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GH10 XynA is the main xylanase identified in the crude enzymatic extract of *Paenibacillus* sp. A59 when grown on xylan or lignocellulosic biomass

Silvina Ghio[a], Ester M. Insani[b], Florencia E. Piccinni[b], Paola M. Talia[b], Daniel H. Grasso[a,c], Eleonora Campos[b,*]

[a] Instituto de Suelos, CIRN, Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina
[b] Instituto de Biotecnología, CICVyA, Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina
[c] Universidad Nacional de Quilmes, Departamento de Ciencia y Tecnología, Buenos Aires, Argentina

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ABSTRACT

A novel bacterial isolate with polysaccharides degrading activity was identified as *Paenibacillus* sp., and named *Paenibacillus* sp. A59. Even though it is a strict mesophile, optimal xylanase activity of the crude enzymatic extract was achieved between 50 °C and 70 °C and more than 60% of the activity was retained after incubation for 48 h at 50 °C, indicating thermotolerance of the enzymes involved. The extract was also active on pre-treated sugarcane residue (SCR) and wheat straw, releasing xylobiose and xylose as the main products, therefore confirming its predominantly xylanolytic activity. By zymograms and mass spectrometry of crude enzymatic extracts of xylan or SCR cultures, a 32 kDa GH10 beta- 1,4- endoxylanase with xylanase and no CMCase activity was identified. We named this enzyme XynA and it was the only xylanase identified under both conditions assayed, suggesting that it is a good candidate for recombinant expression and evaluation in hemicelluloses deconstruction applications. Also, a protein with two S-layer homology domains (SLH) and a large uncharacterized C-terminal domain as well as an ABC substrate binding protein were identified in crude extracts of SCR cultures. We propose that *Paenibacillus* sp. A59 uses a system similar to anaerobic and other Gram positive bacteria, with SLH-domain proteins anchoring polysaccharide-degrading enzymes close to the membrane and the substrate binding protein assisting translocation of simple sugars to the cell interior.

1. Introduction

Lignocellulosic biomass is the most abundant renewable resource in nature. It has three major organic constituents: the polysaccharides cellulose and hemicellulose and a non-fermentable polyphenolic fraction, lignin (Chen 2014). Depending on the biomass source, it may also contain different amounts of pectin, nitrogenous compounds and ash. The complex arrangement of these molecules in plant cell walls requires the synergic action of multiple enzymes for efficient biochemical conversion of polymers into monomeric sugars, the bottleneck for current bioconversion technologies (Himmel et al., 2007; Van Dyk and Pletschke 2012).

The most abundant polysaccharide component, cellulose, is a linear homopolymer of D-glucopyranose units linked by β-1,4 bonds which are deconstructed mainly by the action of endo 1,4 β-D-glucanases (EC 3.2.1.4), exoglucanases or cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (Sweeney and Xu 2012). Xylan, the main hemicellulose in plants, is a branched, heterogeneous polymer with a backbone of β-1,4-linked D-xylopyranose units and different side chain residues such as acetyl groups, arabinose, glucuronic acids, and some other residues (Shallom and Shoham 2003). Because of its branched nature, it is more easily attacked than cellulose, although due to its heterogenity, its complete deconstruction requires the action of multiple enzymes. Endo 1,4-β-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) are the main enzymatic activities required, although α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), and ferulic/coumaric acid esterases (EC 3.1.1.73) have also been shown

* Corresponding author at: Dr. N. Repetto y Los Reseros s/n, (1686) Hurlingham, Buenos Aires, Argentina. Fax: +54 1146210199.
E-mail addresses: campos.eleonora@inta.gob.ar, eleonoracampos@yahoo.com (E. Campos).

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to be important to efficiently depolymerize xylan (Dodd and Cann 2009).

Microbial cellulases and hemicellulases have potential applications in various industries which use lignocellulose as raw material, such as biobleaching of pulp paper, fibers and textiles, enhancement of feed digestibility and production of biofuels and chemicals. Other lignocellulose-based products derived from microbial enzymatic hydrolysis are organic acids (citric, lactic, succinic) and polysaccharides (chitosan, xhantan, bacterial cellulose) (Zamani 2015). For the biobleaching industry, enzymes with xylanase activity and no cellulase activity are necessary. Aerobic bacterial and fungal cellulosic degraders utilize cellulose and hemicellulose mainly through the production of extracellular enzymes that can be recovered from culture supernatants. In particular, the diversity of bacterial enzymes, some of which are tolerant of extreme pH and temperature, can provide increased functions and synergy for lignocellulose bioconversion (Maki et al., 2009).

Paenibacillus sp., reassigned from group 3 of *Bacillus* genus (Ash et al., 1993), has been studied for its ability to hydrolyze a variety of carbohydrates and the potential to produce many extracellular enzymes for industrial applications (Asha et al., 2012). Members of this genus have been described as facultative anaerobic, rod shaped, endospore-forming bacteria, primarily isolated from soil and lignocellulosic sources such as decomposing plant materials and humus-enriched soils (Priest, 2009).

In this study we isolated and characterized a cellulolytic, xylanolytic *Paenibacillus* sp. strain. Our main objectives were to evaluate its potential as a source of enzymes for degradation of lignocellulosic biomass, identify key enzymes responsible of hydrolytic activity and contribute to understanding its polysaccharide degrading system.

2. Materials and methods

2.1. Culture media

Isolates were grown on modified minimal medium (MM) (Hankin and Anagnostakis, 1977; Ghio et al., 2012), 0.1% yeast extract (YE) (Bacto), supplemented with 1% carboxymethyl cellulose (CMC) (low viscosity, SIGMA) or 0.5% beechwood xylan (XY) (SIGMA), as indicated. Agar plates of MM-0.1%YE-0.01% Trypan Blue (SIGMA), 1.5% agar (Bacto) with 1% CMC or 0.5% XY were used for testing cellulolytic or xylanolytic activity, respectively. Congo Red staining was performed as previously reported (Teather and Wood 1982). Selected bacterial isolates were cryopreserved at −80 °C with 20% glycerol. Other cellulosic substrates used were: Whatmann N°1 filter paper strips (1 × 5 cm) (FP), Avicel (AV) (Fluka), sugarcane agricultural residue (SCR) ground to 5 mm particles, SCR pre-treated by steam explosion (197.5 °C, 4.02 min) (SCRse), and wheat straw pre-treated by extrusion (WSe). For anaerobic assays, a rumen fluid medium (RFM) (Dehority 1969) was used, supplemented with cellulosic substrates as indicated.

2.2. Microscopy

Morphology and Gram reactivity of the isolated bacteria were observed using an Olympus optical microscope. Spore formation, cell size analysis and bacterial growth on cellulosic substrates were visualized in a scanning electron microscope (SEM) model FEI Quanta-250 (FEI Co., Netherlands), at the Microscopy Laboratory, CICVyA- INTA. Bacterial cultures grown in Luria Bertani (LB) for 48 h or MM with XY, SCRse or WSe for 72 h were harvested by centrifugation (1000 × *g*), fixed with 2.5% glutaraldehyde for 24 h at 4 °C, washed three-times with sodium phosphate buffer 0.1 mol l^{-1} (pH 7) and stained with osmium tetroxide for 1 h. Then, three washes with water and several steps of dehydration were performed using graded series of ethanol solutions (30–80%). The samples were sputter-coated with gold.

2.3. Physiological and biochemical characterization

Oxygen requirement was determined by growth in closed tubes gassed with carbon dioxide (Grubb and Dehority 1976). Temperature, pH and saline resistance were determined by growth in MM-0.1%YE-0.2%glucose. Biochemical tests were performed by classical methods (Mac Faddin 2003). Starch agar (0.2% soluble starch) and milk agar (2% skim milk) were used for testing amylase and caseinase activity (Logan and De Vos, 2009). MM agar plates supplemented with 1% pectin from citrus peel (SIGMA), 0.001% 4-methylumbelliferyl-β-D-glucuronide (4-MUG) (SIGMA), 2% colloidal chitin (SIGMA) (Hsu and Lockwood 1975) and 2 mmol ml^{-1} 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (SIGMA) with 0.2 mmol l^{-1} copper sulphate were used for testing pectinase, β-glucosidase (cellobiosidase), chitinase and lacasse activities, respectively.

2.4. 16S rRNA gene sequencing and phylogenetic analysis

Total genomic DNA from isolated bacteria was extracted using the Wizard Genomic DNA Extraction kit (Promega). The full length sequence of the 16S rRNA gene was amplified using 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT) universal primers (Lane 1991), with GoTaq (Promega) [95 °C, 4 min, 30 cycles of (30 s, 95 °C; 30 s 56 °C; 2 min, 72 °C), 10 min, 72 °C]. Amplification products were purified and cloned in pGEM-T Easy (Promega). Six clones were fully sequenced with an ABI 3130xl Capillary DNA sequencer (Applied Biosystems, USA) at the Genomics Unit-INTA. Sequences were analyzed with BioEdit software (v 7.2.5) and compared to GenBank Database (http://www.ncbi.nlm.nih.gov/) and Ribosomal Database Project (http://rdp.cme.msu.edu/). Sequence of *Paenibacillus* sp. A59 16S rRNA gene was deposited in GenBank under the accession number KT461879. *GyrB* gene sequence was retrieved from the full genome sequence of *Paenibacillus* sp. A59 (Ghio et al., 2015). Phylogenetic analysis of the 16S rRNA and *gyrB* gene were carried out in MEGA (v 6) (Tamura et al., 2013) and Maximum Likelihood and Neighbor-joining trees were constructed.

2.5. Enzymatic activity determinations

Bacterial cell-free culture supernatants were obtained by centrifugation of cultures grown on different cellulosic substrates at 10000 × *g*, 4 °C for 20 min. When insoluble substrates were used, cultures were first clarified by filtering through 1.2 μm glass-fiber discs (Schleicher & Schuell). The intracellular fraction was obtained by re-suspension of the cell pellet in sodium phosphate buffer pH 6.5, followed by sonication (6 pulses of 10 s, 75 W), and cell debris was removed by centrifugation. Sodium azide (0.4%) was added to all extracts to inhibit bacterial growth. Total protein content was measured by Bradford using Bio-Rad dye reagent and a bovine serum albumin (BSA) standard curve for quantification.

Xylanase and endoglucanase activities were determined in microtube assays by combining 0.1 ml of cell-free culture supernatant and 0.1 ml of XY (1%) or CMC (2%) respectively in phosphate-citrate buffer (pH 6) and incubating for 1 h at 50 °C. Reducing sugars released were measured by dinitrosalicylic acid (DNS) (Miller, 1959) using glucose or xylose standard curves. For all enzymatic assays, one international unit (IU) was defined as the amount of enzyme that released 1 μmol of product per minute under the assay conditions. Specific activity was calculated per mg of total protein (mIU mg^{-1}).