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Identification, heterologous expression and characterization of a novel glycoside hydrolase family 30 xylanase from the fungus *Penicillium purpurogenum*

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ABSTRACT

Penicillium purpurogenum grows on a variety of natural carbon sources and secretes to the medium a large number of enzymes that degrade the polysaccharides present in lignocellulose. In this work, the gene coding for a novel xylanase (XynC) belonging to family 30 of the glycoside hydrolases (GH), has been identified in the genome of the fungus. The enzyme has been expressed in *Pichia pastoris* and characterized. The mature XynC has 454 amino acid residues and a calculated molecular weight of 49 240. The purified protein shows a molecular weight of 67 000, and it is partially deglycosylated using EndoH. Its pH optimum is in the range of 3–5, and the optimal temperature is 45 °C. It is active on both arabinoxylan and glucuronoxylan, similarly to other fungal GH 30 xylanases. It liberates a set of oligosaccharides, which have been detected by thin-layer chromatography, thus indicating that it is an endo-acting xylanase. It hydrolyzes xylooligosaccharides, releasing mainly xylobiose, in contrast to other fungal GH family 30 enzymes which generate chiefly xylose. Highest sequence identity to a characterized family 30 xylanase is found with the enzyme from the fungus *Bispora* sp (53%). This is the first GH 30 xylanase described from a *Penicillium*.

1. Introduction

Xylan is one of the most abundant component of plant cell wall hemicelluloses. It is a heteroglycan constituted by a linear chain of D-xylopyranose units linked β(1 → 4) and substituted by a variety of residues, predominantly methyl glucuronate, L-arabinose and acetate groups. Ferulate or coumarate residues may in turn esterify the L-arabinose moieties. The precise structure of xylan varies depending on its origin [1].

The biodegradation of xylan requires the action of several glycanases and esterases. These enzymes are produced mainly by bacteria and fungi and are mostly extracellular. These enzymes act in a concerted fashion in order to degrade xylan to its individual monosaccharide components [2]. This synergic action implies fine regulatory mechanisms, probably at the transcriptional and translational level, which must coordinate the production of the enzymes at the required time and amounts.

The xylan main chain is hydrolyzed by endoxylanases (E.C. 2.3.1.8) producing xylooligosaccharides of different length [3]. They have been purified, characterized and sequenced from a number of bacteria and fungi [2]. Based on sequence similarities and hydrophobic cluster analysis they have been grouped in families 5, 8, 9, 10, 11, 12, 26, 30, 43, 51, 62 and 98 of the glycoside hydrolase (GH) families defined in the CAZY database [4], [5]. The great majority belong to families 10 and 11; they have been studied the most and differ in their catalytic versatility [6].

Less studied are the endoxylanases form GH family 30. Differences have been found in the properties of the enzymes from bacterial and fungal origin. Examples of the bacterial enzymes are those from *Erwinia chrysanthemi* [7] and *Bacillus subtilis* [8]. They have been called "appendage-dependent GH30 glucuronoxylan hydrolases" [7], since they are specific for glucuronoxylan. Fungal enzymes, XynD of *Bispora* sp [9] and *Trichoderma reesei* XIN IV [10], on the other hand, have a broader specificity, as they are also active on arabinoxylan.

In our laboratory, we have used as model for lignocellulose biodegradation studies the fungus *Penicillium purpurogenum,* an active producer of cellulases and xylanases [11], [12]. Previous work has identified, sequenced and characterized two endoxylanases secreted by this fungus: Xyn A (GH family 10) and Xyn B (GH family 11) [13]. By means of zymograms, the extracellular presence of at least 5 endoxylanases has been detected in culture supernatants [14]. This work describes the properties of a novel xylanase produced by the fungus: XynC (GH

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Table 1
Primers designed for the cloning of *xynC*.

Primer	Sequence	Tm	Direction
FW 0.17	5′-ATGCATCTCTCTATCATTGACAAGC-3′	55.1 °C	Sense
RV 0.17	5′-TTACACAGACACATAAGTAACCATTG-3′	53.2 °C	Antisense
FW 0.17PIC	5′-CTTC**GAATTC**ATGCATCTCTCTATCATTG-3′	55.9 °C	Sense
RV 0.17PIC	5′-TTTT**CTCGAG**TTACACAGACACATAAGTAA-3′	56.1 °C	Antisense

Sequences in bold indicate recognition sites for *Eco*RI (FW 0.17PIC) and *Xho*I (RV 0.17PIC).

family 30). The gene coding for XynC has been identified in the genome of the fungus and the enzyme has been expressed heterologously in *Pichia pastoris* and characterized.

2. Results

2.1. Sequence of the xynC gene and its protein product

Using primers FW 0.17PIC and RV 0.17PIC (Table 1) and *P. purpurogenum* genomic DNA as template, a product of approximately 1400 bp was obtained by PCR, which is similar to the calculated size (1431 bp) of the gene identified from the fungal genome sequence. The PCR product was cloned in pPICZB and transformed into *E. coli* DH5α. Clones with plasmids were isolated from plates containing 25 μg/ml Zeocin. The presence of the insert was confirmed by PCR and the insert was sequenced. The sequence obtained was identical to the sequence of the gene identified from the genome. An analysis of the sequence by AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/) showed that the gene has no introns.

The deduced protein sequence is composed of 476 amino acid residues (Fig. S1). It includes in its amino terminal end a signal peptide of 22 residues as deduced by means of SignalP (http://www.cbs.dtu.dk.services.SignalP/). The mature protein has a molecular mass of 49.42 kDa and a calculated pI of 5.6 as estimated by ExPASy (http://web.expasy.org/). The enzyme possesses 6 potential *N*-glycosylation and 5 potential O-glycosylation sites as shown by NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively.

2.2. Heterologous expression and characterization of XynC

Two *P. pastoris* transformed clones showed enzymatic activity. A negative control, consisting of a *P. pastoris* clone transformed with pPICZB lacking the insert, had no activity. The more active clone (with specific activity of 4.5 U/mg protein) was utilized for further work. The enzyme was purified and the purity was checked with SDS-PAGE (Fig. 1). A band of 67 kDa is observed, suggesting the presence of glycosylation. Treatment with Endo H, which eliminates *N*-glycosylations, reduced the molecular mass to about 55 kDa; the remaining mass difference may be due to O-glycosylation (Fig. 1).

Fig. 2 shows the pH dependence of XynC activity. A pH optimum range between 3.5 and 5 is observed, and a sharp loss in activity occurs at pHs over 5.5. The stability of the enzyme at different pHs can be seen in Fig. 3. After 3 h of incubation, about 60% activity remains, and it is similar at pHs 3 to 5. Fig. 4 shows the activity of XynC at different temperatures; optimal temperature is achieved at 45 °C. The effect of temperature on enzyme stability is presented in Fig. 5. Stability is highest at the lowest temperature tested (25 °C) and activity drops very rapidly at 60 °C.

Activity was assayed using several glucurono- and arabinoxylans as substrates as shown in Fig. 6. Glucuronoxylan from birchwood is the best substrate, but significant activity is also observed with arabinoxylans. In Fig. 7, the hydrolysis products of the action of XynC on birchwood xylan are analyzed by TLC. Xylose liberation is minimal and a set of oligosaccharides of different composition and length is generated, indicating that the enzyme is an endoxylanase. The hydrolysis of a set of xylooligosaccharides was analyzed by TLC as can be seen in Fig. 8. The main product detected is xylobiose, and to a much lesser extent, xylose. No transglycosylation products are observed.

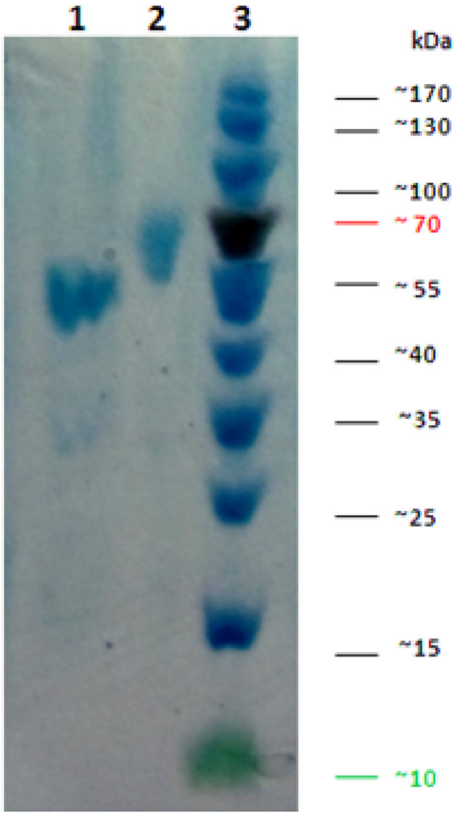

Fig. 1. SDS-PAGE of purified XynC and enzyme treated with *Endo*-H. Lane 1: enzyme treated with Endo H. Lane 2: purified XynC. Lane 3: molecular weight standards (Prestained Protein Ladder, Fermentas).

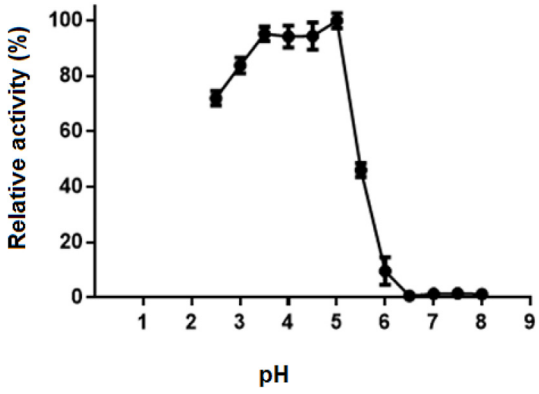

Fig. 2. pH dependence of the activity of XynC. The highest activity is assigned the value 100%. The assays were performed in triplicate.