Microbiological Research 186 (2016) 16–26

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

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*

ARTICLE INFO

Article history:
Received 11 January 2016
Received in revised form 23 February 2016
Accepted 29 February 2016
Available online 3 March 2016

Keywords:
Paenibacillus
Xylanase
GH10
SLH domain
Lignocellulosic biomass

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).

http://dx.doi.org/10.1016/j.micres.2016.02.006
0944-5013/

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}).

The effect of pH on xylanase activity was studied using sodium phosphate-citrate (3.3–8) and glycine-NaOH (8.7–12) buffers at 50 °C and the effect of temperature was evaluated by incubation at pH 6, between 30 °C and 80 °C. Thermal stability of the enzymatic extract was determined by incubation at 50 °C for 48 h and testing residual enzymatic activity in samples taken at regular time points. Percentage of residual activity was calculated considering the highest value as 100%. Beta-glucosidase and β-xylosidase activities were determined using 4-nitrophenyl β-D-glucopyranoside or 4-nitrophenyl β-D-xylopyranoside (SIGMA), respectively. Reactions were performed by combining 0.1 ml of intracellular or cell-free supernatant fraction and 0.1 ml of each substrate in 5 mmol l^{-1} acetate buffer, pH 6, and incubation at 50 °C for 20 min. Reactions were stopped with 500 μl of 2% sodium carbonate and absorbance was determined at 410 nm. A *p*-nitrophenol (*p*NP) curve was used as a standard.

2.6. Identification of sugars released from xylan and lignocellulosic biomass

HPLC analysis of hydrolysis products was carried out on an Agilent 1100 series HPLC with RI detector and an Aminex HPX-87P analytical column (BioRad), at 80 °C. The isocratic mobile phase consisted of water at a flow rate of 1 ml min^{-1}. Concentration of sugars (mg ml^{-1}) was calculated using standard curves of pure compounds (SIGMA). To determine sugars released from xylan, hydrolysis was carried out at pH6 in sodium phosphate-citrate buffer for 1 h, with 1% beechwood xylan as substrate. The enzymatic extract was a cell-free supernatant from a 72 h MM-XY culture, supplemented when indicated with recombinant GH43 β-xylosidase (1.8 mIU) (Campos et al., 2014). The xylan conversion rate was calculated as the ratio of mg of xylose released and initial mg of anhydrous xylose (xylose in xylan).

To evaluate bioconversion of lignocellulosic biomass, 2% (w/v) steam-exploded SCR (SCRse) or extruded wheat straw (WSe) in phosphate-citrate (pH6) buffer were used as substrates in 24 h or 72 h hydrolysis reactions. Enzymatic extract corresponded to a combined mixture (1:1) of supernatants from 72 h MM-XY and MM-CMC bacterial cultures. Substrates, buffers and enzymatic extracts by themselves were used as control.

2.7. Statistical analysis

Significant differences of enzymatic activity assays were determined by analysis of variance (ANOVA) followed by Tukey's test ($p < 0.05$) using InfoStat Software (Di Rienzo et al., 2011).

2.8. Two-dimensional gel electrophoresis, zymography and mass spectrometry

For two-dimensional electrophoresis, a 100 ml culture grown in MM-0.1%YE-0.5%SCR for 72 h was first clarified through a glass fiber filter (1.2 μm), then centrifuged twice at 10000 × g, 20 min at 4 °C and the supernatant was filtered through a 0.2 μm polyethersulfone (PES) filter unit (Thermo Scientific) to obtain a cell-free extract. Proteins were precipitated by a TCA modified method (Ben Guerrero et al., 2015). A second protein precipitation step and removal of interfering substances was done using 2D clean-up kit (GE Healthcare). Proteins were resuspended as indicated by manufacturer and separated by isoelectric focusing (IEF) on an immobilized pH gradient (IPG) strip (7 cm, pH 4–7; Inmobiline dry strip, GE Healthcare), in an Ettan IPGphor 3 System (GE Healthcare). For the second dimension, the strip was equilibrated in buffer containing: 50 mmol l^{-1} Tris–HCl (pH 8.8), 6 mol l^{-1} urea, 30% glycerol, 2% SDS, and 1% DTT; loaded onto SDS-PAGE 10% and run at 80 V for 5 h. Spots were cut from the gel and analyzed by peptide mass fingerprinting (MALDI TOF/TOF mass spectrometry) at the Analytical Biochemistry and Proteomic Unit Service, Pasteur Institute, Uruguay (http://www.pasteur.edu.uy).

For zymography, SDS-PAGE 10–0.2% XY was used (Romano et al., 2013). Parallel SDS-PAGE was run and single bands were cut and analyzed at LANAIS-PROEM, University of Buenos Aires (http://iquifib.org/lanais), in a mass spectrometer MALDI TOF/TOF *plus* (ABI) and, for those peptides with higher signal, MS/MS spectra was obtained. The identity of each peptide was inferred using the MASCOT server (www.matrixscience.com) and against the recently obtained draft genomic sequence of *Paenibacillus* sp. A59 (Ghio et al., 2015).

3. Results

3.1. Isolation, identification and phenotypic characterization of a (hemi) cellulolytic bacterial strain

A cellulolytic, xylanolytic isolate, which we named A59, was obtained from a previously characterized bacterial consortium (Ghio et al., 2012) and was determined to be the organism responsible for the cellulose and xylan degrading activity of the consortium. By 16S rRNA gene sequence analysis, it was identified as a member of the *Paenibacillus* genus, with 99% identity to *Paenibacillus taichungensis* BCRC 17757, *Paenibacillus tundrae* A10b, *Paenibacillus pabuli* (strains HSCC 492 and JCM 9074) and *Paenibacillus xylanilyticus* XYL14, with a sequence coverage of over 98%. The high level of sequence similarity observed does not exclude a possible relationship at the species level with any one of these taxa. Phylogenetic analysis based on 16S rRNA gene, by both Neighbor-joining (Fig. 1) and Maximum Likelihood methods (not shown), showed that strain A59 formed a cluster with *P. taichungensis* BCRC 17757 (gi|343199050|) and *P. pabuli* HSCC 492 (gi|343200166|) supported by a bootstrap value of 0.93, not being able to determine whether it was a new species or a new strain belonging to either of them. Due to the difficulty to distinguish between closely related species by 16S rRNA gene analysis in *Bacillus* species, the *gyr*B gene (coding for the β subunit of DNA gyrase) is an alternative phylogenetic marker (Wang et al., 2007). *Paenibacillus* sp. A59 *gyr*B gene sequence showed a low similarity with the most closely related species (83–93%). Also, the phylogenetic analysis based on this gene placed strain A59 on a separate branch with 0.99 bootstrap support (Fig. 1), suggesting it is a novel species of *Paenibacillus* genus.

Physiological and biochemical assays supported the classification reported above. *Paenibacillus* sp. A59 formed circular, convex and moderate size white colonies in LB agar plates and cells were Gram positive rod-shaped. Terminal or sub-terminal endospores and swollen sporangia were observed by SEM and the average cell size was determined in 2.2 μm length by 0.65 μm width (not shown). Optimal culture conditions were at moderate temperatures, 30 °C in aerobiosis, although growth was also observed in anaerobiosis at 48 h (facultative anaerobe). Even though it was isolated from a pH 7 soil sample, it showed high tolerance to a wide range of pH and high salinity, being able to grow in culture from pH 5 to 10 and up to 7% NaCl (Table 1). It was able to degrade a variety of carbohydrates, such as starch, pectin, glucuronides (4-MUG) and chitin as well as proteins such as casein (Fig. S1), while no hydrolysis of gelatin and urea as well as no oxidation of ABTS was observed.

It showed good growth on carboxymethyl cellulose (CMC) and xylan (XY), reaching 7×10^{11} and 3×10^{11} CFU ml^{-1}, respectively. As expected, lower growth occurred when grown on crystalline cellulose, such as Avicel, or filter paper (7×10^{8} and 2×10^{8} CFU ml^{-1} respectively). It was also able to grow on pretreated sugar cane residue (SCRse) or wheat straw (WSe). Under anaerobic conditions

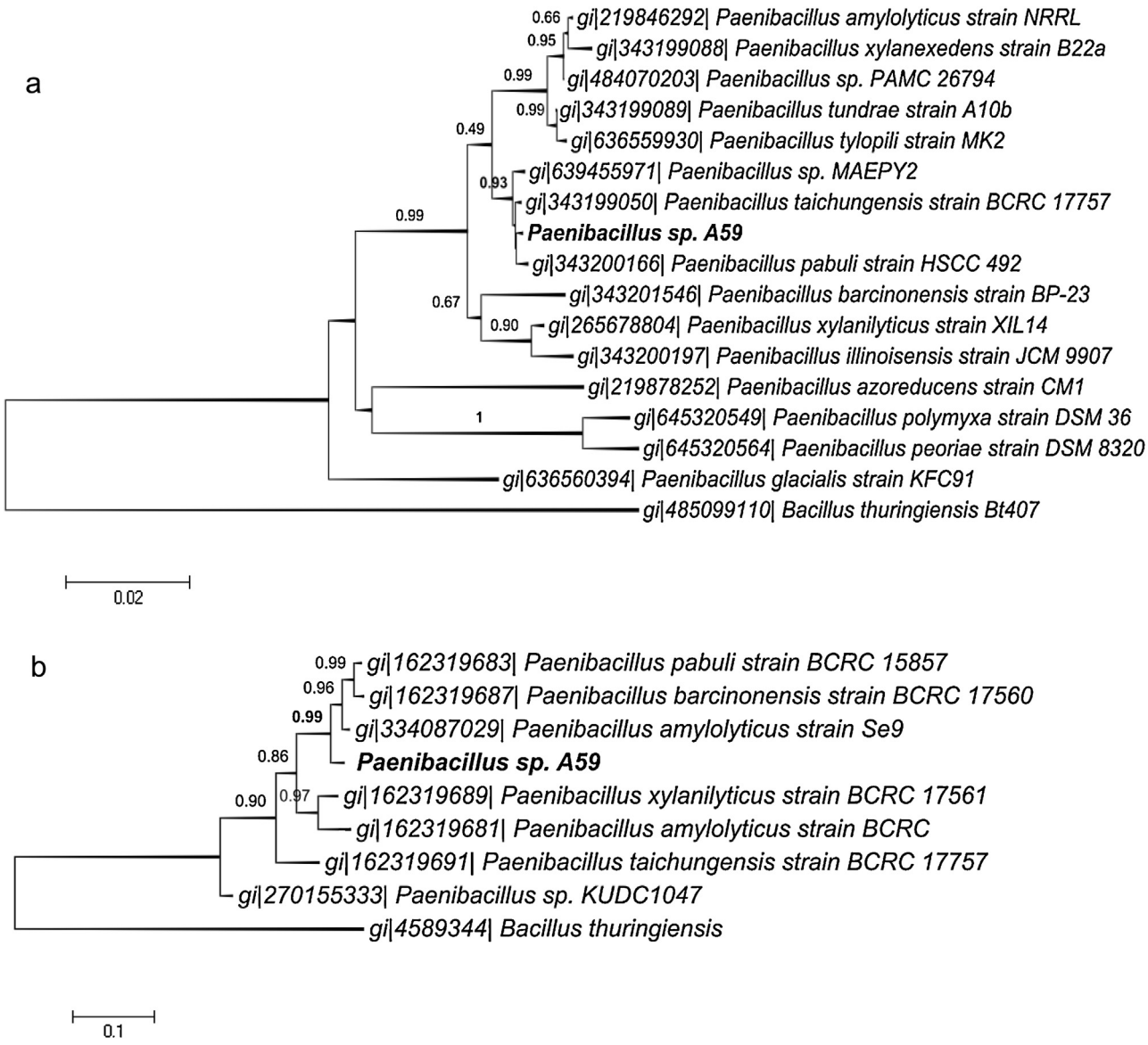

Fig. 1. Phylogenetic relationship of *Paenibacillus* sp. A59 with other species of the genus based on 16S rRNA (Neighbor-Joining) (a) and *gyrB* gene sequences (maximun likelihood) (b), using *Bacillus thuringiensis* as outgroup (MEGA 6.0). The percentage of replicate trees (higher than 0.5) in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Scale bars represent nucleotide substitution per site.

it grew on FP and XY as sole carbon sources, demonstrating its versatility to use (hemi) cellulosic substrates in aerobiosis or anaerobiosis. Active division was observed in cultures of either LB or MM with XY, SCRse or WSe and in some cases, polyhedral features on the cell surface were observed, which correlate with previously reported architecture of endospores coat (Comas-Riu and Vives-Rego 2002; Chada et al., 2003). Cells remained bound to xylan and lignocellulosic substrates, even after several wash steps (Fig. 2).

3.1. Enzymatic activity of crude extracts

The use of crude enzymatic extracts is important when low cost technologies are needed, such as biological pre-treatment and saccharification of biomass for biofuels, biobleaching of paper, pulp and fruit juice extraction, among others (Bhat, 2000). In order to achieve maximal cellulolytic and xylanolytic activity in a crude supernatant extract, bacteria were grown either in MM or LB with CMC, XY or SCR as substrates supplemented with yeast extract as nitrogen source. SCR has a heterogeneous composition: 32.6% cellulose, 23.9% hemicellulose, 20% total lignin, 1.6% acetyl groups, 7.7% ashes and 15.9% extractives (Ballesteros, personal communication) and is therefore a good model for complex lignocellulosic biomass substrates. Endoglucanase and xylanase activities were tested in cell free culture supernatants, which indicate secreted enzymes (hereon referred to as enzymatic extract). The highest xylanase activity was achieved when bacteria were grown in MM-XY (3.66 IU ml^{-1}), while the highest endoglucanase activity was obtained in MM-CMC (0.46 IU ml^{-1}) (Fig. 3). Also, enzymatic extracts from MM-SCR cultures resulted in 2.51 IU ml^{-1} xylanase activity, indicating this low cost substrate can be used to induce xylanolytic activity. In all cases, maximum endoglucanase and xylanase enzymatic activities were reached at 72 h of culture, which corresponded to late log phase of growth. Even though a higher cell count and protein content was obtained when grown on LB rather than MM, enzymatic activity was lower and similar to that obtained in LB cultures without cellulosic substrates (data not shown). It is remarkable that even though cell density was similar in cultures grown on MM-CMC or MM-XY (CMC culture: 7×10^{11} CFU ml^{-1};

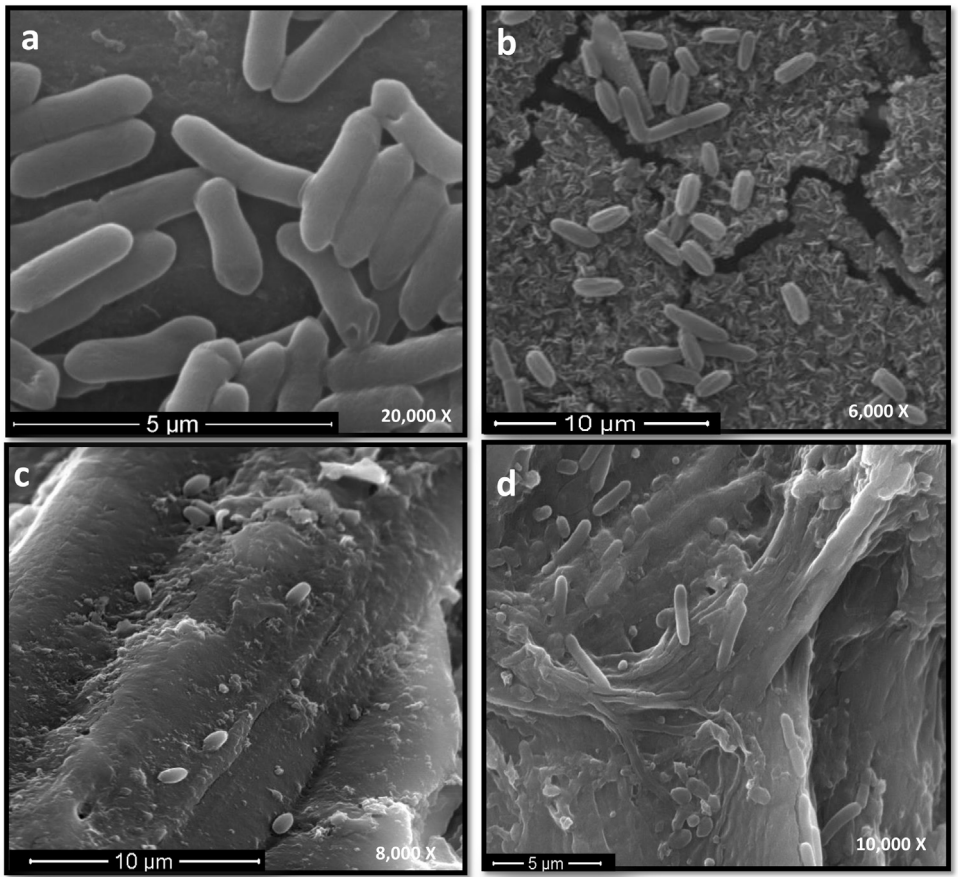

Fig. 2. Scanning electron micrographs of *Paenibacillus* sp. A59 grown on: (a) LB (48 h, 30 °C), (b) MM-0.5% beechwood xylan (72 h, 30 °C), (c) MM-0.5% pre-treated sugarcane residue (SCRse) (72 h, 30 °C) and (d) MM-0.5% pre-treated wheat straw (WSe) (72 h, 30 °C).

Table 1
Physiological and biochemical characterization of *Paenibacillus* sp. A59.

Morphology		Rods
Gram staining		Positive
Average cell size		2.2 µm length x 0.6 µm width
Endospore formation		Terminal or sub-terminal
Sporangium		Swollen
Motility		+
Oxygen requirement		Facultative anaerobe
Catalase test		+
Citrate utilization		–
Growth	Temperature (°C)	30–37
	NaCl (%)	2–7
	pH	5–10
Hydrolysis of	CMC	+
	Xylan	+
	Skim milk	+
	Soluble starch	+
	Pectin from citrus	+
	Urea	–
	Gelatin	–
	4-MUG	+
	ABTS	–
	Colloidal chitin	+

XY culture 3×10^{11} CFU ml^{-1}), there was a significant difference in total secreted proteins (0.01 mg ml^{-1} in CMC and 0.1 mg ml^{-1} in XY), indicating higher induction of protein secretion by a hemicellulosic substrate, such as xylan.

As expected, β-glucosidase and β-xylosidase activities were detected mainly in intracellular extracts. Maximal β-glucosidase activity was achieved when bacteria were grown on CMC (10.8 mIU ml^{-1} ± 0.8) and the highest β-xylosidase activity was achieved when grown on XY (12.3 mIU ml^{-1} ± 0.6), corresponding to specific activities of 0.85 IU mg^{-1} and 0.61 IU mg^{-1}, respectively (total intracellular protein content of 0.12 mg ml^{-1} for CMC culture and 0.2 mg ml^{-1} for XY culture). Also, some β-xylosidase activity (1.5 mIU ml^{-1} ± 1.1) was detected in supernatant from XY culture, while no β-glucosidase activity was evidenced either in MM-XY or MM-CMC culture supernatants. Based on its high xylanolytic potential, temperature and pH dependence assays were carried out in order to characterize the extracellular xylanolytic activity of *Paenibacillus* sp. A59. High relative activity (over 75%) was achieved in a range from pH 5 to 11, with two peaks of optimal activity at pH 6 and pH 10. Optimal temperature occurred between 50 °C and 70 °C, retaining more than 60% activity at 80 °C (Fig. 4) which is remarkable for a mesophile isolate. Moreover, the enzymatic extract retained more than 60% activity after 48 h at 50 °C, indicating thermal tolerance.

The main hydrolysis products released from beechwood xylan with the crude enzymatic extract were xylobiose and xylose, reaching an 8.8% conversion rate into xylose. The conversion rate increased to 29.25% when a GH43 β-xylosidase was added (Campos et al., 2014) (Fig. S2). These results indicated that the secreted enzymes can fully degrade xylan although supplementation with β-xylosidases would improve conversion to xylose.

Whether this conversion would be also attained from complex lignocellulosic substrates was further evaluated. In order to study degradation of the (hemi) cellulosic component of biomass, an enzymatic extract of MM-XY and MM-CMC combined culture supernatants was used. Sugarcane residue pre-treated by steam explosion (SCRse) and wheat straw pre-treated by extrusion (WSe) were subjected to 24 and 72 h hydrolysis. In both cases, the main

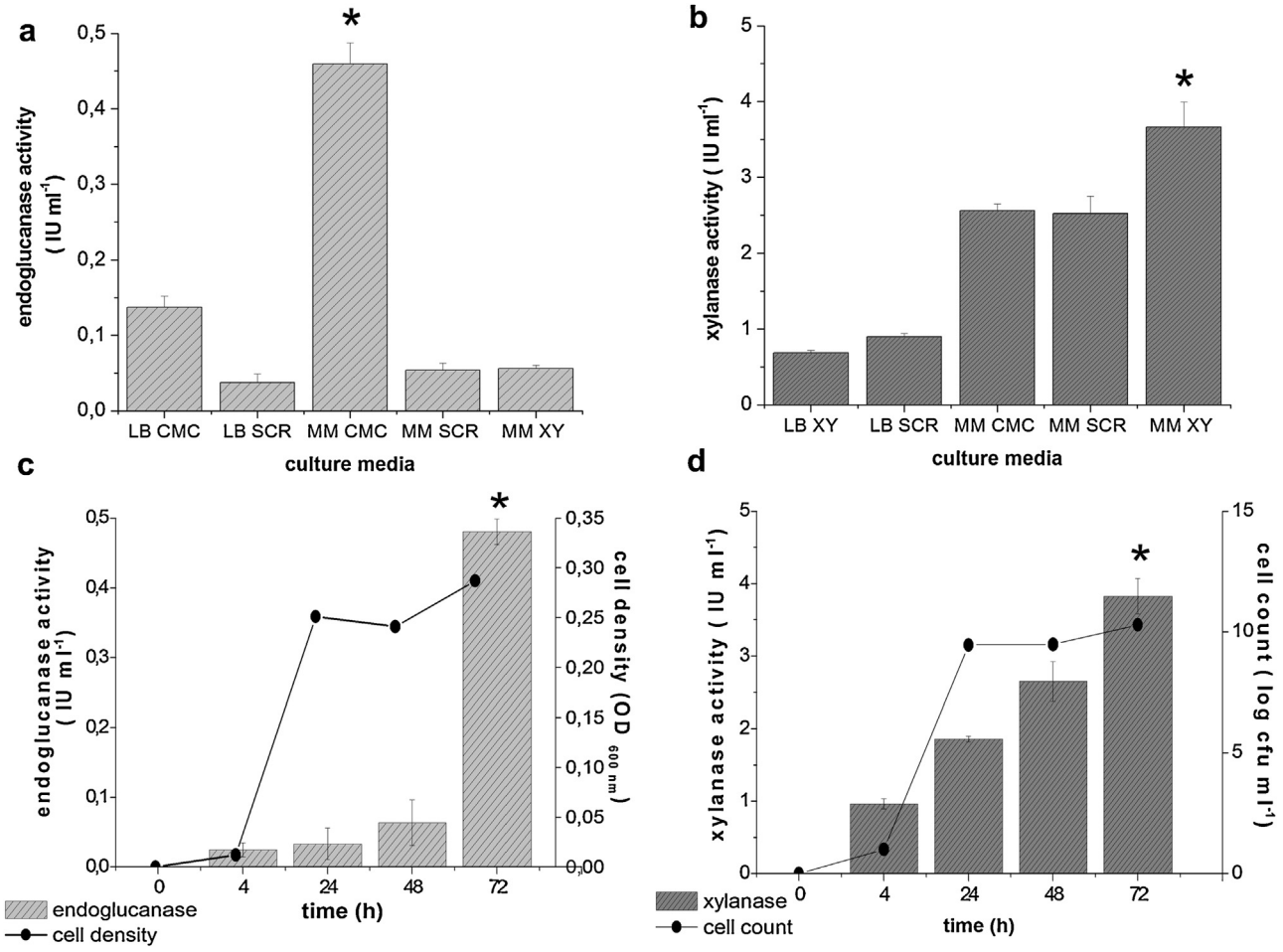

Fig. 3. Optimization of culture conditions for endoglucanase and xylanase activities. Endoglucanase (a, c) and xylanase (b, d) activities of *Paenibacillus* sp. A59 cell free supernatant from cultures in different media or at different time points. Each bar represents the mean ± S.D. ($n = 3$) of IU ml^{-1}. Bacterial growth curves in CFU ml^{-1} (XY) or optical density (CMC) are also shown for each culture. Significant differences were determined by analysis of variance (ANOVA) followed by Tukey's test (* $p < 0.05$). LB (Luria-Bertani broth), SCR (sugarcane residue), MM (minimal medium), CMC (carboxymethyl cellulose), YE (yeast extract) and XY (beechwood xylan).

products released were xylobiose and xylose. Maximum quantities of xylose were obtained in 72 h reactions from both WSe and SCRse (0.25 and 0.20 mg ml^{-1}, respectively). The highest amounts of xylobiose (0.44 mg ml^{-1}) and arabinose (0.17 mg ml^{-1}) were obtained from WSe at 72 h and mannose (0.21 mg ml^{-1}) at 24 h (only from WSe). Cellobiose was released from WSe (0.13 mg ml^{-1}) and traces were also detected from SCRse (0.02 mg ml^{-1}), in both cases only at 24 h hydrolysis, although no glucose was detected at any reaction time (Fig. 5), confirming the predominant xylanolytic activity of the enzymatic extract.

3.2. Identification of secreted proteins

To identify the proteins responsible for the observed activity, the MM-XY enzymatic extract was evaluated on xylan and CMC zymograms. Two bands of approximately 32 kDa and 75 kDa presented xylanase and no CMCase activity and are therefore the potential main enzymes responsible of xylanolytic activity of the enzymatic extract. They were retrieved from a parallel Coomasie-stained PAGE and identified by mass spectrometry as a GH10 β-xylanase and a putative GH13 alpha-amylase, respectively (Fig. 6, Table 2).

The complete GH10 nucleotide and aminoacid sequence was retrieved from the genome sequence of *Paenibacillus* sp. A59, recently released by our group (Ghio et al., 2015). It corresponds to a monocistronic gene, preceded by a predicted promoter (BPROM-Prediction of bacterial promoters) (Solovyev and Salamov, 2011). The protein (GenBank ID WP_053782506.1) has a putative 33 aminoacid N-terminal signal peptide and the theoretical molecular weight of the processed protein is 32 kDa, which is consistent with the experimental data. Two highly conserved glutamate residues involved in the catalytic site were identified at positions 137 (proton donor) and 242 (nucleophile), in accordance with predicted retaining mechanism (Collins et al., 2005). It presented high similarity (90%) to an uncharacterized 1,4-β-xylanase from *Paenibacillus* PAMC 26794 (WP_017691540.1) and 85% similarity with XynA (CBA13561.1) from *Paenibacillus barcinonensis* (Valenzuela et al., 2010) (Fig. S3). We have therefore named it XynA, to maintain the nomenclature. On the other hand, the 75 kDa GH13 alpha-amylase (WP_053784175.1), presented only one matched peptide with a low but acceptable score in MASCOT database and against *Paenibacillus* sp. A59. As there are no GH13 described with xylanolytic activity, recombinant expression of this protein is necessary to confirm this activity.

To have an insight in which proteins are induced by complex lignocellulosic substrates, secreted proteins of a SCR culture were analyzed on CMC and xylan zymograms (Fig. 6c) and in 2D gel electrophoresis (Fig. 6a). A group of undefined bands of high molecular weight (>100 kDa) had endoglucanase and xylanase activities, although they could not be resolved accurately enough to be identified. A band of approximately 32 kDa had only xylanase activity and could correspond to XynA.

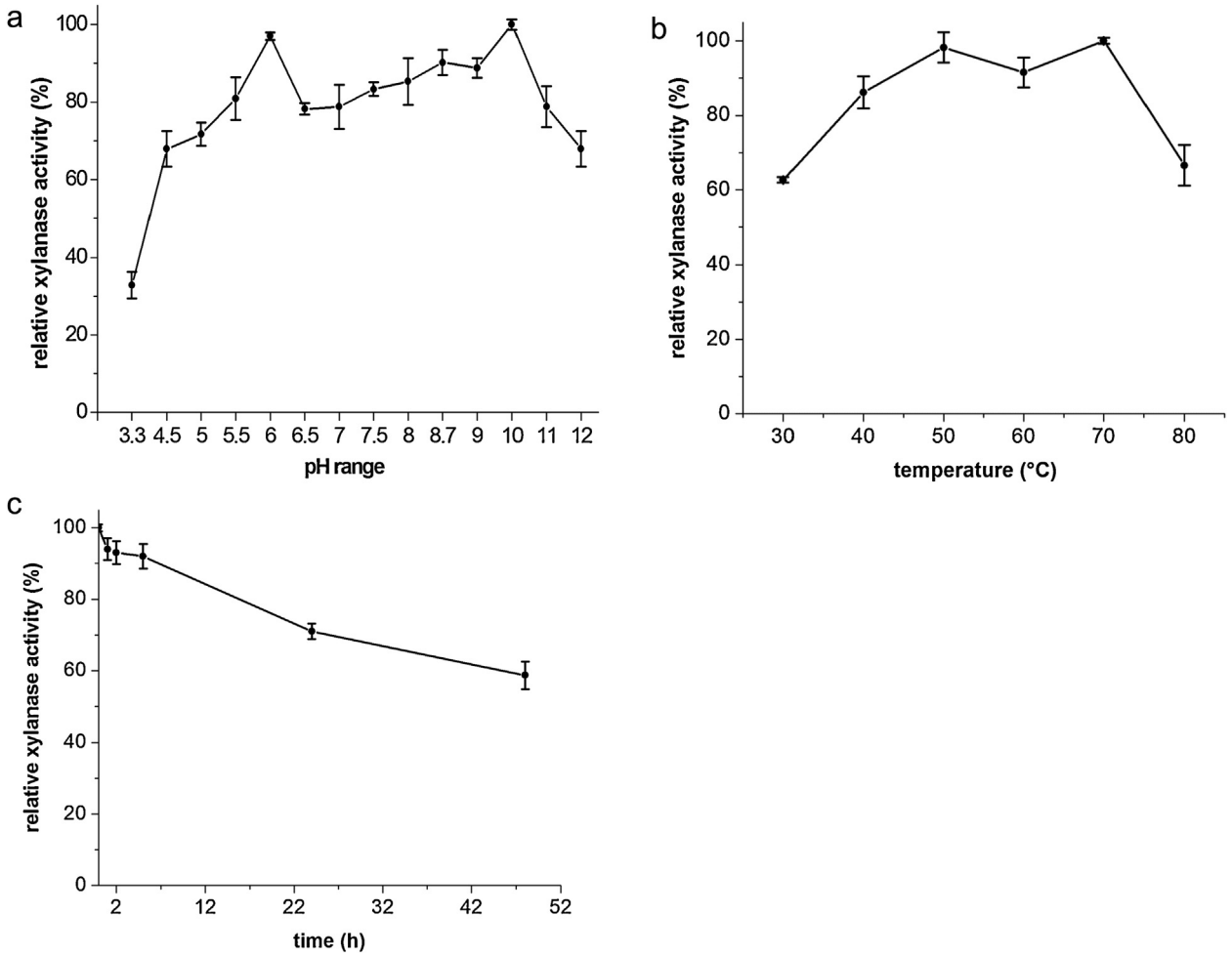

Fig. 4. Xylanase activity of *Paenibacillus* sp. A59 cell free supernatant. Dependence of xylanase activity on pH (a), temperature (b) and thermal stability at 50 °C (c), of enzymatic extracts from 72 h MM-XY culture. Triplicates of each reaction were carried out and relative activities were calculated considering the highest activity (3.66 IU ml^{-1}) as 100%. This assay is one of three biological replicates with equivalent results.

Table 2
Secreted proteins of *Paenibacillus* sp. A59 grown on minimal medium with SCR or XY identified by MALDI TOF/TOF mass spectrometry.

Assay	Spot/band[a]	Identification	Genbank accesion number	Matched peptides	Matched peptide with maximum ion score	Theoretical Ip/MM (kDa)	Experimental Ip/MM (kDa)
2D	16	SLH domain protein	WP_053782863.1	3	K.ATQISGNVYEVDFTANR.L	5.36/127.5	5.5/130
	17	1,4 beta-xylanase GH10	WP_053782506.1	3	K.AEVTQWIQAAGQR.Y	7.15/32	4.0/32
	13	ABC substrate binding	WP_053781964.1	3	K.ESTPSLLTGGVWTYPLAK.H	4.69/67.23	4.5/60
	19	Enolase	WP_053782959.1	3	K.VQLVGDDLFVTNTER.L	4.66/45.77	4.5/40
	34	Endonuclease	WP_053784170.1	3	K.GGSIFFIGDHYNADR.N	4.85/98.43	4.5/30
	23	Peptidase M4	WP_053783199.1	2	R.SLSNPTLYGQPDK.Y	4.86/55.8	5.5/25
1D	A	Alpha-amylase	WP_053784175.1	1	R.WINNDVYVYER.K	6.22/78.2	nd/70
	B	1,4 beta-xylanase GH10	WP_053782506.1	4	K.AEVTQWIQAAGQR.Y	7.15/32	nd/32

[a] Spot numbers are depicted in Fig. 6a and band letters in Fig. 6b. nd: not determined, Ip: isoelectric point, MM: molecular mass.

From the 2D gels, thirty five spots were analyzed by mass spectrometry. Six major proteins were identified: the GH10 XynA previously identified by zymography, one predominant unidentified high-molecular weight protein containing two S-layer homology (SLH) domains (the same identified in the xylan supernatant), an ABC sugar type substrate binding protein, an endonuclease, an enolase and a peptidase (Fig. 6, Table 2). All protein sequences, retrieved from the genome sequence, presented predicted signal peptides which correlate with their localization in the culture supernatant. Other analyzed spots were proteolytic products of these proteins and several spots with different molecular weight and isoelectric point were identified as the same protein, which might be due to the hydrolytic activity of proteases or post-translational modifications (Finnie and Svensson 2002; Nouwens et al., 2003).

The SLH-domain protein of high-molecular weight (127 kDa), contains two N-terminal SLH domains of approximately 65 aminoacids and a large C-terminal uncharacterized region. While proteins with SLH domains have been described as part of cellulosomes and in some cell-surface enzymes of Gram positive bacteria (Desvaux et al., 2006; Blumer-Schuette et al., 2010), their role in polysacharides deconstruction in aerobic bacteria is unclear. A 67 kDa solute-binding protein, with similarity to soluble proteins from ABC type sugar transport system was also identified in the

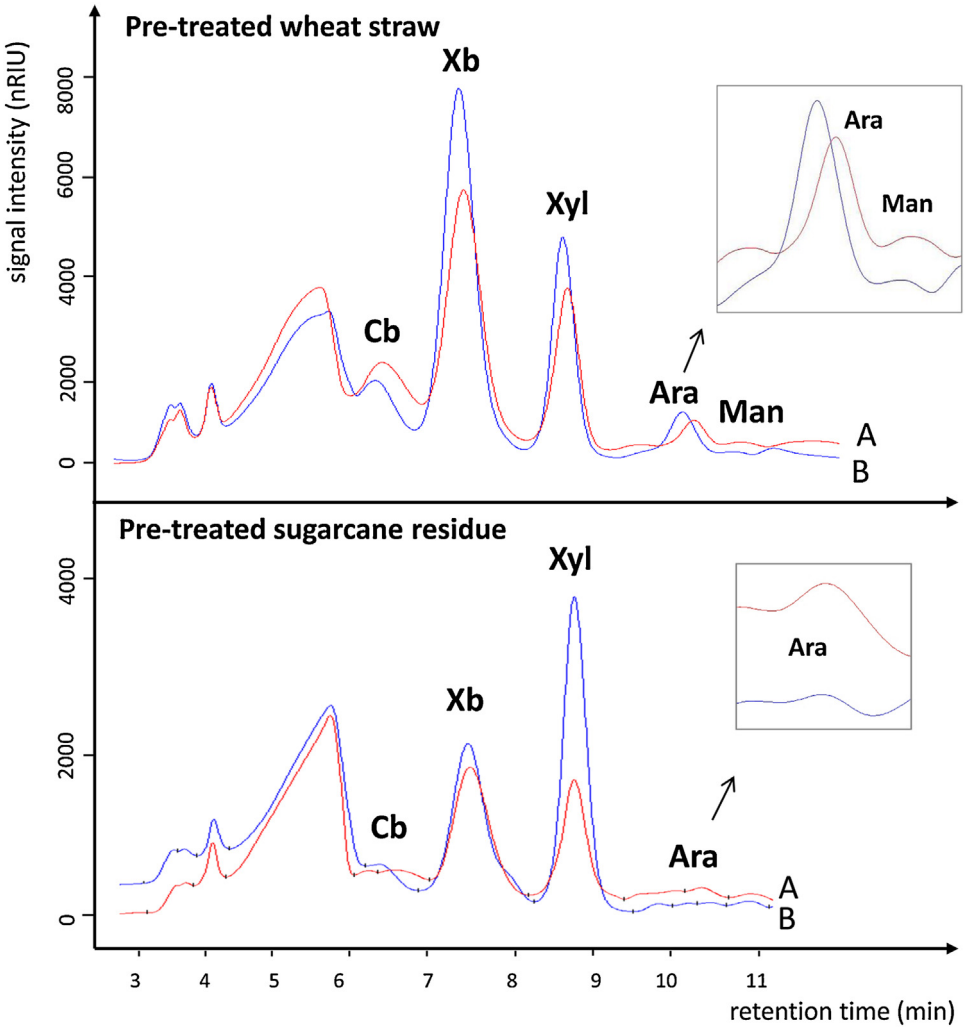

Fig. 5. Hydrolysis products released from pre-treated lignocellulosic biomass by *Paenibacillus* sp A59 crude enzymatic extract determined by HPLC. Hydrolysis reactions were carried out at 50 °C for 24 h (red line: A) and 72 h (blue line: B), using a combined (1:1) extract of supernatant from 72 h MM-XY and MM-CMC cultures. Substrates were 2% steam-exploded sugar cane residue (SCRse) or extruded wheat straw (WSe). Cb: cellobiose, Xb: xylobiose, Xyl: xylose, Ara: arabinose, Man: mannose. Reactions were carried out in duplicate with equivalent results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enzymatic extract. Similar proteins have been proposed to serve as receptors to initiate translocation of solutes through the membrane (Tamn and Saier 1993).

4. Discussion

Lignocellulosic biomass is highly recalcitrant and its bioconversion is considered a way to transform low-value products derived from lignocellulose into products of higher value such as biofuels, chemicals and paper, among others. Enzymatic lignocellulose breakdown has been studied in the last years, focusing on microbial bioprospection in order to explore new and useful enzymatic functions (Alper and Stephanopoulos, 2009). This study presents the identification of key secreted degrading enzymes of a novel *Paenibacillus* isolate with hemicelluloytic activity.

The isolated strain, named A59, showed the main phenotypic features of *Paenibacillus* genus and based on phylogenetic analysis, we proposed it as a new species. *P. pabuli* and *P. taichungensis* were the closest strains to A59 and shared all the tested physiological and biochemical properties (Lee et al., 2008; Priest 2009). However, even though chitinolytic enzymes from these species have been characterized (Juarez-Jimenez et al., 2008; Chen et al., 2010), no cellulolytic or xylanolytic activity has been reported for *P. pabuli* or *P. taichungensis* so far. Therefore, results presented in this work help to clarify the importance of this cluster as a source of carbohydrate hydrolytic enzymes.

In recent years, other *Paenibacillus* strains have been isolated mainly from soil, black liquor and compost samples and characterized for their ability to hydrolyze carbohydrates (Pason et al., 2006; Giacobbe et al., 2014; Liang et al., 2014;). Contrarily to some previously described cellulolytic *Paenibacillus* (Ko et al., 2010; Asha et al., 2012), the strain described in this work (A59) did not grow at temperatures over 45 °C. However, the enzymatic extract showed high thermal stability and an optimal temperature range for its xylanolytic enzymes between 50 to 70 °C, which is highly remarkable for a mesophile isolate. Moreover, although it was isolated from a soil consortium at pH 7, high xylanolytic activity was observed in a range between pH 6 to pH 10, indicating high tolerance to various pH conditions, which is a desirable trait for extracts acting on lignocellulosic biomass. Recently, a thermophilic *Geobacillus* sp. crude extract revealed high thermostable xylanase activity, presenting comparable and better results than commercial enzymes (Bhalla et al., 2015).

Paenibacillus sp. A59 also showed a broad range of hydrolytic activities since it was capable of degrading carboxymethyl cellulose, xylan, starch, pectin, casein and chitin. When grown on (hemi)

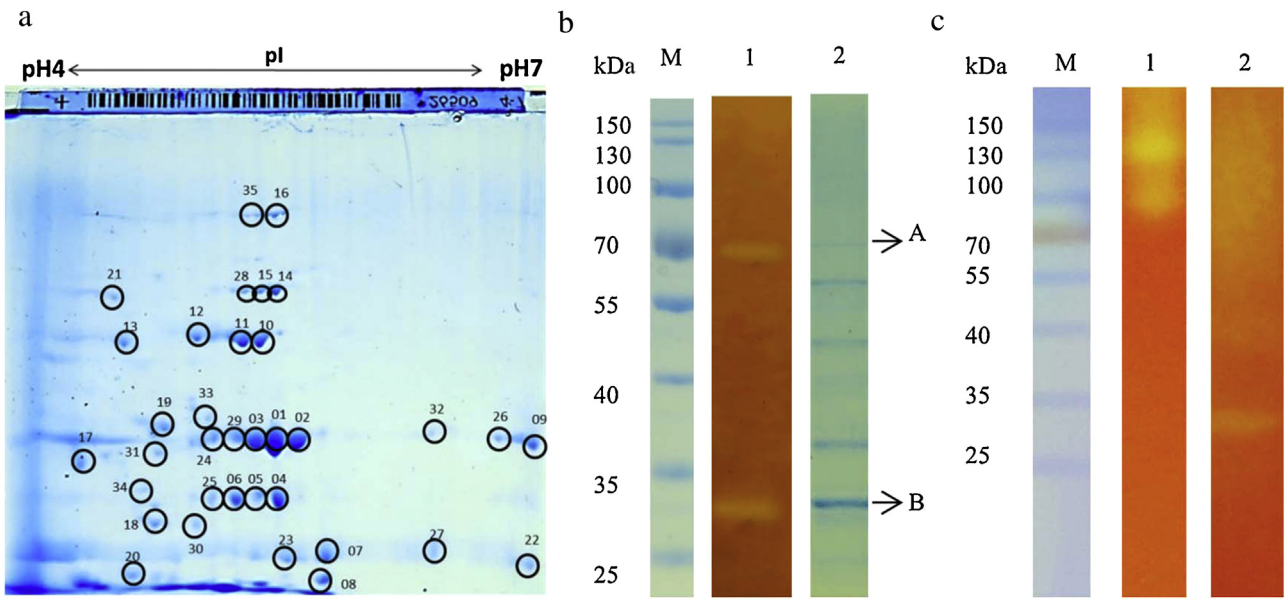

Fig. 6. Analysis of secreted proteins of *Paenibacillus* sp. A59. (a) Two-dimensional gel electrophoresis of secreted proteins from a MM-0.5% SCR culture (72 h). (b) Zymogram of xylanase activity (line 1) and 10% SDS-PAGE of secreted proteins (line 2), from MM-0.5% XY culture supernatant (72 h). (c) Zymogram of endoglucanase (line 1) and xylanase (line 2) activity from MM-0.5% SCR culture. Letters A and B indicate bands analyzed by MALDI-TOF/TOF for protein identification. M: molecular weight marker (Phage Ruler pre-stained protein ladder, Thermo Scientific).

cellulosic substrates its secreted enzymatic extract had xylanase and endoglucanase activities. The level of endoglucanase activity observed was low although it was similar to that from other *Paenibacillus* isolates, such as *Paenibacillus curdanolyticus* B-6 (Pason et al., 2006), *Paenibacillus polymyxa* BE-b40 (Gastelum-Arellanez et al., 2014), *Paenibacillus terrae* ME27-1 (Liang et al., 2014) and *P. polymyxa* EG2 and EG14 (Gorska et al., 2015). However, *Paenibacillus* sp. A59 presented high xylanase activity in its crude enzymatic extract, in similar levels to *Paenibacillus* sp. strain 2S-6 (Ko et al., 2011) and *Paenibacillus campinansensis* BL11 (Ko et al., 2010). Moreover, the enzymatic extract could deconstruct pre-treated complex agricultural biomasses, such as sugarcane residue and wheat straw, releasing di- and mono-saccharides, mainly from hemicelluloses, confirming its xylanolytic activity. Although enzymatic activity of *Paenibacillus* has been previously described, there is little association of enzymatic activity and the key proteins responsible for such activity. Secretome analysis of the crude extract of *Paenibacillus* sp. A59 revealed that the main enzyme with xylanolytic activity in secreted extracts from xylan and SCR cultures was a 32 kDa GH10 endoxylanase which we named XynA, based on its high homology to XynA from *P. barcinonensis* (Valenzuela et al., 2010). Enzymes of this family have broad substrate specificity and versatility, being able to attack not only linear xylans but also decorated heteroxylans and short xylooligosaccharides generating small hydrolysis products (Pollet et al., 2010) and have shown potential for industrial application in biomass deconstruction (Zheng et al., 2014).

Also, apart from XynA, a protein of unclear function with two SLH domains and an ABC type substrate binding protein were identified in the secreted enzymatic extract of SCR cultures. SLH domain proteins have been found as part of the cellulosome scaffold of anaerobic cellulolytic bacteria (Desvaux et al., 2006) and in a few glycosyl-hydrolases from *Paenibacillus* (Lee and Lee 2014; Ito et al., 2003). Their role in polysaccharides degradation of aerobic bacteria is still not fully understood, although it is thought to anchor polysaccharide degrading enzymes bearing SLH domains to the cell surface. Secretome studies of *Caldicellulosiruptor saccharolyticus*, a thermophilic cellulolytic anaerobe with no cellulosomal structure, indicated an SLH domain protein as the most predominant protein in the secreted extract which suggests a possible role of this protein in a non-cellulosomal mechanism of substrate interaction with the cell surface (Blumer-Schuette et al., 2010). Carbohydrate utilization patterns studies in *Caldicellulosiruptor* genus also revealed an ABC type sugar transporter as highly transcribed during growth on complex carbohydrates, along with glycoside hydrolases, suggesting a role for these proteins in the uptake of the degradation products (VanFossen et al., 2009; Blumer-Schuette et al., 2015). Furthermore, proteome analysis of the soluble fraction of a switchgrass-adapted consortium showed the presence of a carbohydrate ABC transporter substrate-binding protein and an S-layer homology domain protein from *Paenibacillus* genus as overrepresented proteins (D'haeseleer et al., 2013).

By analysis of the full genome sequence of *Paenibacillus* sp. A59, we have also identified other predicted xylanases such as a 20 kDa GH11, a 40 kDa GH10 with no signal peptide and a 150 kDa GH10 with two family 4_9 CBMs, a family 9 CBM and three SLH domains. A similar multidomain structure GH10 from *Paenibacillus* D22 was recently characterized and named XynC (Lee and Lee, 2014). The multidomain GH10 that we identified in *Paenibacillus* sp. A59 has a 43% identity with *Paenibacillus* D22 Xyn C, so we cannot confirm at this stage that it presents a similar function. Moreover, we have only identified the 32 kDa GH10 XynA in the xylan and the SCR culture supernatants. Its xylanase (with no CMCase) activity was confirmed by zymography, suggesting it may be the main xylanase responsible for the observed activity. As it did not present CMCase activity, it is a good candidate for further characterization and potential use in biobleaching industry and other hemicelluloses deconstruction processes. The contribution of the other potential xylanases identified in the genome sequence, such as the GH10 multidomain, still needs to be studied.

Also, consistently with the high intracellular β-xylosidase activity observed in this work, 12 predicted GH43 β-xylosidase coding ORFs were identified in the genome sequence of *Paenibacillus* A59 (Ghio et al., 2015).

Therefore, based on the results found in this study, we propose that *Paenibacillus* sp. polysaccharide degrading system involves the interaction of SLH-domain proteins with the cell surface. In cases in which these proteins present a catalytic domain, soluble sugars would be released in close proximity to the bacterial

surface. Other SLH domain proteins, with no recognized catalytic domain (such a as the one identified in this study), could interact with hydrolytic enzymes, such as XynA. Soluble sugars released by hydrolytic enzymes could then be translocated into the cell, assisted by ABC-solute binding proteins. Further research is needed to confirm such interactions.

As a conclusion, we have isolated a novel *Paenibacillus* species and fully characterized its xylanolytic activity, identifying key proteins involved in xylan deconstruction. Results obtained in this study indicate the high potential of the *Paenibacillus* genus as source of xylanolytic and other carbohydrate degrading enzymes and contribute to understanding the mechanism of the polysaccharide degrading system of this recently reclassified genus.

Conflict of interests

Authors declare no conflict of interests.

Acknowledgments

This work was supported by project grants MINCYT PICT2011-2735 and INTA PNAIyAV 1130034. SG and FP are Ph.D. students from the Department of Biological Chemistry (QB), FCEN, Univ. of Buenos Aires, Argentina. SG holds an INTA doctoral fellowship. EC and PT acknowledge CONICET as research career members. We thank Dr. Marcelo Soria for his assistance in phylogenetic analysis and Dr. Maria Cerón for assistance with anaerobic assays. Pretreated biomasses (along with information on their composition) were gently supplied by Dr. Mercedes Ballesteros, from the Center of Energetic, Environmental and Technological Investigations (CIEMAT), Madrid, Spain. We thank Dr. David Wilson for his valuable critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.micres.2016.02.006.

References

Alper, H., Stephanopoulos, G., 2009. Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? Nat. Rev. Microbiol. 7, 715–723.

Ash, C., Priest, F.G., Collins, M.D., 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol. 64, 253–260.

Asha, B.M., Revathi, M., Yadav, A., Sakthivel, N., 2012. Purification and characterization of a thermophilic cellulase from a novel cellulolytic strain *Paenibacillus barcinonensis*. J. Microbiol. Biotechnol. 22, 1501–1509.

Ben Guerrero, E., Arneodo, J., Bombarda Campanha, R., Abrão de Oliveira, P., Veneziano Labate, M.T., Regiani Cataldi, T., et al., 2015. Prospection and evaluation of (Hemi) cellulolytic enzymes using untreated and pretreated biomasses in two argentinean native termites. PLoS One 10 (8), e0136573.

Bhalla, A., Bischoff, K.M., Sani, R.K., 2015. Highly thermostable xylanase production from a thermophilic *Geobacillus* sp. strain WSUCF1 utilizing lignocellulosic biomass. Front. Bioeng. Biotechnol. 3, 84.

Bhat, M.K., 2000. Cellulases and related enzymes in microbiology. Biotech. Adv. 18, 355–383.

Blumer-Schuette, S.E., Lewis, D.L., Kelly, R.M., 2010. Phylogenetic, microbiological, and glycoside hydrolase diversities within the extremely thermophilic, plant biomass-degrading genus *Caldicellulosiruptor*. Appl. Environ. Microbiol. 76, 8084–8092.

Blumer-Schuette, S.E., Alahuhta, M., Conway, J.M., Lee, L.L., Zurawski, J.V., Giannone, R.J., et al., 2015. Discrete and structurally unique proteins (Tapirins) mediate attachment of extremely thermophilic *Caldicellulosiruptor* species to cellulose. J. Biol. Chem. 290, 10645–10656.

Campos, E., Negro Alvarez, M.J., Sabaris Di Lorenzo, G.J., Gonzalez, S., Rorig, M., Talia, P., et al., 2014. Purification and characterization of a GH43 β-xylosidase from *Enterobacter* sp. identified and cloned from forest soil bacteria. Microbiol. Res. 169, 213–220.

Chada, V.G.R., Sanstad, E.A., Wang, R., Driks, A., 2003. Morphogenesis of *Bacillus* spores surfaces. J. Bacteriol. 185, 6255–6261.

Chen, H.B., Kao, P.M., Huang, H.C., Shieh, C.J., Chen, C.I., Liu, Y.C., 2010. Effects of using various bioreactors on chitinolytic enzymes production by *Paenibacillus taichungensis*. Biochem. Eng. J. 49, 337–342.

Chen, H., 2014. Chemical composition and structure of natural lignocellulose. In: Chen, H. (Ed.), Biotechnology of Lignocellulose: Theory and Practice. Chemical Industry Press, China, pp. 25–71.

Collins, T., Gerday, C., Feller, G., 2005. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol. Rev. 29, 3–23.

Comas-Riu, J., Vives-Rego, J., 2002. Cytometric monitoring of growth, sporogenesis and spore cell sorting in *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*). J. Appl. Microbiol. 92, 475–481.

D'haeseleer, P., Gladden, J.M., Allgaier, M., Chain, P.S.G., Tringe, S.G., Malfatti, S.A., 2013. Proteogenomic analysis of a thermophilic bacterial consortium adapted to deconstruct switchgrass. PLoS One 8 (7), e68465.

Dehority, B.A., 1969. Pectin-fermenting bacteria isolated from the bovine rumen. J. Bacteriol. 99, 189–196.

Desvaux, M., Dumas, E., Chafsey, I., Hébraud, M., 2006. Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure. FEMS Microbiol. Lett. 256, 1–15.

Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW, InfoStat versión 2011. Córdoba, Argentina : Grupo InfoStat, FCA, Universidad Nacional de Córdoba.

Dodd, D., Cann, I.K.O., 2009. Enzymatic deconstruction of xylan for biofuel production. Glob. Change Biol. Bioenergy 1, 2–17.

Finnie, C., Svensson, B., 2002. Proteolysis during the isoelectric focusing step of two-dimensional gel electrophoresis may be a common problem. Anal. Biochem. 311, 182–186.

Gastelum-Arellanez, A., Paredes-López, O., Olalde-Portugal, V., 2014. Extracellular endoglucanase activity from *Paenibacillus polymyxa* BEb-40: production, optimization and enzymatic characterization. World J. Microbiol. Biotechnol. 30, 2953–2965.

Ghio, S., Di Lorenzo, G.S., Lia, V., Talia, P., Cataldi, A., Grasso, D., Campos, E., 2012. Isolation of *Paenibacillus* sp. and *Variovorax* sp. strains from decaying woods and characterization of their potential for cellulose deconstruction. Int. J. Biochem. Mol. Biol. 3, 352–364.

Ghio, S., Martinez Cáceres, A., Talia, P., Grasso, D., Campos, E., 2015. Draft genome sequence of cellulolytic and xylanolytic *Paenibacillus* sp A59, isolated from a decaying forest soil from Patagonia Argentina. Genome Announc. 3 (5), e01233–15.

Giacobbe, S., Pepe, O., Ventorino, V., Birolo, L., Vinciguerra, R., Faraco, V., 2014. Identification and characterization of a pectinolytic enzyme from *Paenibacillus xylanolyticus*. Bioresources 9, 4873–4887.

Gorska, E.B., Jankiewicz, U., Dobrzynski, J., Russel, S., Pietkiewicz, S., Kalaji, H., et al., 2015. Degradation and colonization of cellulose by diazotrophic strains of *Paenibacillus polymyxa* isolated from soil. J. Biorem. Biodegrad. 6, 271.

Grubb, J.A., Dehority, B.A., 1976. Variation in colony counts of total viable anaerobic rumen bacteria as influenced by media and cultural methods. Appl. Environ. Microbiol. 31, 262–267.

Hankin, L., Anagnostakis, S.L., 1977. Solid media containing carboxymethylcellulose to detect cellulase activity of microorganisms. J. Gen. Microbiol. 98, 109–115.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 315, 804–807.

Hsu, S.C., Lockwood, J.L., 1975. Powder chitin agar as a selective medium for enumeration of Actinomycetes in water and soil. Appl. Microbiol. 29, 422–426.

Ito, Y., Tomita, T., Roy, N., Nakano, A., Sugawara-Tomita, N., Watanabe, S., et al., 2003. Cloning, expression, and cell surface localization of *Paenibacillus* sp strain W-61 xylanase 5, a multidomain xylanase. Appl. Environ. Microbiol. 69, 6969–6978.

Juarez-Jimenez, B., Rodelas, B., Martinez-Toledo, M.V., Gonzalez-Lopez, J., Crognale, S., Gallo, A.M., et al., 2008. Production of chitinolytic enzymes by a strain (BM17) of *Paenibacillus pabuli* isolated from crab shells samples collected in the east sector of central Tyrrhenian Sea. Int. J. Biol. Macromol. 43, 27–31.

Ko, C.H., Lin, Z.P., Tu, J., Tsai, C.H., Liu, C.C., Chen, H.T., Wang, T.P., 2010. Xylanase production by *Paenibacillus campinasensis* BL11 and its pretreatment of hardwood kraft pulp bleaching. Int. Biodeterior. Biodegrad. 64, 13–19.

Ko, C.H., Tsai, C.H., Tu, J., Yang, B.Y., Hsieh, D.L., Jane, W.N., Shih, T.L., 2011. Identification of *Paenibacillus* sp. 2S-6 and application of its xylanase on biobleaching. Int. Biodeterior. Biodegrad. 65, 334–339.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, New York, pp. 115–147.

Lee, S.H., Lee, Y.E., 2014. Cloning and characterization of a multidomain GH10 xylanase from *Paenibacillus* sp DG-22. J. Microbiol. Biotechnol. 24 (11), 1525–1535.

Lee, F.-L., Tien, C.-J., Tai, C.-J., Wang, L.-T., Liu, Y.-C., Chern, L.-L., 2008. *Paenibacillus taichungensis* sp. nov., from soil in Taiwan. Int. J. Syst. Evol. Microbiol. 58, 2640–2645.

Liang, Y.L., Zhang, Z., Wu, M., Wu, Y., Feng, J.X., 2014. Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region of China and optimization of cellulase production by *Paenibacillus terrae* ME27-1. Biomed. Res. Int., 512497.

Logan, N.A., De Vos, P., 2009. Genus bacillus. In: Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W. (Eds.), Bergeyís Manual of Systematic Bacteriology. The Firmicutes, Volume 3, 2nd edition. Springer, New York, pp. 21–71.

Mac Faddin, J.F., 2003. Pruebas bioquímicas para la identificación de bacterias de importancia clínica, 3ra edición. Ed. Médica Panamericana, Montevideo.
Maki, M., Leung, K.T., Qin, W., 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int. J. Biol. Sci. 5, 500–516.
Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–430.
Nouwens, A.S., Walsh, B.J., Cordwell, S.J., 2003. Application of proteomics to *Pseudomonas aeruginosa*. Adv. Biochem. Eng. Biotechnol. 83, 117–140.
Pason, P., Kyu, K.L., Ratanakhanokchai, K., 2006. Paenibacillus curdlanolyticus strain B-6 xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. Appl. Environ. Microbiol. 72, 2483–2490.
Pollet, A., Delcour, J.A., Courtin, C.M., 2010. Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families. Crit. Rev. Biotechnol. 30, 176–191.
Priest, F.G., 2009. Genus paenibacillus. In: Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W. (Eds.), Bergeyís Manual of Systematic Bacteriology. The Firmicutes, Volume 3, 2nd edition. Springer, New York, pp. 269–295.
Romano, N., Gioffré, A., Sede, S.M., Campos, E., Cataldi, A., Talia, P., 2013. Characterization of cellulolytic activities of environmental bacterial consortia from an argentinian native forest. Curr. Microbiol. 67, 138–147.
Shallom, D., Shoham, Y., 2003. Microbial hemicellulases. Curr. Opin. Microbiol. 6, 219–228.
Solovyev, V., Salamov, A., 2011. Automatic annotation of microbial genomes and metagenomic sequences. In: Li, R.W. (Ed.), Metagenomics and Its Applications in Agriculture, Biomedicine and Environmental Studies. Nova Science Publishers, pp. 61–78.
Sweeney, M.D., Xu, F., 2012. Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. Catalysts 2, 244–263.
Tamn, R., Saier, M.H.J., 1993. Structural, functional and evolutionary relationship among extracellular solute-binding receptors of bacteria. Microb. Rev. 57, 320–346.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA 6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Teather, R.M., Wood, P.J., 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43, 777–780.
Valenzuela, S.V., Díaz, P., Pastor, F.I.J., 2010. Recombinant expression of an alkali stable GH10 Xylanase from *Paenibacillus barcinonensis*. J. Agric. Food Chem. 58, 4814–4818.
Van Dyk, J.S., Pletschke, B.I., 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy. Biotechnol. Adv. 30, 1458–1480.
VanFossen, A.L., Verhaart, M.R.A., Kengen, S.M.W., Kelly, R.M., 2009. Carbohydrate utilization patterns for the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus* reveal broad growth substrate preferences. Appl. Environ. Microbiol. 75, 7718–7724.
Wang, L.T., Lee, F.L., Tai, C.J., Kasai, H., 2007. Comparison of *gyrB* sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. Int. J. Syst. Evol. Microbiol. 57, 1846–1850.
Zamani, A., 2015. Introduction to lignocellulose-based products. In: Karimi, K. (Ed.), In Lignocellulose-based Bioproducts. Biofuels and Biorefinery Technologies. , 1st Ed. Springer, Switzerland, pp. 1–36.
Zheng, H.C., Sun, M.Z., Meng, L.C., Pei, H.S., Zhang, X.Q., Yan, Z., et al., 2014. Purification and characterization of a thermostable xylanase from *Paenibacillus* sp. NF1 and its application in xylooligosaccharides production. J. Microbiol. Biotechnol. 24, 489–496.