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Research paper

Purification and characterization of a GH43 β-xylosidase from *Enterobacter* sp. identified and cloned from forest soil bacteria

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ABSTRACT

The use of lignocellulosic biomass for second generation biofuels requires optimization of enzymatic breakdown of plant cell walls. In this work, cellulolytic bacteria were isolated from a native and two cultivated forest soil samples. Amplification of glycosyl hydrolases was attempted by using a low stringency-degenerate primer PCR strategy, using total soil DNA and bulk DNA pooled from positive colonies as template. A set of primers was designed based on *Acidothermus cellulolyticus* genome, by search of conserved domains of glycosyl hydrolases (GH) families of interest. Using this approach, a fragment containing an open reading frame (ORF) with 98% identity to a putative GH43 beta-xylosidase coding gene from *Enterobacter cloacae* was amplified and cloned. The full protein was expressed in *Escherichia coli* as N-terminal or C-terminal His-tagged fusions and purified under native conditions. Only N-terminal fusion protein, His-Xyl43, presented beta-xylosidase activity. On *p*NPX, optimal activity was achieved at pH 6 and 40 °C and K_m and K_{cat} values were 2.92 mM and 1.32 seg^{-1}, respectively. Activity was also demonstrated on xylobiose (X2), with K_m 17.8 mM and K_{cat} 380 s^{-1}. These results demonstrated that Xyl43 is a functional beta-xylosidase and it is the first evidence of this activity for *Enterobacter* sp.

1. Introduction

The development of alternative fuels is essential to reduce the use of fossil based fuels and carbon dioxide emissions, as well as to face the uncertainty of future petroleum sources. At present, bioethanol is obtained mainly from hydrolysis and fermentation of sugar cane and corn. However, facing the predicted rise in biofuels utilization worldwide, it has become mandatory to develop new sources of biofuels and sustainable technologies. Therefore, there is a growing interest in developing second and third generation biofuels, based on ligno-cellulosic biomass, which includes agricultural residues, wood (especially from softwood), grasses cultivated as specific energy crops and solid urban waste (paper, cardboard, etc.) (Gabrielle 2008; Rubin 2008).

Even though cellulose is one of the most abundant renewable energy sources in nature, its utilization for biofuels has been hampered by the biochemical and structural complexity of plant cell walls. The composition of the lignocellulosic biomass varies depending on the species to be considered, but it is mainly formed by cellulose (35–50%), hemicellullose (20–35%), lignin (10–25%) and other less represented components (Liu et al. 2008). Each cellulose molecule is a linear polymer of β1 → 4D-glucose unit. Hemicellullose is actually a group of pentoses (xylose and arabinose) and hexoses (mannose, glucose and galactose) heteropolymers in a ratio that depends on the type of biomass. Up to now, hemicelluloses are not being industrially fully utilized to generate bioethanol (Agbogbo and Coward-Kelly 2008). Biomass recalcitrance and hydrolytic enzymes are the key limiting steps in obtaining cellulosic ethanol (Merino and Cherry 2007).

In nature, cellulases, hemicelulases and other glycosyl hydrolases are being synthesized by fungi, aerobic and anaerobic bacteria, insects and protozoa (Wilson 2011). They are grouped in families of glycosyl hydrolases based on their hydrolysis mechanism (http://www.cazy.org) (Henrissat 1991; Henrissat and Bairoch 1993) and within each family, there are enzymes presenting different activities, reflected in their EC number (enzyme commission number) (Zhou et al. 2010). Hemicellulases are a group of enzymes defined and classified according to their substrate, hemicelluloses, which are a heterogeneous

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group of branched and linear heteropolymers of xylose, galactose, mannose, arabinose, other sugars and their uronic acids. The synergistic action of multiple enzymes is required to hydrolyze cellulose and hemicelluloses. Nevertheless, hemicellulases have been less studied and characterized than cellulases. They are much more complex, as they represent an array of enzymes such as xylanases, mannases, arabinases (both endo and exo) and their corresponding glycosidases. The best characterized hemicellulases so far are 1.4-β-D-xylanases, either the endo- (EC 3.2.1.8) or exo-type (EC 3.2.1.37) (Shallom and Shoham 2003). Recently, a group of bacterial GH43 beta-xylosidases was thoroughly studied and their activity on different substrates was compared (Jordan et al. 2013).

At present, commercial enzymes for different applications come mainly from fungi (*Trichoderma reesei* and *Aspergillus niger*) (Quinlan et al. 2010). However, for lignocellulosic biomass deconstruction it is necessary the development of new enzymatic extracts or supplement present enzymatic cocktails with a greater number of enzymatic activities that would allow overcoming the inhibition of the reaction due to insoluble substrate or intermediate toxic compounds (Ballesteros 2010). In this regard, a highly promising source of activities is the diversity of bacterial cellulolytic enzymes, recently revised by Mba Medie et al. (2012). A variety of known and unknown species of bacteria from diverse phylogenetic species are capable of hydrolyzing cellulose from a wide range of natural habitats that could be explored in the search for new cellulolytic activities (Zhou et al. 2010).

The general objective of this work was the isolation of cellulolytic bacterial strains from forest soils as a basis for identifying novel cellulases encoding genes. We have chosen a native forest soil sample (NF) and two cultivated forest soil samples, *Pinus taeda* (PT) and *Eucaliptus grandis* (EG), all from the subtropical region of Misiones, Argentina. This native forest region is part of the Atlantic forest and it was chosen because of its high biodiversity and its unique bioma. PT and EG soils were chosen because these species have potential as sources for lignocellulosic biomass feedstocks.

2. Materials and methods

2.1. Soil samples

Samples from the superficial soil layer (0–10 cm) were collected from a native forest (NF1, S26°01′34 W54°26′59) and two cultivated forests (EG and PT, S26°01′08 W54°27′24 and S26°01′10 W54°27′2, respectively) in the month of November (spring, temperatures around 25 °C and high humidity weather conditions) from Misiones, Argentina, and immediately stored at 4 °C. Soils were analyzed by the Soil Analysis Laboratory (LabIS) from Soil Institute (Inst. de Suelos) CIRN, INTA. pH was determined in a 1:2.5 soil:water suspension.

2.2. Most probable number assay (MPN) and culture methods

5 gr of soil samples were suspended in salt solution for cellulolytic enrichment [0.05× Winogradsky salts (K_2HPO_4, 5; NaCl, 2.5; $MgSO_4 \times 7H_2O$, 2.5; $Na_2MoO_4 \times 2H_2O$, 0.005; MnSO4 × $4H_2O$ 0.05; $Fe_2(SO_4)_3$ 0.05 g/L); soil extract 0.02× (prepared by dissolving soil 1/1 (w/v) in distilled water and sterilized by autoclave), NH_4NO_3 1 g/L, $CaCO_3$ 3 g/L]. The resulting solution was sterilized by autoclave and supplemented with cycloheximide 100 μg/mL in order to avoid fungal growth (Zuberer 1994). The suspended soil sample was then filtered by Whatman no. 1, and serial dilutions were made in the same salts solution with filter paper as unique carbon source. Dilutions were done in triplicate and all tubes were incubated at 28 °C for two weeks. Estimation of bacterial cellulolytic active population was done by Most Probable Number method (MPN) (http://www.i2workout.com/mcuriale/mpn/index.html), taking as positive those tubes with filter paper signs of degradation. Subsequent isolation of bacteria was done by repeated streak out of bacteria from positive tubes on minimal media (MM) according to Hankin and Anagnostakis (1977), with some modifications (Ghio et al. 2012): (g/L) K_2HPO_4, 1.67; KH_2PO_4, 0.87; NaCl, 0.05; $MgSO_4 \times 7H_2O$, 0.1; $CaCl_2$, 0.04; $FeCl_3$, 0.004; $Na_2MoO_4 \times 2H_2O$, 0.005; biotin, 0.01; nicotinic acid, 0.02; pantothenic acid, 0.01; NH_4Cl, 1, supplemented with 0.5% CMC (low viscosity-Sigma) and 1.5% agar (MM-CMC). For long term preservation, isolates were grown in LB supplemented with 0,5% CMC, overnight at 28 °C and stored at −80 °C with 20% glycerol.

Table 1
Primers designed for GH amplification.

Primer	Sequence	Tm (°C)
Acm5dr	(5′-STKRAAYCCRAASGTSGTSKW-3′)	56.3
Acgh10f	(5′-ATGCATCACGCCATGCGA-3′)	58.1
Acgh5f	(5′-GTGCCGCGCGCATTGCGGCGA-3′)	71.1
Acgh6f	(5′-ATGCCGCCATCTCAAAAC-3′)	53.6
Acgh48f	(5′-ATGCCAGGATTACGACGGCGA-3′)	61.1
Acgh9f	(5′-ATGCGCTTGACGCGCGCGTGGA-3′)	69.0

2.3. Primer design and amplification of GH

Total DNA from pooled isolates was extracted with Wizard Genomic DNA purification kit (PROMEGA). For GH primer design, protein sequences annotated as glycosyl hydrolases (GH) of families 5, 6, 9, 10 and 48 from *A. cellulolyticus* 11B (GeneBank accession number NC_008578.1) were aligned and conserved motifs were found by Meme application (http://meme.sdsc.edu) (Bailey et al. 2009). Degenerate primer m5dr (Table 1) was designed based on the consensus motif found. Primers for 5′ end were designed for each target gene (Table 1) and checked by Primer3Plus (http://primer3.sourceforge.net). PCR conditions were: 94 °C 2 min, 30 × (94 °C 30 s, 50 °C 30 s, 72 °C 3 min), 72 °C 5 min. Amplification products were visualized in 0.8% agarose with EthBr, purified, cloned in pGEMTeasy vector and sequenced with T7 and SP6 primers. The resulting sequences were assembled and homology was searched by NCBI BLASTx and BLASTn.

As a control reaction, 16S rRNA coding gene was amplified with primers fD1 (5′AGAGTTTGATCCTGGCTCAG3′) and rD1 (5′AAGGAGGTGATCCAGCC3′) (Weisburg et al. 1991), using standard PCR conditions, 95 °C 4 min, 30 × (95 °C 30 s, 53 °C 30 s, 72 °C 2 min), 72 °C, 5 min).

2.4. Cloning, expression and purification of Enterobacter cloacae β-xylosidase

The *E. cloacae* GH43 β-xylosidase gene was amplified from total DNA from isolates using primers 43FBam 5′GGATCCATGGAAATCACTAACCCGATACT3′ and 43RXho 5′CTCGAGTTATCAGRCCGGCTCGTAGGTGAAGTAG3′, for N terminal fusion to 6xHis or primers FxbaCter 5′TCTAGAAATAATTTTAAGAAGGAGATATATGGAAATCACTAACCCGATACT3′ and RxhoCter 5′CTCGAGCGGCTCGTAGGTGAAGTAG3′ for C terminal fusion to 6xHis (restriction sites are shown underlined and initiation and stop codons in bold). These primers were designed based on the sequence of the amplification product previously obtained with the degenerate primers. Primer sequences were also corroborated by comparison with *E. cloacae* subsp. cloacae NCTC9394 draft genome (accession number FP929040.1) (Ren et al. 2010). Expected amplification product was 1626 bp. The amplified gene was cloned in pET28a vector previously digested with *Bam*HI and *Xho*I for Nterminal fusion and *Xba*I/*Xho*I for C terminal fusion. From the obtained recombinant clones, two were randomly selected, sequenced and analyzed for

recombinant protein expression by standard protocols, with IPTG 1 mM induction (Qiaexpressionist). Purification was performed by IMAC with Ni-NTA agarose resin using 50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole as elution buffer. Purified enzyme was conserved at 4° or −20 °C with 40% glycerol (for long term storage). A typical purification resulted in a yield of 20 mg per 50 mL of induced culture.

2.5. Enzymatic assays

Beta-xylosidase activity of both constructs was assayed using *p*-nitrophenyl-β-D-pyranoside (*p*NPX) (Sigma–Aldrich N2132) as substrate in citrate buffer pH = 6 (Poutanen et al. 1987). Activity was calculated according to: UI/mL = (delta Abs410 nm/ε)*10^6/t*7* dilution factor of enzyme, where ε is the molar extinction coefficient of *p*-nitrophenol (15,200 $M^{-1}cm^{-1}$) and t is the incubation time in minutes.

For enzymatic activity characterization of His-Xyl43, β-xylosidase activity was assayed in a mixture (0.2 mL) containing 0.1% *p*-nitrophenyl β-D-xylopyranoside (*p*NPX, Sigma–Aldrich ref. N2132), 50 mM citrate/phosphate buffer (I = 0.3 M, adjusted with NaCl) pH 6 and appropriately diluted enzyme solution. After 5 min, the reaction was stopped by adding 0.2% Na_2CO_3 (0.5 mL) and the A_{400} was measured. A standard curve was prepared by using *p*-nitrophenol (*p*NP). One unit (U) of β-xylosidase activity was defined as the amount of enzyme that liberates 1 μmol *p*NP per minute in the reaction mixture under these assay conditions. Specific activity is defined as the number of international units per milligram of protein. For protein content quantification, BCA protein assay kit (Pierce) was used using bovine serum albumin as standard. For the determination of the optimum pH of His-Xyl43, 50 mM citrate-phosphate buffer (pH 3.5–8) at constant ionic strength (I = 0.3 M with NaCl) was used. Optimum temperature assay was performed at the interval 30–70 °C.

A continuous monitoring method was employed for determining initial-rate reactions for determination of kinetics parameters of the His-Xyl43 acting on *p*NPX substrate. Reactions were monitored at 400 nm for 5 min. For incubation, 10 μL of enzyme (0.97 mg protein/mL) were added to 270 μL of substrate solution (0.5–20 mM of *p*NPX) in citrate/phosphate buffer 50 mM, pH 6 (adjusted with NaCl to I = 0.3 M). Temperature incubation was 40 °C. A standard curve was prepared by using *p*-nitrophenol (*p*NP) at pH 6. Initial rates of catalysis of *p*NPX were fitted to Eq. (1), where v is the initial rate at a specified concentration of *p*NPX, K_{cat} is the rate of catalysis when enzyme is saturated with substrate, S is the substrate concentrations, and K_m is the Michaelis constant. The parameter, K_{cat} was expressed in moles of substrate hydrolyzed per second per mole of enzyme sites (monomers).

$$v = \frac{K_{cat} \times S}{K_m + S} \quad (1)$$

The kinetic parameters K_{cat} and K_m were calculated by nonlinear regression fitting of the the Michaelis–Menten equation using the program OriginPro 7.5 (OriginLab Corporation, Northampton, USA).

Kinetic parameters with substrate 1,4-β-D-Xylobiose (X2) were determined in reaction mixtures 0.2 mL containing 50 mM citrate/phosphate (I = 0.3 M, adjusted with NaCl) at pH and 40 °C, 10 μL enzyme (1 mg/mL), from duplicated 30 min endpoint assays ×2 concentrations varied from 2.5 to 20 mM. Xylose concentrations were determined using an enzyme coupled spectrophotometric assay (Megazyme Xylose-kit). Data were fit to Eq. (2) (Hill equation) where n is the Hill coefficient.

$$v = \frac{K_{cat} \times S^n}{K_m^n + S^n} \quad (2)$$

Table 2
Soil characteristic and bacterial total cellulolytic activity.

Soil sample	pH	Organic matter content (%)	MPN[d]
NF[a]	6.67	4.24	$>1.1 \times 10^4$
PT[b]	4.84	2.43	2.9×10^1
EG[c]	4.61	4.59	0.4×10^1

[a] Native forest.
[b] *Pinus taeda* soil sample.
[c] *Eucalyptus grandis* soil sample.
[d] Most probable number assay.

2.6. Sequencing

All sequencing reactions were performed using an ABI 3130xl Capillary DNA sequencer (Applied Biosystems, USA) at the Genomics and Sequencing Department of Biotechnology Institute, INTA Castelar.

2.7. Nucleotide sequence accession number

The nucleotide sequence of the *xyl43* gene described here has been submitted to GenBank database and assigned accession no. JX569191.

3. Results

3.1. Enrichment and identification of cellulolytic bacteria from forest soils

Soil samples from decaying native (NF) and cultivated (*P. taeda*-PT and *E. grandis*-EG) woods were collected and presence of cellulolytic bacteria was assessed by filter paper degradation and quantified by Most Probable Number (MPN) assay. Organic matter content and pH was determined for all soil samples. As expected, soils from *Pine* and *Eucalyptus* cultivated forests were more acidic than soil from native forest. MPN assay revealed that the native forest soil had a higher content of cellulolytic microorganisms (Table 2).

Isolation of bacteria was attempted from the filter paper degradation positive tubes of MPN assay, by successive streak out on minimal media (MM) agar plates supplemented with CMC 0.5% as sole carbon source (MM-CMC), at 28° under aerobic conditions (no growth was observed on MM, without CMC). CMCase activity of isolates was identified by degradation halo, revealed by Congo red staining (Fig. 1). All isolates were also able to grow on minimal media with Avicel 0.5% (MM-Avi) as sole carbon source. Also, xylanase activity of isolates was assayed by Congo red staining and degradation halo visualization on minimal media supplemented with xylan from beechwood 0.5% as sole carbon source (MM-Xy) (Fig. 1). When using xylan as substrate, degrading halos were less clear than on CMC. For both substrates tested, only those isolates showing a clear halo in three independent experiments were considered positive. These results indicated a high potential for hydrolytic activities of interest in native forest isolates.

3.2. Amplification of GH with a mixed degenerate/specific primer approach

In order to attempt amplification of cellulase coding genes from bacterial isolates total genomic DNA (as a bulk) and directly from soil samples (metagenomic DNA), a low stringency PCR approach was used. *A. cellulolyticus* 11B genome (GeneBank accession number NC_008578.1) was used to identify glycosyl hydrolase sequences of interest and for primer design (Barabote et al. 2009). Although it had not been identified as part of the bacterial consortia isolated (data not shown), it was chosen based

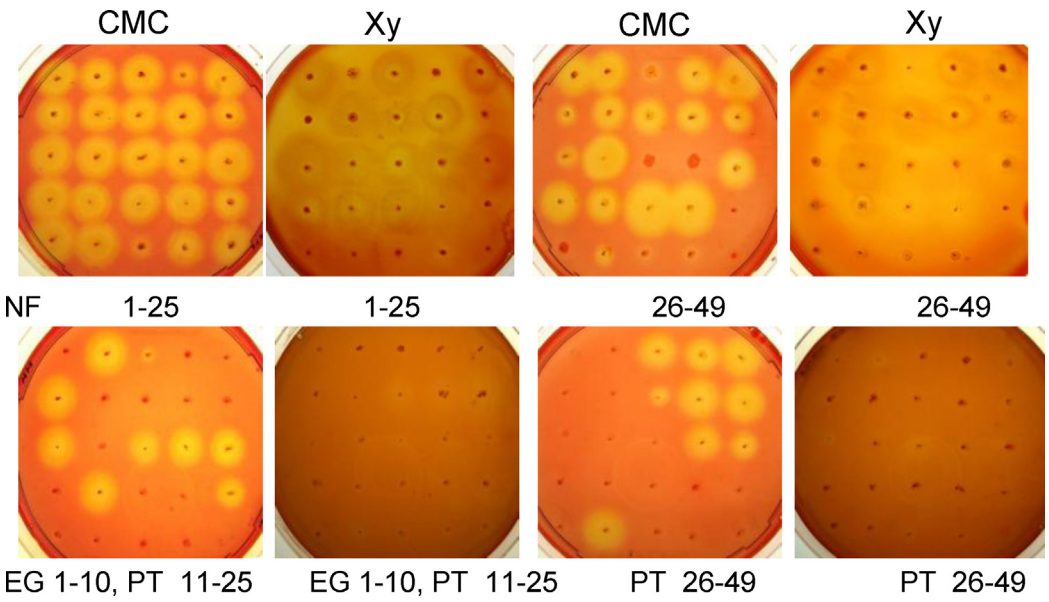

Fig. 1. Bacterial isolates on MM-CMC agar (CMC) or MM-Xylan agar (Xy), stained with Congo red. DH5α was used as negative control in both plates (position number 50). NF, EG and PT: isolates from Native Forest soil, *Eucalyptus grandis* and *Pinus taeda* plantations, respectively.

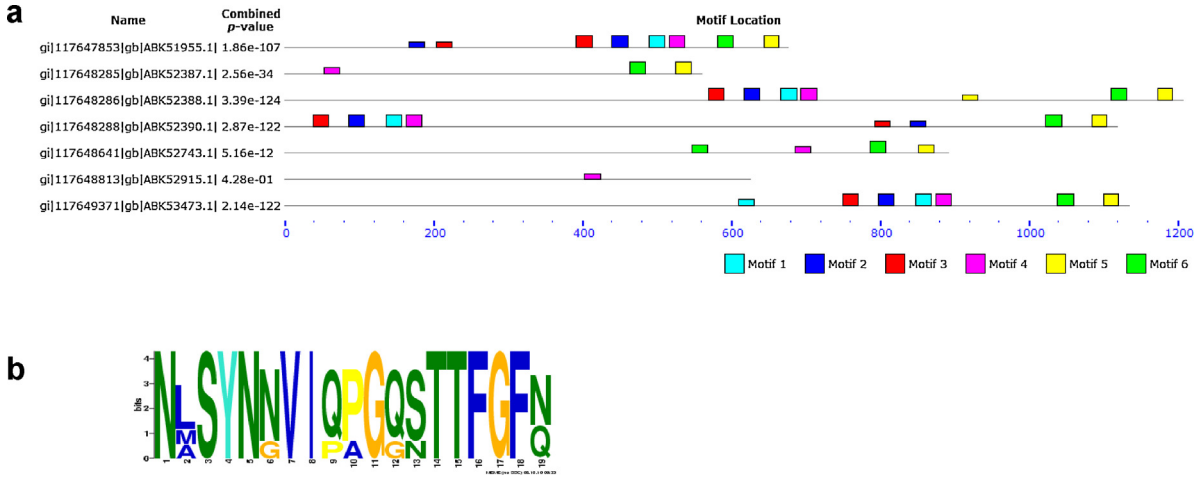

Fig. 2. Identification of conserved motives by MEME application. (a) Aligned sequences and relative motif location. (b) Sequence logo of Motif 5.

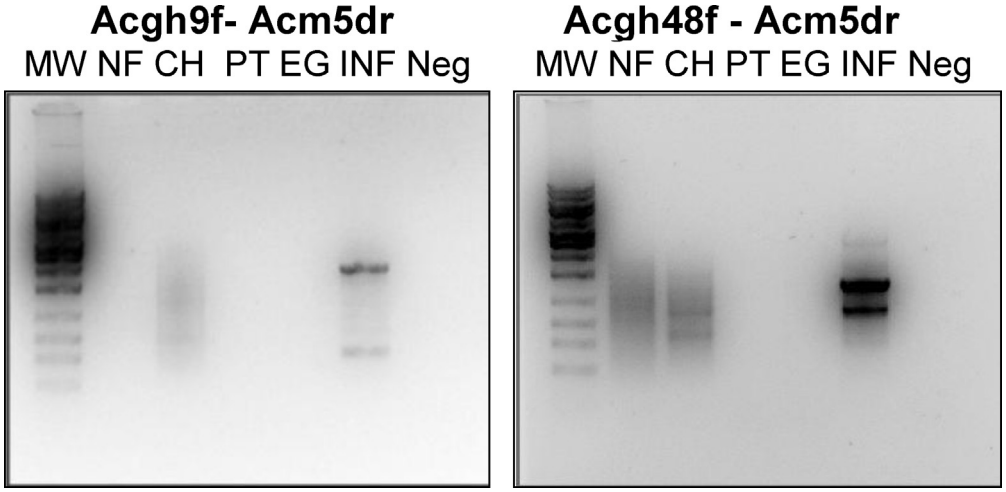

Fig. 3. Amplification of GH. Primers that produced discrete amplification products were Acgh9f and Acgh48f with degenerate reverse primer Acm5dr. MW: molecular weight marker (Axygen 1 kB), NF, CH, PT, EG: soil total metagenomic DNA from Misiones native forest, Chaco native forest, *Pinus taeda*, *Eucalyptus grandis*, respectively. INF: total bulk DNA from Misiones native forest isolates. Neg: no DNA.

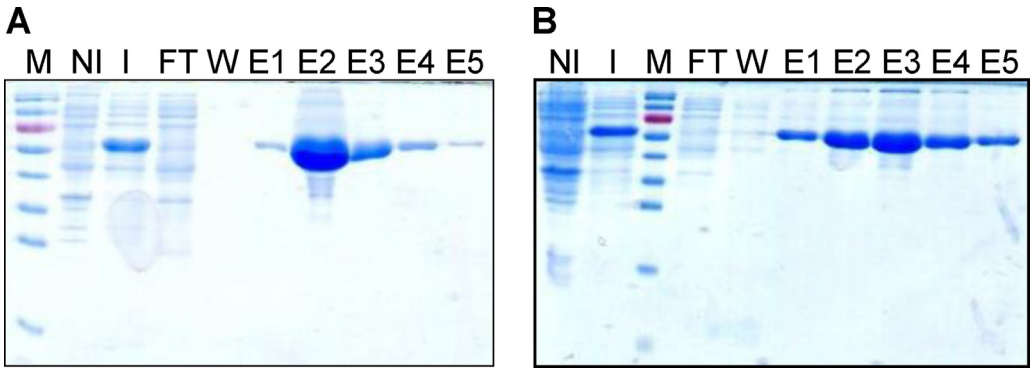

Fig. 4. Purification of Xyl43. Coomasie stained SDS-PAGE. (A) N-terminal fusion (6xHis-Xyl43). (B) C-terminal fusion (Xyl43-6xHis). M: Pre-stained broad range molecular weight marker (Pierce); NI: not induced; I: induced-soluble fraction; FT: flow through; W: wash with 10 mM imidazole; E1 a E5: elution fractions with 250 mM imidazole.

on the fact that it is a model cellulolytic bacteria from soil, its genome is well annotated and it had been previously identified by our group in a soil sample from native forest using a metagenomic approach (Talia et al. 2012). From eleven identified GH protein sequences annotated as belonging to glycosyl hydrolases families GH3, GH10, GH18 (2), GH5, GH6, GH48, Gh9, GH15-related, GH9, GH3 (accession numbers gi|117647801, gi|117647853, gi|117648096, gi|117648274, gi|117648285, gi|117648286, gi|117648288, gi|117648641, gi|117648813, gi|117649371, gi|117649720, gi|117649165, respectively), seven sequences (underlined in previous enumeration) were successfully aligned and conserved motives of twenty aminoacids were identified using MEME application (Bailey et al. 2009). A conserved motif (motif 5) from the carboxi-terminal end of the protein was chosen and from this motif, a sequence coding for six highly conserved aminoacids (S/NTTFGF) was selected and a reverse primer was tailor-designed taking into account the corresponding nucleotide sequence (Fig. 2). As no conserved aminoacid motives were found in the amino terminal end of the proteins, individual primers for each target sequence were designed, based on the corresponding nucleotide sequence, as specified in materials and methods. Metagenomic DNA from all soil samples and total DNA from pooled isolates from NF, PT and EG were used as templates. Also, metagenomic DNA from another type of native forest (from Chaco region, Argentina) (Talia et al. 2012) was included in the assay. Using low stringency PCR conditions, discrete amplification products were obtained using forward primers Acgh48f or Acgh9f with reverse primer Acgh5dr; but only from total genomic DNA from bacterial isolates from NF (Fig. 3). No discrete products were obtained with the other forward primers or from any of the metagenomic DNA samples used, even though DNA was of high molecular weight. As control, 16S RNA coding gene was amplified successfully from all templates, using fd1 and rd1primers, which showed that there were no PCR inhibitors in the samples (data not shown).

Discrete bands corresponding to amplification products were purified from agarose gel, cloned in pGEMTeasy vector and sequenced. One of the amplified fragments (using primers Acgh9f–Acgh5dr) was selected for further work as it contained a complete ORF, which we named *xyl43*, with 98% identity at nucleotide level with a gene annotated as β-xylosidase (*xyl*1) in *E. cloacae* subsp. cloacae NCTC9394 genome (accession number FP929040.1) (Ren et al. 2010). The other two amplification products obtained with primers Acgh48f–Acm5dr, did not have homology with glycosyl hydrolases.

The predicted aminoacid sequence of Xyl43 (536 aminoacids) was subjected to BLAST analysis (blastp, nr database, http://blast.ncbi.nlm.nih.gov) and the protein showed the highest similarities (identity percentage and GeneBank accession numbers between parentheses) with a GH43 beta-xylosidase from *E. cloacae* subsp. cloacae (99%, gi|295098416), with a phage xylosidase/arabinosidase from *Enterobacter* sp. SST3(98%, gi|401676827) and a beta-xylosidase from *Enterobacter hormaechei* ATCC 49162 (97%, gb|EGK61536.1). Also, *xyl43* gene showed high similarity to an orthologous gene described in *Escherichia coli* K12 (encoded in a defective prophage), which is absent from *E. coli* DH5alfa and BL21 strains (Blattner et al. 1997). The approximate 100 nucleotides upstream and downstream of the *xyl43* ORF contained in the amplification product, showed partial homology with a sugar (glycoside-pentoside-hexuronide) transporter (upstream) and a transcriptional regulator of IclR family (downstream). This data is in agreement with the genomic organization of this region in *E. cloacae*, which suggests that the sugar transporter and the β-xylosidase form a transcriptional unit.

These results strongly indicated that *xyl43* coded for a β-1,4-D-xylosidase (EC 3.2.1.37) from *E. cloacae*, belonging to GH43 CAZy family (http://afmb.cnrs-mrs.fr/CAZY). The predicted aminoacid sequence of Xyl43 revealed a theoretical molecular weight of 60.84 with an isoelectric point of 5.53 and parameters of high stability (http://web.expasy.org/cgi-bin/protparam/protparam). The sequence does not present a signal peptide (http://www.cbs.dtu.dk/services/SignalP-3.0/), so it is expected to be intracellular. The aminoterminal portion of the protein presents a conserved domain of 300 aminoacids, common to β-xylosidases, that involves the active site. There is also another conserved domain of unknown function (classified as DUF1349 superfamily) in the carboxiterminal end of the protein involving the last 150 aminoacids.

3.3. Cloning and heterologous expression of Xyl43

In order to evaluate if this gene encoded a functional enzyme, we designed primers to express Xyl43 as a His-tagged fusion, either to the amino or to the carboxi-terminal end. Plasmid DNA from pGEMTe-amplicon Acgh9f–Acm5dr, was used as template. The amplification product of the expected size (1600 bp) was purified, cloned in pGEMTeasy vector, then digested with BamHI/XhoI (N-terminal fusion construct) or XbaI/XhoI (C-terminal fusion construct) and cloned in pET28a vector previously digested with the corresponding enzymes. The nucleotide sequence was verified and expression of both constructs was achieved. The predicted molecular weight (MW) and isoelectric point (IP) of the recombinant proteins His-Xyl43 (N-terminal fusion) and Xyl43-His (C-terminal fusion) were MW = 64.4 kDa/IP = 5.85 and MW = 61.66/IP = 5.7, respectively. Purification under native conditions was achieved for both proteins (Fig. 4). Elution fractions were then tested for β-xylosidase activity.

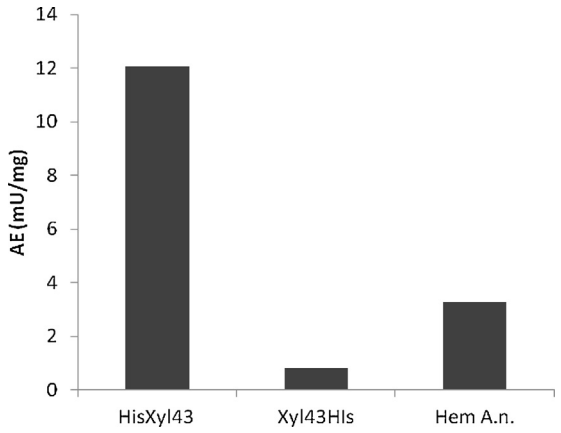

Fig. 5. Beta-xylosidase activity of recombinant Xyl43 at pH = 6. His-Xyl43, aminoterminal fusión, Xyl43-His, carboxiterminal fusion. Hem A.n: commercial *Aspergillus niger* hemicellulase extract (Sigma). Each block is the media of two independent determinations. These results are one of three equivalent assays.

3.4. Enzymatic activity of Xyl43

The enzymatic activity of the recombinant proteins obtained was assayed using β-D-xilopyranoside as substrate (*p*NPX) and measuring *p*-nitrophenol (*p*NP) release by $Abs_{400\,nm}$ in what is the standard activity assay for β-xylosidases (Ghose and Bisaria 1987; Poutanen et al. 1987). In order to compare both constructs, enzymes were diluted to 5 mg/mL each. As a positive control, a commercial hemicellulases extract of *A. niger* (Sigma) was used. Only the aminoterminal fusion His-Xyl43 presented activity, suggesting that carboxiterminal fusion impaired activity or correct folding of the protein. Specific activity of recombinant His-Xyl43 was approximately 12 mU/mg at pH 6 (Fig. 5). In order to test the effect of pH and temperature on β-xylosidase activity of His-xyl43, different pH and temperatures were assayed. His-Xyl43 β-xylosidase activity peaked between 5.5 and 7, with the highest activity measured at pH 6 in citrate/phosphate buffer at 50 °C (Fig. 6a), while the optimum temperature for this enzyme was at 40 °C (Fig. 6b). Kinetic studies of β-xylosidase activity with *p*NPX as substrate showed Michaelis–Menten kinetic (Fig. 6c). The K_m values were 2.92 mM and the K_{cat} 1.32 seg^{-1}. However, when acting on xylobiose substrate (X2) at pH 6.0, the saturation curve displays positive cooperativity of X2 binding (Fig. 6d). A Hill coefficient of 2.8 was determined. The K_{cat}^{X2} values were 380 s^{-1} and K_m^{X2} 17.8 mM.

4. Discussion

Large efforts are under way worldwide to develop renewable energy sources alternative to fossil fuels. One attractive alternative is by generating bioethanol from lignocellulosic biomass. In order to achieve so, it is necessary to efficiently hydrolyze plant cell walls to fermentable sugars. This hydrolysis requires multiple enzymatic activities, involving mainly cellulases and hemicellulases. At present, commercial enzymatic extracts mostly from *Trichoderma* and *Aspergillus* are being used to evaluate different pretreatment methods and biomass candidates. However, in order to achieve a sustainable process at a reasonable cost, more efficient and diverse enzymatic activities are required. Therefore, the discovery of new enzymes is a key aspect of these efforts and prospection of biomass degrading bacterial communities is an approach to identify potential candidates.

In this study, microbiological cultures of soil samples from native and cultivated forest soils from a subtropical region of the Atlantic rainforest (Misiones, Argentina) were enriched in cellulose-degrading bacteria. Our results indicated that the native forest soil sample (NF) was the richest source of cellulolytic bacteria, both in terms of absolute activity (indicated by MPN assay) as well as in bacterial diversity (data not shown).

In an attempt to amplify and clone genes encoding glicosyl hydrolases, a strategy of degenerate primers and low stringency PCR was used, with total DNA from the enrichment-isolates, as well as total metagenomic DNA from soil samples as templates. As a result an amplification product was obtained from bacterial enrichment isolates from NF. This fragment contained a complete ORF with 98% identity with a GH43 beta-xylosidase coding gene from *E. cloacae*, which we named *xyl43*. When Xyl43 was expressed as His-tagged recombinant protein in *E. coli*, only the aminoterminal fusion presented beta-xylosidase activity, indicating that the carboxiterminal fusion may interfere either with appropriate folding or with active site. His-Xyl 43 showed β-xylosidase activity not only on a standard substrate, such as *p*NPX, but also on xylobiose (X2), demonstrating that this is a functional beta-xylosidase, as determined by the Enzyme Commission (Cantarel et al. 2009).

Bacteria that digest plant cell walls encode multiple cellulases, together with hemicellulolytic and pectinolytic enzymes. In a recent thorough in silico genome analysis of 1495 bacteria listed in CAZy database, bacteria were classified based on the different combinations of cellulolytic, hemicellulolytic and pectinolytic enzymes that they encode. Nevertheless, only a small amount of enzymes from these bacteria have been actually characterized (Mba Medie et al. 2012). Noteworthy, *E. cloacae* is classified as a cellulose synthesizing bacteria, as it encodes a putative bacterial cellulose synthesis (*bcs*) operon. Analysis of genomic sequences of subspecies of *E. cloacae* revealed numerous GH and GT coding genes (Cazy database, www.cazy.org). However, these correspond mostly to predicted enzymes as activity has not been proved. In this work, we have amplified a gene with high identity to an *E. cloacae* GH43 β-xylosidase from a soil bacteria consortium with very high cellulolytic activity and have demonstrated the activity of the recombinant protein. These results indicate that although *Enterobacter* genus has not been previously regarded as of cellulolytic importance, it should be further explored as source of cellulolytic enzymes. By analysis of *E. cloacae* genome, and confirmed by the sequence of the full amplification product cloned, *xyl43* appears to be part of an operon with a sugar transporter. As Xyl43 does not have a signal peptide, this transporter could be implicated in the internalization of small sugars for their full degradation. Characterization of this system could shed light on *E. cloacae* xylanolytic activity and its participation in soil communities. In line with our results, an extracellular xylanase was isolated from an environmental thermoalkalophilic *Enterobacter* sp. (Khandeparkar and Bhosle 2006). Therefore, classification of *Enterobacter* genus regarding activity in cellulose/hemicelluloses deconstruction may need to be revised.

Beta-xylosidases are hemicellulases that hydrolyze beta-1,4 bonds between two units of xylose in xylobiose and short xylooligoscharides; activity that is essential for complete breakdown of xylans as they are necessary to avoid product inhibition of xylanases and therefore for final degradation of vegetal cell walls (Poutanen et al. 1987; Saha 2003). In second generation biofuels research, adding beta-xylosidases improved the enzymatic hydrolysis of cellulose and hemicelluloses in solids after pre-treatment of corn stover, probably by facilitating access to cellulose (Qing and Wyman 2011). These enzymes are part of microbial xylanolytic systems (Badal and Bothast 1999) and can be grouped into families 39, 43 and 52 of the general glycosyl hydrolase classification (Shallom and Shoham 2003). However, only a very small amount of β-xyosidases has been expressed as recombinant proteins and even less are commercially available (Fekete and Kiss 2012; Jordan et al. 2007, 2013; Moraïs et al. 2012; Smaali et al. 2009; Zhou et al. 2012). Based on their mechanism of action, GH43 β-xylosidases

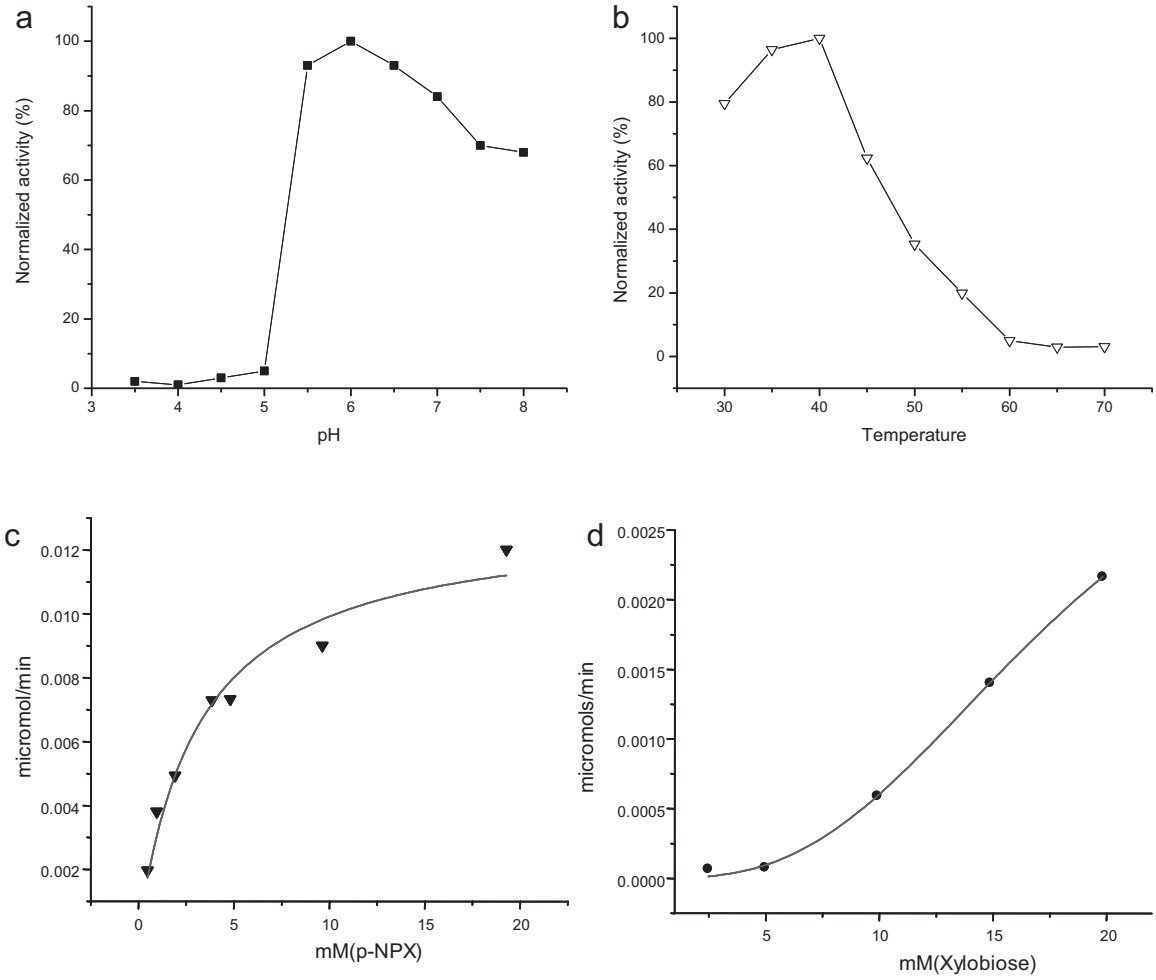

Fig. 6. His-Xyl43 β-xylosidase activity. (a) Effect of pH at 50 °C. (b) Effect of temperature at pH 6. (c) Nonlinear regression fitting to the Michaelis–Menten equation of hydrolysis rate versus *p*-NPX concentration. (d) Nonlinear regression fitting to the Hill equation of hydrolysis rate versus xylobiose concentration.

are a very attractive source of high-performing β-xylosidases for deconstruction of plant biomass (Jordan et al. 2013).

Among the examples reported in the literature, a β-xylosidase from *Thermobifida fusca* (TfBXyl43) was expressed and fully biochemically characterized, demonstrating that it could hydrolyze artificial and natural substrates (xylobiose and xylotriose) from the non-reducing end as well as present transxylosidase activity (Fekete and Kiss 2012). Also studies on a β-xylosidase from another strain of *T. fusca* demonstrated that both modules (Gh43 and a C-terminal module A) were necessary for enzyme activity (Moraïs et al. 2012). In this regard, sequence analysis of *E. cloacae* Xyl43 characterized in this work, also revealed two distinct modules (a GH43 and an uncharacterized module in the C-terminal portion of the protein). Whether both modules are necessary for activity will need to be investigated and could help to establish a function for the uncharacterized module.

In spite of available biochemical information on purified β-xylosidases, comparison of overall activity remains elusive, basically due to differences on assay methods and availability of natural substrates (most of which are not commercial). Overall, results on artificial substrate *p*NPX are considered a common starting point for enzyme characterization although demonstration of hydrolysis of xylobiose and/or xylooligosacharides is required to confirm beta-xylosidase activity (Cantarel et al. 2009). Specific activity of β-xylosidases of purified proteins from fungal extracts has been reported to be highly variable, ranging from very low activities (about 2 U/mg) to 50 U/mg in *Fusarium* sp. (Saha 2001, 2003) and above 500 U/mg in *Aureobasidium* sp. (Hayashi et al. 2001), on *p*NPX. For inducing expression of these fungal enzymes, adding the substrate was required. Regarding bacterial enzymes, specific activity of recombinant β-xylosidase from *Caulobacter* has been reported to be of 1.5 U/mg (Graciano et al. 2012). A beta-xylosidase from *Selenomonas ruminatium* demonstrated high activity on xylooligosaccharides and is a promising candidate for biotechnological engineering for improved kinetic parameters (Jordan et al. 2011). In a thorough comparison of best performing GH43 beta-xylosidases (three of which are commercially available), K_{cat} ranged from 17 to 82 s^{-1} on *p*NPX and from 38 to 407 s^{-1} on X2. K_m ranged from 0.5 to 6.9 mM on *p*NPX and 1.44–5.21 mM on X2 (Jordan et al. 2013). We have shown that recombinant *E. cloacae* Xyl43 has activity on *p*NPX and X2. Under the conditions assayed, K_m and K_{cat} were 2.92 mM and 1.32 s^{-1} on *p*NPX and 17.8 mM, 380 s^{-1} on X2, respectively. These results showed that this enzyme is a real β-xylosidase and that its activity is in range with other GH43 bacterial enzymes. It is the first evidence of β-xylosidase activity from an *Enterobacter* sp. gene. Evaluation on xylooligosacharides DP 3–6 and pre-treated biomass as well as inhibition assays will determine its potential for industrial applications. Moreover, directed evolution experiments may lead an enzyme with improved kinetic parameters.

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