2.7×88 cm), pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0. Fractions (5.7 ml) were collected at a flow rate of 22.8 ml h^{-1}. Fractions with xylanase activity were pooled, dialyzed against distilled water, concentrated by freeze-drying and stored for later use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [14], using 12% gels. After electrophoresis, protein bands were silver-stained by the method of Blum *et al* [3]. The molecular weight was estimated by SDS-PAGE. The following molecular weight markers were used: bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa).

Amino acid analysis was carried out in a Hitachi L-8500

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amino acid analyzer which employs a post-column nynhidrin derivatization system. Samples were hydrolyzed in a PicoTag workstation using vapor phase HCl (6 N) at 109°C for 24 h. The amino acid composition was used for a homology search against the SWISS-PROT database composition using the AACompIdent software (http://expasy.hcuge.ch/chzol/aacompi.html).

A dried unbleached hardwood kraft pulp from *Eucalyptus grandis* (Bahia Sul Co, Mucuri, Brazil) was treated with 2 IU of xylanase for 3 h in 50 mM sodium acetate buffer pH 5.0–6.0 at 50°C and consistency of 10% (10 g of pulp in water [pulp humidity] content). After the enzyme treatment (X), the pulp samples were subjected to alkali (E), oxygen (O) and hydrogen peroxide (P) sequence treatment. The pulp in the control sequence was subjected to the same treatment with no enzyme added. Kappa number and viscosity were determined by TAPPI test methods T-236 os-76 and T-230 su-63, respectively.

A small xylanase produced by solid-state cultures of *Trichoderma harzianum* strain C was purified by only a two-step procedure. The crude extract was subjected to ultrafiltration using a 10 kDa cut-off point membrane. Xylanase activity was detected in the concentrated crude extract and ultrafiltrate. Several xylanases were described to pass through the ultrafiltration membrane [9,10,27]. The ability of xylanases to penetrate an ultrafiltration membrane is suggested to be due to their compact structure or to the non-uniformity of membrane pore size [9]. The ultrafiltrate was further fractionated by gel filtration on Sephacryl S-100. Two peaks of xylanase activity, designated XYL1 and XYL2, were resolved by gel filtration (Figure 1). SDS-PAGE of xylanase XYL2 revealed a homogeneous protein of approxinately 18 kDa (Figure 2). The purification of xylanase XYL2 showed total activity and yield of 13.32 IU and 1.2%, respectively. This low yield value could be credited to a greater level of xylanase activity in the concentrated crude extract and the presence of other xylanase activity (XYL1) in the ultrafiltrate. Furthermore, β-xylosidase and side-chain enzyme activities were not detected in the ultrafiltrate. As stated elsewhere, these enzymes are important for the complete hydrolysis of xylan [1,4,5]. Therefore, since xylan-degrading enzymes act synergistically to degrade the substrate, the yield value above was underestimated [9]. Furthermore, comparison of this value

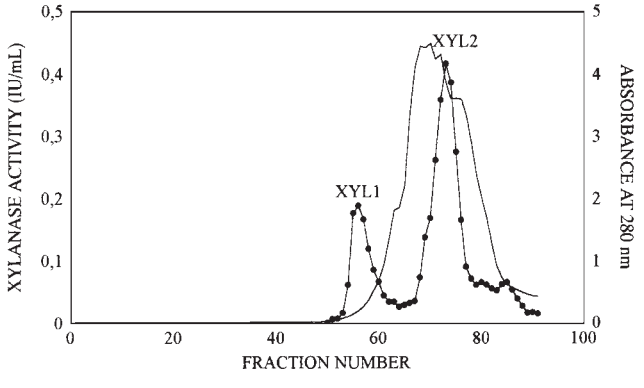

Figure 1 Purification of xylanase XYL2 from the ultrafiltrate of *Trichoderma harzianum* strain C by gel filtration chromatography on a Sephacryl S-100 column. —●— Xylanase activity; — ABS 280 nm.

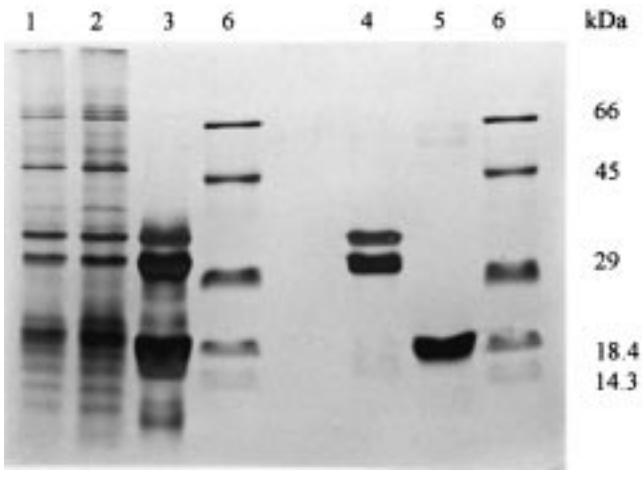

Figure 2 SDS-PAGE of xylanase XYL2 from *Trichoderma harzianum* strain C. Lane 1, crude extract; lane 2, concentrated crude extract; lane 3, permeate; lane 4, xylanase XYL1; lane 5, xylanase XYL2; lane 6, molecular weight standards (from the top): bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa).

with those reported for xylanases from other sources is not very meaningful because of variability in xylanase assays [10].

Comparison of some properties of xylanase XYL2 and β-xylanases from other *Trichoderma* species are summarized in Table 1. The purified enzyme was most active at pH 5.0 and 45°C. In general, the activity of xylanases isolated from *Trichoderma* is highest at pH and temperature ranges of 3.5–6.5 and 45–65°C, respectively [1,22,23,28,29]. The thermostability of xylanase XYL2 at 50°C and pH 5.0 was higher than that reported for some *Trichoderma* species [22,29]. The enzyme activity was reduced to 80% after 4 h of incubation. Xylanase XYL2 presented a higher affinity for insoluble oat spelt xylan than for highly substituted oat spelt arabinoxylan. The apparent K_m values with soluble and insoluble oat spelt and birchwood xylans as the substrates were 13.66 mg ml^{-1}, 5.89 mg ml^{-1}, 8.34 mg ml^{-1} and 16.85 mg ml^{-1}, respectively. In comparison to soluble birchwood xylan, the lower K_m against the soluble form suggests that the enzyme cleaves main chain linkages only in the immediate vicinity containing acetyl residues as side chains. In this case, the substitutent is required for the proper orientation of the substrate in the catalytic site [5].

Xylanase XYL2 did not show β-xylosidase, α-arabinofuranosidase, β-glucanase, β-mannosidase, β-mannanase or cellulase activity. The specificity for xylan as substrate is an important parameter for its use in pulp bleaching, whereas in this process the enzyme has to be cellulase-free [1]. The low molecular weight and high specificity for substrate suggest that xylanase XYL2 belongs to the family G/11 [24].

Some percent amino acid compositions of β-xylanases from *Trichoderma* are listed in Table 2. The purified enzyme did not show a composition similar to xylanases produced by *Trichoderma harzianum* E58, *Trichoderma koningii* or *Trichoderma viride* [7,13,25,27–29]. It appears to be distinct because of its high content of

Table 1 Some physico-chemical properties of β-xylanases from *Trichoderma* species

Species	Strain	Enzyme	Molecular weight (kDa)[a]	Optimum pH	Optimum temp (°C)	pI
T. harzianum	C	XYL2	18.0	5.0	45	–
T. harziamum	E58	A	20.0	5.0	50	9.4
		B	22.0	4.5–5.0	45–50	8.5
		C	29.0	5.0	60–65	9.5
T. koningii	G-39		21.5	5.5	60	8.9
T. koningii	IMI 73022		29.0	4.9–5.8	60	7.2
T. lignorumi			20.0	6.5	45	8.7
T. lignorumi			21.0	3.5	45	5.1
T. reesei	QM 9414		20.0	5.3	–	9.0
T. reesei	VTT-D-80133		23.0	4.0–5.0	–	–
T. reesei	VTT-D-80133		32.0	4.0–5.0	–	4.2
T. viride	Onozuka		16.0[b]	5.5–6.0	–	–
T. viride	Onozuka		17.8[b]	4.8	59	9.2
T. viride	Sigma V		22.0	5.0	53	9.3
T. viride	Meicellase		–	3.5	50	–

[a]Molecular weight estimated by SDS-PAGE.
[b]Molecular weight estimated by gel filtration.

Table 2 Percent amino acid composition of β-xylanases from *Trichoderma* species

Amino acid residue	*T. harzianum* E58 A	*T. koningii* G-39	*T. viride*			*T. harzianum*		
			ATCC 52438	Cellulysin	Sigma V	E58 B	E58 C	C XYL2
Ala	4.6	5.5	3.9	3.8	3.7	11.2	10.7	3.19
Arg	3.5	4.7	3.7	3.6	3.2	2.5	3.2	3.78
Asp + Asn	11.6	11.8	14.3	12.1	12.2	10.7	12.3	9.57
Cys	0	1.0	0	0	0	5.1	0.8	8.85
Glu + Gln	5.2	7.6	5.0	4.9	5.3	8.1	8.3	25.92
Gly	13.9	16.2	14.6	14.7	14.8	13.2	8.3	8.58
His	2.3	1.7	1.8	1.3	2.1	2.0	2.4	3.16
Ile	5.2	3.2	4.2	5.7	5.3	4.6	4.7	3.24
Leu	2.9	4.8	3.8	4.7	2.6	4.1	8.7	5.82
Lys	2.3	2.1	1.6	1.4	2.1	2.5	5.9	4.39
Met	0.6	0.6	0	0	0.5	1.0	0.8	1.28
Phe	4.0	3.8	4.3	4.0	3.7	2.5	2.4	10.65
Pro	3.5	5.3	3.3	3.4	3.2	6.6	3.6	0.00
Ser	11.6	11.6	11.8	10.6	12.7	7.6	9.5	4.38
Thr	9.2	9.4	7.7	6.5	9.0	4.6	4.3	2.91
Trp	3.5	–	3.9	3.5	3.2	0	3.2	–
Tyr	9.2	4.6	8.7	7.8	9.5	5.6	4.7	0.00
Val	6.9	5.8	7.2	6.2	6.9	8.1	6.3	4.28
Total number	173	–	–	–	189	197	253	156

Table 3 Effect of xylanase activity from *T. harzianum* strain C on pulp bleaching

Samples	Kappa number	Viscosity (cp)	Efficiency of delignification (%)	Selectivity
Control[a]	12.8	29.3	3.0	0.54
Ultrafiltrate[a]	12.3	28.8	6.8	0.96
Xylanase XYL2[a]	12.0	29.5	9.1	1.89
Control[b]	9.6	20.9	27.3	0.84
Ultrafiltrate[b]	9.2	20.6	30.3	0.90
Xylanase XYL2[b]	9.0	20.5	31.8	0.94

Unbleached pulp kappa number of 13.2 and viscosity of 31.0 cp.
[a] Pulp prebleaching.
[b] Pulp bleaching followed by alkaline, oxygen and hydrogen peroxide treatments.

glutamate/glutamine, phenylalanine and cysteine, and a low content of glycine and serine. Proline and tyrosine were not detected. A high level of cysteine residues can be essential to activity. They may be involved in the hydrogen bond(s) with the substrate [4]. The other possibility is that such residues may participate in covalent glycosyl-enzyme intermediate formation [4]. Xylanase XYL2 was activated by l-cysteine, l-tryptophan, dithioerythritol, β-mercaptoethanol and glycerol. The degrees of activation were 124%, 114%, 114%, 152.5% and 113%, respectively. l-Tryptophan was reported to be essential for binding of the xylanase to its substrate [2]. The activation of xylanase XYL2 by thiol-containing reagents (l-cysteine, dithioerythritol and β-mercaptoethanol) suggests that such reagents are involved in maintaining the integrity of the native enzyme conformation generally or of the active site in particular [9]. Furthermore, the interaction between glycerol and water would

be responsible for the strength of hydrophobic interactions among nonpolar amino acids, thereby activating the xylanase. In contrast to some xylanases from *T. harzianum* strain E58, xylanase XYL2 did not have a high alanine content [28,29]. The amino acid composition of xylanases from *T. viride*, *Aureobasidium pullulans* and *Aspergillus japonicus* was substantially different from that of xylanase XYL2, containing higher levels of glycine, asparagine/aspartate, threonine and serine [7,16,20].

Treatment prior to alkali delignification resulted in kappa number reduction and an increase in delignification effect (Table 3). Moreover, the pulp viscosity was not substantially altered, indicating that xylanase XYL2 facilitates lignin removal with high specificity. The pulp pretreatment of xylanase XYL2 was more efficient than the control (performed without xylanase activity) and ultrafiltrate sample. It presented a better bleaching selectivity. In comparison to xylanases from different sources [8,12,19,27], Xylanase XYL2 from *T. harzianum* strain C was an efficient system for hardwood kraft pulp bleaching. This enzyme can enhance the bleaching efficiency of nonchlorine-based bleaching reagents, including hydrogen peroxide. The enzyme's low molecular weight may contribute to an efficient diffusion into small pores in wood, and access to the hemicellulose-lignin-cellulose matrix [10,24].

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