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Biochemical characterization of an endoxylanase from *Pseudozyma brasiliensis* sp. nov. strain GHG001 isolated from the intestinal tract of *Chrysomelidae* larvae associated to sugarcane roots

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ABSTRACT

Endo-xylanases play a key role in the hydrolysis of xylan and recently they have attracted much attention due to their potential applications on the biofuel and paper industries. We isolated a *Pseudozyma brasiliensis* sp. nov. strain from the intestinal tract of *Chrysomelidae* larvae that parasitize sugarcane roots. This basidiomycetous yeast produces a xylanase designated PbXynA which was purified and characterized. The molecular weight of PbXynA is 24 kDa, it belongs to the GH11 family and its optimum pH and optimum temperature are 4.0 and 55 °C, respectively. PbXynA has as secondary structure predominantly β-sheets and sigmoidal kinetic behavior with elevated speed conversion from substrate-to-products (V_{max} = 2792.0 μmol product/min/mg protein). It is highly activated by bivalent cations such as Ca^{2+}, however in the presence of Cu^{2+} xylanase activity was inhibited. It has a high specific activity and produces xylooligosaccharides that have a variety of industrial applications, indicating PbXynA has a great biotechnological potential.

1. Introduction

Lignocellulosic materials account for approximately 50% of the biomass in the world [1]. Degradation and conversion of lignocellulosic biomass are attracting attention because of its potential for the development of a sustainable and environmentally friendly bioenergy, biorefining and biomaterials industry [2]. Hemicellulose is the second most abundant renewable biomaterial available after cellulose representing 20–35% in lignocellulosic polymers [1]. Hemicelluloses consist of heteropolymers such as xylan, glucomannan, galactoglucomannan and arabinogalactan [3]. Xylan is a linear polymer of β-D-xylopyranosyl units linked by (1–4) glycosidic bonds and is the major hemicellulose. The complete hydrolysis of this heteropolysaccharide requires a set of enzymes, which the endo-1,4-β-xylanase is the most abundant [4].

Xylanases, the enzymes responsible for xylan backbone breakdown, have been classified into different GH (glycoside hydrolase) families: 5, 8, 10, 11, 16, 26, 30, 43, and 62 [1], but the major group can be classified into families 10 and 11 based on their amino-acid sequence similarities [5]. GH11 family is one of the best characterized GH families and it displays several interesting properties like high substrate selectivity and high catalytic efficiency, small size (around 20 kDa), variety of optimum pH and optimum temperature, making them suitable for various conditions in many applications [1]. Xylanases have played an important role in many industrial processes and have been applied as additives to enhance the quality of baked goods, animal feeds, and bleachers to kraft pulp [6]. Recently, they have received much attention owing to their use in degradation of lignocellulosic biomass for biofuel production [5]. The xylanolytic systems are extensively studied in filamentous fungi (*Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp., *etc.*) and bacteria (*Bacillus* sp., *Streptomyces* sp.) but there are very few yeast examples [7].

Pseudozyma brasiliensis sp. nov. strain GHG001 is a basidiomycetous yeast, closely related to *Pseudozyma fusiformata* and *Pseudozyma vetiver*. *Pseudozyma* spp. are able to produce xylanases [7] and biosurfactants such as mannosylerythritol lipids [8–13]. We are currently looking for yeast strains that colonize the intestinal

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tract of insects that colonize sugarcane roots and evaluating them for the more efficient use of either single pentoses or their polysaccharides, such as xylose or xylan. We have isolated from the intestinal tract of a *Chrysomelidae* a strain that can grow very well in xylose as sole carbon source and produces high levels of xylanase when grown in xylan. Thus, in our studies we purified and characterized a xylanase (PbXynA) produced by this *P. brasiliensis* sp. nov. strain. Although there are hundreds of studies describing the characterization of eukaryotic xylanases, in this work we reported a G11 endo-1,4-xylanase showing a higher specific activity than the eukaryotic xylanases previously studied. Moreover, the PbXynA showed important biochemical characteristics such as a sigmoidal kinetic behavior, high activation by bivalent ions and application in xylooligosaccharides production, showing a strong biotechnological potential and commercial value.

2. Materials and methods

2.1. Isolation of P. brasiliensis sp. nov. strain GHG001

Chrysomelidae at last larval stages were collected from *Saccharum officinarum* roots in sugarcane plantations located in Ribeirão Preto, São Paulo State, Brazil. The larvae were anesthetized at 4 °C for 10 min, then externally sterilized with 70% ethanol for about 1 min, and rinsed twice with sterilized water. The guts of different larvae were dissected and the intestinal juices were transferred to a sterile flask containing 1 ml of cold sterile water. A small aliquot of each gut sample (50 μl) was then inoculated into 250 ml Erlenmeyer flask with 50 ml of minimal medium (MM) composed of 50 ml/l salt solution (6 g/l $NaNO_3$, 1.5 g/l KH_2PO_4, 0.5 g/l KCl and 0.5 g/l $MgSO_4$) and 1 ml/l trace elements (10 g/l EDTA, 4.4 g/l $ZnSO_4{\cdot}H_2O$, 1.0 g/l $MnCl_2{\cdot}4H_2O$, 0.32 g/l $CoCl_2{\cdot}6H_2O$, 0.315 g/l $CuSO_4{\cdot}5H_2O$, 0.22 g/l $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1.47 g/l $CaCl_2{\cdot}2H_2O$ and 1 g/l $FeSO_4{\cdot}H_2O$) pH 6.5 with 1% xylose, supplemented with a cocktail of anti-bacterial antibiotics (100 μg/μl ampicillin, 34 μg/μl kanamycin, 20 μg/μl tetracycline and 35 μg/μl chloramphenicol). The inoculum was incubated at 30 °C under agitation at 130 rpm for enrichment of microorganisms that could use xylose more efficiently. Two weekly transfers were performed by changing the medium and after the second transfer, 100 μl of tenfold dilution were spread on solid MM with 1% xylose, and single colonies were repeatedly streaked out until pure cultures were obtained. These isolates were inoculated in 125 ml Erlenmeyer flasks with 25 ml of liquid YPD (20 g/l peptone, 10 g/l yeast extract and 20 g/l dextrose) and incubated under shaking at 30 °C for 2 days. Among the isolates strain GHG001 was selected and the extraction of genomic DNA followed the protocol proposed by Sambrook and Russell [14], and genome sequencing was carried out on an Illumina HiSeq2000 instrument (unpublished data).

2.2. Xylan/xylose/glucose induction and determination of enzymatic activities

P. brasiliensis were inoculated (1×10^6 cells/ml) in 0.67% YNB medium and 2% xylan, 2% xylose or 1% glucose as carbon sources, at 30 °C for 12, 24, 48, 72, 96 and 120 h. An aliquot was used for spectrophotometric determination of optical density at 590 nm, the supernatant was harvested by centrifugation and was kept at −20 °C for enzymatic analysis. The xylanase enzymatic assay was performed using Azo-Xylan (Birchwood; from Megazyme International, Bray, Ireland) as substrate, according to the manufacturer's protocols. Briefly, supernatant containing enzymes from xylan, xylose or glucose induced *P. brasiliensis* sp. nov. was mixed with 100 mM sodium acetate buffer (pH 4.5) in an appropriate volume. Reaction mixtures consisted of 0.5 ml of buffered enzyme preparation and 0.5 ml of substrate solution (1% w/v Azo-Xylan (Birchwood)). The samples were incubated at 40 °C for 10 min, and the reactions were interrupted by adding 2.5 ml of ethanol (95%, v/v) with vigorous stirring. Non-hydrolyzed ethanol-precipitated substrate was removed by centrifugation at 1000 × *g* for 10 min, and the absorbance of the supernatant was measured at 590 nm. Enzymatic activity was determined using Mega-Calc™ software (Megazyme International). One unit of enzymatic activity was defined as the amount of enzyme required to release 1 mM D-xylose-reducing sugar equivalent per minute from arabinoxylan (pH 4.5) at 40 °C.

2.3. Supernatant activity assay

Supernatant activity was assayed using xylan from beechwood, rye arabinoxylan, β-mannan, β-glucan, laminarin, pectin, arabinogalactan, arabinan from sugar beet, xyloglucan from tamarind and carboxymethylcellulose (CMC), all at a 5% final concentration. The released reducing sugars were quantified by a dinitrosalicylic method [15]. One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute. Specific activity was expressed as U/mg protein.

2.4. Xylanase production and purification

Xylanase production was performed in 1 l Erlenmeyer flasks with 200 ml of the production medium. The flasks were inoculated with 10^6 yeasts/ml and incubated at 30 °C on a rotary shaker (250 rpm). The cell growth was harvested after 96 h by centrifugation (4000 rpm, 20 min) and the supernatant was used as a crude enzyme preparation. The supernatant was concentrated by precipitation with 65% ammonium sulfate saturation. The concentrated enzyme sample was loaded on Superdex 75 column pre-equilibrated with 50 mM sodium acetate buffer, pH 4.0, and the fractions (1.5 ml/tube) were collected. The fractions were analyzed for xylanase activity and by 12% SDS-PAGE followed by staining with 0.1% Coomassie Brilliant Blue R-250 (w/v) in methanol/acetic acid/water (v/v/v) (4:1:5). One distinct xylanase peak was identified which was designated as PbXynA.

2.5. Mass spectrometry

The PbXynA corresponding band was separated by SDS-PAGE, and after extensive washing was submitted to *in situ* trypsin gel digestion with 0.5 μg of modified trypsin (Promega Corporation, Madison, WI, USA). An aliquot of the resulting peptide mixture was separated by C18 (75 μm × 100 mm) RP-nanoUPLC (nanoAcquity, Waters) coupled with a Q-Tof Ultima mass spectrometer (Waters) with nanoelectrospray source at a flow rate of 0.6 μl/min. The gradient was 2–90% (v/v) acetonitrile in 0.1% (v/v) formic acid over 45 min. The instrument was operated in the 'top three' mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. The spectra were acquired using software MassLynx v.4.1 (Waters Corporation, MA, USA) and the raw data files were converted to a peak list format (mgf) by the software Mascot Distiller v.2.3.2.0, 2009 (Matrix Science Ltd.). The MS/MS profile was searched against predicted protein sequences using engine Mascot v.2.3 (Matrix Science Ltd.) with carbamidomethylation as fixed modification, oxidation of methionine as variable modification, one chymotrypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions.

2.6. Enzyme characterization

Enzymatic activity was measured colorimetrically using xylan from beechwood as substrate and the reducing sugars determined according to Miller procedure [15]. The reaction mixture, consisting of 50 μl substrate (5%, w/v) in 50 mM sodium acetate buffer, pH 4.0 and 10 μl enzyme solution at 1724.53 U/ml, that was incubated at 55 °C in a thermocycler for 10 min. The reaction was stopped by adding 100 μl of DNS and immediately boiled for 5 min. The reducing sugars released as a result of enzyme activity were measured at 540 nm. One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars per minute. To determine the optimum pH and temperature profiles, the enzymatic reaction was carried out at different pH in McIlvaine's buffer system (pH 2.0–10.0) and various temperatures (25–80 °C).

The PbXynA kinetic constants were determined using xylan from beechwood (1.2–60 mg/ml) as substrate at pH 4.0 and 55 °C and the kinetic data were fitted using the SigrafW software [16]. The protein content was measured at 280 nm by Nanodrop and calculated by molar extinction coefficient. The effect of metal ions (Zn^{2+}, Mn^{2+}, Fe^{2+}, Na^{+}, Mg^{2+}, K^{+}, Cu^{2+}, Co^{2+} and Ca^{2+}) on xylanase activity was analyzed adding 10 mM ions at final concentration to the assay and a xylanase-bivalent ions free solution as control (Chelex 100, from Bio-Rad).

2.7. CD spectroscopy and thermal denaturation

Far-UVCD spectra of PbXynA were measured between 195 and 250 nm in 50 mM sodium acetate buffer, pH 4.0 at 25 °C with a Jasco (Hachioji City, Tokyo, Japan) J-810 spectropolarimeter using 1 mm-path-length cuvette and a protein concentration of 0.16 mg/ml. For each measurement, a total of eight spectra were collected, averaged and corrected by subtraction of a buffer blank and ellipticity was reported as the mean residue molar ellipticity (θ; deg cm^2 dmol^{-1}). Thermal denaturation was carried out using a 1 mm path-length cuvette at the same protein concentration in the same buffer, and measuring the CD signal change at 224.8 nm over the temperature range 20–100 °C at 2 degree intervals with a settling time of 60 s. The transition temperatures were estimated from the inflection points of the first derivative of the CD signal.

2.8. Capillary zone electrophoresis of oligosaccharides

The oligosaccharides released by the enzyme action on the xylan from beechwood was derivatized with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) by reductive amination as described [17]. Capillary zone electrophoresis (CZE) of oligosaccharides was performed on a P/ACE MQD instrument (Beckman Coulter) equipped with laser-induced fluorescence detection. A fused-silica capillary (TSP050375, Polymicro Technologies) of internal diameter of 50 μm and total length of 31 cm was used as separation column for oligosaccharides. Electrophoresis conditions were 15 kV/70–100 μA at a controlled temperature of 20 °C. Oligomers labeled with APTS were excited at 488 nm and emission was collected through a 520 nm band pass filter.