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**The acetyl xylan esterase II gene from *Penicillium purpurogenum* is differentially expressed in several carbon sources, and tightly regulated by pH.**

**RENATO CHÁVEZ<sup>1,3</sup>, KAREN SCHACHTER<sup>1,4</sup>, CLAUDIO NAVARRO<sup>1,5</sup>, ALESSANDRA PEIRANO<sup>1</sup>, PAULINA BULL<sup>1</sup> and JAIME EYZAGUIRRE<sup>1,2</sup>**

<sup>1</sup>Laboratorio de Bioquímica, Departamento de Genética Molecular y Microbiología, Pontificia Universidad Católica de Chile, Santiago, Chile.

<sup>2</sup> Departamento de Ciencias Biológicas, Universidad Andrés Bello, Santiago, Chile. .

<sup>3</sup>Current Address: Instituto de Biotecnología, (INBIOTEC), León, Spain .

<sup>4</sup>Current Address: Department of Biochemistry, Michigan State University, East Lansing, Michigan, U.S.A.

<sup>5</sup>Current Address: Departamento de Ciencias Biológicas, Universidad de Santiago, Santiago, Chile.

Corresponding author: Dr. Jaime Eyzaguirre. Laboratorio de Bioquímica. Pontificia Universidad Católica de Chile. Casilla 114-D, Santiago, Chile. Telephone: (56 2) 686-2664 - Fax: (56 2) 222-5515. Email: [jcyzaq@bio.puc.cl](mailto:jcyzaq@bio.puc.cl)

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### ABSTRACT

The expression of the acetyl xylan esterase II (*axeII*) gene from *Penicillium purpurogenum* is repressed by glucose and induced by xylan, as well as to a small degree by xylose and xylitol. This gene is expressed at neutral pH, but not under alkaline or acidic conditions, in agreement with previous findings for other xylanolytic genes of this organism. This is the first report showing pH regulation of an *axe* gene.

**Key terms:** acetyl xylan esterase; gene expression; *Penicillium purpurogenum*

### INTRODUCTION

Xylan is a plant heteropolysaccharide constituted by linear chains of  $\beta$  (1 $\rightarrow$ 4) linked D-xylopyranose units with different substituents that vary in type and amount depending on the source. Due to its complex structure, the biodegradation of xylan requires the concerted action of a number of glycanases and esterases (collectively called xylanases), which are produced by a variety of bacteria and fungi (18). Acetate residues are removed from the main chain of xylan by acetyl xylan esterases (AXEs) (E.C. 3.1.1.6). The soft-rot fungus *Penicillium purpurogenum* is an active producer of a variety of xylan-degrading enzymes (1, 7, 10), including at least two AXEs (AXEI and AXEII), which have been purified and characterized (9). The crystalline structure of AXEII (11), the nucleotide sequence of its gene, *axeII* (13), and its chromosomal location (5) have recently been determined.

With respect to the regulation of xylanase gene expression, work has been carried out in arabinofuranosidases,  $\beta$ -xylosidases and endoxylanases, primarily in fungi such as *Aspergillus* and *Trichoderma* ([12](#), [16](#), [22](#), [24](#)). In *Penicillium purpurogenum*, we have recently shown differences in the expression of two endoxylanases ([6](#)), which could be related to the presence of regulatory elements such as CreA (a negatively acting regulatory protein mediating carbon catabolite repression) ([8](#)), XlnR (a transcriptional activator) ([21](#)), and PacC (a pH regulator) ([19](#)).

Despite their importance in xylan degradation, little work has been carried out on the expression of acetyl xylan esterases. To the best of our knowledge, except for the work on the *axeA* gene from *Aspergillus niger* ([20](#)), there are no other studies on fungal *axe* gene expression in the literature. For a better understanding of the xylanolytic system of *P. purpurogenum*, we analyzed the expression pattern of *axeII* under several experimental conditions.

## MATERIALS AND METHODS

### *Microorganism and culture media*

*P. purpurogenum* ATCC N<sup>o</sup> MYA-38 was kept on 2.4% potato dextrose agar plates (Difco), and spores were inoculated and grown in Mandels' liquid medium ([15](#)) as described previously ([3](#)) using different carbon sources (at 1%) and pH conditions as indicated in each experiment.

### *RNA isolation*

Total RNA was extracted following a described procedure ([4](#)). Briefly, the mycelium was homogenized with a volume of Trizol (GibcoBRL) in a potter homogenizer with Teflon pestle. After phenol/chloroform extractions, RNA was precipitated with isopropanol and dissolved in 10 mM Tris-HCl, 1 mM EDTA pH 7.5. The RNA was kept at -70°C until further use.

### *Northern blot*

Northern blots were performed using an *axeII* probe as described previously ([5](#)). As loading control, a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene probe was used. It was generated by PCR using the following primers: N<sup>o</sup> 1814 (5'-TC (C/T)GCTGATGCC(C/T)ATGTT-3') and N<sup>o</sup> 1815 (5'-CCCACTCGTTGTCGTACCA-3') and *P. purpurogenum* DNA as template. The primers were designed based on highly conserved regions in the sequence of GAPDH of other species.

### *Inverse PCR*

Genomic DNA (0.5  $\mu$ g) was cut with *HindIII* and purified in a Wizard column (Promega), and 0.5  $\mu$ l of T4 DNA ligase (400 U/  $\mu$ l) was added to 30  $\mu$ l of the eluted DNA for an autoligation reaction (16<sup>o</sup> C for 12 hours) in a final volume of 50  $\mu$ l. Five  $\mu$ l of the autoligated mixture was used as a template in a PCR reaction using the divergent primers JE-126 (5'- CCGAAGGAAGGGTTTTGGCTAATGAAG-3') and JE-127 (5'-GAATACTTCCTT-CACCGCGAGACTTA-3'). The PCR conditions were: DNA 0.5 to 0.6 ng, 20 mM Tris-HCl pH 8.75, 10 mM KCl, 10 mM ammonium sulfate, 2 mM MgSO<sub>4</sub>, 0.1% Triton X100, 100  $\mu$ g/ml BSA, dATP, dCTP, dGTP and TTP 50  $\mu$ M each, 5 pmoles of each primer, 0.625 U Taq polymerase and 0.0375 U of Pfu polymerase (Stratagene). The following PCR program was used: 1 min

denaturation at 95°C, followed by 35 cycles of 1 min 95°C, 1 min 54°C, 1 min at 72°C, and a final 10 min incubation at 72°C.

#### *Western blots*

For western blots, we followed Bollag & Edelstein's method (2), using antibodies prepared against purified AXE II (9).

#### *Induction of axeII expression*

The transcription of *axeII* was analyzed by growing *P. purpurogenum* in several carbon sources, using a strategy described previously (6). Briefly, the fungus was grown in fructose (a non-inducing and non-repressing sugar) for 17 hours to increase the mycelial mass; it was then transferred to fresh Mandels' medium containing the carbon source at 0.1%. After the time indicated in each experiment, total RNA extraction and northern blot analyses were performed. In the case of the pH experiments, *P. purpurogenum* was grown on fructose and transferred to Mandels' medium buffered with 30 mM citrate (pH 4.6 to 4.8), 30 mM 2-(N-morpholino) ethane sulfonate (pH 6.6 to 6.7) or 30 mM Tris-HCl (pH 7.8) and containing 0.1% oat spelt xylan as carbon source.

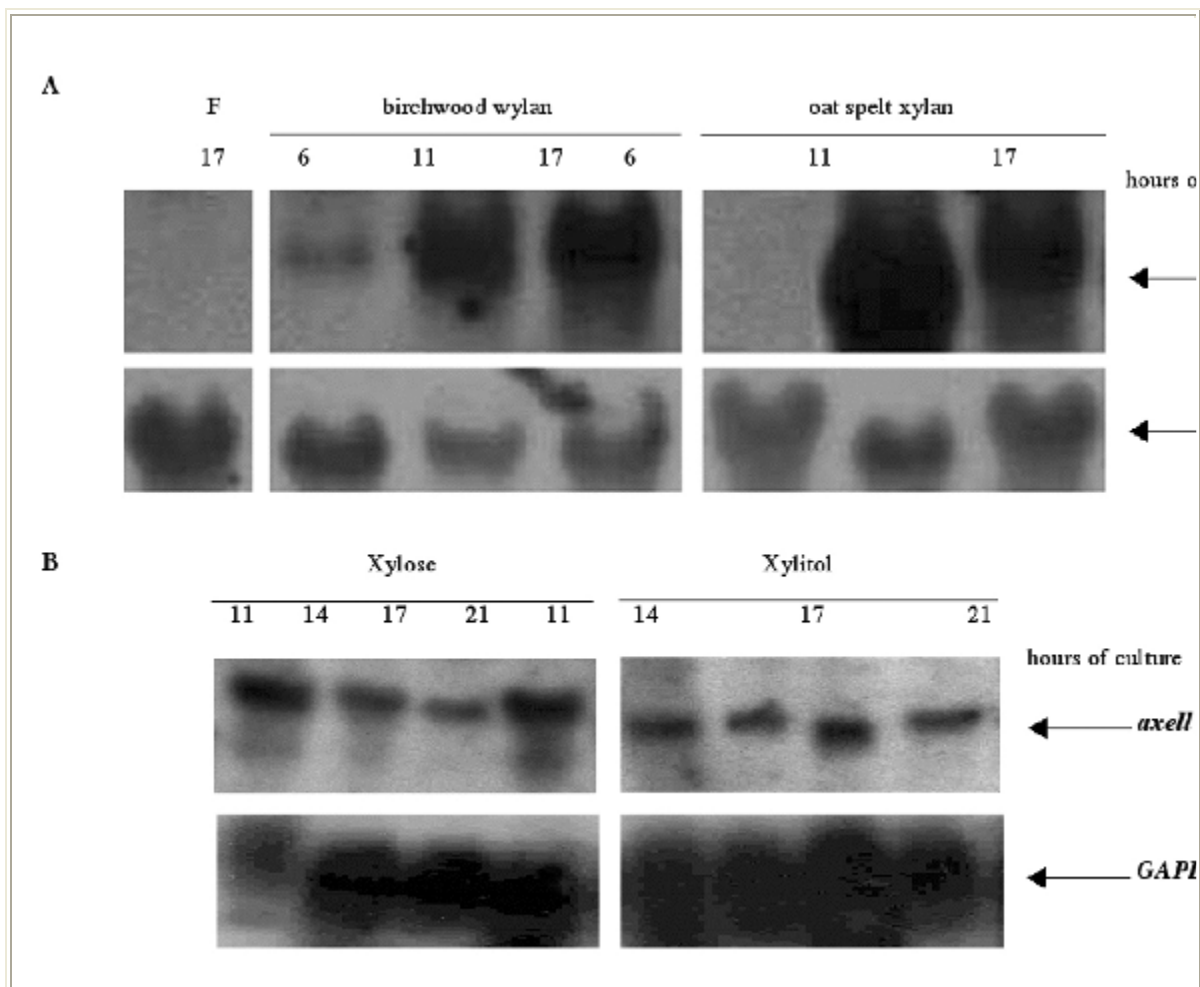
#### *DNA sequencing strategy*

A strategy utilizing inverse PCR and the Universal Genome Walker kit (Clontech) and based on the procedures described for other xylanase promoters (6) was used. Genomic DNA of *Penicillium purpurogenum* was cut with HindIII, and the fragments were ligated to an adapter as recommended by the kit manufacturer. Sequencing was performed on both strands using an Applied Biosystem ABI PRISM 310 DNA sequencer.

## RESULTS AND DISCUSSION

#### *Expression of axeII mRNA and AXE II activity by P. purpurogenum grown in different carbon sources*

The northern blot in [Figure 1](#) shows that the concentration of *axeII* mRNA is highest when the fungus is grown in oat spelt xylan, lower in birchwood xylan, and weaker in xylose or xylitol. No *axeII* mRNA was detected in either glucose or fructose (data not shown). Induction by acetylated oat spelt xylan (a powerful inducer of total AXE activity (9)) also gives a strong signal (data not shown).



**Figure 1.** (A) Northern blots of total RNA extracted from mycelium after growth in the presence of D-fructose (lane F) for 17 h and then transferred to media containing 0.1% oat spelt xylan or 0.1% birchwood xylan. (B) Similar experiment, but mycelia were transferred to 0.1% xylose or 0.1% xylitol. A human  $\beta$ -actin (6) or a *P. purpurogenum* GAPDH gene probe were used as loading controls.

Translation of the *axeII* gene was analyzed by western blot (Fig. 2). Under the conditions utilized, expression was only detected in the medium with oat spelt xylan. This is due probably to a lower sensitivity of the immunoblot assay as compared to the northern blot.

The data suggest that glucose exerts carbon repression (probably mediated by CreA) and that xylan, and to a lesser extent its degradation products (xylose and xylitol), are able to induce *axeII* expression, probably by a XlnR-mediated mechanism. Interestingly, van Peij *et al.* (20) have shown that the *axeA* gene from *A. niger* is induced by birchwood xylan, but not by xylose. Since *P. purpurogenum* produces at least two acetyl xylan esterases, it will be of interest to determine whether the expression pattern of *axeI* differs from that of *axeII*.

The same expression pattern described for *axeII* was observed for the endoxylanase B gene (*xynB*) from the fungus, but not for the endoxylanase A (*xynA*) gene, which is only

expressed in oat spelt xylan (6). These results suggest that the xylanolytic system from *P. purpurogenum* contains at least two kinds of enzymes: those that are broadly induced by xylan and their metabolites (*axeII* and *xynB*), and others (*xynA*) that are induced only by some carbon sources. Whether this differential expression is due to a different chemical structure of the inducers, to properties of the promoter sequences, or both, remains to be determined.

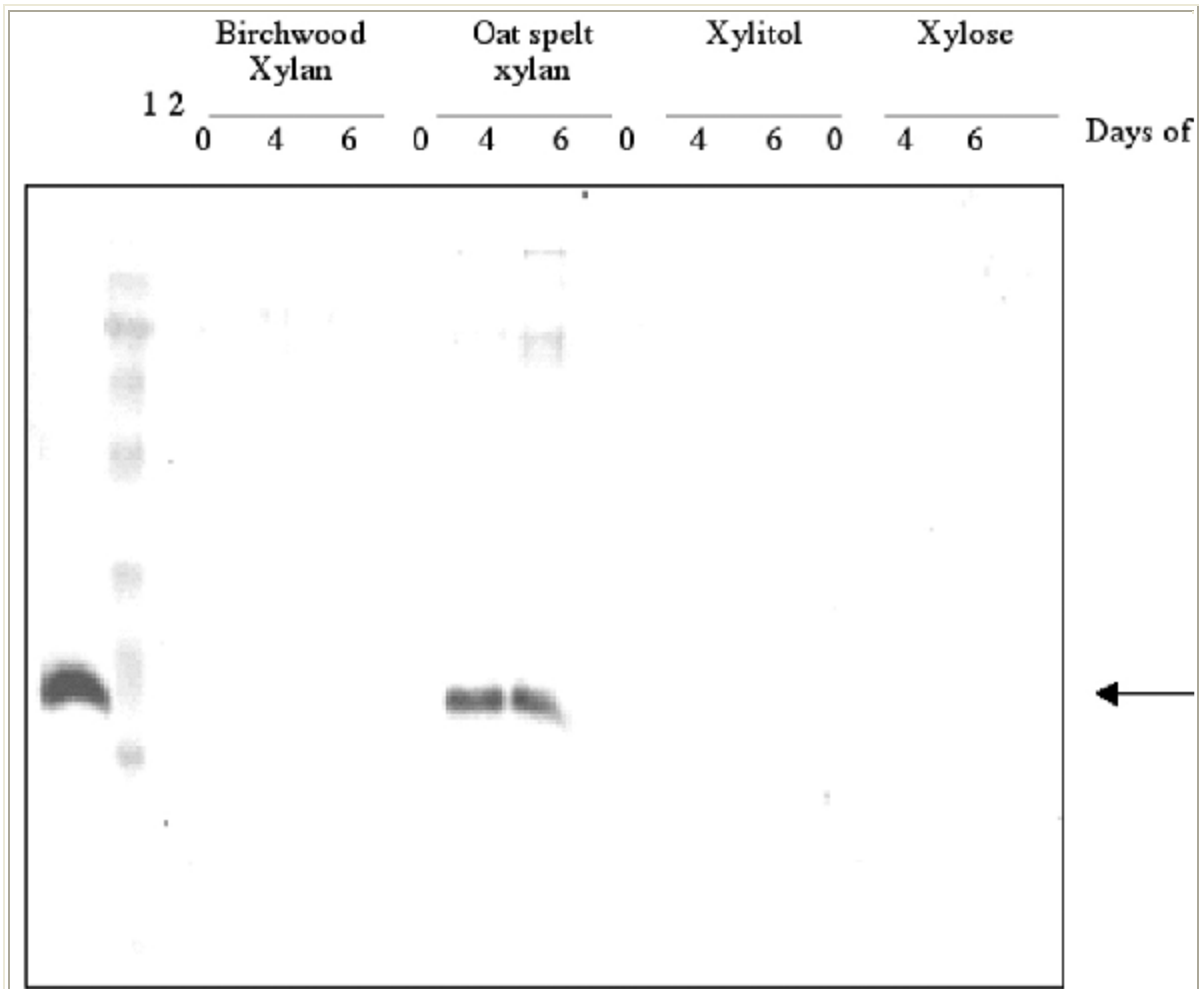
#### *Expression of AXE II at different pH values*

In order to investigate whether the *axeII* gene is regulated by pH, we analyzed the transcription of the gene under acidic (pH 4.6 to 4.8), neutral (pH 6.6 to 6.7) and alkaline (pH 7.8) growth conditions. At 11 and 14 hours of incubation, total RNA from mycelia was extracted and analyzed by northern blot. Figure 3 shows that the *axeII* transcript only appears at neutral pH. This result indicates a tight regulation of *axeII* expression by pH. To our knowledge, this is the first report showing pH regulation for an *axe* gene.

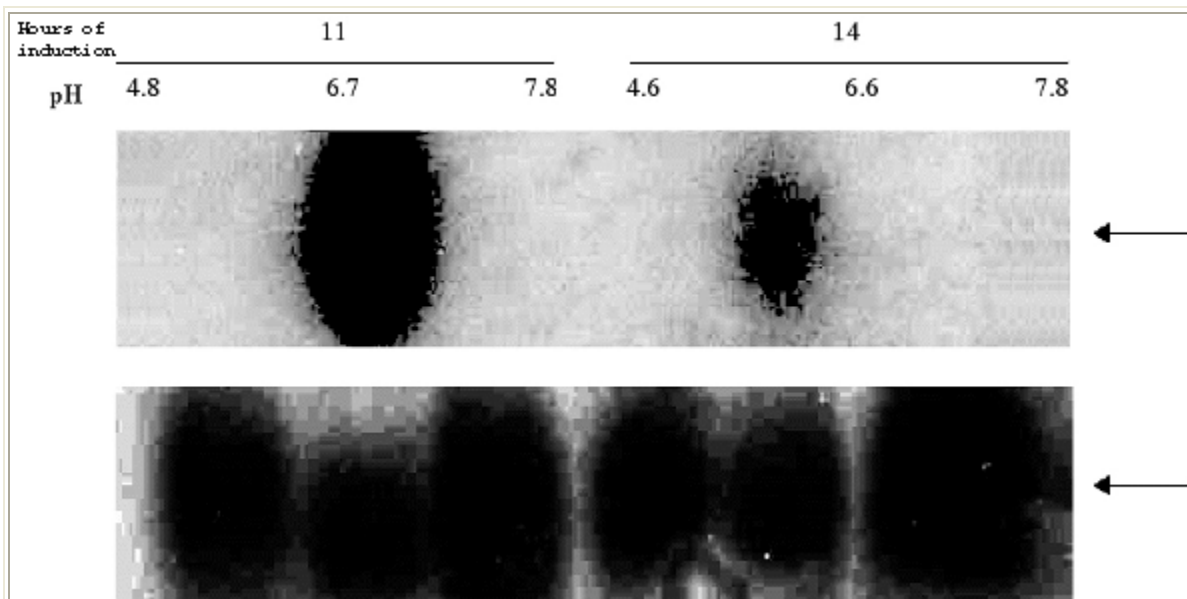
Steiner et al. (17) showed that high xylanolytic activities are detected in cultures of *P. purpurogenum* grown at neutral pH, but not at acidic or basic pH. These results agree with the expression patterns detected by Chávez et al. (6) for *xynA* and *xynB*. Both genes are expressed only at neutral pH. Here we show that the *axeII* gene has the same expression pattern. The expression of xylanases at neutral pH correlates with the growth of the fungus at different pHs: *P. purpurogenum* shows good growth in xylan at neutral pH, but not at extreme pHs (6). These results suggest that the xylanolytic system of *P. purpurogenum* may be induced only at neutral pH. This is an interesting difference with respect to other fungi. For example, *A. nidulans* produces two endoxylanases, XlnA and XlnB, which are expressed at alkaline or acidic pH, respectively (14). The same organism has a  $\beta$ -xylosidase, the induction of which seems to be insensitive to pH changes (16), and similar results have been described for *Penicillium chrysogenum*, whose endoxylanase gene (*xyIP*) is expressed independent of the environmental pH (23).

#### *Sequence analysis of the P. purpurogenum axe II promoter*

A nucleotide sequence of 508 bp of the upstream region of the *axeII* gene was obtained. It reveals the presence of two CreA, one XlnR and two PacC putative consensus-binding sites (Fig. 4), suggesting that the transcription of *axeII* may be regulated by the carbon source and by pH. These findings agree with the results shown above. More experiments need to be performed (e.g. gel shift analysis, in vivo reporter gene expression directed by different promoter fragments) in order to demonstrate that these putative consensus-binding sites are indeed functional.



**Figure 2.** Western blot of supernatants of *P. purpurogenum* cultures grown in the indicated carbon sources. Lane 1: purified sample of AXE II. Lane 2: pre-stained molecular weight standards.



**Figure 3.** Northern blots of total RNA extracted from mycelium after growth in 0.1 % oat spelt xylan at different pHs. The indicated pH values were recorded at harvesting time. A GAPDH gene probe was used as loading control.

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ATCCGGCAAATGAGGTTGAACCTGCATTTCTTCACGGCAG -468
ATCAATGCGGGGAAGCAGTCGAGGCCTTACGCAGATCCCTC -427
      CreA

CTATTCGAGGCCCCCCAGCCGAGACACCACCGTAACTCTATC -386
      CreA

ATGTCTCACCCCTCAATTTGCTTATTTTGCCTGGCGGAATCG -345
      PacC

TCCCGAAACGCCGATCGATTCAGCCTGATTGAATCAATACG -304
ACTTCTTCACGGCTAAATGAAGCAAGTTGCCTGCCAAGTCT -263
      X1nR                PacC

CAATTGAAACTTCAAGCTTCATCTTCATTAGCCAAAACCCT -222
TCCTTCGGTCAAAGGGATCATCAACTCGATCTATTCGAGAT -181
GCTTCACGGAAACATGGAACAAATTTTTGTTTCGAATCATC -140
AGTTTAAGAGAGTTCTTCTATAAATCATTGACTAAGTCTCG -99
CGGTGAAGGAAGTATTCACGAGCATTATCTCTATCATCCA -58
AGCCTTGATTACCACCATCTATTTTGACCCTTTTCAGTTAT -17
CAACGTAACAAGAAAATG 3

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**Figura 4.** Sequence of 508 bp of the *axeII* promoter. Putative binding sites for regulatory proteins are indicated below their respective sequece (bold). Start codon (ATG) is shown in bold and italics. The nucleotide sequence has been

deposited in GenBank under the accession number AF528538.

## ACKNOWLEDGEMENTS

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***Sociedad de Biología de Chile***

**Canadá 253, piso 3º, Dpto. F.**

**PO Box 16164**

**Santiago - Chile**

**Tel.: (56-2) 22093503**

**Fax: (56-2) 22258427**



**[socbiol@biologiachile.cl](mailto:socbiol@biologiachile.cl)**