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ARTICLE

# Recombinant Expression of an Alkali Stable GH10 Xylanase from *Paenibacillus barcinonensis*

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Xylanase A from *Paenibacillus barcinonensis*, a new species isolated from a rice field, has been cloned and expressed in *Escherichia coli*. Purified recombinant xylanase showed high activity on xylans from hardwoods and cereals, and exhibited $K_m$ and $V_{max}$ of 2.93 mg/mL and 50.67 U/mg on birchwood xylan. Xylanase A was highly active at 60 °C in alkaline pH values up to 9.5 and remained stable for at least 3 h in alkaline conditions. The amino acid sequence deduced from *xynA* revealed that it is a single domain xylanase belonging to the GH10 family. Thin layer chromatography analysis showed that the enzyme released a mixture of hydrolysis products including substituted xylooligomers from cereal arabinoxylans, while xylose, xylobiose, and aldotetraouronic acid were the main products released from glucuronoxylan from birchwood. The enzyme released a complex mixture of xylooligomers for acetylated xylan from eucalyptus, revealing its potential to depolymerize this widely used resource in the pulp and paper industry.

**KEYWORDS: Biotechnology; xylanase; alkali stable; enzymatic hydrolysis; bioconversion; *Paenibacillus***

## INTRODUCTION

Xylan is a main structural polysaccharide in plant cell walls that constitutes approximately one-third of the renewable organic carbon on earth (*1*). The catabolic breakdown of xylan thus represents a critical step in the recycling of carbon in nature and has been targeted as a subject of intense research as renewable energy resources (*2*). Xylans contain a xylose $\beta(1 \rightarrow 4)$ linked backbone, which depending on the plant source, can be variably substituted by side chains of arabinosyl, glucuronosyl, methylglucuronosyl, acetyl, feruloyl, and *p*-coumaroyl residues. Biodegradation of xylan is a complex process that requires the coordinate action of several enzymes, among which xylanases (1,4-$\beta$-D-xylan xylanohydrolase; EC 3.2.1.8), cleaving internal linkages on the $\beta$-1,4-xylose backbone, play a key role. On the basis of amino acid sequence similarity and hydrophobic cluster analysis, xylanases have been classified into two main families of glycosyl hydrolases, GH10 and GH11 (*3*, *4*), although several recently characterized xylanases, that could complement the activity of GH10 and GH11 enzymes, have been described in glycosyl hydrolases families 5, 7, 8, and 43 (*1*). The complex chemical nature and heterogeneity of xylan can account for the multiplicity of xylanases produced by microorganisms (*5*).

Xylanases have biotechnological application in many industrial processes, although xylan bioconversion depends on the efficiency of the xylanase selected (*6*). Pulp bleaching is one of the most highlighted industrial application of xylanases, where they enhance the effectiveness of bleaching chemicals, thereby reducing the consumption of chlorine containing chemicals and consequently reducing the generation of toxic wastes (adsorbable organic halogens, AOX) and the environmental impact of pulp bleaching (*7*, *8*). Xylanases are also successfully used as additives to improve the digestibility of feed (*6*), in the bakery industry to improve the quality of bread (*9*), while their use to obtain xylooligosaccharides to be used as prebiotics is one of the most recent and promising applications of these enzymes (*10*, *11*).

*Paenibacillus barcinonensis* is a recently identified new species, isolated from rice fields in the Ebro river delta in Spain, and selected for its high xylan degrading activity (*12*, *13*). It shows a multiple enzyme system for xylan degradation, which allows it to utilize xylan as the only carbon source. XynA is the main xylanase of *P. barcinonensis*, as it is the most abundant enzyme among the proteins secreted by the strain when grown on xylans. XynA was previously tested on eucalyptus kraft pulp bleaching, showing high effectiveness as a bleaching aid that allowed important reductions in the chlorine dioxide dose needed to reach target brightness (*14*). We describe in this article the cloning of the *xynA* gene from *P. barcinonensis* and its heterologous expression in *Escherichia coli*. The recombinant enzyme has been purified and biochemically characterized. This makes possible the production of the enzyme in recombinant hosts, devoid of background cellulase activity, for its biotechnological applications.

## MATERIALS AND METHODS

**Bacterial Strains and Plasmids.** *Paenibacillus barcinonensis* was grown as described previously (*12*). *Escherichia coli* BLR(DE3) and pET3b were used as host strain and plasmid vector, respectively, to express *xynA*. Cultures from *E. coli* BLR(DE3)/pET3XynA were grown on tetracycline and ampicillin supplemented LB broth (50 μg/mL each) at 37 °C for 16 h. Cells were disrupted by sonication for small culture volumes (up to 10 mL) or by French press for higher volumes (1 or 2 L).

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**Table 1.** Oligonucleotide Primers Used[a]

| | |
|---|---|
| deg1XynA[b] | AAYGGNAAYATHATHGCNGGNCARGTNCC |
| deg2XynA[b] | TAYTGGAAYCARGTNACNCCNGARAAY |
| XynA-A1[b] | TGGTCGATGGCATCGGTATTCAA |
| XynA-A2[b] | AATCCCAGCCTGTCTGCCCGTCT |
| XynA-upstream[b] | TGCGAGCGTACCTACCGTC |
| XynA-downstream[b] | AGCGTCCTGCGATGCAATG |
| XynAFwNdeI[c] | GGAGGAATCATATGTTGAAGTC |
| XynARvBamHI[c] | CGACTCGGATCCAGATCAAG |

[a] Restriction sites introduced in oligonucleotide sequences are underlined. [b] Oligonucleotides used in sequencing. [c] Oligonucleotides used in the cloning of *xynA* in pET3b.

**Nucleic Acid Manipulations.** Genomic DNA of *P. barcinonensis* was isolated with Genomic DNA Extraction Kit (Biotools). It was digested by *Eco*RV, *Pvu*II, *Dra*I, or *Stu*I restriction enzymes, and the fragments obtained were linked to commercial adaptors of Universal Genome Walker Kit (BD Biosciences). The DNA samples resulting from ligation were used as templates for PCR amplification with pairs of primers from the commercial kit and deduced from the N-terminal sequence of xylanase A, previously determined (*14*) (**Table 1**). One of the fragments amplified showed a DNA sequence encoding for the known N-terminal sequence of the enzyme. This DNA sequence allowed the design of new primers to obtain the whole *xynA* sequence by gene walking. The following primers XynAFwNdeI (GGAGGAATCATATGTTGAAGTC) and XynARvBamHI (CGACTCGGATCCAGATCAAG), including restriction sites for *Nde*I and *Bam*HI, respectively, were used to amplify the whole *xynA* and to clone it in the polylinker of expression vector pET3b under the control of the T7 promoter. In this construction, no extra amino acids were added to XynA, which maintained its original signal peptide. The primers used in genome walking and in cloning were purchased from Sigma-Aldrich and are listed in **Table 1**.

Plasmid DNA was purified with commercial Ilustra Plasmidprep Mini Spin Kit (GE Healthcare). PCR amplifications were performed with Pfu DNA polymerase (Stratagene) or Taq DNA polymerase (New England Biolabs). Restriction enzymes and ligases were purchased from Fermentas, New England Biolabs, and Roche Diagnostics. All kits were used according the instructions of the suppliers. *E. coli* was transformed as described (*15*). The DNA sequence was obtained by automated fluorescence sequencing with an ABI PRISM dye terminator cycle sequencing ready reaction mix (Perkin-Elmer) in a 377 Perkin-Elmer DNA sequencer. Sequence similarity was analyzed through BLAST (*16*).

**Enzyme Assays.** Xylanase activity was assayed essentially as described (*17*). The polysaccharides tested as substrates were birchwood, oat spelt and beechwood xylan, methylglucuronoxylan, CMC, starch, pectin, lichenan, polygalacturonic acid (Sigma Chemical), rye or wheat arabinoxylan (Megazyme), and Avicel (Fluka). The assay mixtures contained 1.5% of polysaccharide in a final volume of 0.1 mL of 50 mM phosphate buffer at pH 6.5. The mixture was incubated at 60° for 15 min. Color development was measured at 520 nm. To study the activity on aryl-glycosides, the enzyme was incubated with substrate at 50 °C in a final volume of 0.5 mL of 100 mM phosphate buffer at pH 6.5 until the solution became yellow. Substrate concentration was 0.5% for *pNP*-$\beta$-xyloside, *oNP*-$\beta$-xyloside, *pNP*-$\beta$-glucopyranoside, *pNP*-$\alpha$-arabinofuranoside, or *pNP*-$\alpha$-arabinopyranoside (Sigma Chemical). The reaction was stopped by the addition of 1 mL of 1 M $Na_2CO_3$, and color development was measured at 400 nm. One unit of enzymatic activity was defined as the amount of enzyme that releases 1 $\mu$mol of reducing sugar equivalent, *p*-nitrophenol, or *o*-nitrophenol per min under the assay conditions described.

To study the effect of temperature and pH on enzyme activity, we used the same assay conditions described above, but incubation was performed at different temperatures or pH. The following buffers were used: 150 mM sodium citrate, pH 3.0−4.0; 150 mM sodium acetate, pH 4.0−6.0; 150 mM sodium phosphate, pH 6.0−7.0; 150 mM Tris-HC1, pH 7.0−9.0; and 150 mM sodium glycine, pH 9.0−11.0. For the study of thermostability, enzyme samples were preincubated for different intervals at several pH values and temperatures, and residual xylanase activity on birchwood xylan was determined under the standard assay conditions.

Kinetic parameters ($V_{max}$ and $K_m$) were determined under optimal assay conditions using 0−30 mg mL$^{-1}$ of birchwood xylan as substrate. $K_m$ and $V_{max}$ were determined by fitting hyperbolic Michaelis−Menten curves with the program SigmaPlot, version 4.00 (Jandel Scientific).

**Purification of XynA.** XynA was purified from *E. coli* BLR(DE3)/pET3XynA cultures. The recombinant strain was grown on 2 L of LB broth supplemented with ampicillin and tetracycline (50 $\mu$g/mL each) at 37 °C for 16 h. To avoid the formation of inclusion bodies because of enzyme overexpression, cultures were not induced by IPTG. In these conditions, 21 xylanase units/mL of culture were obtained. Cells were collected following disruption by a French press. Cell extracts were treated with streptomycin sulfate to eliminate nucleic acids by ultracentrifugation, and proteins were concentrated by precipitation with 20% ammonium sulfate. Concentrated extracts were subjected to cation exchange chromatography (Tricorn MonoS 5/50 GL, GE-Healthcare) on a fast protein liquid chromatography system (AKTA FPLC, GE-Healthcare). Bound proteins were eluted with a 0−1 M NaCl gradient.

**Gel Electrophoresis and Zymograms.** Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed in 12% gels, essentially as described (*18*). For the detection of xylanase activity, 0.2% birchwood xylan was included in gels before polymerization, and zymograms were developed as described (*17*). Samples were heated for 10 min at 50 °C in sample buffer before being applied to gels. After electrophoresis, gels were washed for 30 min in 2.5% triton X-100, then were incubated for 30 min in 50 mM phosphate buffer at pH 6.5, and finally incubated at 55 °C for 10 min in the same prewarmed fresh buffer. Gels were then stained with 0.1% Congo Red for 15 min and washed with 1 M NaCl until xylanase bands became visible. Gels were then immersed in 10% (v/v) acetic acid and photographed. Isoelectric focusing (IEF) was performed as described (*19*).

**Thin Layer Chromatography.** Products from xylan and xylooligosaccharide hydrolysis were analyzed on silica gel plates (Merck, Darmstadt). Xylose and birchwood xylan were from Sigma Chemical, xylooligosaccharides and wheat xylan were from Megazyme, and *Eucalyptus* xylan was a gift of J. Carlos Parajó. The solvent used was chloroform−acetic acid−water (3:6:1). Hydrolysis products were detected by spraying the plates with an ethanol-concentrated sulfuric acid mixture (95:5) (*20*).

**Nucleotide Sequence Accession Number.** The DNA sequence of the *xynA* gene was submitted to the EMBL database under accession number FN430833.

## RESULTS

**Cloning of *xynA* Gene.** N-Terminal sequence of xylanase A was previously determined (*14*), allowing the design of specific primers to PCR amplify a small portion of its encoding gene using as a template a mixture of *Paenibacillus barcinonensis* genomic DNA fragments linked to commercial adaptors. By gene walking from this *xynA* portion, the sequence of a 3000 bp DNA fragment containing a 960 bp open reading frame encoding a xylanase of 320 amino acids was obtained. *xynA* was flanked upstream by a putative 2′,3′-cyclic-nucleotide 2′-phosphodiesterase precursor (71% identity with YP_002775154 from *Brevibacillus brevis*) and downstream by an open reading frame with similarity to a copper amine oxidase domain protein (38% identity with YP_003243369 from *Geobacillus* sp.). These flanking open reading frames do not seem to belong to the same transcriptional unit of *xynA*, as a putative promoter sequence showing a −35- (TTCAAA) and −10 (TATAAA) regions is found between the open reading frame located upstream and *xynA*, while the downstream open reading frame is transcribed in the opposite direction.

Six nucleotides upstream of the ATG initiation codon of *xynA*, a potential Shine−Dalgarno sequence (GGAGG), were found. The deduced amino acid sequence of XynA shows an N-terminal region of 30 amino acids with the features of a signal peptide. The predicted molecular weight and pI of the mature xylanase are 32.62 kDa and 8.65, respectively. Alignment of the deduced amino acid sequence of *xynA* to sequences contained in databases showed that the cloned enzyme is a single domain xylanase with

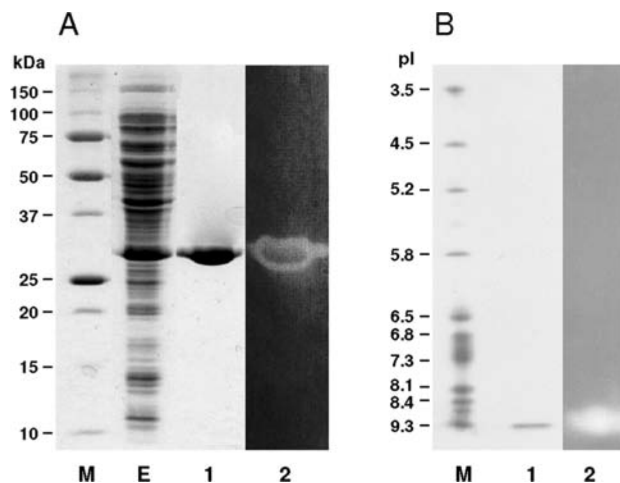


**Figure 1.** Electrophoretic analysis of xylanase A. (**A**) Analysis by SDS−PAGE. (**B**) Isoelectric focusing. Lanes: 1, protein staining of purified XynA; 2, zymogram of the purified enzyme; E, protein staining of cell extracts of *E. coli* BLR(DE3)/pET3XynA; M, molecular weight or pI standard proteins.

similarity to enzymes belonging to the GH10 family. The highest similarity was found to be to the catalytic domain of a xylanase from an uncultured bacterium (*21*) which shares 75% identity, while xylanase B from *Paenibacillus* sp. KCTC8848P (*22*) and Xyn10A from *Clostridium acetobutylicum* ATCC 824 (*23*) share identities of 73 and 65% with the cloned enzyme, respectively. Putative catalytic residues, Glu144 and Glu251, were found in XynA in the position corresponding to conserved regions of GH10 xylanases (*24*, *25*).

**Production of Recombinant XynA in *Escherichia coli*.** Two specific primers were designed to clone the whole *xynA* encoding sequence in plasmid vectors in *Escherichia coli*. Several expressing vectors and host strains were tested, but in many of the recombinant clones constructed, the yield of active xylanase was low, probably because of the formation of inclusion bodies by an excessive production of the enzyme. Among the recombinant strains constructed, *E. coli* BLR(DE3)/pET3XynA, which showed the highest enzyme activity, was chosen to produce active xylanase. Cultures of the strain were collected, cells were disrupted by a French press, and cell extracts obtained were used to purify the xylanase. The alkaline pI of XynA allowed the purification to homogeneity of the enzyme in a single step, by cationic exchange chromatography in buffers at pH 7.0, where XynA was the only protein detected in the bound fraction. It was eluted from the column with a NaCl gradient, and the purified xylanase was analyzed by SDS−PAGE, isoelectric focusing, and zymography, showing an apparent molecular weight of 31 kDa and a pI of 9.3 (**Figure 1**), in agreement with the molecular weight and pI deduced from the nucleotide sequence. The properties of recombinant xylanase A were similar to those reported for the enzyme produced in the *P. barcinonensis* original host (*14*), indicating a proper folding and signal peptide processing of the protein in the recombinant host.

**Characterization of Xylanase Activity.** The hydrolytic activity of the recombinant enzyme on various substrates was determined (**Table 2**). The enzyme was highly active on glucuronoxylans and arabinoxylans, showing the highest activity on birchwood xylan (47 U/mg). It showed reduced activity on aryl xylosides, while carboxymethyl cellulose (CMC), Avicel, starch, polygalacturonic acid, or lichenan were not hydrolyzed by the enzyme. The kinetic constants of the enzyme were determined on birchwood xylan as substrate, showing a $K_m$ and $V_{max}$ of 2.93 mg/mL and 50.67 U/mg, respectively.

**Table 2.** Substrate Specificity of XynA

| substrates | activity (U/mg) |
|---|---|
| birchwood xylan | 47.13 |
| oat spelt xylan | 31.70 |
| beechwood xylan | 41.23 |
| wheat arabinoxylan | 32.46 |
| rye arabinoxylan | 24.44 |
| 4-*O*-metylglucuronoxylan | 22.17 |
| polygalacturonic acid | ND[a] |
| starch | ND[a] |
| Avicel | ND[a] |
| carboxymethyl cellulose (CMC) | ND[a] |
| lichenan | ND[a] |
| pectin | 10.83 |
| pNPX | 3.30 |
| pNPG | ND[a] |
| oNPX | 7.50 |
| pNPAp | 3.20 |
| pNPAf | 0.10 |

[a] Not detected.

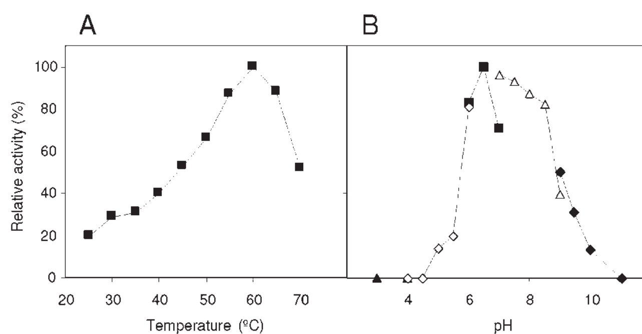


**Figure 2.** Effect of temperature and pH on Xylanase A activity. (**A**) Effect of temperature on XynA activity. Activity assays were performed under standard conditions at pH 6.5 and temperatures ranging from 25 to 70 °C. (**B**) Effect of pH on XynA activity. Activity assays were performed under standard conditions at 60 °C and pH ranging from 3.0 to 11.0 with citrate buffer (▲), acetate buffer (◇), phosphate buffer (■), Tris buffer (△), and glycine buffer (◆).

Analysis of the effect of temperature and pH on the enzymatic activity of XynA showed that optimal conditions for xylan hydrolysis were 60 °C and pH 6.5 (**Figure 2**). However, the xylanase showed a good level of activity at alkaline pH, exhibiting more than 60% of its maximum activity at pH 8.5 and showing high activity at pH 9.5 and 60 °C (**Figure 2**). Stability assays showed that the enzyme was highly stable at alkaline conditions, retaining 77% of initial activity after incubation at pH 8.5 and 50 °C for 3 h (**Figure 3**).

**Products of Xylan Degradation by XynA.** The hydrolysis products from xylooligosaccharides and xylans from birchwood, wheat, and *Eucalyptus* were analyzed by thin layer chromatography (TLC). XynA was not active on xylobiose, while longer oligosaccharides were hydrolyzed to xylobiose and xylose as main products (**Figure 4**). Birchwood glucuronoxylan was cleaved to xylobiose, aldotetraouronic acid ($MeGlAX_3$), and xylose as main hydrolysis products in long-term incubations (**Figure 5**). Main products from wheat arabinoxylan were xylose, xylobiose, and oligosaccharides that migrated among linear xylooligomers, indicating that they were variably substituted with arabinose side chains. A more complex hydrolysis pattern was obtained from acetylated *Eucalyptus* xylan, which was degraded to a mixture of products ranging from long size oligosaccharides up to products of higher mobility than xylose, including products of intermediate

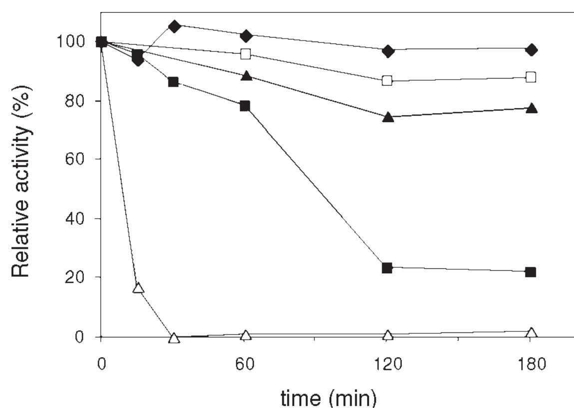


**Figure 3.** Stability of xylanase A. Thermal stability of XynA at 50 °C and pH 6.5 (♦), 7.5 (□), or 8.5 (▲). Thermal stability of XynA at pH 6.5 and 55 °C (■) or 60 °C (△).

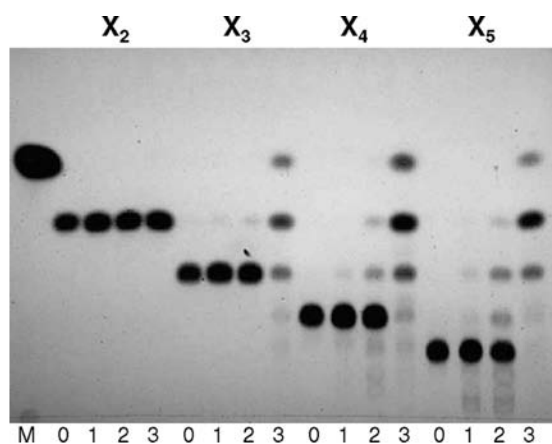


**Figure 4.** Hydrolysis products from xylooligosaccharides. XynA (0.15 $\mu$M) was incubated at 50 °C and pH 6.5 with 1.5% xylobiose ($X_2$), xylotriose ($X_3$), xylotetraose ($X_4$), or xylopentaose ($X_5$). Samples were taken at times 0 (lanes 0), 15 min (lanes 1), 60 min (lanes 2), or overnight (lanes 3) and analyzed by thin layer chromatography. Lane M contains a xylose size marker.

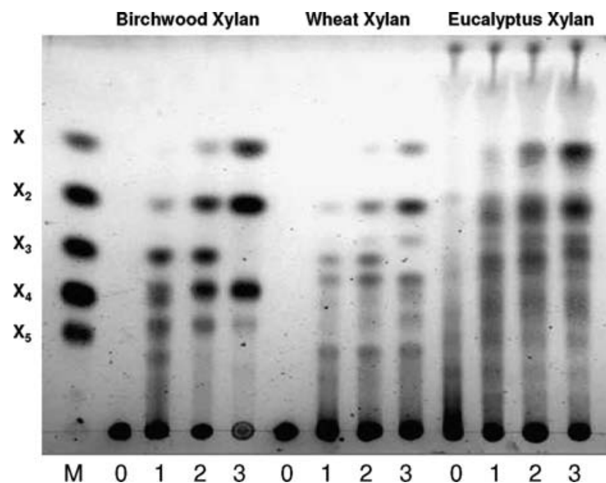


**Figure 5.** Hydrolysis products from xylans. XynA (1.5 $\mu$M) was incubated at 50 °C and pH 6.5 with 1.5% birchwood xylan, wheat xylan, or *Eucalyptus* xylan. Samples were taken at times 0 (lanes 0), 15 min (lanes 1), 60 min (lanes 2), or overnight (lanes 3) and analyzed by thin layer chromatography. Lane M contains size markers of xylose (X), xylobiose ($X_2$), xylotriose ($X_3$), xylotetraose ($X_4$), and xylopentaose ($X_5$).

mobility, in agreement with the high level of side chain substitution and acetylation of the *Eucalyptus* xylan tested.

## DISCUSSION

Xylanase A from *Paenibacillus barcinonensis* was previously evaluated on the bleaching of *Eucalyptus* kraft pulp, showing a remarkable bleach boosting effect, which gives the enzyme a high potential for application in the pulp and paper industry (*14*). However, the commercial exploitation of the xylanase requires, besides its high effectiveness, the improvement of enzyme yield by the producer microbial host to minimize production costs to competitive prices. Additionally, for a bleaching application the enzyme has to be devoid of contaminant cellulase activity. This makes *Paenibacillus barcinonensis* a poor candidate as xylanase producer for pulp bleaching, as it has an important cellulase activity background. As a strategy to produce the enzyme for this purpose, we isolated the XynA encoding gene and cloned it in *Escherichia coli* under the control of an expressing vector. This also facilitated the purification of the enzyme, which was biochemically characterized.

The recombinant enzyme produced in *E. coli* showed high activity and stability in alkaline conditions. Although the enzyme showed maximum activity at pH 6.5, it was highly active up to pH 9.5 and remained stable after 3 h of incubation at pH 8.5. The profile of activity and stability at alkaline pH of XynA is clearly different from that of homologous GH10 xylanases, as the xylanase from the uncultured bacterium described by Li et al. (*21*) and Xyn10A from *Clostridium acetobutylicum* (*26*) showed low activity above pH 8.0 or were not active at all in these conditions; while biochemical traits of the homologous xylanase from *Paenibacillus* sp. KCTC8848P (*22*) have not been reported. The pH profile of xylanase A, together with its optimum temperature of 60 °C, make it a good candidate for pulp bleaching, an application where enzymes active and stable at alkaline pH and high temperature, conditions of the industrial process, are required (*27−29*).

TLC analysis of mode of action of XynA on birchwood glucuronoxylan showed that the enzyme behaves as a typical GH10 xylanase, releasing a mixture of products including aldotetraouronic acid as the shortest glucuronic acid substituted oligomer (*30*). Analysis of hydrolysis pattern on wheat arabinoxylan indicated that XynA is able to cleave in proximity to arabinose side chains, similar to what has been reported for GH10 xylanases from *Cellvibrio mixtus* and *Aspergillus aculeatus*, that release arabinoxylobiose and arabinoxylotriose from cereal xylans (*31*, *32*). The production of arabinoxylooligosaccharides (AXOS) is an important focus of research because of their increasing use as ingredients in functional foods and prebiotics (*11*). On the other hand, the enzyme degraded acetylated xylan from *Eucalyptus* to a complex mixture of oligomers, that should contain both acetyl and glucuronic acid substitutions. At this regard, acetylated xylooligomers have been reported among the hydrolysis products from acetylated glucuronoxylan from beechwood (*33*). The high activity of XynA on *Eucalyptus* pulp, in agreement with its bleach boosting effect previously reported, is a clear indication of the potential of the enzyme for its application in the pulp and paper industry in template climate countries, where *Eucalyptus* is the main resource for pulp production.

The recombinant clone constructed produced a high level of xylanase activity devoid of the cellulase background produced by the original host. This facilitates the production of XynA to study the optimization of its application in the pulp industry and to test it in other biotechnological applications. However, the industrial production of the enzyme will require a further improvement of

xylanase yield and the expression of the enzyme in a secreting host to facilitate its purification or concentration. In this way, we have developed several cloning vectors for xylanase expression in cellulase deficient strains of *Bacillus subtilis* (*34*). Current experiments are in progress to construct recombinant strains that produce and secrete high levels of XynA, to test its biotechnological application in industrial processes where alkali active and stable enzymes are required.

## ACKNOWLEDGMENT

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