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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.

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

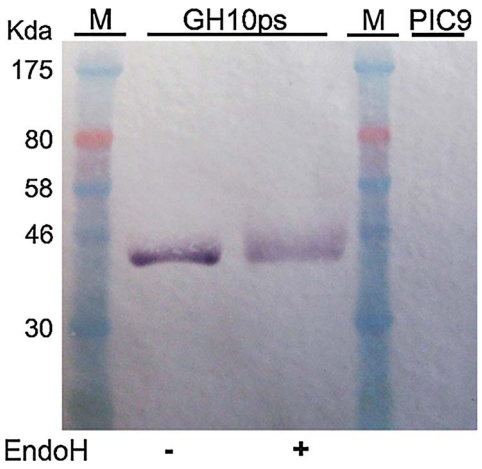

Fig. 1. Electrophoretic characterization of recombinant GH10ps. Western blot of crude extracellular *P. pastoris* protein extracts expressing GH10ps before (−) and after (+) treatment with EndoH endoglycosidase and extracellular extracts of *P. pastoris* transformed with control empty vector (PIC9),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

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