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ORIGINAL PAPER

Purification and characterization of two sugarcane bagasse-absorbable thermophilic xylanases from the mesophilic *Cellulomonas flavigena*

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Abstract We report the purification and characterization of two thermophilic xylanases from the mesophilic bacteria *Cellulomonas flavigena* grown on sugarcane bagasse (SCB) as the only carbon source. Extracellular xylanase activity produced by *C. flavigena* was found both free in the culture supernatant and associated with residual SCB. To identify some of the molecules responsible for the xylanase activity in the substrate-bound fraction, residual SCB was treated with 3 M guanidine hydrochloride and then with 6 M urea. Further analysis of the eluted material led to the identification of two xylanases Xyl36 (36 kDa) and Xyl53 (53 kDa). The pI for Xyl36 was 5.0, while the pI for Xyl53 was 4.5. Xyl36 had a K_m value of 1.95 mg/ml, while Xyl53 had a K_m value of 0.78 mg/ml. In addition to SCB, Xyl36 and Xyl53 were also able to bind to insoluble oat spelt xylan and Avicel, as shown by substrate-binding assays. Xyl36 and Xyl53 showed optimal activity at pH 6.5, and at optimal temperature 65 and 55°C, respectively. Xyl36 and Xyl53 retained 24 and 35%, respectively, of their original activity after 8 h of incubation at their optimal temperature. As far as we know, this is the first study on the thermostability properties of purified xylanases from microorganisms belonging to the genus *Cellulomonas*.

Keywords *Cellulomonas flavigena* · Mesophilic · Sugarcane bagasse · Thermal stability · Xylanase

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Introduction

Xylan is the major hemicellulosic component in plant cell walls. Xylan, a complex highly branched heteropolysaccharide, varies in structure between different plant species, and the homopolymeric backbone chain of 1,4-linked β-D-xylopyranosyl units can be substituted to varying degrees with uronic acid, hexoses and side chains of other pentoses. Due to the structural heterogeneity of xylan, the complete hydrolysis requires the coordinated action of several enzymes. Xylanases (1,4-β-D-xylan xylanohydrolase E.C.3.2.1.8) that cleave internal linkages on the β-1,4-xylopyranose backbone play a crucial role in this process [2, 4].

Xylanases are produced in significant amounts by bacteria and filamentous fungi to degrade xylan as a source of energy [2, 42]. Xylanases have significant applications in a wide range of industrial processes such as food, animal feed and paper and pulp industries [2, 16]. Studies on xylanases and cellulases have also been focused on the recovery of fermentable sugars from lignocellulosic biomass for ethanol production, as an alternative energy source. However, the cost of production and the low yields of these enzymes are major problems for industrial applications [2, 4]. Therefore, the hydrolysis of lignocellulosic materials, such as sugarcane bagasse (SCB), wheat straw, corncobs, etc., by cellulose- and hemicellulose-hydrolyzing microbial strains has received an increasing interest in research.

Xylanases, stable and active at high temperature and alkaline conditions, are suitable for biotechnological applications. One of the ways to identify enzymes, which are thermally stable is to exploit natural sources, but the stability of the enzyme can also be increased by

chemical modification, cross-linking, immobilization, treatment with additives and protein engineering [11]. For this reason, the study of the complete xylanolytic enzyme system from both mesophilic and thermophilic xylanolytic microorganisms is important to satisfy the need of xylanases for different industrial applications.

Most of the xylanases have a modular structure consisting of two domains: a catalytic domain (CD), responsible for the hydrolysis reaction, and a carbohydrate-binding module (CBM), mediating binding of the enzymes to the substrate, thus improving the hydrolysis of insoluble substrates [26]. CBM has been found also in other glycosyl hydrolases like cellulases [19, 40], β-glucosidases [22], α-arabinosidases, acetylesterases and mannanases [13]. CBMs, originally identified as "thermostabilizing" modules in xylanases from thermophilic bacteria [7, 10], have been found also in mesophilic bacteria such as *Cellulomonas fimi* [8] and *Bacillus* sp. [6].

Cellulomonas species are mesophilic, aerobic and Gram-positive bacteria found in the soil and known to produce an array of enzymes involved in the degradation of components of the plant cell wall, mainly cellulases and xylanases [2, 42]. *C. fimi*, the most studied microorganism from the genus *Cellulomonas*, produce four xylanases and the respective encoding genes (*xyn*A, *xyn*B, *xyn*C and *xyn*D) have been cloned [3, 14]. Studies on the extracellular xylanase activity by *Cellulomonas flavigena* have been focused mainly on the activity detected in the soluble fraction of the culture supernatant of bacteria grown on insoluble substrates such as carbon sources [1, 23, 27, 41]. However, a study on the localization of xylanase activity in *Cellulomonas* sp. IIbc strain, grown on SCB pith, showed that the xylanase activity associated with SCB is ten times higher than the activity in the soluble fraction of the bacterial culture supernatant [33], although none of the enzymes responsible for the substrate-bound xylanolytic activity were identified.

In this work, we report the purification and characterization of two thermostable xylanases, from the mesophilic bacteria *C. flavigena* CDBB-531, identified for their ability to bind to residual SCB used as the only carbon source to support the microorganism growth.

Materials and methods

Microorganism and chemicals

Cellulomonas flavigena CDBB-531 was obtained from the National Collection of Microbial Cultures (CINVESTAV, México).

Birchwood xylan, oat spelt xylan and bovine serum albumin were purchased from Sigma. Avicel PH 101, guanidine hydrochloride and urea were obtained from Fluka. Chemicals used for SDS-PAGE and gel filtration chromatography were purchased from Bio-Rad. All other chemicals were of reagent grade and obtained from Sigma.

Growth conditions

C. flavigena CDBB-531 was grown in a mineral medium base containing (l^{-1}) 5.5 g NaCl; 2.5 g $(NH_4)_2SO_4$; 0.1 g $CaCl_2{\cdot}2H_2O$; 0.1 g $MgSO_4{\cdot}7H_2O$; 5.3 g K_2HPO_4 and 10.6 g KH_2PO_4. For seeding cultures, the mineral medium base was supplemented with 0.2% (w/v) yeast extract and 1% (v/v) glycerol. These cultures were grown at 37°C for 24 h in an orbital shaker at 150 rpm (Aquaterm Water Bath Shaker, New Brunswick Scientific). Bacterial growth was measured turbidimetrically at 600 nm and converted to dry cell weight by means of a calibration curve. Samples of fermented broth containing SCB were filtered through a nylon cloth (105 μm) to remove residual SCB [41]. All results are the average of at least three independent experiments.

Enzyme production

For enzyme production, 5 ml of seeding culture was added to 100 ml of mineral medium base containing 0.2% (w/v) yeast extract and 1% (w/v) SCB. The cultures were incubated (37°C for 48 h at 150 rpm) and then centrifuged at low speed (1,650*g*, 4°C, 10 min) in order to separate the residual SCB. Then, the supernatant was centrifuged at 5,000*g* at 4°C for 15 min to separate the cells from the culture supernatant. Both the residual SCB and the culture supernantant free of cells were assayed for protein and xylanase activity. All results are the average of at least three independent experiments.

Protein and xylanase activity assay

Protein was quantified by the Lowry method [21] using bovine serum albumin as standard. xylanase activity was measured by determining the release of reducing sugars from 0.25% birchwood xylan solution prepared in 0.1 M citrate–phosphate buffer of pH 6.5, as described [32], but with incubation at 60°C instead of 40°C [27]. Reducing sugars were assayed by the dinitrosalicylic acid method [25] using xylose as standard. One international unit (IU) of xylanase activity released 1 μmol of xylose equivalent per mg of protein per min.

Purification of Xyl36 and Xyl53

For enzyme purification, residual SCB was resuspended in five volumes of 1 M NaCl and then centrifuged at 5,000*g* at 4°C for 15 min. This washing step was repeated with distilled water, instead of 1 M NaCl. Then, residual SCB was resuspended in two volumes of 3 M guanidine hydrochloride and incubated at 4°C for 18 h with shaking at 150 rpm. The supernatant was recovered by centrifugation (5,000*g*, 4°C, 15 min), dialyzed against 15 mM sodium acetate buffer of pH 6 at 4°C and concentrated by lyophilization. The pellet was resuspended in five volumes of 50 mM sodium acetate buffer of pH 6 and centrifuged at 5,000*g* at 4°C for 15 min. Then, the pellet was resuspended in two volumes of 6 M urea and incubated at 4°C for 18 h with shaking at 150 rpm. The supernatant was recovered by centrifugation (5,000*g*, 4°C, 15 min), dialyzed against 15 mM sodium acetate buffer of pH 6 at 4°C and concentrated by lyophilization. Both guanidine hydrochloride and urea extracts were independently fractionated by gel filtration chromatography on a Bio-Gel A-1.5 m column equilibrated with 50 mM sodium acetate buffer of pH 6. Fractions with xylanase activity were pooled, dialyzed and concentrated by lyophilization.

Molecular weight, pI and zymogram analysis

Proteins were analyzed by SDS-PAGE on 10% polyacrylamide gels according to the Laemmli method [17]. Broad range protein standards were used to estimate molecular mass (MW). The isoelectric points were determined using the Mini-Protean 2.0 electrophoresis cell (BioRad), according to the manufacturer's instructions. Zymogram analyses were carried out as previously described [36] on polyacrylamide gels co-polymerized with 0.1% (w/v) Remazol Brilliant Blue-Xylan.

Optimal pH and temperature

The optimal pH was determined by incubating the enzymatic preparation in 0.25% birchwood xylan solution prepared in 0.1 M citrate–phosphate buffer (pH 4–7.5) at 60°C. The optimal temperature of the xylanases was determined by incubating the protein extract at different temperatures (30–70°C) in 0.25% birchwood xylan solution prepared in 0.1 M citrate–phosphate buffer pH 6.5.

Thermostability assay

The purified enzymes were incubated at their optimal temperature in 0.1 M citrate–phosphate buffer, pH 6.5, in the absence of substrate. The residual xylanase activity was determined at 60 min intervals for 8 h.

Kinetic parameters

The kinetic parameters for the purified Xyl53 and Xyl36 were determined under optimal conditions for activity using birchwood xylan as substrate, according to the Lineweaver-Burk method [20].

Substrate-binding assay

Binding assays were carried out as described [9] with some modifications as follows. An appropriate dilution of enzymes with insoluble oat spelt xylan or Avicel was performed at 4°C for 2 h with 2% (w/v) insoluble oat spelt xylan or 2% (w/v) Avicel in 10 ml of 0.1 M phosphate buffer of pH 7. Incubation samples, taken every 20 min during 2 h, were centrifuged (5,000*g*, 4°C for 5 min) and the supernatants assayed for xylanase and protein. It was considered that at time zero, 100% of xylanase activity and protein were located in the supernatant. The percentage of substrate-bound enzyme was determined by comparing xylanase activity and protein content in the incubation supernatants.

Results and discussion

Xylanase activity and cell growth

C. flavigena CDBB-531 produced a high titer of extracellular xylanase activity when grown at 37°C on SCB as substrate [30]. During *C. flavigena* CDBB-531 growth, the activity of the substrate-bound xylanases reached a maximum at the end of the exponential phase of growth after 15 h of incubation, then decreased slowly during the stationary phase (Table 1). Xylanase activity in the soluble fraction increased slightly throughout the whole fermentation (Table 1). Thus, these data indicate that the production of extracellular xylanases by *C. flavigena* CDBB-531 is associated with cell growth. The production of extracellular cellulase activity by *C. flavigena* is also associated with cell growth [3, 34].

Localization of xylanase activity

Quantification of xylanase activity during the *C. flavigena* CDBB-531 growth showed that the enzymatic activity was 4–15 times higher in the substrate-bound fraction (insoluble fraction) than in the culture supernatant free of cells (soluble fraction) throughout the

Table 1 Production and localization of xylanase activity by *C. flavigena* grown on SBC

Time (h)	Cell growth (g/l)[a]	Residual SCB (g)[b]	Total xylanase activity (IU)[c]	Total xylanase activity SBC-bound (IU)	Total xylanase activity free (IU)	Total xylanase activity SBC-bound (%)	Total xylanase activity free (%)	Xylanase activity per gram of SBC (IU)	Xylanase activity free per gram (IU)	Xylanase activity SBC-bound/free (IU/g_SBC)/(IU/g)
0	0.20	0.50	0	0	0	0	0	0	0	0
5	0.42	0.48	88.69	8.43	80.26	9.50	90.50	17.56	1.78	9.83
10	1.32	0.43	110.25	14.24	96.01	12.92	87.08	33.12	2.22	14.92
15	3.44	0.35	131.81	14.62	117.19	11.09	88.91	41.77	2.65	15.74
20	4.50	0.20	192.96	9.18	183.78	4.76	95.24	44.79	3.96	11.29
25	5.12	0.15	208.80	6.41	202.39	3.07	96.93	43.03	4.29	10.03
30	5.42	0.12	201.02	4.93	196.09	2.45	97.55	40.08	4.31	9.30
35	5.50	0.12	213.44	4.38	209.06	2.05	97.95	37.13	4.57	8.11
40	5.54	0.10	275.54	3.48	272.06	1.26	98.74	34.13	5.91	5.78
45	5.5	0.09	278.10	3.01	275.09	1.08	98.92	32.72	6.09	5.37
50	5.5	0.09	288.90	2.35	286.55	0.81	99.19	26.98	6.33	4.26

[a] Cell cultures were grown at 37°C at 150 rpm in a mineral medium base containing 0.2% (w/v) yeast extract and 1% (w/v) SCB. The bacterial growth was measured turbidimetrically at 600 nm and converted to dry cell weight by means of a calibration curve

[b] Residual SCB was filtered through a nylon cloth (105 μm) and quantified according to Vega-Estrada et al. [41]

[c] Xylanase assay was measured by determining the release of reducing sugars from 0.25% birchwood xylan in 0.1 M citrate–phosphate buffer of pH 6.5 [32] at 60°C. Reducing sugars were assayed by the dinitrosalicylic acid method [25] using xylose as standard. One international unit (IU) of xylanase activity released 1 μmol of xylose equivalent per mg of protein per min

whole fermentation (Table 1). Xylanase activity bound to residual SBC (41.77 IU/g) was 15 times higher than the enzymatic activity in the culture supernatant free of cells (2.65 IU/g) after 15 h of incubation (Table 1). By 50 h, these numbers were 26.98 and 6.33 IU/g, respectively, which correspond to the lowest level of xylanase activity detected in the substrate-bound fraction throughout the whole fermentation (Table 1). At this point, the enzymatic activity in the insoluble fraction was only four times higher than that detected in the soluble one and the remaining amount of residual SBC was only 20% of the original. Thus, indicating that a considerable amount of xylanolytic activity, produced and secreted by *C. flavigena* CDBB-531 grown on SCB has affinity to this substrate. In agreement with these data, Rodriguez et al. [33] found that the xylanase activity associated with SCB is ten times higher than the activity in the soluble fraction of the culture supernatant of *Cellulomonas* sp. IIbc strain grown on SCB pith.

Purification of Xyl36, Xyl53 and zymogram analysis

To identify some of the *C. flavigena* CDBB-531 enzymes responsible for the xylanase activity associated with the substrate-bound fraction, the substrate-bound xylanase activity was eluted from residual SCB by treatment with 3 M Guanidine hydrochloride and 6 M Urea. First, the residual SCB was treated with 3 M Guanidine hydrochloride and the eluted material was fractionated by gel filtration chromatography. This procedure allowed the purification of *C. flavigena* CDBB-531 xylanase with an approximate MW of 36 kDa, named Xyl36 (Fig. 1, lane 3). Then, the residual SCB was resuspended in 6 M urea and subjected to gel filtration chromatography, leading to the purification of a second *C. flavigena* CDBB-531 xylanase with an approximate MW of 53 kDa, named Xyl53 (Fig. 1, lane 2). Zymogram analysis of purified Xyl53 and Xyl36 showed a single band of activity (Fig. 1, lanes 4, 5), which corresponds to the purified enzymes on the SDS-PAGE analysis (Fig. 1, lanes 2, 3).

pI determination

Both Xyl53 and Xyl36 are acidic xylanases since the enzymes showed pI values of 4.5 and 5.0, respectively. Xyl53 follows the relationship between molecular weight and pI proposed by Wong et al. [42], in which low MW (<22,000) xylanases are basic proteins, whereas high MW (>43,000) xylanases are acidic proteins. Xylanases with MW between 22,000 and 43,000 showed basic or acidic pI [12, 15], like Xyl36 with pI of

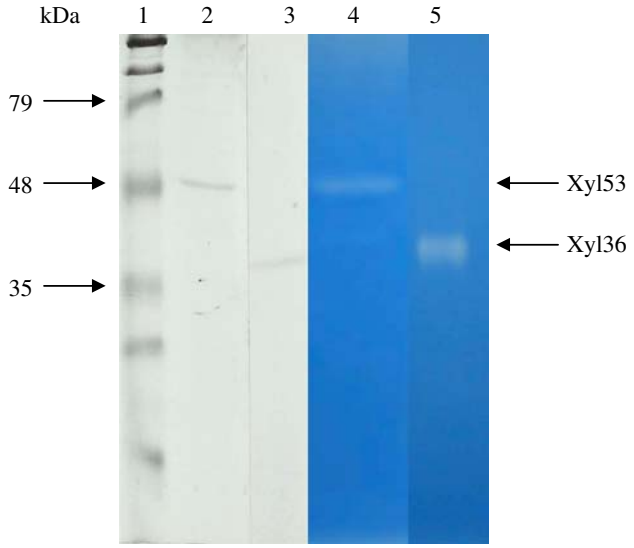

Fig. 1 Protein and zymogram analysis of Xyl53 and Xyl36. About 10% SDS-PAGE analysis of purified enzymes. *Lane 1* MW protein standards; *lane 2* Xyl53; *lane 3* Xyl36; *lane 4*, *5* Zymogram analysis of Xyl53 and Xyl36, respectively

5.0. Interestingly, a 35 kDa xylanase with pI of 8.5 was purified from the culture supernatant of *C. flavigena* grown on SBC [27]. Xyl53 could correspond to the *C. flavigena* 56 kDa xylanase previously reported [23], although they showed pI values of 4.5 and 6.14, respectively.

Effect of temperature and pH on xylanase activity

Xyl36 and Xyl53 were active on birchwood xylan in the neutral pH range, with optimal activity at pH 6.5 (Fig. 2). A decline in activity was observed at pH values below and above pH 6.5 (Fig. 2). Optimal activity near neutral pH has been described for many bacterial xylanases [24, 28]. The optimal temperature for Xyl53 activity was found to be 55°C, although the enzyme exhibited 90% of its activity in the range from 50 to 60°C (Fig. 2). Xyl36 showed optimal temperature for activity at 65°C. The xylanolytic activity of Xyl36 dropped at temperature values above 65°C, and only 20% of the enzymatic activity observed at 65°C was detected at 70°C (Fig. 2). Interestingly, Xyl36 and Xyl53 from *C. flavigena* CDBB-531 displayed optimal activity at temperature values 7–25°C higher than those observed (40–45°C) for xylanases from *C. fimi* [14] and *C. uda* [31]. There are few reports of thermophilic xylanases isolated from a mesophilic microorganism such as *C. flavigena* CDBB-531; there are only two reports for xylanases with optimal temperatures of 65°C from the mesophilic microorganisms *Trichoderma harzianum* [38] and *Streptomyces sp.* [44].

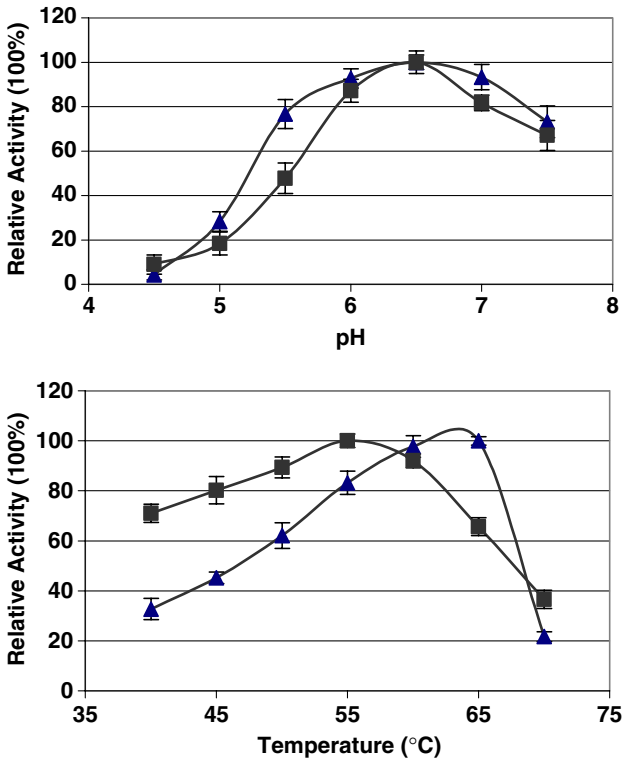

Fig. 2 Effect of pH and temperature on the xylanase activity of Xyl53 and Xyl36. **a** pH effect. Xyl53 (*filled square*) and Xyl36 (*filled triangle*) were incubated at pH ranging from 4 to 7.5 (60°C) for 10 min. **b** Temperature effect. Xyl53 (*filled square*) and Xyl36 (*filled triangle*) were incubated at temperatures ranging from 30 to 70°C for 10 min in 0.1 M citrate–phosphate buffer of pH 6.5

Kinetic parameters

Xyl36 and Xyl53 showed K_m and V_{max} values of 1.95 mg/ml and 17.37 IU/mg, and 0.78 mg/ml and 48.82 IU/mg, respectively. These K_m values are comparable to those reported for xylanases from *C. fimi* [14], but lower than those reported for xylanases from *Clostridium acetobutylicum* [18] and *T. harzianum* [38]. The *C. flavigena* xylanases of 35 kDa [27] and 56 kDa [23] showed K_m and V_{max} values of 3.3 mg/ml and 333 IU/mg, and 1.2 mg/ml and 322 IU/mg, respectively. Thus, it seems unlikely that Xyl36 and Xyl53 could correspond to the *C. flavigena* xylanases previously reported [23,27], due to differences in both pI values (above) and kinetic parameters.

Thermal stability

Thermostability of xylanases Xyl36 and Xyl53, determined by studying the time-dependent thermal inactivation at their optimal temperature (Fig. 3), showed that 60% of the Xyl36 and 50% of the Xyl53 enzyme activity was lost after 1 h at 65 and 55°C, respectively.

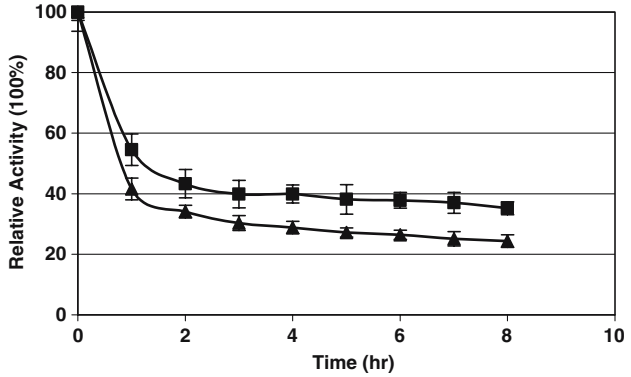

Fig. 3 Thermal stability of Xyl53 and Xyl36. Xylanases were incubated in 0.1 M citrate–phophate buffer of pH 6.5 at 65°C for Xyl36 (*filled triangle*) and 55°C for Xyl53 (*filled square*)

By 2 h, these numbers were 65 and 60%, respectively. Xyl36 and Xyl53 retained 24 and 35% of their original activity, respectively, after 8 h of incubation at 65 and 55°C, respectively (Fig. 3). Thermostable enzymes are classified into three groups, defined by their range of temperature stability: moderately thermostable (45–65°C), thermostable (65–85°C) and extremely thermostable (>85°C) [43]. Thus, Xyl36 and Xyl53 could be classified in the range of moderately thermostable xylanases, since both present a half-life of inactivation after 1 h of incubation at their optimal temperature (65 and 55°C, respectively). Interestingly, xylanases from the mesophilic *Aspergillus foetidus* MTCC [37] and two xylanases from the thermotolerant *Aspergillus caespitosus* lost 100% of their original activity after 1 h of incubation at 60°C [35]. Also, a xylanase from another mesophilic fungus, *Schizophyllum commune*, lost 75% of its initial activity at 50°C after 5 h of incubation [29].

Substrate-binding assay of Xyl36 and Xyl53

Xyl36 and Xyl53 were identified for their ability to bind to SCB, used as the only carbon source to support the *C. flavigena* CDBB-531 growth. To determine whether Xyl36 and Xyl53 have an affinity for other polysaccharides, their ability to bind to Avicel or insoluble oat spelt xylan was tested by substrate-binding assays. The absorption patterns observed were very similar for both enzymes; thus, indicating that Xyl36 and Xyl53 were able to bind to both substrates. However, Xyl36 and Xyl53 showed more affinity to insoluble oat spelt xylan than to Avicel (Table 2), particularly, in the case of Xyl36. The capability of Xyl36 and Xyl53 to bind to microcrystalline cellulose (Avicel) and to xylan-rich polysaccharides (residual SCB and insoluble oat spelt xylan) could be due to the presence of at least one CBM involved in enzyme binding to the insoluble

Table 2 Substrate-binding assay of Xyl 36 and Xyl53 to Avicel and insoluble oat spelt xylan

	Avicel[a]	Insoluble Xylan[b]
Xyl36	81.4 ± 2.1	72.5 ± 3.2
Xyl53	85.6 ± 2.8	78.3 ± 2.7

The assay was carried out using 2% (w/v) Avicel or insoluble oat spelt xylan in 0.1 M phosphate buffer of pH 7.0 at 4°C

[a, b] The percentage of residual xylanase activity after 60 min of incubation

substrates. Xylanolytic enzymes that bind to insoluble xylan and crystalline cellulose have been reported in *C. fimi* [26] and *Thermospora fusca* [5]. Also, mannanases from *B. subtilis* [39] and *Trichoderma reesei* [13] are able to bind to three different substrates mannan, insoluble xylan and cellulose.

The relatively high optimal temperature displayed by Xyl36 and Xyl53 (65 and 55°C, respectively) could be due to the presence of a CBM domain involved in the binding of Xyl36 and Xyl53 to avicel and xylan-rich polysaccharides and performing as a thermostabilizing module, like those reported in *Clostridium thermocellum* and *C. fimi* [7, 10].

In summary, we report the purification and characterization of two neutral thermophilic and moderately thermostable xylanases, named Xyl36 and Xyl53, from the mesophilic *C. flavigena* CDBB-531, with affinity to Avicel and xylan-rich polysaccharides. In view of these enzymatic properties, Xyl36 and Xyl53 have potential applications in biodegradation of hemicellulosic materials and they constitute good candidates for improvement of their catalytic properties by chemical modification, immobilization and protein engineering methods.

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