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Heterologous production and characterization of a thermostable GH10 family endo-xylanase from *Pycnoporus sanguineus* BAFC 2126

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ABSTRACT

Xylanases are key enzymes for agricultural biomass saccharification for the production of cellulosic ethanol. Success in enzymatic lignocellulose bioconversion is restricted by enzyme production costs, activity and stability under harsh reaction conditions, and their performance when interacting into cellulolytic cocktails. In this work, we present the heterologous expression and enzymatic characterization of a novel endo-β-1,4 xylanase of glycoside hydrolase family 10 (GH10ps) from the white-rot basidiomycete *Pycnoporus sanguineus* BAFC 2126. Recombinant expression of GH10ps in *Pichia pastoris* showed that it is a robust enzyme active at a wide range of pHs and temperatures, and with a half-life of 3 h at 70 °C and a stability higher than 48 h at 60 °C. Recombinant GH10ps was also capable of releasing xylooligosaccharides and xylose from pretreated agricultural waste biomass and also complemented commercial cellulases in lignocellulose bioconversion to fermentable sugars.

1. Introduction

Bioconversion of lignocellulose requires the combined action of multiple enzymatic activities including cellulases, hemicellulases and lignin-modifying enzymes. Among them, hemicellulases constitute a diverse group due to the heterogeneity and complexity of hemicellulose polymer, the second most abundant reserve of organic carbon after cellulose. The most common hemicellulose backbone found in hard wood and annual plants is xylan (20–35%), a polymer chain of β-1,4-linked xylopyranosyl units with mostly arabinose and glucuronic acid as substituents [1]. Release of fermentable pentoses from xylans is performed by the concerted action of endo-1,4-β-xylanases (EC 3.2.1.8) that cleave the β-1,4-glycosidic bonds between D-xylose residues in the main chain [1–4], de-branching enzymes such as α-L-arabinofuranosidases (EC 3.2.1.55) [5] and α-D-glucuronidases (EC 3.2.1.139) that remove side groups, and β-xylosidases (EC 3.2.1.37) that release xylose from the reducing end of short xylooligosaccharides and xylobiose [2,6]. Described endo-xylanases include members of several glycosil hydrolase (GH) families, classified according to amino acid sequence homologies and structure analysis (http://www.cazy.org/, [7]), but most of them are grouped in families GH10 and GH11 [6]. GH11 xylanases show a β-jellyroll folding, an overall MW < 30 KDa and high specifity to xylan [2,8] while GH10 xylanases show a $(\beta/\alpha)_8$ or TIM-barrel folding, MW > 30 KDa, are less selective for substrates and are generally active on xylooligosaccharides of low degree of polymerization [2,9]. Endo-1,4-β-xylanases have a wide range of biotechnological applications not only in the bioconversion of plant lignocellulose biomass into fermentable sugars for the production of biofuels, but also in pulp bleaching, food and animal feed industries [3,8]. Since industrial use of xylanases requires of robust enzymes active and stable at high temperatures and extreme pHs [10,11], much effort is done to characterize novel enzymes efficient in harsh processes conditions.

Xylophagous fungi constitute an important source of fibrolytic enzymes capable of performing efficient degradation of all components of plant cell walls. Among them, white rot basidiomycetes of genus *Pycnoporus* are widely studied for their ability to secrete thermostable laccases as part of their ligninolytic system [12], and also as good producers of cellulolytic and hemicellulolytic extracts. Cellobiose dehydrogenase and xylanase activities were characterized in extracellular extracts of *Pycnoporus cinnabarinus* [13], while endoglucanase, β-glucosidase, xylanase, mannanase, α-glactosidase, α-arabinofuranosidase, polygalacturonase activities where characterized in *P. sanguineus* PF-2 extracellular extracts and tested for sugarcane bagasse sacharification [14].

Recent availability of *P. cinnabarinus* CIRM BRFM 137 complete genome [15], *P. coccineus* CIRM BRFM 310 transcriptome and

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secretome [16], *P. sanguineus* BAFC2126 transcriptome [17] as well as comparative analysis of transcriptomes and secretomes of the three species grown on diverse agricultural biomass substrates [18] have shown that these fungi have a large number of candidate genes encoding lignocellulases and allowed the cloning and characterization of accessory enzymes for lignin degradation such as glioxal oxidases [19], a glucose dehydrogenase [20] and aryl alcohol quinone oxidoreductases [21] from *P. cinnabarinus*.

In this work we describe the cloning and heterologous production of a thermostable endoxylanase of glycoside hydrolase family 10 (GH10) from *P. sanguineus* BAFC 2126 and test its ability to supplement commercial cellulases in the saccharification of pretreated agricultural plant biomass.

2. Material and methods

2.1. Fungal strain and culture conditions

Pycnoporus sanguineus (Polyporaceae, Aphyllophorales, Basidiomycetes) strain BAFC 2126 was obtained from the BAFC Mycological Culture Collection of the Department of Biological Sciences, Faculty of Exact and Natural Sciences, University of Buenos Aires. Stock cultures were maintained on malt extract agar slants at 4 °C. Fungal culture was performed in GA medium (2% glucose, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l KH_2PO_4, 0.6 g/l K_2HPO_4, 0.09 mg/l $MnCl_2{\cdot}4H_2O$, 0.07 mg/l H_3BO_3, 0.02 mg/l $Na_2MoO_4{\cdot}H_2O$, 1 mg/l $FeCl_3$, 3.5 mg/l $ZnCl_2$, 0.1 mg/l thiamine hydrochloride, 3 g/l asparagine monohydrate and 1 mM $CuSO_4$), initial pH 6.5. Incubation was carried out statically at 28 ± 1 °C. Cultures were harvested at day 21 and filtered mycelia used for total RNA extraction as previously described [17].

2.2. Cloning of xyl10ps cDNA

Complete *xyl10ps* nucleotide sequence was assembled *in silico* based on the identity of the partial sequence previously identified in *P. sanguineus* BAFC 2126 (GenBank GAKI01004661, [17]) and putative homologues in *Pycnoporus (Trametes) cinnabarinus* BRFM137 (GenBank CCBP010000044) and *Trametes versicolor* FP-101664 SS1 (GenBank XM008037780). Prediction of signal peptide and processing site in translated protein was performed using SignalP 4.0 software (http://www.cbs.dtu.dk/services/SignalP/). Resulting coding sequence was used for design of forward (GH10Fw 5′-GAATTCGTCGCTGTCTG-3′) and reverse (GH10Rv 5′-TCTAGATTACGCCAGAGC- 3′) cloning primers, containing adaptors with EcoRI and XbaI restriction endonucleases recognition sites, respectively.

Total RNA of *P. sanguineus* BAFC 2126 was used as template for cDNA synthesis using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) and oligodTVN primer, according to manufacturer's instructions. PCR amplification of *xyl10ps* cDNA sequence encoding mature protein GH10ps was performed using Pfu DNA polymerase (Fermentas) and GH10Fw/GH10Rv primers. Fragment of the expected size (1095 bp) was gel-purified and cloned in pGEM®-T Easy Vector (Promega, Madison, WI, USA) for sequencing (Macrogen Inc). For expression of mature GH10ps fused to an N-terminal 6xHIS tag in *P. pastoris*, EcoRI/XbaI restriction product was cloned in compatible EcoRI/AvrII sites of vector pPICNHIS to obtain plasmid pNHISGH10ps. *P. pastoris* pPICNHIS vector was derived of pPIC9 backbone (Invitrogen Life Technologies, Inc.) by inserting a 6-histidine coding sequence in open reading frame downstream of *Saccharomyces cerevisiae* α-mating factor signal sequence.

2.3. Recombinant GH10ps expression in P. pastoris and purification

Recombinant vector pNHISGH10ps was linearized with DraI restriction enzyme and used for transformation of *P. pastoris* strain GS115 (Invitrogen Life Technologies) by electroporation. Recombinant clones reverting histidine auxotrophy were first selected on minimal medium MD plates (0.34% yeast nitrogen base without amino acids, 10 g/l $(NH_4)_2SO_4$, 2% dextrose and 2% agar) and then by secreted xylanolytic activity on minimal medium MM plates (0.34% yeast nitrogen base without amino acids, 10 g/l $(NH_4)_2SO_4$, and 2% agar) supplemented with 1% oat spelt xylan (Sigma Aldrich). Induction of AOX1 (*P. pastoris* alcohol oxidase 1) promoter was achieved by adding 100 µl of 100% methanol to plate lid and xylan degradation halos were revealed with Congo Red differential staining [22]. Transformed clones showing xylan degradation were selected and conserved on MD or YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) agar slants.

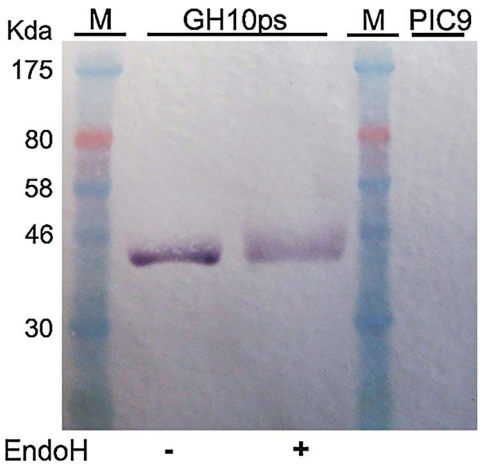

Fig. 1. Electrophoretic characterization of recombinant GH10ps. Western blot of crude extracellular *P. pastoris* protein extracts expressing GH10ps before (−) and after (+) treatment with EndoH endoglycosidase and extracellular extracts of *P. pastoris* transformed with control empty vector (PIC9), revealed with anti-his tag antibody. M: Colorplus™ Prestained Protein Marker (New England Biolabs).

Histidine tagged recombinant protein production in *P. pastoris* and purification by affinity chromatography was performed in the same conditions as previously described [23].

2.4. Polyacrylamide gel electrophoresis and immunoblotting

Recombinant GH10ps xylanase in crude cell-free extracts and purification fractions was separated by reducing 12% SDS-PAGE and identified by Coomassie Blue staining or transferred to 0.45 µm nitrocellulose membranes (Bio-Rad Laboratories Inc, USA). Western blot was performed by probing the membranes with 0.1 µg/ml of polyclonal rabbit anti-HIS antibody (Genescript, USA) followed by 1:15000 dilution of alkaline phosphatase-linked goat anti-rabbit antibody (Sigma Chemical Co., USA). Phosphatase activity was revealed by a chromogenic reaction using 5 bromo-4 chloro-3 indolyl phosphate and nitroblue tetrazolium as substrates (Sigma Chemical Co., USA).

2.5. Deglycosylation assay

N-glycan removal of recombinant xylanase was performed on denatured protein (0.5% SDS, 40 mM DTT, 100 °C, 5 min) by incubation with endoglycosidase H (Endo Hf, New England BioLabs, USA) according to manufacturer's instructions. The deglycosylated enzyme was separated by SDS-PAGE and analyzed by Western blot.

2.6. Enzyme activity determination assay

Xylanase activity was estimated by monitoring the release of reducing sugars equivalents from 1% beechwood xylan (Sigma Chemical Co., USA) in Mc Ilvaine's buffer (citrate-phosphate), pH 5 at 60 °C using 3,5-dinitrosalicylic acid (DNS) method [24]. One unit of enzymatic activity was defined as the amount of enzyme releasing 1 µmol of xylose

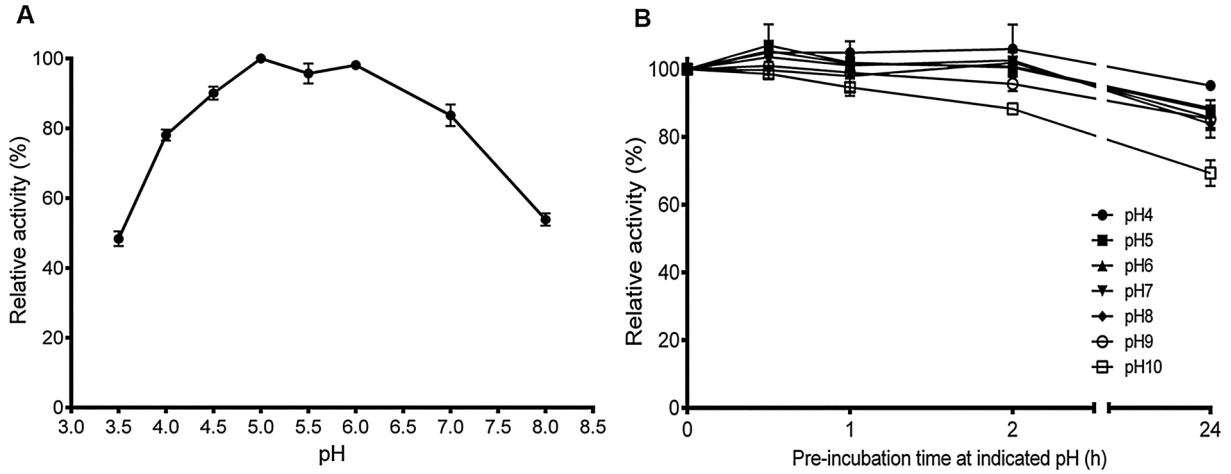

Fig. 2. pH activity and stability of GH10ps A) pH profiles determined in citrate-phosphate buffer at 30 °C Activity is represented as% relative to the maximum, assigned as 100%. B) Xylanolytic activity after pre-incubation of purified GH10ps for 24 hs at the indicated pHs. Activity is represented as% of initial time (To), assigned as 100%. Error bars correspond to standard deviation for triplicates.

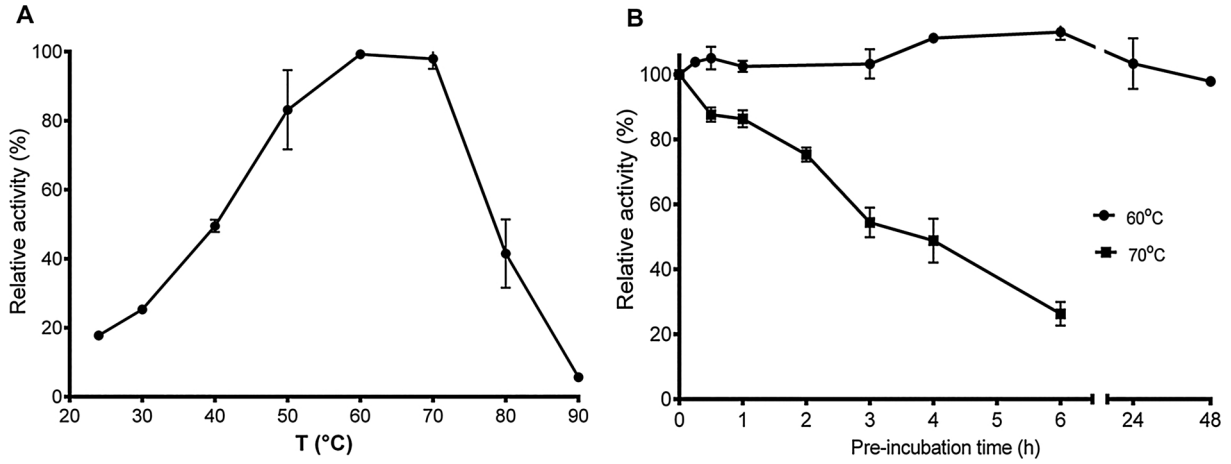

Fig. 3. Temperature activity profile and thermostability of GH10ps. A) Temperature profile using xylan as substrate at pH 5. B) Xylanolytic activity after pre-incubation of purified GH10ps at 60 °C at the indicated times in citrate-phosphate buffer pH 5. C) Xylanolytic activity after pre-incubation of purified GH10ps at 70 °C at the indicated times in citrate-phosphate buffer pH 5. Activity is represented as% of initial time (To), assigned as 100%. Error bars correspond to standard deviation for triplicates.

Table 1
Soluble sugars released from beechwood xylan and extruded wheat straw (Wse) after treatment with GH10ps and Cellulase, determined by HPLC.

Substrate	Xylobiose (mg/ml)	Xylose (mg/ml)	Cellobiose (mg/ml)	Glucose (mg/ml)	Arabinose (mg/ml)
Xylan + GH10ps	0.5	< 0.1	ND	ND	ND
Wse + GH10ps	0.3	< 0.1	ND	ND	ND
Wse + Cellulase	ND	ND	0.1	0.2	ND
Wse + GH10ps + Cellulase	0.3	0.2	0.2	0.3	0.3

(as reducing equivalents) per min at the indicated pH and temperature. For temperature and pHs profiles, reactions were performed either in citrate-phosphate buffer pH 5 at 24 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C or in citrate-phosphate pH 3, 3.5, 4, 4.5, 5, 5.5, 6, and 7 at 30 °C. Thermostability assays were performed pre-incubating purified xylanase at 60 °C or 70 °C in citrate-phosphate buffer pH 5 for the indicated times and the release of reducing equivalents determined at 60 °C and pH 5. For pH stability assays, xylanase was pre-incubated at 25 °C in citrate-phosphate buffer pH 4, 5, 6, 7 or Glycine-NaOH buffer pH 8, 9, 10 and activity determined at 60 °C, pH 5.

2.7. Identification of sugars released from xylan and lignocellulosic biomass

To determine sugars released from xylan and evaluate bioconversion of lignocellulosic biomass, hydrolysis was carried out in Mc Ilvaine's buffer pH 5 for 20 min at 60 °C with 1% beechwood xylan as substrate or in Mc Ilvaine's buffer pH 6 for 16 h at 60 °C with 2% (w/v) steam-exploded sugar cane (SCRse, 197,5 °C, 4 min) or extruded wheat straw (WSe, extruded at 70 °C, NaOH 6%) as substrates and GH10ps (final concentration of 0.014 mg/ml, 4.4 U/ml, in a final reaction volume of 1 ml). In combined assays, commercial *Aspergillus* sp. extracts (Carezyme 1000L, Novozymes/Sigma Chemical Co., USA, 4870 U/g- data provided by manufacturer) were used as cellulase source. The final cellulase concentration in the reaction was 120 mg/ml (equivalent to 584 U/ml), in a 1 ml final volume reaction. Analysis of carbohydrates released by hydrolysis was performed in a Agilent 1100 series HPLC with RI detector using a Aminex HPX-87P analytical column (BioRad), at 80 °C with water as mobile phase at a flow rate of

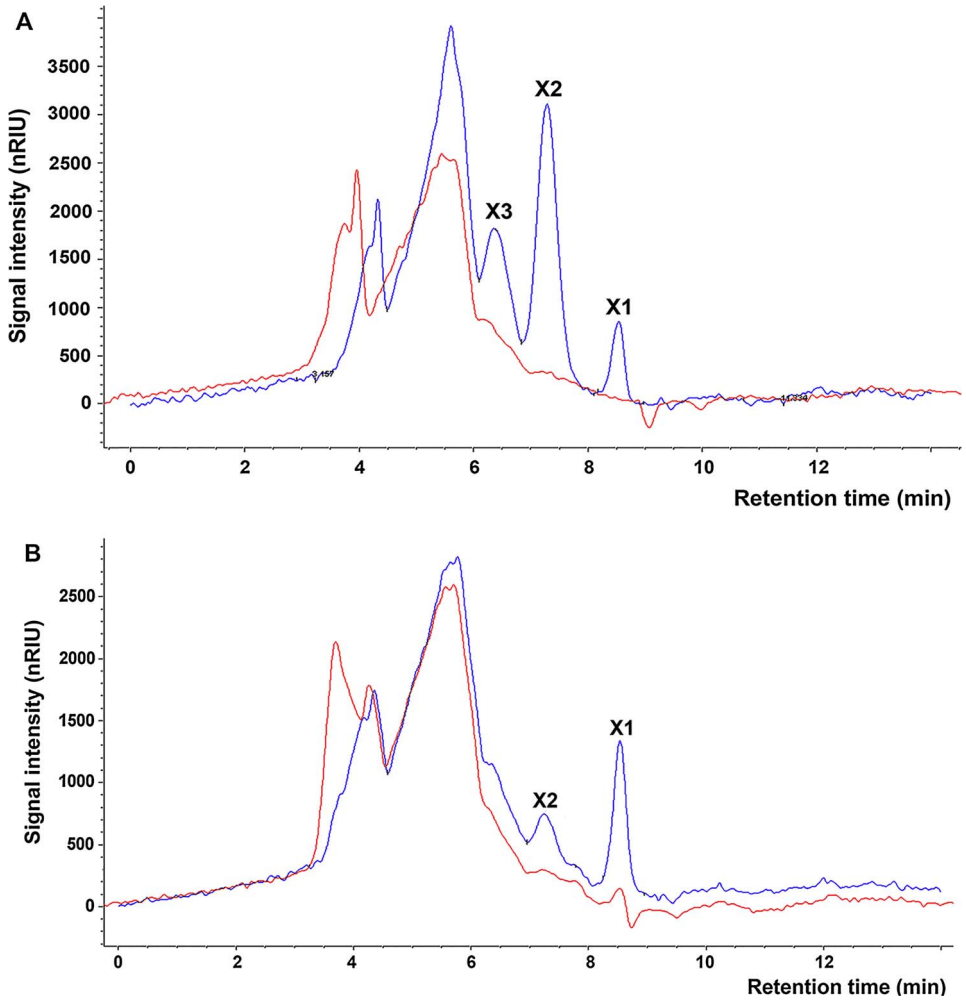

Fig. 4. HPLC analysis of hydrolysis products of pretreated biomass by GH10ps. Hydrolysis reaction was performed at 60 °C, pH 6 for 16 hs with 2% steam-exploded sugar cane (SCRse) (A) or extruded wheat straw (Wse) (B) as substrates (Blue lines). X3: Xylotriose, X2: xylobiose, X1: xylose. Red lines: control HPLC profiles for substrates without the addition of GH10ps. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.5 ml/min, as previously described [25]. Concentration of sugars was determined using standard curves of pure compounds (Sigma Chemical Co., USA).

2.8. Biomass composition

Pre-treated lignocellulosic biomass, as well as determination of its composition, was kindly provided by Dr. Mercedes Ballesteros, from CIEMAT, Madrid. Sugar cane straw was subjected to steam explosion (197.5 °C; 4.02 min) and its composition was 51% cellulose, 15% hemicellulose, 26% lignin (acid soluble), 13% ashes. Wheat straw was pre-treated by extrusion (conditions: 70 °C, NaOH 6%, 2 mm) and its composition was 47% cellulose, 30% hemicellulose, 20% lignin (acid insoluble), 1.3% lignin (acid soluble), 2.1% ashes.

2.9. Data availability

Data of *P. sanguineus* mature *xyl10ps* coding sequence was deposited at DDBJ/EMBL/GenBank under the accession number MF981011.

3. Results and discussion

3.1. Cloning of xyl10ps coding sequence from P. sanguineus BAFC 2126

In a previous study of *P. sanguineus* BAFC 2126 transcriptome, we identified a partial RNA sequence (GenBank GAKI01004661) encoding a predicted protein with high amino acid identity with fungal β-1,4-endo4-endoxylanases of glycoside hydrolase family 10 [17]. Using this sequence information and the highest identity sequences reported for the genomes of the related species *Pycnoporus (Trametes) cinnabarinus* BRFM137 (GenBank CCBP010000044, [15]) and *Trametes versicolor* FP-101664 SS1 (GenBank XM008037780, [26]), we reconstructed *in silico* the complete coding sequence for a putative GH10 β-1,4-endo4-endoxylanases including a predicted 20 amino acid signal peptide. Cloning and sequencing of cDNA (*xyl10ps*) for mature protein (GH10ps) showed it encoded a polypeptide of 360 amino acids with a theoretical molecular weight of 38 KDa. *In silico* analysis of protein sequence confirmed the presence of a C-terminal catalytic domain characteristic of glycoside hydrolase family 10, containing the two conserved glutamic acid residues (E189 and E295) as putative general acid/basic residue and catalytic nucleophile, respectively [27,28]. A fungal carbohydrate-binding module CBM1 containing the 4 conserved cysteine residues involved in disulfide bridge formation and conserved aromatic residues (W4, Y30 and Y31) involved in ligand recognition [29–31] is located at N-terminal and linked to catalytic domain by a flexible linker rich in proline-threonine residues. Two N-X-S/T sequons (positions 50 and 88 of mature protein) were found in protein GH10ps, however N-glycosylation is expected to be impaired by the presence of a proline in position X [32].

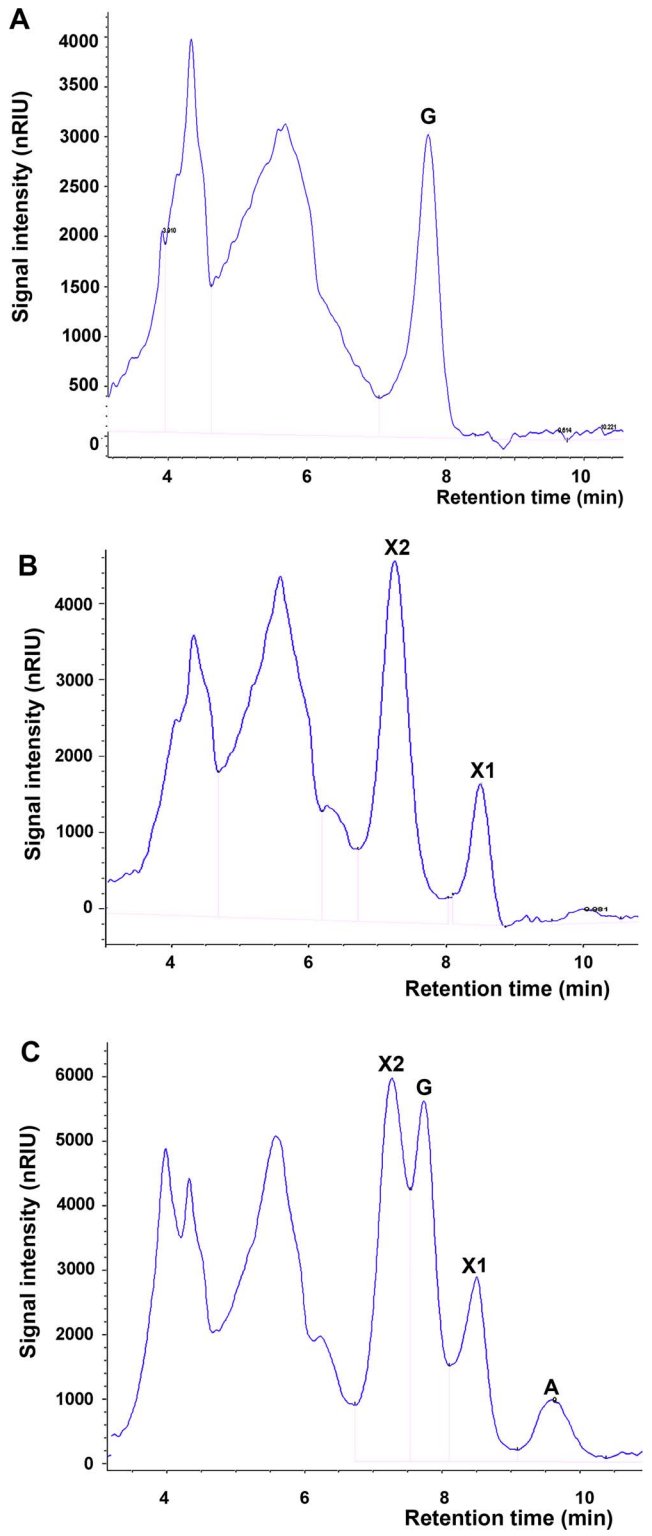

Fig. 5. HPLC analysis of hydrolysis products of pretreated Wse by GH10ps and cellulases. Hydrolysis reaction was performed at 60 °C, pH 6 for 16 hs with 2% extruded wheat straw (Wse) as substrate with the addition of Carezyme1000L (A), GH10ps (B) or both (C). X2: xylobiose, X1: xylose, G: glucose, A: arabinose.

3.2. Production and biochemical characterization of recombinant GH10ps protein

For production and biochemical characterization of GH10ps, we expressed the mature protein in *P. pastoris*, as a fusion to a 6-histidine tract for affinity purification and to the signal peptide of *Saccharomyces cerevisiae* α-mating factor for secretion. Xylanolytic activity (4660 ± 620 U/l, pH 5, 60 °C) was observed in extracellular crude culture extracts of transformed *P. pastoris* clones, confirming expression and secretion of active recombinant GH10ps xylanase. Analysis of culture supernatants by Western blot revealed with anti-HIS antibody showed a single protein band with estimated molecular mass of 39 KDa corresponding to the predicted molecular weight for recombinant 6xHIS-GH10ps fusion (Fig. 1 lane −). Treatment of protein extract with endoglycosidase H showed no changes in mobility, confirming N-X-T/S sites of GH10ps were not N-glycosilated in *P. pastoris* (Fig. 1 lane +). Recombinant GH10ps was single-step purified by Ni-NTA affinity chromatography and elution fractions showing a single band in Coomasie blue stained acrylamide gel were pooled and used for biochemical characterization.

For pH activity profile, the optimum was between 5–6 and remained above 50% in a range of pH 3.5–8 (Fig. 2A), as commonly observed for other fungal endo-xylanases [1,8]. Regarding stability, GH10ps retained more of 85% of activity after 24 hs of incubation at pH 4–10 (Fig. 2B), showing the high flexibility of this enzyme for industrial application in a wide range of pH conditions.

Temperature activity profile for GH10ps showed a characteristic bell-shaped curve with an optimum of 60 °C to 70 °C and with a decrease in activity observed for temperatures below 40 °C and above 80 °C (Fig. 3A), Since *Pycnoporus* species are known to express thermostable laccases [12,33] and industrial enzymatic saccharification of lignocellulose is performed at temperatures equal or above 50 °C, we tested thermostability of recombinant GH10ps. No decrease in residual activity of the recombinant enzyme was observed when it was pre-incubated at 60 °C for at least 48 hs while pre-incubation at 70 °C resulted in a half-life of 3 hs (Fig. 3B and C). Stability of recombinant GH10ps resulted to be higher than described for thermostable xylanases from other mesophilic fungi such as *Gloeophyllum trabeum* GtXyn10 (half-life of 1 h at 70 °C, [34]), *Aspergillus fumigatus* Af-XYNA (half-life of < 30 min at 70 °C, [35]) or *Phanerochaete chrysosporium* XynA and XynC that were inactivated after 30 min at 70 °C [36]. Also, thermostability of GH10ps is comparable to that found in GH10 xylanases from thermophilic fungi such as *Malbranchea pulchella* ($T_{1/2}$ > 24 hs at 65 °C, [37]), *Humicola insolens* (XynA $T_{1/2}$ < 30 min at 65 °C, [38]) or *Myceliophthora* sp. [39], and from thermophilic bacteria *Alicyclobacillus acidocaldarius* (GH10-XA retained 40% and 100% of activity after 1 h or 3.5 days at 75 °C and 65 °C, respectively, [40]).

Main products of beechwood xylan hydrolysis by recombinant enzyme were determined by HPLC, showing the release of xylose (xyl-1), xylobiose (xyl-2) and xilooligosaccharides (XOS) of higher polymerization degree, confirming endo-xylanase activity of GH10ps (Table 1).

Since GH10ps showed a conserved CBM1 domain, we also evaluated activity on carboxymethylcellulose, although no reducing sugar release was observed after 20 min of incubation in optimal conditions (pH 5, 60 °C). This cellulase-free activity of GH10ps is valuable for testing its application in pulp treatment, since cellulose fibers should remain intact in the bleaching process [1]. On the other hand, cellulose-binding role of CBM1 was previously demonstrated on other fungal endo-xylanases of GH10 family such as *Malbranchea pulchella* MpXyn10A [37] and *Talaromyces cellulolyticus* Xyl10A [41], suggesting a function related to anchoring the enzyme on the cellulose fiber and therefore favouring the xylanase catalytic module to get closer to the hemicellulosic substrate.

3.3. Hydrolytic activity on lignocellulosic biomass

Conversion of lignocellulosic agricultural residues by recombinant GH10ps was evaluated by the determination of released products of hydrolysis by HPLC. Incubation of sugarcane residue pre-treated by steam explosion (SCRse) and wheat straw pre-treated by extrusion (WSe) with GH10ps for 16 hs at 60 °C showed the release of xylose and

xylobiose as the main products of hydrolysis in both cases, indicating GH10ps is able to hydrolyze xylan from lignocellulosic biomass (Fig. 4A and B).

Development of minimal enzymatic cocktails for efficient conversion of lignocellulose into fermentable sugars is one of the main objectives to achieve economically viable bioethanol production from agriculture biomass [42,43]. Consequently, we evaluated the hydrolytic activity of GH10ps in presence of commercial cellulases. HPLC analysis of WSe hydrolysis products showed supplementation of cellulases of *Aspergillus* sp. with GH10ps extracts not only allowed the release of fermentable pentoses, not achieved by the cellulases alone, but also resulted in an increase in glucose release respect of the treatment with cellulases (Fig. 5), demonstrating synergic interaction. This is in accordance with previous reports that have shown that endoxylanases can interact synergically with cellulases, improving hydrolysis of many pretreated lignocellulosic substrates [44–47], supporting the potential of GH10ps for biomass saccharification.

4. Conclusions

In this work we performed the cloning, recombinant expression and purification of a novel endo-xylanase of GH10 family from *P. sanguineus*. Recombinant GH10ps proved to be a versatile enzyme, showing activity at a wide range of temperatures and pHs, and stable at 60 °C and 70 °C and at a broad pH range. In addition, GH10ps was active on insoluble lignocellulosic substrates and was able to supplement commercial cellulases in pretreated agricultural biomass saccharification, demonstrating its potential to test its application in minimal cocktails with reduced enzyme loading for production of second generation bioethanol.

E-supplementary data of this work can be found in online version of the paper.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.procbio.2018.01.017.

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