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Functional characterization of *Penicillium occitanis* Pol6 and *Penicillium funiculosum* GH11 xylanases

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A B S T R A C T

Xylanases are hemicellulolytic enzymes, which are responsible for the degradation of heteroxylans constituting the lignocellulosic plant cell wall. Xylanases from the GH11 family are considered as true xylanases because of their high substrate specificity. In order to study in depth a crucial difference in the thumb region between two closely related xylanases from *Penicillium* in terms of kinetic parameters and inhibition sensitivity, the GH11 xylanases from *Penicillium occitanis* Pol6 (PoXyn3) and from *Penicillium funiculosum* (PfXynC) were heterologously expressed in *Pichia pastoris.* The PoXyn3 and PfXynC cDNAs encoding mature xylanases were cloned into pGAPZαA vectors and integrated into the genome of *P. pastoris* X-33 under the control of the glyceraldehyde 3-phosphate dehydrogenase constitutive promoter. PfXynC was expressed as a His-tagged recombinant protein and purified from the supernatant homogeneity by a one-step purification protocol using immobilized metal affinity chromatography. The recombinant PoXyn3 was purified using a single anion-exchange chromatography. The purified recombinant enzymes were optimally active at 45 °C and pH 4.0 for PoXyn3 and 40 °C and pH 3.0 for PfXynC. The measured kinetic parameters (k_{cat} and V_{max}) showed that PfXynC was five times more active than PoXyn3 irrespective of the substrate whereas the apparent affinity (K_m) was similar. The recombinant enzymes showed distinct sensitivity to the *Triticum aestivum* xylanase inhibitor TAXI-I.

Introduction

Enzymatic hydrolysis of the plant cell wall release small carbohydrates that are utilized as an energy and carbon source by a range of organisms. This degradative process is of fundamental biological and industrial importance. Endo-(1,4)-β-xylanases (xylanases; EC 3.2.1.8) depolymerize the xylan backbone, a significant component of the plant cell wall, by cleaving the β-(1,4) glycosidic bonds between D-xylose residues in the main chain to produceshort xylo-oligosaccharides [1]. Based on amino acid sequence similarities, xylanases are mainly classified into families GH10 and GH11 of glycoside hydrolases [2]. The two families have different molecular structures, molecular weights, and catalytic properties. Family GH10 generally consists of higher molecular weight proteins (>30 kDa) with a $(\beta/\alpha)_8$ barrel structure, whereas family GH11 consists of lower molecular weight proteins (20–30 kDa) with a β-jelly-roll fold [3]. Enzymes with xylanolytic activities are also found in GH families GH5, GH8 and GH43 [4], but only GH11 family is monospecific, consisting solely of "true xylanases" exclusively active on xylan. The three-dimensional structure of GH11 xylanases has been described as a "partly closed right hand". This hand contains a thumb-like structure that connects the B7 and B8 β-strands, which is the most mobile region of the xylanase family 11 [7]. The movement of the loop affects the movement of the connecting strands and could be essential for the function of the enzyme. The thumb has also been identified as the target for some plant proteinaceous inhibitors [7–8]. Xylanases have been studied for their role in several industrial food processes, mainly in baking, wheat processing, and as supplements in animal feed production [3]. They are routinely used as bread-improvers, but the observed effects on bread volume, crumb texture, and flavour vary depending on the xylanase tested [5]. These differences could be due to the variability in xylanase specificity towards arabinoxylan but also to the presence of xylanase protein inhibitors in cereals [6]. To date, two main types of xylanase inhibitors with different structures and specificities have been described [6].

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The xylanase inhibitor protein (XIP)-type inhibitors can inhibit both GH10 and GH11 xylanases, whereas the *Triticum aestivum* xylanase inhibitor (TAXI)-type inhibitors are active against bacterial and fungal GH11 xylanases, but not against GH10 xylanases [9].

The methylotrophic yeast *Pichia pastoris* is a host system, which has been widely used in both academic and industrial laboratories for the production of a variety of heterologous proteins [10]. This system has many of the advantages of eukaryotic expression, such as the ability to carry out proteolytic processing, folding, disulphide bond formation, and glycosylation. A wide variety of proteins have been produced successfully using the tightly controlled methanol-inducible alcohol oxidase 1 (AOX1) promoter, including the classical human pancreatic lipase [11]. Another *Pichia* expression systems based on the strong constitutive promoter of *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene (GAP) have recently become available [12]. The GAP promoter is a convenient alternative to the inducible AOX1 promoter for the heterologous expression of genes giving products which are not toxic to *P. pastoris*, or when the use of methanol, a highly toxic solvent, has to be ruled out.

Penicillium fungi are primarily saprophytic in nature, and numerous species have been exploited in commercial food production (e.g., cheeses) and for the antibiotic penicillin. They have also been used for the production of xylanolytic enzymes [13]. The *Penicillium occitanis* Pol6 mutant secretes two xylanases termed PoXyn2 and PoXyn3[1], which were purified from culture supernatant and characterized in terms of biochemical properties [14,15]. Recently, PoXyn2 was successfully expressed in the methylotrophic yeast *P. pastoris* X-33 [16]. The PoXyn3 belongs to the GH11 family and is an extracellular, acidophilic xylanase with an unusual naturally deleted portion in the thumb, which was revealed by sequence analysis and modeling of the 3D structure (Fig. 1) [14]. PoXyn3 is closely related to the native *Penicillium funiculosum* xylanase C (PfXynC) with a more conventional thumb that was previously characterized notably with respect to its inhibition by XIP and TAXI [17]. In the present study, we report the expression and purification of *Penicillium* xylanases, PoXyn3 and PfXynC, in *P. pastoris* and the biochemical and enzymatic characterization of both recombinant enzymes in terms of kinetics towards arabinoxylan and inhibition sensitivity to TAXI-I.

Materials and methods

Strains, plasmids, culturing conditions

The pGEX/1λT expression vector harboring PfXynC cDNA was from [18] and the PoXyn3 cDNA cloned in the pGEMT-easy vector from [14].

The *P. pastoris* host strain X-33 and *P. pastoris* transfer vector pGAPZαA were from Invitrogen (San Diego, CA). *P. pastoris* liquid cell cultures were grown in buffered YPD medium containing 10 g yeast extract, 20 g Bacto-peptone, 20 g D-glucose, and 10 mM sodium phosphate buffer with the pH initially adjusted to 6.8. The YPDS medium used for recombinant yeasts culture was YPD medium to which 18.2 g sorbitol per liter was added. To prepare plates for solid cell cultures, 2% agar (w/v) was added to the YPD medium.

All cloning steps were performed in *Escherichia coli*, DH5α cultured in low salt Luria–Bertani medium in the presence of 25 μg/ml Zeocin (Invitrogen).

Cloning of PoXyn3 and PfXynC in pGAPZαA expression vector

From PfXynC cDNA, a 591-bp DNA fragment was amplified by PCR with forward primer (PF1: 5′-CATTACTGTGAATTCCAATCAATCACGACCAGCCAG-3′) and reverse primer (PF2: 5′-CATTACTGTTCTAGACCGGACACTGTGATGGTACTA-3′). Similarly from PoXyn3 cDNA, a 483-bp DNA fragment was amplified by PCR with forward (PO1: 5′-CATTACTGTGAATTCATGAAACTGTTTCTGGCGGCG-3′) and reverse (PO2: 5′-CATTACTGTTCTAGATTAGCTCGCCACAATGGTCGCGCT-3′) primers. Primers include *EcoRI* sites (underlined) immediately upstream of the mature sequences (without signal peptides) and *XbaI* site downstream of the stop codon in the case of PfXynC. The *EcoRI–XbaI* digested PCR products were purified and inserted into the pGAPZαA. *P. pastoris* expression vector, (pGAPZαA–PfXynC, 3691 bp; pGAPZαA–PoXyn3, 3583 bp) downstream of the GAP constitutive promoter as described by Sias [19]. Plasmid DNAs were isolated from *E. coli* cell cultures using the alkaline lysis procedure [20] and purified using the Wizard Plus Midipreps DNA Purification System (Promega). Restriction enzyme digestion and ligation steps with T4 DNA ligase were performed as recommended by the enzyme suppliers. Plasmid DNAs were introduced into *E. coli* DH5α cells by electroporation using a gene pulser (Bio-Rad). The DNA nucleotide sequence was determined using the dideoxynucleotide chain termination method using an ABI PRISM 3100 Avant (Applied Biosystem) sequencer, according to the instructions of the manufacturer.

Transformation of P. pastoris and selection of PoXyn3 and PfXynC-best-producing transformants

Electrocompetent *P. pastoris* X-33 cells were prepared using standard methods [21] and their transformation was performed by electroporation [20]. Prior to the yeast transformation, both recombinant plasmids were linearized by BglII. The recombinant yeast clones were selected on YPDS plates containing 100 μg/ml zeocin [20]. The colonies were subsequently screened by performing direct PCR on yeast colonies to confirm the correct integration of each PoXyn3 and PfXynC DNAs into the genome [20]. For the screening of the best-producing transformants, positive clones were grown in 250-ml Erlenmeyer flasks containing 50 ml YPD medium with Zeocin, at 30 °C for 30 h under shaking at 100 rpm. All yeast cultures had an initial optical density (600 nm) of 0.2. After centrifugation (10 min at 3000*g*), the culture supernatants were stored at 20 °C. The presence of recombinant xylanases in the culture supernatants was checked by performing SDS–PAGE. The three days time-course of recombinant xylanases secretion into the culture supernatant was determined using three transformants.

Production of recombinant PoXyn3 and PfXynC xylanases in P. pastoris

A preculture with the best-producing transformants was performed for 24 h in a 250-ml Erlenmeyer flask containing 50 ml YPD medium. This culture was used to inoculate larger cultures at an optical density (600 nm) of 1, and grown in a 1-l Erlenmeyer flask containing 200 ml YPD medium at 30 °C at 100 rpm. The cultures were stopped after 96 h and the cells were pelleted by centrifugation at 3000*g* for 20 min.

Purification and characterization of recombinant PoXyn3 and PfXynC

The recombinant PfXynC xylanase was purified from the culture supernatant after cultivation for 96 h. Cell free medium was concentrated and interchanged with buffer A: 10 mM Tris–HCl buffer (pH 7.5) by ultrafiltration through a 10-kDa membrane (Millipore, USA). The total sample volume obtained (10 ml) was then mixed with 5 ml of the Ni_2-nitrilotriacetate resin (Qiagen, CA, USA) equilibrated with Tris–HCl buffer (Buffer A). The mixture was loaded onto a chromatographic column and washed with 200 ml of buffer

[1] *Abbreviations used:* PoXyn3, *Penicillium occitanis* xylanase 3; PfXynC, *Penicillium funiculosum* xylanase C; GH11, glycoside hydrolase family 11; AOX1, alcohol oxidase 1

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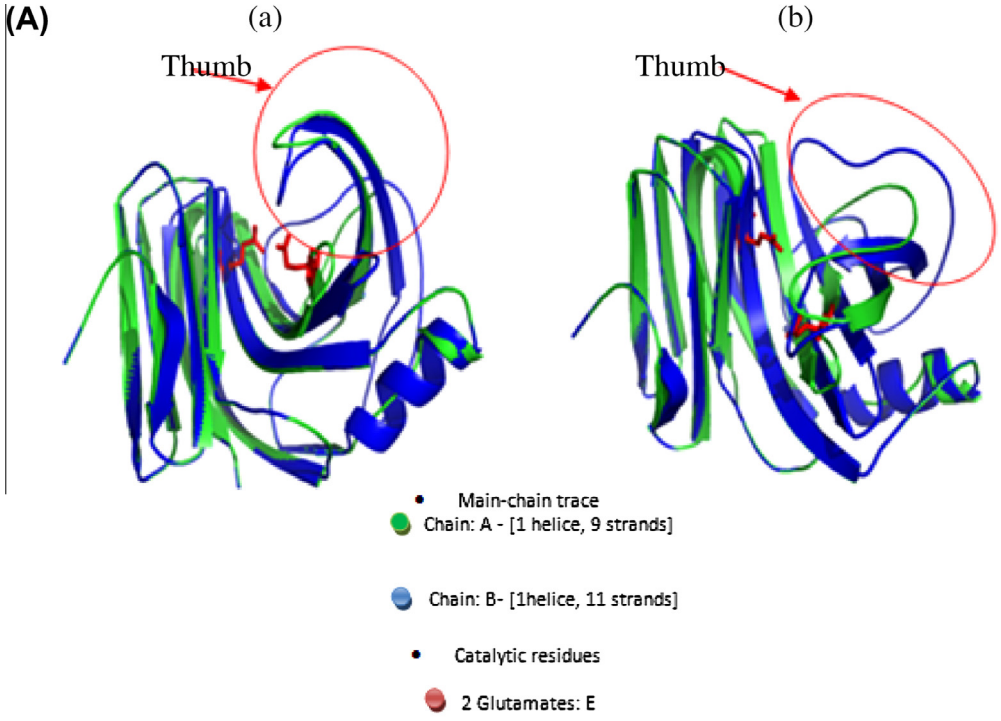

(B)

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Xyn3 de P.occitanis Pol6  TYESGINNGYYYTIWINGGGEVTYINGDNGEYSVIWVDCGDFTSGKGWNPANAQTVTYSG 60
XynC de P.funiculosum     TSQTGINNGYYYSFWT-GGGEVTYINGDNGEYSVIWVDCGDFTSGKGWNPANAQTVTYSG 59
Clustal Consensus         * ::********: ** ****************************************** 57


Xyn3 de P.occitanis Pol6  EFNPSGNAYLAVYGWTT--LVEFFLHLIWTYFWPGHHLP-------------------AN 99
XynC de P.funiculosum     EFNPSGNAYLAVYGWTTDPLVEYYILESYGTYNPSSGLTSLGQVTSDGGTYDIYSTQRVN 119
Clustal Consensus         *****************  ***:::   :  : *.  *.                  .* 85
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Fig. 1. (A) Three-dimensional structure of PfXynC (A) and PoXyn 3(B). The protein schematic is color ramped from the N-terminus (blue) to the C terminus (green). These structural representations were all drawn with Swiss pdb viewer (http://swiwwmodel.expasy.org). (B) Alignment of parts of the cDNAs of Xyn3 from *P. occitanis* Pol6 and XynC from *P. funiculosum* around the sequence delimiting the thumb region. The dashes (–) indicate the naturally deleted region in the Xyn3 sequence.

A. The recombinant PfXynC xylanase was eluted with a linear imidazole gradient from 0 to 500 mM in buffer A.

The recombinant PoXyn3 xylanase was purified by using an anion exchange chromatography on a PBE (Poly Buffer Exchanger) 94 column. The column was equilibrated and eluted in Tris–HCl 20 mM, pH 8; 20 mM NaCl. The flow rate was 30 ml/h, and the absorbance of the eluant was monitored at 280 nm.

In both cases, the eluted fractions containing the recombinant proteins were pooled, concentrated and assayed for xylanase activity using the 3,5-dinitrosalicylic acid (DNS) assay (see below).

The presence of glycan chains in the purified xylanase was checked by the anthrone–sulfuric acid method using glucose as a standard [22].

To determine the optimal pH and temperature profiles, the enzymatic reaction was carried out at different pHs and temperatures. The effect of selected metal ions (5 mM) on the activity of the purified enzyme was investigated by adding the divalent metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}) to the reaction mixture. The effect of an enzyme inhibitor on xylanase activity was also studied using 5 mM ethylenediaminetetraacetic acid (EDTA). Activity of the enzyme in the absence of metal ions or EDTA was used to define 100% activity under standard conditions. For the determination of apparent Michaelis–Menten constants, the initial velocities of the enzymes with increasing concentrations (0.1–10 mg ml^{-1}) were measured at 30 °C in McIlvaine's buffer, pH 5.5, with substrate concentrations ranging from 2 to 20 mg ml^{-1}. Due to the heterogeneous nature of polymeric substrates, their molar concentrations could not be calculated. Consequently, only an apparent value for the Michaelis constant, K_m (app), was determined. The kinetic parameters were estimated using weighted nonlinear squares regression analysis with the Grafit software (Biosoft, Cambridge, UK).

Xylanase activity and inhibition assays

Both purified xylanases activities were measured using the DNS assay [23] as described in [6]. One unit of xylanase activity was defined as the amount of protein that released 1 µmol of xylose/min at 30 °C and pH 5.5.

Inhibition assays of the *Penicillium* xylanases were determined using low viscosity wheat arabinoxylan (LVAX) at 30 °C and pH 5.5. Recombinant PfXynC and PoXyn3 were incubated with TAXI-I [24] up to an inhibitor/enzyme molar ratio of 30:1. All assays were carried out in triplicate.

Purification of TAXI I

TAXI-I (*T. aestivum* Xylanase Inhibitor Type I) was purified from wheat flour according to [24].

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Results and discussion

Expression and purification of PfXynC and PoXyn3 xylanases in P. pastoris

In this report, we expressed and characterized two family GH11 xylanases from *Penicillium* species. Both *P. occitanis* and *P. funiculosum* xylanases were efficiently produced in *P. pastoris* X33. In fact, the 672 bp and 483 bp cDNAs coding for PfXynC and PoXyn3 xylanases were amplified by PCR and fused in-frame to the α-factor secretion signal of the pGAPZαA expression vector and the GAP promoter-driven constitutive expressions of these two proteins were achieved by integrating linearised pGAPZαA–PoXyn3 and pGAPZαA–PfXynC plasmids into the X-33 wild type *P. pastoris* genome at the *GAP* locus. The presence of xylanases inserts in these Zeo-resistant transformants was checked by PCR. Eighteen positive clones were randomly chosen for both xylanases cultures. One of the two clones was introduced into *P. pastoris* X33. The transformants were screened on YEPD plate containing 0.1 mg/ml Zeocin and further confirmed by PCR using 5′- and 3′-AOX primers.

Purification of the recombinant PfXynC was facilitated by the presence of six His residues at the N-terminus of the enzyme, allowing purification of the xylanase using a one-step Ni–NTA affinity chromatography. PoXyn3 did not contain an His-tag and was readily purified by anion exchange chromatography. Both enzymes were purified to homogeneity with purification yields of 49% and 43% and a purification yield of 2.17 and 1.14-fold for PoXyn3 and PfXynC, respectively (Table 1). SDS–PAGE analysis of the purified enzymes showed the presence of one band corresponding to an apparent molecular mass of about 30 and 20 kDa for PfXynC and PoXyn3, respectively (Fig. 2).

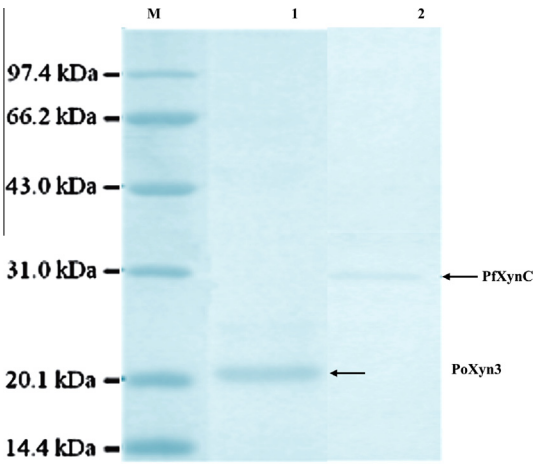

Fig. 2. SDS–PAGE of recombinant PfXynC and PoXyn3 secreted by *P. pastoris*. Lane M: protein marker (Fermentas). Lane 1: 15 µl of purified culture supernatant of PoXyn3; lane 2: 10 µl of purified mature PfXynC. The protein gels were stained with Coomassie brilliant blue G250.

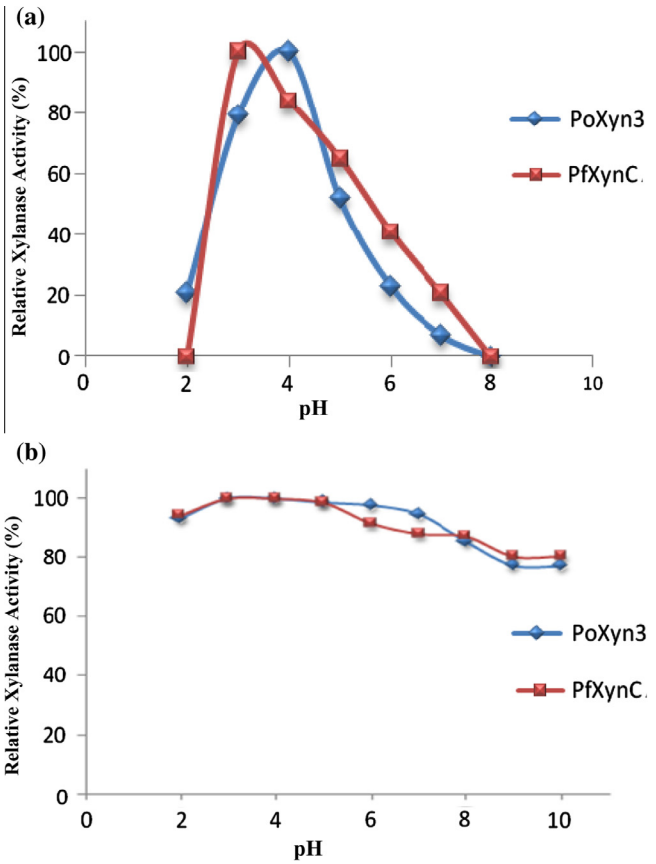

Fig. 3. Effect of pH on (a) activity and (b) stability of the purified xylanases PoXyn3 and PfXynC. The xylanase activity was assayed in the pH range 2–10 using buffers at different pH values at 45 °C and 40 °C for PoXyn3 and PfXynC, respectively. The maximum activity obtained at pH 3.0 was taken as 100%. The pH stability was determined by incubating the enzymes in different buffers at 4 °C for 24 h and the residual activity was measured at optimum pH and temperatures for both xylanases. The activity of the enzyme before incubation was taken as 100%.

Biochemical characterization of recombinant xylanases

The N-terminal sequences of PoXyn3 and PfXynC (T-Y-E-S-G-T-N and T-S-D- I-T-Q-N, respectively) were identical to the corresponding deduced amino acid sequences, indicating a correct cleavage of the α-factor by the Kex2. The extent of glycosylation of the recombinant xylanases was determined by the anthrone method. PoXyn3 showed a 17.7% glycosylation of compared to 10.8% for the native one whereas PfXynC glycosylation was slightly higher than the expressed one in *E. coli* (which were not glycosilated) [18].

The optimal temperature of the purified xylanases was determined at pH 3.0 and 4.0 for 60 min for PoXyn3 and PfXynC, respectively at different temperatures (30–80 °C). The purified enzymes showed optimal temperatures at 40 and 45 °C for PoXyn3 and PfXynC, respectively (Fig. 4a). To determine the optimal pH, PfXynC and PoXyn3 were incubated at their optimum temperatures for 10 min at different pHs (2–10), showing an optimal pH of 3.0

Table 1
Purification of recombinant xylanases PoXyn3 and PfXynC from *P. pastoris*.

Xylanases	Purification step	Total activity (UT)	Total protein (mg)	Specific activity (UI/mg)	Recovery (%)	Purification (x-fold)
PoXyn3	Crude extract	2823	173.4	16.28	100	1
	PBE-94	1380	39	35.38	48.88	2.17
PfXynC	Crude extract	4443	19.2	231.4	100	1
	His-Trap	1918.5	7.23	265.35	43.18	1.14

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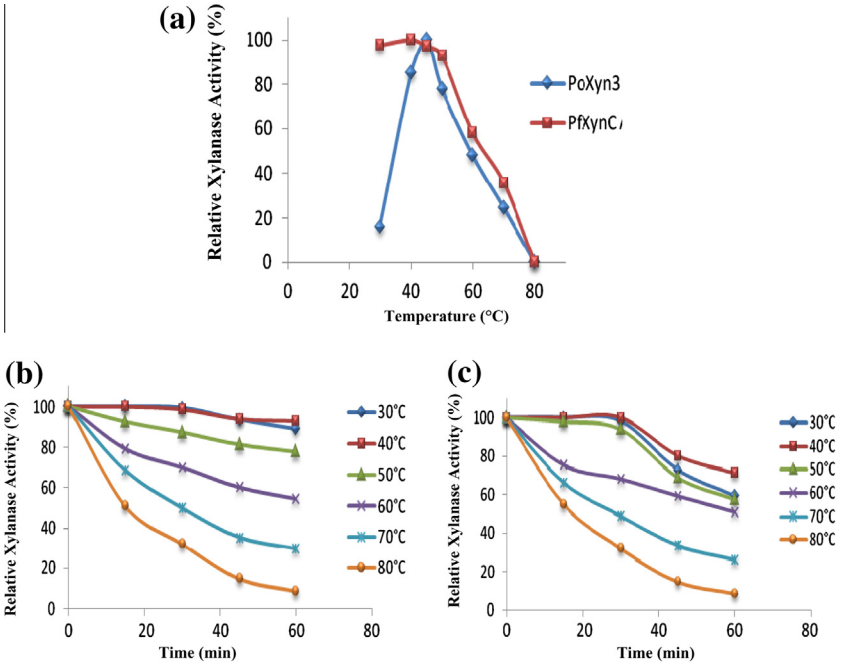

Fig. 4. Effect of temperature on (a) activity and (b) stability of the purified xylanases PoXyn3 and (c) stability of the purified xylanases PfXynC. The temperature profiles were determined by assaying xylanases activity at temperatures from 30 to 80 °C. The activity of the enzymes at optimum temperatures were taken as 100%. The temperature stabilities for both enzymes were determined by incubating each purified enzyme at temperatures from 30 to 70 °C for 60 min. Aliquots were withdrawn and residual enzyme activities were measured under standard assay conditions. The non-heated enzyme was taken as 100%.

and 4.0, respectively (Fig. 4a). Furthermore, the purified enzymes were highly stable within a broad pH range. The enzymes retained 100% of their activity over a wide pH range from 2.0 to 10.0 (Fig. 3b).

The purified PoXyn3 enzyme was stable at temperatures below 30 °C. The enzyme retained about 70%, its initial activity after 60 min incubation at 60 °C (Fig. 4b). The PfXynC enzyme displayed almost the same profile across increasing temperatures, retaining more than 80% of its initial activity after 60 min incubation at 50 °C suggesting that these xylanases are mesophilic enzymes.

The effect of selected bivalent metal ions on the recombinant xylanases was investigated. Under optimum conditions determined above, a slight activity enhancement of 105–107% and of 101–106% for PoXyn3 and PfXynC, respectively, in the presence of 5 mM Co^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+} metal ions was observed (Table 2). However, in the presence of 5 mM Ca^{2+} and Mg^{2+} metal ions, the enzymes activities were reduced to 89% and 93% for PoXyn3, respectively, and to 95% and 91% for PfXynC, respectively from their initial activity. Preincubation of the enzyme with a 5 mM EDTA did not affect the activities; the enzymes recovered their original activities following addition of any of the bivalent metal ions.

Overall, PoXyn3 and PfXynC recombinant xylanases were respectively similar to native PoXyn3 and to PfXynC expressed in *E. coli* in terms of molecular mass, pH, and temperature optimum and specific activity [14,18].

Table 2
Effect of metal ions (5 mM) on the activity of the xylanases PoXyn3 and PfXynC.

Treatment	Relative PoXyn3 activity (%)	Relative PfXynC activity (%)
None metal ions (5 mM)	100	100
EDTA	94.57	94.09
Ca^{2+}	89.34	95.26
Co^{2}+	105.19	101.29
Cu^{2+}	105.12	105.12
Fe^{2+}	107.63	106.64
Mg^{2+}	93.15	91.24
Zn^{2+}	105.83	103.28

Kinetic parameters, substrate and inhibition specificity

Although PfXynC and PoXyn3 share 89.3% amino acid homology, the 3D modeling of the two xylanases revealed critical differences in the thumb region (Fig. 1B) [10], the two enzymes showed different enzymatic and inhibition profiles. PoXyn3 showed a five-fold decrease in catalytic turnover compared to PfXynC, but likely the PoXyn3 remained active despite of its deleted thumb. In fact, the kinetics parameters of PfXynC and PoXyn3 xylanases were determined using oat spelt xylan, wheat arabinoxylan (LVAX, Low Viscosity ArabinoXylan) and birchwood xylan. PfXynC showed

Table 3
Kinetic parameters of purified recombinant xylanases PoXyn3 and PfXynC in different substrates.

Substrate	Enzyme	V_m (U/mg)	K_m (mg/ml)	K_{cat} (sec^{-1})	K_{cat}/K_m (ml sec^{-1} mg^{-1})
Oat spelt xylan (1%)	PoXyn3	806.3 ± 0.9	14.13 ± 0.4	629.689	44.53
	PfXynC	2540 ± 140	14.08 ± 0.7	1231	87.42
LVAX (1%)	PoXyn3	902.7 ± 0.7	14.54 ± 0.1	715.658	49.42
	PfXynC	8559 + 0.1	14.4 + 0.7	2939	204
Birchwood (1%)	PoXyn3	784.4 ± 0,5	14.01 ± 0.1	594.360	42.42
	PfXynC	2729 ± 0.4	14.08 ± 0.2	3024.14	214.78

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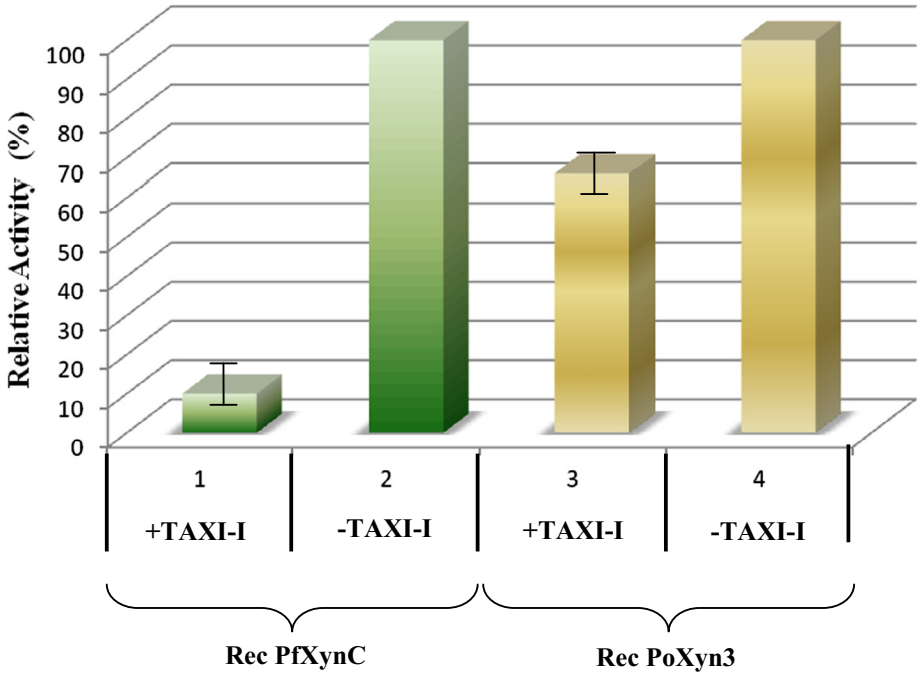

Fig. 5. Inhibition profile of PfXynC and PoXyn3 by TAXI-I. Values represent mean ± SE of three independent experiments.

Table 4
Inhibition Kinetic of PoXyn3 and PfXynC toward TAXI-I inhibitor used at 50nM.

	PoXyn3		PfXynC	
	[TAXI-I] = 0 nM	[TAXI-I] = 100 nM	[TAXI-I] = 0 nM	[TAXI-I] = 100 nM
Vmax (µmole/min)	28.57	28.57 ± 0.34	9.80	9.80 ± 1.32
Km^{app} (mg/ml)	10	–	4,34	–
Km'^{app} (mg/ml)	–	166.6 ± 0.12	–	89,2 ± 0.09

the highest catalytic efficiency on LVAX with a k_{cat}/K_m of 204 s^{-1} (Table 3) while PfXynC hydrolysed LVAX at a higher rate but with a lower apparent affinity compared to PoXyn3 (Table 2). The k_{cat} was almost five times higher in the case of PfXynC than the ones of PoXyn3 irrespective of the nature of the substrate, whereas the apparent affinity was similar between the two recombinant xylanases. The PfXynC expressed in *E. coli* [18] showed similar kinetic parameters, which confirms that post-translational modifications in *P. pastoris* did not affect folding and/or activity of the recombinant protein.

The ability of the proteinaceous wheat inhibitor, TAXI-I, to inhibit PoXyn3 and PfXynC was further evaluated. Using LVAX as substrate, no decrease in xylanase activity was observed up to a TAXI-I/PoXyn3 ratio of 30:1 (Fig. 5) (Table 4) for PoXyn3 whereas PfXynC was strongly inhibited. In fact, PfXynC overexpressed in *P. funiculosum* has been shown to efficiently degrade birchwood xylan and soluble wheat arabinoxylans and its activity was strongly inhibited by three xylanase inhibitor proteins from wheat; XIP-I, TAXI I and TAXI II [17] suggesting that post-translational modifications in *Pichia* do not affect its inhibition sensitivity. Similar results were obtained with XynA, especially with the XIP inhibitor, from *Penicillium griseofulvum* [18]. Although TAXI-I typically inhibits fungal and bacterial xylanases, the inability of TAXI-I to inhibit *P. occitanis* xylanase can be explained by the structural determinants of inhibitor specificity, in particular, around the "thumb" region of the enzyme, a key determinant of the xylanase–inhibitor interaction [25,26]. The lack of twenty amino acids in the thumb of the PoXyn3 [14] would cause this short loop to adopt a strikingly different conformation that would introduce steric clashes with TAXI-I, thus, preventing inhibitor binding. This result stresses the importance of the tip region located immediately after the conserved Gly (Fig. 1B)[25]. In fact, the structure of the TAXI-I-*A. niger* xylanase complex (1T6G.pdb) reveals a direct interaction of the inhibitor with the active site region of the enzyme and further substrate-mimicking contacts with binding subsites filling the whole substrate-docking region [27].

Since PoXyn3 is a fungal xylanase that belongs to the GH11 family, it may be the target of other plant xylanase inhibitors such as XIP-I and TAXI-II [6]. We can predict that the absence of key aminoacids within the thumb region of PoXyn3 may affect binding to XIP-I [25,26]. From an enzymological point of view, PoXyn3 thus represent an attractive model to study structure–function relationships in the GH11 xylanase family. These reported differences in the action pattern of *Penicillium* xylanases on arabinoxylan, together with their distinct sensitivity towards TAXI-I, are to be taken into consideration for specific food processing applications.

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