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Biochemical Characteristics of *Penicillium crustosum* FP 11 Xylanase II and an Assessment of the Properties of Xylanases Produced by the Genus *Penicillium*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors JWFB, VCA and JCL designed the study and wrote the first draft of the manuscript. Author CLDT wrote the protocol. Authors JLCS and AM managed the analyses of the study. Author RCGS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Penicillium crustosum FP 11 produces two extracellular xylanase, which are designated xylanase I and II, and are induced by corn stover. In this work, xylanase II was purified 40-fold with a recovery yield of 9.2% using DEAE-Sephadex and Sephadex G-75 gel filtration, and the biochemical characteristics of the enzyme were compared with other xylanases produced by the genus *Penicillium*. Xylanase II exhibited a single band on SDS–PAGE, and had an apparent molecular mass of 28 kDa. The optimal temperature and pH of xylanase II activity were 50ºC and 5.5, respectively. Xylanase II had activities of 61, 53 and 55% in the presence of Mg^{2+}, DTT and β-mercaptoethanol, respectively; however, the enzyme was strongly inhibited by 5 mM Cu^{2+}, EDTA, and SDS. Hydrolysis of beechwood xylan released mainly xylose and short-chain xylo-

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oligosaccharides as final products. Thus, an assessment of the enzymatic properties of xylanase II showed that its biochemical characteristics are best suited for the saccharification of lignocellulosic biomass into fermentable sugars.

Keywords: Penicillium crustosum FP 11; biochemical characterization; purification; xylo-oligosaccharides.

1. INTRODUCTION

Xylanases (E.C. 3.2.1.8) are enzymes that randomly cleave the β-1,4 linkages that connect xylose molecules of the xylan backbone [1,2], a component of the plant cell wall [3]. Xylanases have several biotechnological applications, which are determined by their biochemical characteristics. Active alkaline xylanases at elevated temperatures may be employed in the pulp and paper industry [4], while active acid xylanases at moderate temperatures can be used to clarify juices by reducing turbidity [5] via saccharification, converting lignocellulosic biomass into fermentable sugars for the production of ethanol [6] and baking by improving the characteristics of bread [7]. In addition, xylanases hydrolyze lignocellulosic biomass to produce xylo-oligosaccharides (XOS), which can be used as prebiotics and to stimulate bacterial growth beneficial to the human host [8]. These xylanases are produced by various microorganisms, such as filamentous fungi, bacteria, and yeast. Currently, the xylanases from microorganisms are some of the most industrially important enzymes produced, and the demand for fungal xylanases is even greater than for similar enzymes produced by other microorganisms, such as bacteria and yeast. This is due to the large capacity of fungi to secrete enzymes into the extracellular environment [9].

In this context, it is important to search for microorganisms capable of expressing high levels of these enzymes for the large-scale development of fermentation processes, to enhance xylanase purification strategies, and for the decrease costs [10,11,12]. Among the filamentous fungi, members of the genus *Penicillium* have been described as key xylanase producers [13,14]. However, there are few reports regarding enzyme production with the fungus *Penicillium crustosum*. In a previous study, two xylanolytic enzymes were induced in *P. crustosum* FP 11 with corn stover, xylanase I and II. Xylanase I has previously been purified and characterized [15]. Thus, in the present study, extracellular xylanase II produced from *P. crustosum* was purified and its biochemical characteristics were compared with other xylanases produced by the genus *Penicillium*.

2. MATERIALS AND METHODS

2.1 Chemicals

All the chemicals used were of reagent grade. The following substances were used: *Potato Dextrose Agar* (HiMedia Laboratories), salts used in the culture medium (Synth and Merck**),** dinitrosalicylic acid, beechwood xylan, bovine serum albumin, DEAE-Sephadex, Sephadex G-75 , Coomassie Brilliant Blue R-250**,** (Sigma – Aldrich, Brazil) Triton X-100, Congo red (Merck) TLC silica plate (Alufolien DC-Kieselgel 60, Merck®).

2.2 Microorganism and Culture Conditions

The fungus, *P. crustosum,* was previously isolated from the Atlantic forest biome of Paraná-Brazil and taxonomically identified. Its sequence has been deposited in GenBank (access number: KM065878). The fungus was maintained on potato dextrose agar (PDA) medium at 4°C. *P. crustosum* spores were used to inoculate modified Czapek liquid medium, which was supplemented with 2% (w/v) corn stover as a carbon source, as described by Silva et al [16]. The fungus was cultivated under stationary conditions at 28°C. After 6 days, cultures were filtered, and the enzymatic extract obtained was used to purify xylanase.

2.3 Enzyme Assay and Protein Determination

Xylanase activity was measured by determining reducing sugar content using dinitrosalicylic acid (DNS), as described by Miller [17]. Fifty microliters of the enzyme was incubated in 50 µL beechwood xylan (1%) in 50 mM sodium acetate, pH 5.5 for 10 min at 50°C. Then, 100 µL DNS reagent was added to the reaction mixture and it was boiled 5 min. One unit of activity (U) was defined as the amount of enzyme needed to release 1 µmoL reducing sugars per minute

using xylose as a standard. Specific activity was expressed in units (U) per mg protein. Protein quantification was performed according to the Bradford [18] using bovine serum albumin (BSA) as a standard. Proteins that had been eluted from chromatographic columns were monitored by measuring absorbance at 280 nm.

2.4 Xylanase Purification

The crude extract obtained from the *P. crustosum* corn stover culture was centrifuged for 10 min at 2,500 rpm at 4°C, and the supernatant was dialyzed for 18 h. The enzymatic extract was equilibrated to a final concentration of 20 mM in Tris-HCl buffer (pH 7.5) and was loaded on a DEAE-Sephadex ion-exchange chromatography column (10 × 2.0 cm) as described by Dapper et al. [19]. Then, 2.5 mL fractions were collected at a flow rate of 1 mL min^{-1}, and resin-bound proteins were eluted using a NaCl gradient (0.05 to 1.0 M). Fractions exhibiting xylanase activity were pooled and dialyzed for 18 h. The sample was then concentrated and 950 µL was loaded onto a Sphadex G-75 gel filtration column and eluted with 20 mM acetate buffer (pH 5.5) at a flow rate of 0.5 mL min^{-1}, and a pure xylanase sample was obtained.

2.5 SDS-PAGE and Zymogram

SDS-PAGE electrophoresis was performed according to methods described by Laemmli [20] on 10% acrylamide. After electrophoresis, the gel was cut into two parts, each containing one set of samples. The first gel was stained with Coomassie Brilliant Blue R-250 to assess protein homogeneity, and the other was used for zymogram analysis. The gel was incubated in 0.5% (v/v) Triton X-100 for 30 min at room temperature. After being washed with distilled water, the gel was incubated in 1% (w/v) xylan (prepared in 50 mM sodium acetate buffer, pH 5.5) at 50°C for 30 min. Then, the gel was stained with 0.5% (w/v) Congo red for 10 min at room temperature and distained with 1.0 M NaCl to reveal bands corresponding with enzymatic activity (light zone). The gel was kept in 1% acetic acid solution.

2.6 Influence of Temperature and pH on Xylanase Activity

The optimal pH for enzymatic activity was determined by performing an enzyme assay using 1% xylan beechwood for 10 min at 50°C using citrate-phosphate buffer in the 3.0–8.0 pH range. Xylanase stability at a particular pH was measured using pH 4.5, 5.5, 6.5, and 7.0 citrate-phosphate buffer for up to 48 h, without the substrate. Aliquots were withdrawn at an interval of 0–48 h to measure residual activity.

The optimal temperature for enzymatic activity was determined by incubating the enzyme with xylan (in 50 mM sodium acetate, pH 5.5) for 10 min at varying temperatures of 35–75°C. Thermal stability of xylanase was measured by incubating the enzyme at temperatures of 30°C, 40°C, 50°C, and 60°C. Aliquots were withdrawn at 0–180 minute intervals to determine residual activity.

2.7 Determination of Kinetic Analysis

The kinetic parameters (Km and Vmax) of *P. crustosum* xylanase II were determined using 1–24 mg/mL beechwood xylan. Enzymatic activity was measured using the standard dosage. Km and Vmax values were estimated via linear regression from double-reciprocal plots according to methods described by Lineweaver and Burk [21] using Origin 8.0 software.

2.8 Analysis of Hydrolyzed Products

End-products of xylanase hydrolysis were determined using thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC). First, 500 µL of the enzyme was mixed with 500 µL xylan beechwood (1%, w/v) and incubated at 50°C. Aliquots were withdrawn after 0, 30, 60, 120 160 min, or 12 and 24 h (for HPLC analysis), and subsequently boiled at 100°C for 5 min. Mixtures not containing enzyme were used as negative controls. TLC samples were spotted on silica plates and butanol:pyridine:water (7:3:1 v/v) was used as a solvent system according to Correa et al. [22] Products of hydrolysis were detected by spraying with 0.1% (w/v) orcinol in sulfuric acid:methanol (1: 9 v/v), followed by heating at 100°C for a few min. For HPLC analysis, aliquots of 20 uL were quantified using HPLC (Shimadzu model RID 20-A) using a refractive index detector and a 200 x10 mm RSO-Oligosaccharide Ag+ (4)% column maintained at 75°C with Mili-Q water as a mobile phase (0.6 mL/min flow rate). The standards used were xylose (X1), xylobiose (X2), xylotriose (X3) and xylotetraose (X4).

2.9 Effect of Metal Ions and Additives on Enzyme Activity

Regarding the effect of various compounds (Co^{2+}, K^{+}, Na^{+}, Sn^{2+}, Ba $^{2+}$, Ca^{2+}, Hg^{2+}, Fe^{2+},

Mg^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, EDTA, SDS, Triton X-100, DTT, β-mercaptoethanol, ethanol, and acetone) on xylanase activity, the final concentration of compounds within the reaction mixture were 1 mM and 5 mM (metal ions and reducing agents) and 1% and 5% (detergents and organic solvents). The residual activity was determined by standard enzymatic assay.

3. RESULTS AND DISCUSSION

3.1 Xylanase Purification

P. crustosum expressed two xylanases, named xylanase I (not bound to DEAE) and xylanase II (bound to DEAE). After the sample was eluted from the DEAE-Sephadex ion-exchange column, Xylanase II was eluted from the column using a 0.3–0.48 M NaCl gradient. Xylanase II was purified 40-fold with a recovery yield of 9.2% after Sephadex G-75 gel filtration, as shown in Table 1. Enzyme purity was confirmed via zymogram and the molecular mass of the enzyme was estimated using SDS-PAGE, which revealed a single band with an apparent molecular mass molecular weight of 28 kDa that was greater than xylanase I (23.4 kDa) (Fig. 1). The molecular mass of this xylanase was different than those of other xylanases produced by *Penicillium* strains (Table 2), but was similar to the 30 kDa weight previously determined for *P. funiculosum* PfXynC xylanase [23] and PoXyn2 of *P. occitanis* Pol6 [24].

3.2 Effect of Temperature on Xylanase Activity and Stability

The optimal temperature determined for *P. crustosum* xylanase II was the same obtained for xylanase I of 50°C (Fig. 2A), which was the temperature previously shown to be optimal for *P. canescens* xylanases [25] and *P. rolfsii* c3-2(1) IBRL [26]. However, optimal temperatures of 40°C and 80°C have also found for some fungal xylanases of *P. occitanis* Pol6 [24] and *P. funiculosum* [27], respectively. A great variability with regard to optimal temperature for enzymatic activity exists among the *Penicillium* mesophilic fungal xylanases. In fact many are most active at elevated temperatures, such as 75°C for *P. pinophlum* C1 xylanase [28] and 80°C for *P. funiculosum* XynD [27].

Table 1. Summary of steps used to purify xylanase II from *P. crustosum* grown on corn stover

steps	Total activity (U)	Total proteins (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification factor (fold)
Crude supernatant	3,572.0	692	5.16	100	1
DEAE-Sephadex	1,047.2	31.45	33.29	29.3	6.45
Gel filtration (Sephadex-G75)	328.65	1.59	206.70	9.2	40.06

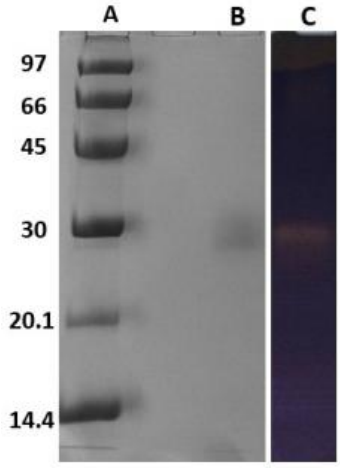

Fig. 1. SDS-PAGE and zymogram of xylanase II purified from *P. crustosum*
Lane A: low molecular weight marker: phosphorylase b (97 kDa); albumin (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa); and α-lactalbumin (14.4 kDa). Lane B: xylanase after Sephadex G-75. Lane C: xylanase II activity zymogram

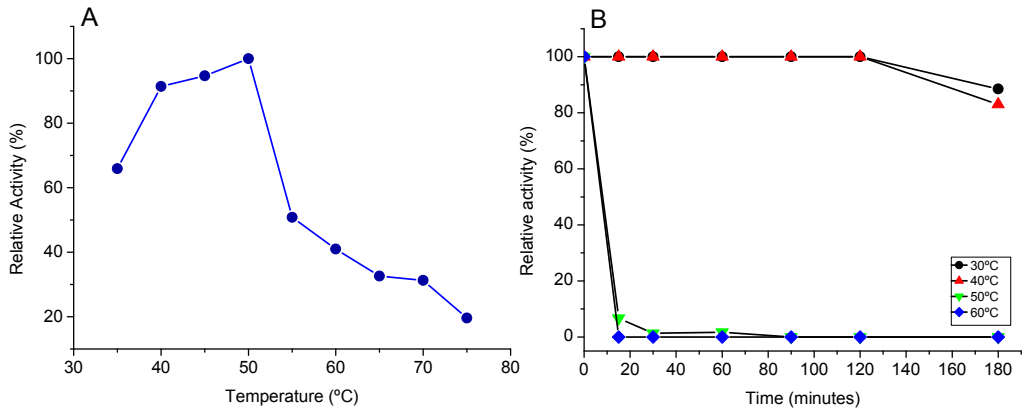

Fig. 2. Optimum temperature (A) and thermal stability (B) for purified xylanase II activity
The optimal temperatures were determined at temperatures between 35°C and 75°C, using 1% beechwood xylan in a 50 mM sodium acetate buffer (pH 5.5) as a substrate. Thermal stability was determined by measuring residual enzyme activity after incubation at 30°C, 40°C, 50°C, and 60°C for up to 180 min

Xylanase II exhibited 100% thermal stability at 35–40°C temperatures, and its activity declined after a 2 h incubation period (Fig. 2B). At temperatures of 50–60°C, the enzymatic activity decreased and became inactivated after 15 min. Thus, xylanase II has the potential to be used for the saccharification of lignocellulosic materials and XOS production, which demand mild temperatures.

3.3 Influence of pH on Xylanase Activity and Stability

P. crustosum xylanase II exhibited maximal activity at pH 5.5 (Fig. 3A) slightly higher than that of xylanase I (Table 2) [15]. Similar pH values were also obtained from xylanases of *P. chrysogenum* FS010 [29]. However, most *Penicillium* xylanases reported (Table 2) have maximum enzymatic activity at acidic pH, ranging from pH 2.0 for *P. occitanis* [30] to pH 6.0 for the xylanases produced by *P. janczewskii* [31], *P. oxalicum* GZ-2 [6] and XynB of *P. citrinum* GERM P-15944 [32]. However, others functioned optimally at neutral and alkaline-range pH. For instance, xylanase A produced by *P. purpurogenum* [33] functions optimally at pH 7.0, *Penicillium sp*. SS1 [34] at pH 8.0, and *P. citrinum* at pH 8.5 [35].

Xylanase II exhibited pH stability in the range of 5.5–7.0, the enzyme retained more than 80% of its activity for up to 48 h. However, it was most highly stable at pH 7.0, and 90% of its enzymatic activity was retained after 48 h (Fig. 3B). According to Törrönen and Rouvinen [36], xylanases with alkaline pH activity may be active at a broad pH range of 4.0–8.0, while xylanases that function optimally at acidic pH have a narrower range of 3.0–6.0.

3.4 Kinetic Parameters

The apparent Km and Vmax values determined here, using beechwood xylan as substrate, were 1.1 mg/mL and 1,064 U/mg, respectively. A Km of 1.0 mg with beechwood xylan was also obtained for xylanase Xyn10B produced by *P. oxalicum* [6]. A comparison with the Km value obtained for *P. crustosum* xylanase II shows that this enzyme has a relatively greater affinity for beechwood xylan. However, xylanases with even greater Km have previously been identified, including *P. oxalicum* GZ-2 Xyn 10B and *P. oxalicum* B3-11(2), which produced Km values of 3.0 mg/mL [6] and 4.6 mg/mL [37], respectively.

3.5 Analysis of Hydrolyzed Products

The means by which purified xylanase hydrolyzed beechwood xylan was assessed using TLC (Fig. 4A). These analyses showed that xylanase II hydrolyzes β-1,4 linkages of both inner and outer ends of the xylan substrate, and mainly releases X1, X2 and X3 as products. This is similar to reactions of XynA and XynB from *P. citrinum* FERM P-15944 [32,38] and the xylanase from *Penicillium* sp. SS1 [34]. Some enzymes produce exclusively X2 and X3 products, such as

P. crustosum FP 11 xylanase I [15], *P. canascens* xylanase [39], and a large number of other *Penicillium* xylanases released X1, X2, X3, X4, X5, and xylooligosaccharides (Table 2). Using HPLC, xylose (2.76 and 3.14 mg/mL) and xylobiose (5.61 and 5.96 mg/mL) were obtained after 12 and 24 h, respectively (Fig. 4B). However, the amount of xylotriose (0.95 mg/mL) and xylotetraose (0.03 mg/mL) did not differ after 12 and 24 h (Fig. 3B). Similar results were obtained with XynA and XynB from *P. citrinum* FERM P-15944 [32,38] and xylanase from *Penicillium* sp. SS1 [34]. The production of short-chain xylo-oligosaccharides and xylose by *P. crustosum* xylanase II reveals that the enzyme is an endo and exo-xylanase, and could promote the saccharification of lignocellulosic biomass into fermentable sugars.

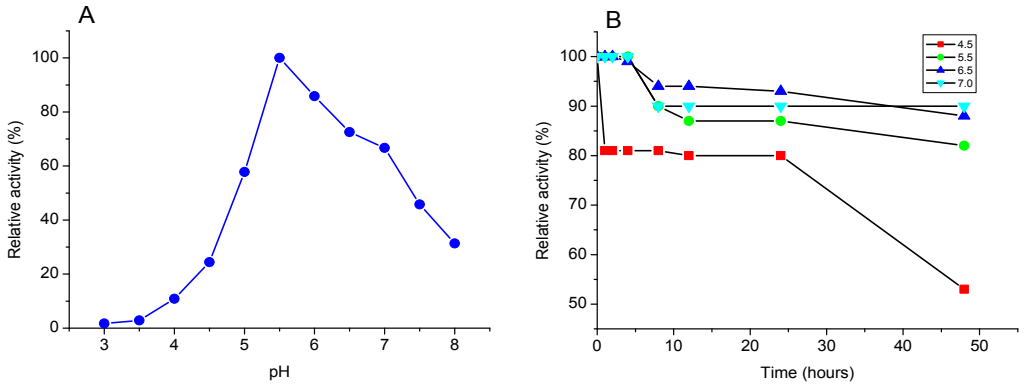

Fig. 3. Optimal pH (A) and pH stability (B) of *P. crustosum* xylanase II

Optimal pH was assessed by incubating the enzyme in pH 3.0–8.0 citrate-phosphate buffer with 1% xylan beechwood substrate. pH stability was determined by measuring residual enzymatic activity after incubation in citrate-phosphate buffer at pH 4.5, 5.5, 6.5, and 7.0 for up to 48 h

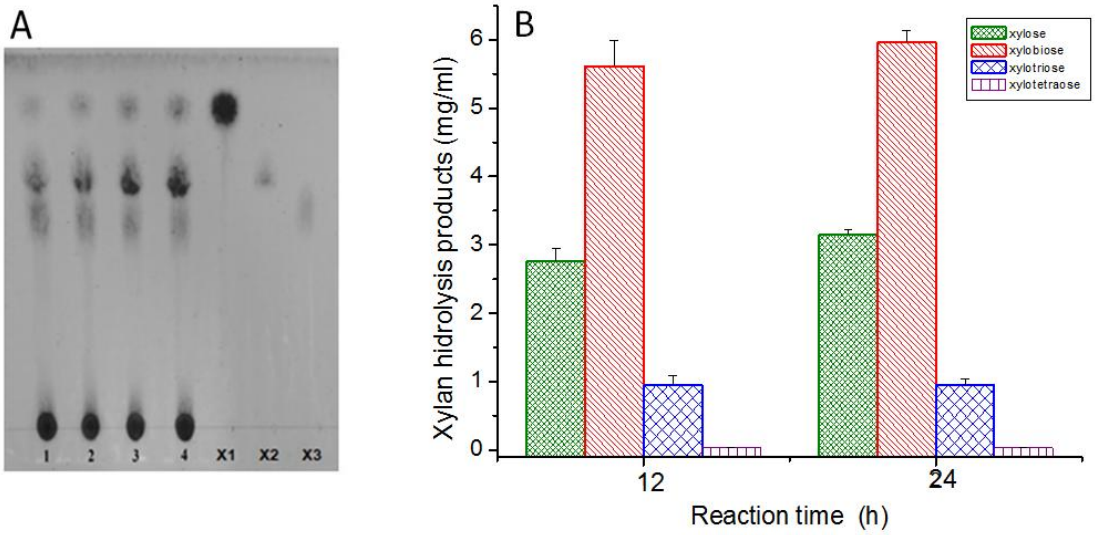

FIG. 4. Analysis of the products of *P. crustosum* xylanase ii hydrolysis using thin layer chromatography (a)

The enzymatic reaction was stopped after 30, 60, 120 and 160 min, as shown in lanes 1, 2, 3 and 4, respectively. The standards included xylose (x1), xylobiose (x2), xylotriose (x3) and xylotetraose (x4). Hplc analysis of products of beechwood xylan hydrolysis after 12 and 24 h (b).

Table 2. Properties of xylanases produced by fungi of the genus *penicillium*

Penicillium Strain	Enzyme name	Molecular mass (kDa)	Optimum pH	pH stability	Optimum Temperature (°C)	Thermal stability	Xylan hydrolysis products	References
Penicillium crustosum FP 11	Xylanase II	28	5.5	5.5-7.0	50	30-40	X1, X2, X3	This work
Penicillium crustosum FP 11	Xylanase I	23.4	5.0	4.0-7.0	50	40-50	X2, X3	[15]
Penicillium oxalicum GZ-2	XYN11A	21.3	3.0	3.0-5.0	50	50-60	X4, X5, X6	[42]
Penicillium janczewskii	Xyl 1	30.4	6.0	2.5-10.0	65	50-65	X3, X4, XOS	[31]
Penicillium pinophilum C1	XYN10C1	40	4.0-5.5	3.0-6.5	75	70-80	X1 and X3	[28]
Penicillium occitanis Pol6	PoXyn3	20	4.0	2.0-10.0	40	30-50	NR	[23]
Penicillium funiculosum	PfXynC	30	3.0	2.0-10.0	45	30-50	NR	[23]
Penicillium oxalicum GZ-2	Xyn10A	36	6.0	5.0-7.0	40	40-45	X2, X3, X4	[6]
Penicillium oxalicum GZ-2	Xyn10B	43	6.0	5.0-7.0	70	50-70	X2, X3, X4	[6]
Penicillium oxalicum GZ-2	Xyn11A	23	4.0	4.0-5.0	50	40-45	X2, X3, X4, X5	[6]
Penicillium oxalicum GZ-2	Xyn11B	32	5.0	4.0-5.0	50	40-45	X2, X3, X4, X5	[6]
Penicillium ramulosum N1	xylanase	25	3.0	NR	55	NR	NR	[44]
Penicillium canescens	XylE	40	5.5-6.0	4.0-8.0	70	65-75	X2, X3	[39]
Penicillium citrinum	xylanase	25	8.5	4.0-10.0	50	30-50	NR	[35]
Penicillium sp. SS1	xylanase	NR	8.0	6.0-10.0	50	50-60	X1, X2, X3, XOS	[34]
Penicillium occitanis Pol6	PoXyn2	30	3.0	2.0-9.0	50	30-50	NR	[24]
Penicillium sp. F63	Xyn11F63	21	4.5	4.5-9.0	40	40-45	X1, X2, X3, X4	[45]

Penicillium Strain	Enzyme name	Molecular mass (kDa)	Optimum pH	pH stability	Optimum Temperature (°C)	Thermal stability	Xylan hydrolysis products	References
CGMCC 1669								
Penicillium funiculosum	XynD	46	4.0-5.5	4.0-5.5	80	70-80	X2,X3, X4	[29]
Penicillium occitanis	PoXyn2	NR	2.0-4.0	NR	65	50-65	NR	[30]
Penicillium oxalicum B3-11(2)	PoxynA	32	5.0	2.0-7.0	50	30-50	X2, X3, X5	[37]
Penicillium chrysogenum FS010	Xyl	38	5.5	4.5-8.0	25	25-35	NR	[29]
Penicillium purpurogenum	Xylanase A	33	7.0	6.0-7.5	60	NR	NR	[33]
Penicillium purpurogenum	Xylanase B	23	3.5	6.0-7.5	50	NR	NR	[33]
Penicillium sclerotiorum	Xylanase 1	23.9	2.5	1.6-3.0	50	40-50	X2, XOS	[43]
Penicillium sclerotiorum	Xylanase 2	33.1	4.5	3.0-7.0	55	45-55	X3, XOS	[43]
Penicillium citrinum FERM P-15944	XynA	20	5.0	2.0-10.0	55	NR	X1, X2, X3, X4	[32]
Penicillium citrinum FERM P-15944	XynB (Endo-1,4-b-xylanase)	31.6	6.0	3.0-10.0	50	NR	X1, X2, X3, XOS	[38]

NR: NOT REPORTED

3.6 Effect of Potential Inhibitors and Activators on Xylanase II Activity

The effects of potential inhibitors and activators on purified xylanase II from *P. crustosum* is shown in Table 3. Xylanase II activity was stimulated 61.8, 53.6 and 55.7% in the presence of 5 mM Mg^{2+}, DTT and β-mercaptoethanol, respectively (Table 3). Querido et al. [40] also reported *P. expansum* xylanase activation by Mg^{+2}. Enzymatic activation by thiol group-containing compounds, such as DTT and β-mercaptoethanol confirms the presence of a reduced thiol group of cysteine residue in this enzyme [41]. However, some compounds inhibited the enzymatic activity of *P. crustosum* xylanase, especially Hg^{2+} and Cu^{2+}. Inhibition of xylanases by Hg^{2+} has been described by Liao et al. [42] in a xylanase from *Penicillium oxalicum* as well as *P. sclerotiorum* xylanase [43] Similarly, Cu^{2+} ions have widely been reported inhibitors of xylanases [28].

Table 3. Effect of potential inhibitors and activators on *P. crustosum* xylanase II activity

Compounds	Residual activity[a] (%)	
	1 mM	5 mM
Control	100	100
$CoCl_2$	109.3 ± 0.02	100.1 ± 0.02
KCl	89.9 ± 0.02	77.1 ± 0.04
NaCl	93.1 ± 0.02	68.4 ± 0.06
$SnCl_2$	52.6 ± 0.05	19.0 ± 0.01
$BaCl_2$	81.9 ± 0.06	71.4 ± 0.01
$CaCl_2$	103.7 ± 0.01	120.6 ± 0.01
$HgCl_2$	8.9 ± 0.01	0
$FeCl_2$	81.5 ± 0.04	61.5 ± 0.02
$MgCl_2$	100.3 ± 0.02	161.8 ± 0.03
$CuSO_4$	43.4 ± 0.02	5.2 ± 0
$MnCl_2$	108.2 ± 0.02	108.5 ± 0.04
$ZnSO_4$	82.2 ± 0.02	80.7 ± 0.01
EDTA	83.9 ± 0.40	0
ethanol	70.5 ± 0.02	37.0 ± 0.01
Acetone	86.7 ± 0.01	41.0 ± 0.02
SDS	20.4 ± 0.01	16.7 ± 0.01
Triton X100	88.2 ± 0.01	58.9 ± 0.03
β-mercaptoethanol	167.0 ± 0.04	1 55.7 ± 0.03
DTT	141.8 ± 0.05	153.6 ± 0.01

[a]*Average of three individual experiments*

4. CONCLUSION

P. crustosum xylanase II exhibited biochemical properties different from xylanase I, but similar to many *Penicillium* xylanases. The enzyme was optimally active at acidic pH and was stable throughout an acidic to neutral pH range. The thermal stability of xylanase II was optimal at mild temperatures. An assessment of hydrolyzed products of the enzyme revealed that it exhibited both endo and exo-xylanase activity. However, an increased proportion of short-chain xylo-oligosaccharides and xylose were produced. Thus, an assessment of the enzymatic properties of xylanase II showed that its biochemical characteristics are best suited for the saccharification of lignocellulosic biomass into fermentable sugars for the production of bioethanol.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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