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Sequence analysis, cloning and over-expression of an endoxylanase from the alkaliphilic *Bacillus halodurans*

M. Alejandra Martínez[1,2,*], Osvaldo D. Delgado[2], Mario D. Baigorí[1] & Faustino Siñeriz[1]
[1]*PROIMI, Planta Piloto de Procesos Industriales Microbiológicos, Av. Belgrano y Pasaje Caseros, T-400 1MVB, Tucumán, Argentina*
[2]*Department of Biotechnology, Centre for Chemistry & Chemical Engineering, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*
**Author for correspondence (E-mail: alejandra.martinez@webmail.unt.edu.ar)*

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Abstract

The BhMIR32 *xyn11A* gene, encoding an extracellular endoxylanase of potential interest in bio-bleaching applications, was amplified from *Bacillus halodurans* MIR32 genomic DNA. The protein encoded is an endo-1,4-*β*-xylanase belonging to family 11 of glycosyl hydrolases. Its nucleotide sequence was analysed and the mature peptide was subcloned into pET22b(+) expression vector. The enzyme was over-expressed in a high density *Escherichia coli* culture as a soluble and active protein, and purified in a single step by immobilised metal ion affinity chromatography with a specific activity of 3073 IU mg^{-1}.

Introduction

Xylanases have a number of biotechnological applications, both alone and in combination with other enzymes. Most relevant applications of xylanases are their use in the paper and pulp industry, in bread-making, for the saccharification of ligno-cellulosic biomass, and in the production of acidic xylo-oligosaccharides having potential pharmacological benefits (Beg *et al.* 2001, Christakopoulos *et al.* 2003).

Hydrolysis of the xylan backbone is performed mainly by the action of endoxylanases (endo-1,4-ß-D-xylan-xylanohydrolase EC 3.1.2.8). A large number of these enzymes from different micro-organisms have been described and mostly grouped into two families of glycosyl hydrolases, GHF 10 (or F) and GHF 11 (or G) based on sequence similarities and hydrophobic cluster analysis (Henrissat 1991, Collins *et al.* 2002). GFH 11 of endoxylanases are characterised as being well-packed proteins of around 20 kDa. Due to their small size and compact folding, family 11 xylanases can easily penetrate into the cellulose fiber network; this makes these enzymes of particular interest in biobleaching (Törrönen & Rouvinen 1997, Oakley *et al.* 2003).

An endo-1,4-ß-D-xylan-xylanohydrolase with a molecular weight of about 20 kDa, showing high stability in alkaline conditions at 50 °C was reported by Breccia *et al.* (1998). This enzyme was isolated from an organism belonging to *B. halodurans* species (Martínez *et al.* 2002), and was found to be a promising candidate for biobleaching of kraft pulp according to preliminary tests done at Holzforschung Institute in Austria (unpublished data). Here, we report its sequence analysis, cloning and over-expression in *E. coli* BL21 (DE3). The BhMIR32 *xyn11A* nucleotide sequence was deposited in the GenBank/EMBL/DDBJ database under accession no. AY170624.

Materials and methods

Bacterial strains and growth conditions

Bacillus halodurans MIR32 was grown as described previously (Martínez *et al.* 2002). *E. coli* NovaBlue and *E. coli* BL21 DE3 pLysS (Novagen, EMD Biosciences Inc, Darmstadt, Germany) were used as cloning and expression hosts, respectively, and grown in LB medium containing 70 μg carbenicillin ml^{-1} and 32 μg chloramphenicol ml^{-1}, when required.

Plasmids, genomic DNA and cloning procedures

Chromosomal DNA was prepared as previously described (Martínez *et al.* 2002). Plasmid preparations, standard recombinant DNA techniques, and *E. coli* transformation using the $CaCl_2$ procedure were done according to Sambrook *et al.* (1989). DNA fragments were gel purified using the Qiagen Extraction Kit (Qiagen NV, the Netherlands) and vector pET22b(+) was used for xylanase cloning and expression (Novagen). All enzymes used were from Promega (Promega Corp., WI).

A genomic DNA fragment containing BhMIR32 *xyn11A* was PCR-isolated from *B. halodurans* MIR32 with primers ExFrNX (5′-TTTA AGGCTGGTACGAGGTGAA-3′) and ExRvNX (5′-CGGATGAGTGATAGATGCCAACG-3′). This fragment was used to isolate a 546 bp sequence corresponding to the mature peptide of the xylanase, BhMIR32 *Δ(pS)xyn11A*, which was amplified with primers NXNco (5′-CGCG GGACCATGGATAATACCTATTGGCAATAT TGG-3′) and NXXho (5′-TTTCTC CTCGAGCC AAACCGTCACATTCGATCT-3′). PCR conditions were: an initial denaturation step at 94 °C, 4 min, followed by 10 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1.5 min and 25 cycles decreasing the annealing temperature to 48 °C, with a final extension strep at 72 °C for 7 min. Both the plasmid and BhMIR32 *Δ(pS)xyn11A* fragment were gel purified, digested separately with *Nco*I plus *Xho*I, ligated, and used to transform *E. coli* NovaBlue cells.

As a rapid screen, several clones were grown and samples of cell free culture media (extracellular fraction) and disrupted cells (intracellular fraction), were tested for xylanolytic activity on agar plates with the coloured substrate RBB-xylan (4-*O*-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R, Sigma) prepared in 50 mM Tris/HCl (pH 7.5). 25 μl of both, extracellular and intracellular fractions in wells made in the agar to test activity after 20 min incubation at 37 °C; among the clones showing activity (clear halos), one was selected and its recombinant plasmid, designated pNX33, was confirmed to bear the expected sequence and then used to transform *E. coli* BL21 (DE3) pLysS, the expression host.

Shake-flask cultivations and sample treatment

E. coli BL21 cells harbouring pNX33 were incubated in LB medium with antibiotics and shaken at 200 rpm overnight at 37 °C. Then 5 ml of the culture were used to inoculate 100 ml fresh LB medium and grown until an OD_{600nm} of 0.7 was reached, and induce to xylanase expression by adding 1 mM IPTG. The growth was continued for 6 h sampling (5 ml) every hour. The resultant cell pellets were recovered after centrifugation, resuspended in the same volume of 20 mM Tris/HCl (pH 8.0) and frozen (−20 °C). Recovery of the xylanase was facilitated by the lysozyme expressed from the co-plasmid pLysS and completed by sonication (3×120 s, 0.5 cycle, 60 W cm^{-2}) with an UP400S sonicator (Dr. Hielscher GmbH, Stahnsdorf, Germany). Cell debris was separated by centrifugation ($20{,}000 \times g$, 20 min at 4 °C) and the upper phase (soluble protein fraction) was used as source of the enzyme for further analyses. The sample obtained at the end of cultivation was analysed to search where the enzymatic activity was mainly located, according to the protocols described by the supplier of the pET Expression System (Novagen).

Over-expression of xylanase

For enzyme production, a high density *E. coli* BL21 (DE3) culture was obtained in a defined medium with a fed-batch cultivation strategy according to Åkesson *et al.* (2001). Inoculum was prepared in LB medium and used to inoculate a 5 l bioreactor (Inceltech LH series 210, Toulouse, France) with a working volume of 2.5 l. The pH was kept at 7.0 by titration with

28% NH_4OH, temperature was 37 °C and dissolved O_2 was controlled at 30% by adjusting the agitation. After 4 h of cultivation, the medium was intermittently fed with a peristaltic pump to keep a basal level of glucose concentration in the culture broth. Xylanase production was induced 2 h later by addition of IPTG (1 mM), and the cultivation was continued for 5 h. Samples were taken every hour for monitoring cultivation parameters.

Data search and sequence analysis

The BLAST 2.0 sequence similarity search tool (http://www. ncbi.nlm.nih.gov/) was used to align related nucleotide and/or protein sequences using standard search algorithms. Properties of translated proteins based on the primary structure analysis were predicted using the ProSite (http://www.expasy.ch/tools/scnpsite.html) and SignalP V 3.0 (http://www.cbs.dtu.dk/services/SignalP/) on-line programs.

Purification of recombinant xylanase enzyme

Purification of the xylanase was performed by immobilized metal affinity chromatography (IMAC) on a Cu^{2+} loaded iminodiacetic acid-Sepharose CL-6B column (20 × 2 cm) (Hermanson *et al.* 1992). Chromatography was done at room temperature using a BioLogic FPLC system (BioRad). Binding of the enzyme to the column was done in 20 mM Tris/HCl (pH 8.0), while elution was performed using the same buffer containing 500 mM NaCl and 200 mM imidazole. Active fractions were pooled and dialysed, freeze dried and resuspended in the same volume of 10 mM Tris/HCl (pH 6.8).

Analytical methods

Cell growth was monitored turbidimetrically at 600 nm and correlated to cell dry weight (CDW) determined gravimetrically. Residual glucose was detected with the enzymatic GOD/POD kit (Wiener Lab, Rosario, Argentina). Xylanase activity was measured by limited hydrolysis of birchwood xylan using the dinitrosalicylic acid method as previously described (Breccia *et al.* 1998); 1 unit of enzyme activity is defined as the amount of enzyme releasing 1 μmol of reducing sugar equivalent to xylose per minute under the assay conditions. Protein content was determined by the bicinchoninic acid (BCA) method using bovine serum albumin as a standard (Sigma). SDS-PAGE was performed on a 15% running gel (Laemmli 1970) and resolved proteins were visualized by staining with Coomassie Brilliant Blue G250 (Merck, Darmstadt, Germany).

Results and discussion

Sequence of the xylanase from B. halodurans *MIR32*

A search for related proteins was done based on the characteristics and *N*-terminal sequence of the pure xylanase (Breccia *et al.* 1998), named BhMIR32 Xyn11A according to a scheme proposed by Henrissat *et al.* (1998), and the complete genome of the related strain *B. halodurans* C-125 in Extremobase (http://www.jamstec.go.jp/genomebase/micrHome_bha.html). This search revealed an ORF, bh0899, from *B. halodurans* C-125 (accesion no. AP001510) encoding a predicted protein with characteristics similar to those of BhMIR32 Xyn11A. These data were used to design the primers to isolate BhMIR32 *xyn11A*, the use of which resulted in a single band of a 1343 bp PCR product (accession no. AY170624). Using the BLAST algorithm from NCBI only one hit (100%) was produced, corresponding to the homologous gene from *B. halodurans* C-125. Since the full length gene from strain C-125 has not been cloned and fully characterised (Honda *et al.* 1985), we decided to carry out these studies on BhMIR32 *xyn11A*.

Analysis of the 1343 bp fragment containing BhMIR32 *xyn11A* showed an open reading frame of 663 bp. An AGGAG motif similar to the consensus ribosome binding site for *Bacillus* spp. was found eight nucleotides upstream of the probable translation start codon ATG, which was also complementary to the 3′ terminus of the 16S rRNA gene from *B. halodurans* C-125 (Takami *et al.* 1999). The initiation site is preceded by a well conserved −10 element TATAAT located at position −129, which is similar to the *B. subtilis* consensus promoter that perfectly matches the consensus promoter recognised by σ^A

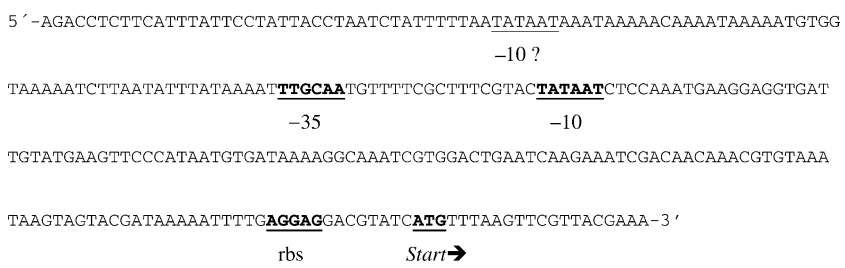

Fig. 1. Promoter region of BhMIR32 *xyn11A*. This region corresponds to nt 1–274 in the deposited GenBank sequence (accession no. AY170624). The ATG start codon, the putative ribosome-binding site and −10 and −35 regions are underlined. A second plausible −10 element was found at position −207 but we could not identify any appropriately spaced −35-like sequence. Initiation of multiple transcripts is a common feature in several prokaryotic promoters and may explain this −10 region upstream of putative promoter.

factor (Moran 1993) (Figure 1). This sequence was also found as a possible promoter element in other thermophilic (Vecerek & Venema 2000) and alkaliphilic (Gupta *et al.* 2000) *Bacillus* spp. The element located at −129 is more likely to be the −10 promoter region since a TTGCAA sequence located 17 bp upstream probably represents the −35 region, with four matches out of six with the σ^A consensus sequence, TTGACA, from *B. subtilis*.

Complete genome analysis of *B. halodurans* C-125 showed that genes coding for RNA polymerase and sigma factors belonging to σ^{70} family (including σ^A) are highly similar between *B. halodurans* and *B. subtilis* species (Takami *et al.* 2000). It is therefore reasonable to consider that promoter sequences recognised by those factors are also similar. Conclusions concerning putative promoter sequences are as well supported by our analysis of BhMIR32 *xyn10A*, which encodes the alkaline xylanase produced by *B. halodurans* MIR32 (accession no. AF534180). The putative promoter sequences detected 400 bp upstream of the start codon are in agreement with experimental data of *xynA* from *B. halodurans* C-125 (accession no. D00087).

BhMIR32 *xyn11A* encodes a 1,4-β-D-xylan xylanohydrolase of 210 amino acids with a molecular mass of 23 kDa and an isoelectric point of 10 (Compute pI/MW, ExPASy). However, the sequence also predicts a putative signal peptide of 3 kDa (Figure 2), the removal of which would result in a mature protein of 20 kDa. These predictions are consistent with experimental data previously obtained from the purified protein (Breccia *et al.* 1998).

Structural and phylogenetic relationships among 82 GHF 11 protein sequences revealed that this group of enzymes shows a single domain and clear amino acid similarity, all having a common fold with two conserved catalytic residues, with glutamate (E) residues located opposite each other in an open active side cleft (Sapag *et al.* 2002). Although neither XynN from *B. halodurans* C-125 nor BhMIR32 Xyn11A were included in that study, all conserved sequences were found in these proteins. The key catalytic residues of BhMIR32 Xyn11A are E in positions 104 and 197 (Figure 2).

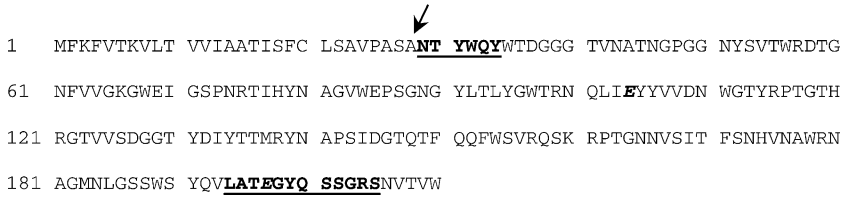

Fig. 2. Deduced amino acid sequence of the endo-β-1,4-xylan-xylanhydrolase encoded by BhMIR32 *xyn11A*. The arrow indicates the predicted cut of the signal peptidase between positions 28 and 29 (ASA-NT) and the first 6 amino acids of the mature peptide as experimentally determined are underlined and in bold face. *E* (glutamate) residues in italics, equivalent to E 78 and E 172 in *B. circulans* xylanase (Sapag *et al.* 2002), would be involved in the glycosidic bond disruption. Structural consensus pattern defined for GHF11 is indicated in positions 194–205 (CAZy, http://afmb.cnrs-mrs.fr/CAZY/index.html).

Cloning and expression of xylanase mature peptide in E. coli

Restriction sites *Nco*I and *Xho*I in the PCR fragment encoding the mature peptide were used to the in-frame cloning into b(+)pET 22, and to pace BhMIR32 Δ*(pS)xyn11A* under the control of the T7*lac* hybrid promoter (IPTG inducible). This construct was also in-frame with the *pelB* leader to allow the export of the mature protein into the periplasmic space and to facilitate its folding in the expression host. In addition, the NXXho reverse primer was designed to by-pass the native stop codon allowing the fusion of a His_6 tag at the *C*-terminus of the protein to facilitate its purification by affinity chromatography.

From the clones showing clear halos in RBB-Xylan-agar, the one carrying pNX33 was selected after verifying its sequence, and used for expression and enzyme production. The extracellular fraction (cell free medium) had no activity, while all of the xylanase activity was obtained from the cellular fraction after cell disruption. Further analysis of xylanolytic activity in the cellular fractions obtained after 5 and 6 h of induction in the batch culture, done according to standard protocols, showed that the periplasmic fraction contained more than 95% of the detected enzymatic activity.

Over-expression or recombinant xylanase

For enzyme over-production from *E. coli* harbouring pNX33 plasmid, after a batch period of batch cultivation in the bioreactor, a fed-batch cultivation mode was started. Glucose was maintained at a low level (approximately 1.5 g l^{-1}) so as to minimise acetic acid formation (Åkesson *et al.* 2001). After 2 h of feeding, when the OD_{600nm} was around 21 (10 g CDW l^{-1}), the culture was induced with IPTG and additionally incubated during 5 h. The final cell density reached an OD_{600nm} of 57 (25.5 g CDW l^{-1}). The total xylanase activity obtained was of about 2200 IU ml^{-1} of culture volume, corresponding to a specific activity of 618 IU mg^{-1} total protein (Table 1).

Purification of the recombinant xylanase from E. coli

After IMAC purification, BhMIR32 Xyn11A migrated as a single band with a relative molecular mass of 20 kDa (Figure 3). Its specific activity was of 3073 IU mg^{-1}, with a yield of 73% and a purification factor of 5 (Table 1). The biochemical properties of the protein recovered were similar to those of the native enzyme previously described (Breccia *et al.* 1998).

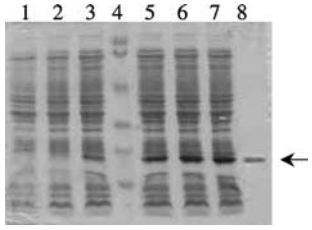

Fig. 3. SDS-PAGE for recombinant *E. coli* BL21 (DE3) pLysS: pNX33 growth under fed-batch conditions. Lane 1: time 0, before IPTG induction; 2: 1 h post-induction; 3: 2 h post-induction; 4: low range size marker; 5: 3 h post-induction; 6: 4 h post-induction; 7: 5 h post-induction and 8: after IMAC purification. Purified xylanase band is indicated by an arrow.

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Table 1. Purification of the recombinant *B. halodurans* xylanase from fed-batch culture.

Purification step	Total activity (IU ml^{-1})	Protein (mg ml^{-1})	Specific activity (IU mg^{-1})	Purification factor	Yield (%)
Soluble fraction after cell disruption	2176	3.52	618	1	100
IMAC	1598	0.52	3073	5	73

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