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Purification and properties of a low molecular weight 1,4-β-D-glucan glucohydrolase having one active site for carboxymethyl cellulose and xylan from an alkalothermophilic *Thermomonospora* sp. ☆

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Abstract

A low molecular weight 1,4-β-D-glucan glucohydrolase from an extracellular culture filtrate of *Thermomonospora* sp. was purified to homogeneity. The molecular weight of the purified enzyme was 14.2 kDa by MALDI-TOF analysis and is in agreement with SDS–PAGE and gel filtration chromatography. The purified enzyme exhibited both endocarboxymethyl cellulase and endoxylanase activities. A kinetic method was employed to study the active site of the enzyme that hydrolyzes both carboxymethyl cellulose and xylan. The experimental data coincide well with the theoretical values calculated for the case of a single active site. Conformation and microenvironment at the active site was probed with fluorescent chemo-affinity labeling using *o*-phthalaldehyde as the chemical initiator. Formation of isoindole derivative resulted in complete inactivation of the enzyme to hydrolyze both xylan and CMC as judged by fluorescence studies corroborating a single active site for the hydrolysis of xylan and CMC.

Keywords: Cellulase; Xylanase; OPTA; *Thermomonospora* sp.; CMC; Xylan; Single active site

Cellulose is a fibrous, insoluble, and crystalline polysaccharide made up of β-1-4-linked D-glucopyranosyl residues and constitutes the major structural component of plant cell walls. It is the most abundant biopolymer on earth and approximately 10^7 tons is synthesized per annum. Cellulose can be degraded by the coordinated action of cellulolytic enzymes, such as endoglucanase, cellobiohydrolase, and β-glucosidase [1]. Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in plant cell walls. It is a heterogeneous polysaccharide consisting of β-1-4-linked D-xylosyl residues on the backbone but also containing arabinose, glucuronic acid, and arabinoglucuronic acid linked to D-xylose backbone. Xylanases are enzymes that degrade the xylan backbone into small oligomers [2]. Most microbes are thought to play a prominent role in cellulose hydrolysis in nature, have evolved strategies that bring the cell close to the cellulose surface, and give the cellulolytic organism "first access" to hydrolysis products [3,4]. The removal of certain polysaccharides by one species or group of microbes may improve the accessibility of a second group to cellulose [5]. In this regard, it is interesting that some cellulolytic bacteria can actively depolymerize certain hemicelluloses, particularly xylans [6–8]. In nature, these cellulolytic species utilize xylanases and pectinases to gain access to cellulose [9].

Thermomonospora is a filamentous thermophilic soil bacterium and an important species degrading cellulose

☆ *Abbreviations:* kDa, kilodalton; MALDI -TOF, matrix-associated laser desorption ionization time-of-flight; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CMC, carboxymethyl cellulose; OPTA, *o*-phthalaldehyde; HPLC, high performance liquid chromatography.

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and hemicellulose in plant residues [10,11]. In the present investigation, we report the purification and properties of 1,4-β-D-glucan glucohydrolase with a molecular weight of 14.2 kDa from *Thermomonospora* sp. For the first time based on the kinetic analysis, experimental evidence showed that the purified enzyme has a single active site for the substrates CMC and xylan.

Materials and methods

Microorganism and culture conditions

Thermomonospora sp. is an alkalothermophilic actinomycete having optimum growth at pH 9 and 50 °C used in this study and was isolated from self-heating compost from Barabanki district, Uttar Pradesh, India [12].

Enzyme assays. Both assays were carried out by mixing the suitably diluted enzyme with 0.5 ml substrate (1% xylan for xylanase activity and 1% CMC for carboxymethyl cellulase activity) in a final volume of 1 ml and incubating the mixture at 50 °C for 30 min. The reducing sugar released was determined by the dinitrosalicylic acid method [13].

One unit of xylanase activity was defined as the amount of enzyme that produced 1 μmole of xylose equivalent per minute from xylan, under assay conditions. One unit of carboxymethyl cellulase activity was defined as the amount of enzyme that produced 1 μmole of glucose equivalent per minute from CMC, under assay conditions. Protein concentration was determined according to the method of Bradford, using bovine serum albumin as standard [14].

Production and purification of 1,4-β-D-glucan glucohydrolase

Thermomonospora sp. was grown for 120 h in modified media containing 1% yeast extract, 4% cellulose paper powder, and 0.1% Tween 80. The pH of the medium was adjusted to 9 after autoclaving with sterile 10% Na_2CO_3. The culture and insoluble cellulose paper powder were removed by centrifuging at 5000 rpm for 10 min. All purification steps were carried out at 4 °C unless otherwise stated. The broth was subjected to fractional ammonium sulfate precipitation (30–55%). The precipitate was dissolved in 0.05 M sodium phosphate buffer, pH 7, dialyzed against the same buffer with several changes for 24 h, and applied to DEAE–Sephadex A50 column (6 × 20 cm) previously equilibrated with 0.05 M sodium phosphate buffer, pH 7. The elution was carried out by a linear gradient of sodium chloride (0–1.0 M) in 0.05 M sodium phosphate buffer, pH 7. The fractions having maximum specific activity were pooled and concentrated by ultrafiltration through Amicon UM-10 membrane. The concentrated sample was applied to Biogel P100 column (2.5 × 100 cm) that was equilibrated with 0.05 M sodium phosphate buffer, pH 7. Elution was carried out using the same eluant at a flow rate of 12 ml/h and 2 ml fractions were collected. The fractions having maximum specific activity were pooled and concentrated. The purity of the enzyme was checked by SDS–PAGE followed by silver staining [15].

Determination of molecular weight 1,4-β-D-glucan glucohydrolase

The molecular weight of the enzyme was determined by gel filtration chromatography using Sephadex G-75 previously equilibrated with 50 mM phosphate buffer, pH 7, which was calibrated using the following marker proteins: albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700). The void volume of the column was determined by the elution volume of blue dextran. The presence of subunits in the protein was determined by 12% SDS–PAGE.

Matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of the purified enzyme sample was carried out using applied Biosystems instrument.

Identification of optimum pH, temperature, and stability of purified 1,4-β-D-glucan glucohydrolase

Estimation of carboxymethyl cellulase and xylanase activities at different pH (4–10) and temperature (40–100 °C) values was carried out under standard assay conditions to determine optimum pH and temperature for enzyme activity. The pH stability of the enzyme was measured by incubating 5 IU enzyme at 50 °C in buffer of desired pH for 1 h. The temperature stability was determined by incubating 5 IU enzyme at different temperatures. The samples were removed at regular intervals and the residual activity was estimated under standard assay conditions.

End product analysis of hydrolysis of CMC and xylan

Five milligrams of CMC was incubated with 0.5 IU enzyme in a reaction volume of 1 ml for various time intervals (3 and 7 h) at 50 °C. The reaction was terminated by the acidification of the reaction mixture with 1 N HCl to pH 6.0 followed by 3 min incubation in boiling water bath. The samples were then centrifuged to remove unhydrolyzed CMC. The same procedure was carried out for hydrolysis of xylan.

HPLC. A sugar–PAK column—300 mm × 6.6 mm (ID)—(Waters, Analytical) was used for resolution of cello-oligosaccharides. The column was eluted with 1×10^{-4} M Ca-EDTA buffer at a flow rate of 0.4 ml/min at 70 °C. The products were determined by the measurement of refractive index changes measured in millivolts. The retention times and the areas of the individual peaks after correction for the baseline were compared with those of the standard cello-oligosaccharides run under identical conditions. The same procedure was followed for the end product analysis of hydrolysis of xylan.

Kinetic analysis

We have used a kinetic method elaborated by Keleti et al. [16] to evaluate whether endoglucanase hydrolyzing both CMC and xylan has one or two active sites. The initial velocities are calculated as μmole/min/ml in the simultaneous presence of both substrates at concentrations obeying the relationship

$$[\mathrm{CMC}]\ K_m^{\mathrm{xylan}} + [\mathrm{xylan}]\ K_m^{\mathrm{CMC}} = \mathrm{K} = 10\ (\mathrm{mg/ml})^2,$$

where the value of K was chosen arbitrarily to achieve concentration ranges around K_m values. For computations, $K_m^{\mathrm{xylan}} = 3$ mg/ml and $K_m^{\mathrm{CMC}} = 6$ mg/ml determined separately were used. The Michaelis constants and maximum velocities (V_{max}) of the cellulase and xylanase activities of the enzyme were estimated from Lineweaver–Burk plots of their initial velocities of saccharification of the substrates.

If we consider an enzyme with one active center acting concomitantly with substrate S_1 and the alternative substrate S_2, the two substrates will compete with each other for the same active site (Scheme 1) [16]:

$$\mathrm{E} + \mathrm{S}_1 \leftrightarrows \mathrm{ES}_1 \rightarrow \mathrm{E} + \mathrm{P}_1$$

$$\mathrm{E} + \mathrm{S}_2 \leftrightarrows \mathrm{ES}_2 \rightarrow \mathrm{E} + \mathrm{P}_2$$

Scheme 1.

In this case, the equation for the initial velocity (V_0) is represented by

$$V_0 = (V_1K_2[\mathrm{S}_1] + V_2K_1[\mathrm{S}_2]/K_1K_2 + K_1[\mathrm{S}_2] + K_2[\mathrm{S}_1]) \quad (1)$$

where V_1 and V_2 are maximum velocities, and K_1 and K_2 the dissociation constants for CMC and xylan, respectively.

Assuming two different active sites for the substrates S_1 and S_2, the mechanism of action can be represented by Scheme 2:

$$E + S_1 \leftrightarrows ES_1 \rightarrow E + P_1$$

$$E + S_2 \leftrightarrows ES_2 \rightarrow E + P_2$$

$$ES_1 + S_2 \leftrightarrows ES_1S_2 \rightarrow ES_1 + P_2$$

$$ES_2 + S_1 \leftrightarrows ES_1S_2 \rightarrow ES_1 + P_1$$

Scheme 2.

If the two active sites are independent, the initial velocity can be represented by

$$V_0 = \frac{V_1K_2[S_1] + V_2K_1[S_2] + (V_1 + V_2)[S_1][S_2]}{K_1K_2 + K_1[S_2] + K_2[S_1] + [S_1][S_2]}. \quad (2)$$

If the two substrates mutually influence each other's binding:

$$V_0 = V_1K_2[S_1] + V_2K_1[S_2]/K_1K_2 + K_1[S_2] + K_2[S_1] + [S_1][S_2] + (V_1 + V_2)[S_1][S_2]/K_1K_3 + K_4[S_2] + K_3[S_1] + [S_1][S_2], \quad (3)$$

where K_3 and K_4 are the dissociation (or Michaelis) constants of S_2 and S_1 in the presence of S_1 and S_2, respectively.

Chemo-affinity labeling studies using OPTA

1,4-β-D-Glucan glucohydrolase (50 μg), in 0.05 M phosphate buffer, pH 7, was incubated with *o*-phthalaldehyde in methanol at 25 °C. The formation of enzyme-isoindole derivative was followed spectrophotometrically by monitoring the increase in fluorescence at 420 nm with an excitation wavelength fixed at 338 nm. The aliquots were removed at intervals to check the residual carboxymethyl cellulase and xylanase activities of the enzyme.

Results and discussion

Purification of 1,4-β-D-glucan glucohydrolase

The purification of 1,4-β-D-glucan glucohydrolase from *Thermomonospora* sp. is summarized in Table 1. The enzyme was purified by fractional ammonium sulfate precipitation (30–55%) followed by sequential chromatography on DEAE–Sephadex A-50 ion exchange and Biogel P100 gel filtration column. The cellulase and xylanase activities were almost completely coincident with each other and with the protein peak. The purity of enzyme was analyzed by SDS–PAGE and was electrophoretically homogeneous, thus revealing a single 14.3 kDa band (Fig. 1A). The purified 1,4-β-D-glucan glucohydrolase was also analyzed by MALDI-TOF mass spectrometry, which revealed a sharp peak of an average molecular mass of 14.2 kDa confirming the homogeneity of the enzyme (Fig. 1B). The endoglucanases from *Thermomonospora* reported to date are of the molecular weight in the range of 30–108 kDa [17–19]. The first time

Table 1
Purification of 1,4-β-D-glucan glucohydrolase from *Thermomonospora* sp.

Step	Total protein (mg)	Total enzyme activity (IU)				Ratio	% Recovery	Fold purification
		Xylanase	Specific activity (IU/mg)	Cellulase	Specific activity (IU/mg)	Xylanase:cellulase		
Culture filtrate	640	11,700	18.28	700	1.1	16.61	100	1
Ammonium sulfate precipitation	208	8784	42.23	528	2.54	16.63	75	2.30
DEAE–Sephadex	35.4	6055	171.04	366.2	10.34	16.53	51	9.40
Biogel P 100	0.25	242.52	970.09	14.7	58.79	16.49	9	53.44

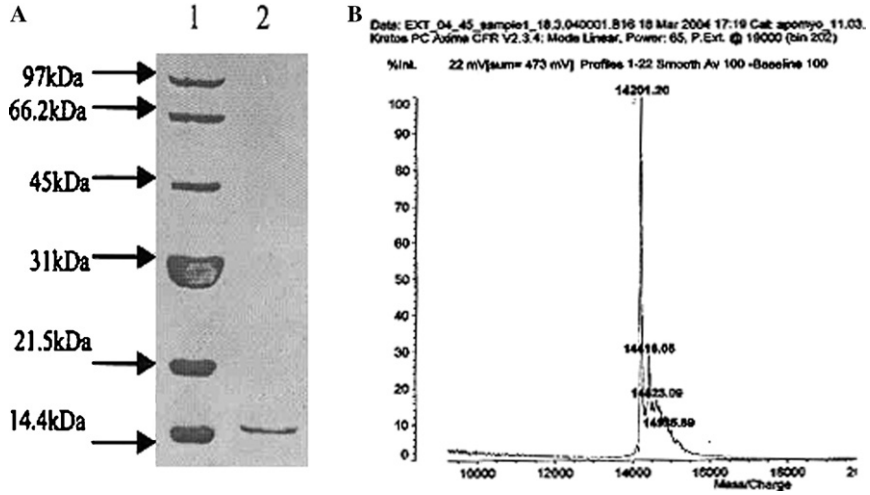

Fig. 1. (A) Twelve percent SDS–PAGE of standard molecular marker proteins in lanes 1 and 2 contained purified 1,4-β-D-glucan glucohydrolase was visualized by silver staining. (B) MALDI-TOF mass spectrometry of purified enzyme.

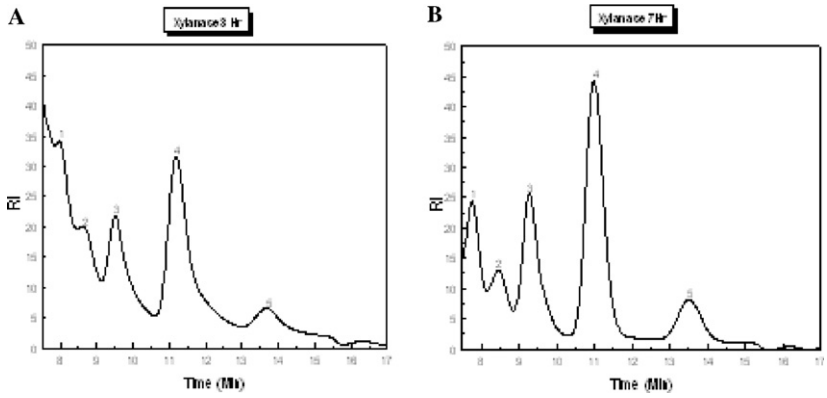

Fig. 2. HPLC analysis of the end products of hydrolysis of xylan using sugar–PAK column. The column was eluted with 1×10^{-4} M Ca-EDTA buffer at a flow rate of 0.4 ml/min at 70 °C. (1) Xylopentaose, (2) xylotetraose, (3) xylotriose, (4) xylobiose, and (5) xylose.

we report on the lowest molecular weight endoglucanase from a novel alkalothermophilic *Thermomonospora.*

The isoelectric point of the enzyme was determined to be 4.33 by isoelectric focusing, indicating that it is an acidic protein.

Characterization of 1,4-β-D-glucan glucohydrolase

The enzyme was active in a wide range of pH from 5 to 8 with optimum activity at pH 7 for both cellulase and xylanase activities. It was stable in an expansive range of pH 5–8 with more than 85% activity. The temperature optimum of the purified enzyme was 50 °C. The enzyme was highly thermostable, retaining complete activity of hydrolyzing xylan and CMC at 50 °C for more than 15 h and half-life of 20 min at 80 °C.

Mode of action of enzyme

The products formed by hydrolysis of CMC and xylans by purified enzyme were analyzed separately by HPLC. The HPLC profiles show the formation of cello-oligosaccharides and glucose from hydrolysis of CMC and xylo-oligosaccharides and xylose from xylan. The enzyme produced comparatively larger amounts of cello-oligosaccharides and glucose from CMC in 7 h while xylo-oligosaccharides and xylose from xylan suggest the endomode of action (see Figs. 2 and 3).

Kinetic studies of carboxymethyl cellulase and xylanase activities

During purification, the ratio of carboxymethyl cellulase to xylanase activity remained constant, indicating that both the activities reside on the same protein. Therefore, the question arises of whether the two activities of the enzyme are localized on the same or on two different active sites. The following data indicated that the enzyme at the same active site hydrolyzes CMC and xylan: (i) the enzyme showed same pH optima for CMC and xylan hydrolysis, (ii) the enzyme was stable at 50 °C for 12 h for both substrates, (iii) both the activities are stable in wide pH range of 5–8, (iv) both activities were inhibited by *o*-phthaldialdehyde, (v) the mode of action of the enzyme for hydrolysis of both the substrates is of endotype. However, the K_m for the

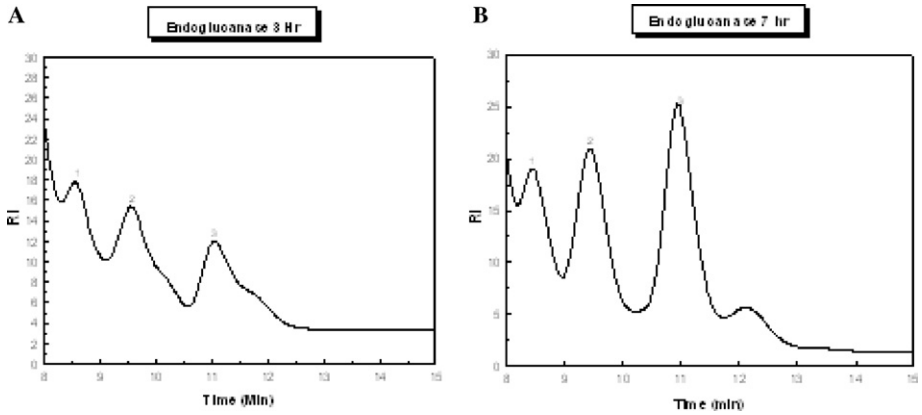

Fig. 3. HPLC analysis of the end products of hydrolysis of CMC using sugar–PAK column. The column was eluted with 1×10^{-4} M Ca-EDTA buffer at a flow rate of 0.4 ml/min at 70 °C. (A,B) (1) Cellotriose, (2) cellobiose, and (3) glucose.

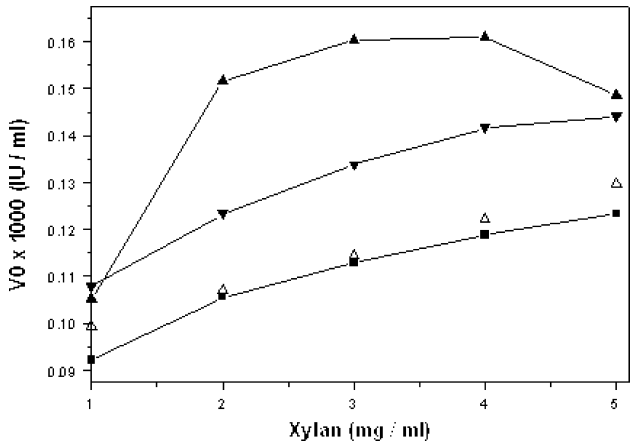

Fig. 4. Plot of V_0 vs. [S] at different concentrations of CMC and xylan. In each case, 0.2 U/μg of the enzyme was added to reaction mixture. The concentration of only one substrate is indicated on the abscissa. (■) Experimental points, (△) single active site Eq. (1), (▲) two substrates mutually influencing each other's binding Eq. (3), and (▼) two independent active sites Eq. (2).

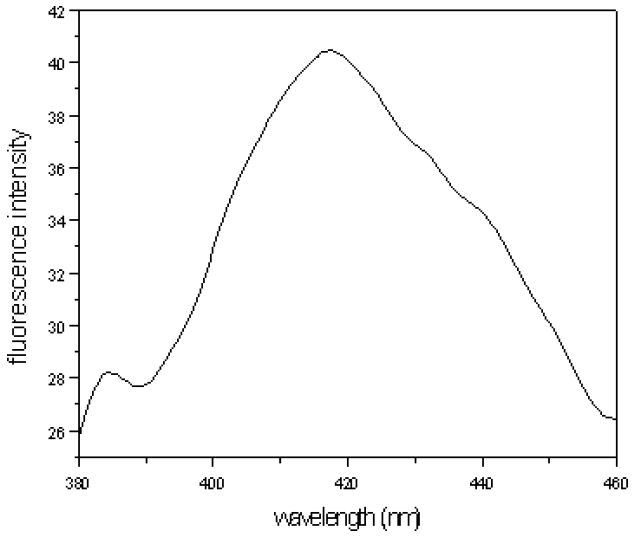

Fig. 5. Isoindole fluorescence of endoglucanase modified by OPTA. Endoglucanase sample (50 μg) was incubated with 1 mm OPTA for 10 min at 25 °C. The isoindole fluorescence was monitored at λ_{excit} 338 nm and λ_{emiss} 415 nm.

two activities are widely different, viz. 6 mg/ml for CMC and 3 mg/ml for xylan. The kinetic analysis by the method of Keleti et al. [16] was employed to analyze whether one or two sites are responsible for hydrolysis of CMC and xylan (Fig. 4).

If CMC and xylan were hydrolyzed at two different active sites, the rate of hydrolysis of two substrates should be equal to the sum of their individual rates. Conversely, if there is a single site, these two substrates will show competitive inhibition with respect to each other, and hence the rate of hydrolysis by the enzyme with the mixture of substrates would be lower than the sum of the rates with individual substrates. Table 2 summarizes the calculated and the actual experimental values (see Fig. 5).

The observed values of overall rate of hydrolysis agreed better with the theoretical values calculated from Eq. (1) than with those calculated from Eqs. (2) and (3) (Fig. 4). The results of the kinetic analysis are consistent with assumption of a single active site.

Chemo-affinity labeling studies

Conformation and microenvironment at the active site of 1,4-β-D-glucan glucohydrolase was probed with fluorescent chemo-affinity labeling using *o*-phthalaldehyde as the chemical initiator. *o*-Phthalaldehyde is a bifunctional reagent that forms isoindole derivative by cross-linking the proximal thiol and amino groups [20]. *o*-phthalaldehyde reacted with 1,4-β-D-glucan glucohydrolase yielding a stable fluorescent derivative, which exhibited an absorbance characteristic of isoindole (420 nm) with the complete loss of activity of the enzyme to hydrolyze both CMC and xylan (see Fig. 5). Complete inactivation of the 1,4-β-D-glucan glucohydrolase by *o*-phthalaldehyde might be due to the formation of isoindole derivative by cross-linking the proximal thiol and amino groups located at or near the active site of the enzyme.

Cellulases from fungi and anaerobic bacteria have been discovered which show the interrelationship with

Table 2
Overall reaction velocities of 1,4-β-D-glucan glucohydrolase with mixtures of various concentrations of xylan and CMC

Individual rates				Rate of mixtures	
Xylan		CMC		Observed	Calculated
Concentration (mg/ml)	A_{540}	Concentration (mg/ml)	A_{540}	A_{540}	A_{540}
1	0.0984	10	0.1376	0.0921	0.2360
2	0.1368	8	0.1223	0.1055	0.2591
3	0.1626	6	0.1128	0.1128	0.2754
4	0.1874	4	0.1076	0.1188	0.3062
5	0.2061	2	0.0921	0.1233	0.3294

xylanase in its induction, regulation at transcriptional level, and inactivation by inhibitors [21–26]. It has been reported that few endoglucanase [1,4-β-D-glucan glucohydrolase] components of *Trichoderma viride* [27] and *Irpex lacteus* show xylanase activity [28]. However, the number of bifunctional enzymes which have both cellulase and xylanase activities known to date is rather low, and information on their properties is scarce. The results of the present study suggest that an interrelationship exists between the xylanase and cellulase activities with probable ecological significance. The identical pH and temperature optima, similar stability, inhibition by OPTA reagent, and kinetic analysis are in favor of the single active site for the hydrolysis of carboxymethyl cellulose and xylan by 1,4-β-D-glucan glucohydrolase.

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