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ORIGINAL PAPER

Regulation of xylanase in *Aspergillus phoenicis*: a physiological and molecular approach

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Abstract Microbial xylanolytic enzymes have a promising biotechnological potential, and are extensively applied in industries. In this study, induction of xylanolytic activity was examined in *Aspergillus phoenicis*. Xylanase activity induced by xylan, xylose or β-methylxyloside was predominantly extracellular (93–97%). Addition of 1% glucose to media supplemented with xylan or xylose repressed xylanase production. Glucose repression was alleviated by addition of cAMP or dibutyryl-cAMP. These physiological observations were supported by a Northern analysis using part of the xylanase gene *ApXLN* as a probe. Gene transcription was shown to be induced by xylan, xylose, and β-methylxyloside, and was repressed by the addition of 1% glucose. Glucose repression was partially relieved by addition of cAMP or dibutyryl cAMP.

Keywords Fungi · *Aspergillus phoenicis* · Xylanase · cAMP · Gene transcription

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Introduction

Xylans are hemicelluloses present in the cell wall and in the middle lamella of plant cells, being the second most abundant non-cellulosic polysaccharide in nature [4]. Xylans are composed of variable proportions of monosaccharide units, such as D-xylose, D-mannose, D-glucose, L-arabinose, D-galactose and D-glucuronic acid. Xylans are cleaved by *endo*-1,4-β-xylanase (EC 3.2.1.8), which hydrolyzes glycosidic bonds in the xylan backbone, thus reducing the degree of polymerization of the substrate. This enzyme can be obtained from bacteria, yeasts and filamentous fungi, but filamentous fungi are more attractive because they secrete higher levels of these enzymes into the medium, as compared to yeast and bacteria [23]. Xylanases have different industrial applications, such as to manufacture animal food [34], bread [3], textile [2, 5], and clarification of juices [38] and cellulose pulp [29].

An extensive literature exists on the regulation of fungal xylanases, especially from species of *Aspergillus* and *Trichoderma*. Most strains are induced either by xylan/xylobiose or by xylose and xylan/xylobiose, and carbon catabolite repression by glucose is reported as well [12]. Nevertheless, few studies detail the transcriptional regulation of xylanase and the participation of the cAMP in the repression by glucose. Morosoli et al. [17] report that the xylanase from *Cryptococcus albidus* is induced by xylan or β-methylxyloside, a non-metabolizable inducer, and that xylose represses the production of the enzyme. In this microorganism, addition of exogenous cAMP elicits a 1.5- to 2-fold increase in xylanase production in the presence of the inducer, but does not relieve the repression caused by xylose. This cyclic nucleotide also affects the cells growth rate. These authors suggest that a 15-nucleotide sequence located upstream from the xylanase gene could be part of a

cAMP regulatory site. Catabolic repression is also described by Ruijter and Visser [26] in *Aspergillus nidulans* and *A. niger*. These authors suggest that the CREA protein plays a major role in carbon repression, by binding to specific sequences in the promoter of many target genes, and inhibiting their transcription. More recently, was isolated a transcriptional activator of xylanases (XlnR) from *Aspergillus niger* [21] and a transcriptional activator (AoXlnR) in *Aspergillus oryzae* which is XlnR homolog. Northern blot analysis reveals the controls of the expression of genes encoding xylanolytic enzymes [13]. Repression of xylanolytic genes by glucose has been assigned to the protein CREA in *A. niger* [7] and to its homolog Cre1 in *Trichoderma reesei* [31]. CREA protein modulates the expression of XlnR induced by xylose in *A. niger* [36] and *A. nidulans* [24].

Two specific xylanases belonging to family 11 were reported in *Trichoderma reesei*, Xyn I and Xyn II, with different biochemical properties, but highly homologous in sequence [27, 33]. It was reported that avicel, sophorose and L-sorbose induced a novel specific xylanase (Xyn III) in *T. reesei* PC-3-7 mutant. Molecular analysis indicates that the gene *xynIII* may be coordinately expressed with cellulase genes [19, 39].

The aim of this work was to analyze the effect of several carbon sources as inducers or repressors of the xylanolytic activity of *Aspergillus phoenicis*, and the effect of cyclic AMP on the catabolic regulation of the enzyme, using biochemical and molecular approaches.

Materials and methods

Organism and culture conditions

The fungus *A. phoenicis* RP-02 was isolated from soil samples from Ribeirão Preto (São Paulo State, Brazil) and identified according to Raper and Fennell [25] and Klich and Pitt [9]. The organism was maintained at 30 °C on slants of solid Vogel [35] medium supplemented with 0.75% yeast extract and 0.75% peptone. Liquid cultures (50 mL contained in 250 mL culture flask) were started with a conidia inoculum (10^7 conidia mL^{-1}) in Vogel minimal liquid medium (VML medium) [35] supplemented with different carbon substrates, as indicated for each experiment. The incubations were carried out at 42 °C, at 100 rpm of agitation.

Extraction of xylanolytic enzymes

Mycelia were harvested by filtration, rinsed with distilled water, blotted on filter paper and stored at −15 °C until used. The mycelial mass was disrupted in a mortar with acid-washed sea sand at 4 °C. After addition of 10 mL citrate–phosphate buffer [14], pH 4.0, cell disruption was continued for more 15 min. The slurry was then centrifuged (12,100×*g*, 15 min), and the supernatant was used to determine mycelial xylanase activity (as described below) and protein levels according to Lowry et al. [11] using bovine serum albumin as standard. The culture filtrate was dialysed overnight against the same buffer, and used to determine extracellular activity.

Determination of xylanase activity

Xylanase activity was assayed by measuring the reducing sugar released from birchwood xylan [15]. The reaction mixture consisted of 250 µl of 1% xylan, 150 µl of McIlvine buffer, pH 4.0 and 100 µl of crude cell extract. The reaction mixture was incubated at 50 °C for 15 min. One enzymatic unit was defined as the amount of enzyme that released 1 µmol xylose per minute under the assay conditions.

PCR amplification and isolation of a DNA fragment encoding xylanase

Degenerated oligonucleotides were designed based on an amino acid sequence alignment of xylanase proteins from different organisms. These oligonucleotides were used to amplify by PCR a DNA fragment from genomic DNA [37]. The primers F1 (5′-ATYGARTACTACATYGTBGA-3′), F2 (5′-TACTTRGCYGTBTACGGHTGG-3′), R1 (5′-GTRGCVACRATCTGGTAGTT-3′) and R2 (5′-GCCCARGCGTTGAARTGGT-3′) corresponded to oligonucleotide sequences encoding highly conserved amino acid sequences in *Aspergillus*. One fragment of the expected size (approximately 300 bp) was amplified using the primers F2 and R1. The nucleotide sequence was determined in both strands, and is available at GenBank under Accession no. DQ306885. Nucleotide and protein sequences were analyzed at the BCM Sequence Launcher web site. Sequence alignment was performed by using the ClustalW 1.8 program, and the identities and similarities were determined by using the BoxShade 3.21 program.

Growth conditions for gene expression assay

The xylanase gene expression under different growth conditions was analysed by Northern blot. For that, *A. phoenicis* mycelia were pre-cultivated in 50 mL of VML medium supplemented with 1% glucose during 72 h at 42 °C. One aliquot (3 mL) was withdrawn, filtered, frozen in dry ice, and stored at −80 °C for further processing (control). The remaining culture was filtered, transferred to media of different composition, as indicated in the legend of Fig. 3,

and incubated during 6 h at 42 °C. After incubation, the mycelia were harvested and the total RNA was prepared [30]. RNA (10 µg) was separated by electrophoresis on an 1.2% formaldehyde–agarose gel, and transferred to neutral nylon membranes (Hybond-N, Amersham) in 2X SSPE [28]. The blot was probed with the genomic DNA fragment (approximately 300 bp), radiolabelled by random priming, in 5 mL of Ultrahyb hybridization solution (Ambion) at 42 °C. After overnight hybridization the blot was washed twice for 10 min in 2X SSPE, 0.1% SDS and twice for 15 min in 0.1X SSPE, 0.1% SDS and exposed to X-ray film.

Results

Carbon regulation and effect of cyclic AMP on xylanase production

In preliminary experiments the fungus was initially grown up to72 h in VML medium supplemented with 1% glucose. After 72 h the level of glucose in the culture medium dropped below 0.05 mg/mL, and some xylanolytic activity was already detectable in this carbon-derepressed culture. On the other hand, for 48-h-old cultures, glucose concentration was about 0.15–0.20 mg/mL, and xylanolytic activity was very low, almost below detection. After being exposed to inducers of xylanolytic activity (xylose or xylan), the carbon catabolite-derepressed 72-h-old mycelium produced three times more xylanolytic activity than the 48-h-old mycelium, which might be still affected by glucose repression (data not shown), thus, 72-h-old cultures were used for the next experiments.

The 72-h-old cultures were harvested, rinsed and resuspended in fresh VML medium supplemented with different carbon sources (Table 1), and incubated for an additional 48 h. As compared with the control, there was an additional increase in mycelium mass for all the conditions assayed, except when lactose or *β*-methylxyloside was used as carbon sources (data not shown). Significant enzymatic levels were detected in the cultures supplemented with xylose, xylan of different origins and *β*-methylxyloside. Under these conditions the *endo*-1,4-*β*-xylanase was predominantly liberated to the medium (93–97%). Addition of glucose to media supplemented with these inducers strongly repressed the xylanolytic activity. Ribose, a pentose sugar, did not induce the xylanase activity, and some induction was observed in the presence of arabinose. In the fungus *A. sydowii* no induction was observed in the presence of ribose, however, only a slight induction was described in the presence of arabinose [6]. In order to distinguish induction of xylanase activity from either enzyme stabilisation and/or pro-enzyme activation, cycloheximide was added to the induced cultures. A drastic reduction of the enzymatic levels was observed, thus confirming "de novo" synthesis (data not shown).

Table 1 Production of extracellular xylanase by *Aspergillus phoenicis* on different carbon sources

Carbon source (1%)	Extracellular xylanase (U mg protein^{-1})
Control[a]	1.15
Arabinose	8.90
Fructose	3.31
Galactose	1.70
Glucose	2.37
Mannose	2.50
Ribose	2.22
Xylose	152.00
Xylose + glucose	40.00
Cellobiose	1.52
Raffinose	1.42
Lactose	0
Maltose	5.90
Sucrose	4.14
Starch	13.10
Xylan (birchwood)	172.40
Xylan (oat spelt)	171.40
Xylan (beechwood)	173.00
Xylan + glucose	90.00
Xylitol	3.14
β-Methylxyloside	152.30
β-Methylxyloside + glucose	25.00

The fungus was previously grown on VLM medium supplemented with 1% glucose, during 72 h, at 42 °C. After this period the mycelium was washed with distilled sterile water and re-incubated in media supplemented with 1% different carbon sources, during 48 h at 42 °C

[a] VLM medium without addition of carbon source

After that, we decided to examine the effect of cyclic AMP on the expression of xylanase. First, the fungus was cultivated for 72 h in VML supplemented with 1% glucose at 42 °C, and then transferred for 6 h to fresh liquid media, as follows: (1) induction medium (0.1% xylan or xylose); (2) induction medium + 0.1% glucose; (3) induction medium + 0.1% glucose + 100 µM cAMP or dibutyryl-cAMP; (4) induction medium + 100 µM cAMP or dibutyryl-cAMP. When glucose was added to the medium supplemented with xylan or xylose a marked reduction on the culture filtrate activity (Fig. 1a, c) and the mycelial extract activity (Fig. 1b, d) was observed (75 and 66%, respectively), in agreement with the previous results. Glucose repression was partially relieved when cAMP or dibutyryl-cAMP was added (Fig. 1).

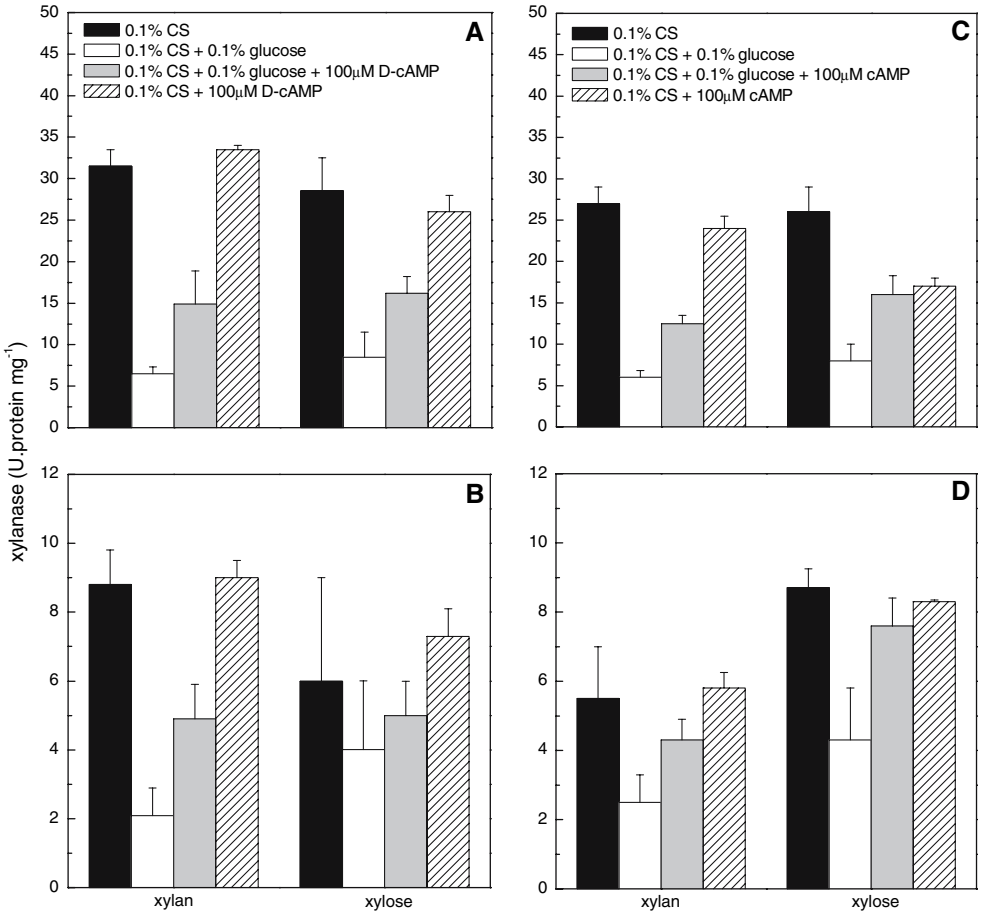

Fig. 1 Effect of glucose, dibutyryl-cAMP or cAMP on xylanase production. The culture media were supplemented with xylan or xylose (*CS*—carbon source). Extracellular activity (**a**, **c**); mycelial extract activity (**b**, **d**). Cultures **a** and **b** were added by 0.1% CS; 0.1% CS + 0.1% glucose; 0.1% CS + 0.1% glucose + 100 μM dibutyryl-cAMP or 0.1% CS + 100 μM dibutyryl-cAMP. Cultures **c** and **d** were added by 0.1% CS; 0.1% CS + 0.1% glucose; 0.1% CS + 0.1% glucose + 100 μM cAMP or 0.1% CS + 100 μM cAMP. The fungus was previously grown on Vogel medium supplemented with 1% glucose, during 72 h, at 42 °C and transferred to the different media during 6 h. $N = 4$

Considering these results, the effect promoted by cyclic AMP could be on gene expression, either at transcription level or involving posttranslational modifications. To address this question the gene transcription was analysed by Northern blot.

Isolation of a DNA fragment encoding xylanase in *A. phoenicis*

A 300 bp fragment was amplified and after DNA sequencing it showed to encode part of an *A. phoenicis* xylanase gene. We propose the name *Apxln* for the gene and ApXLN for the corresponding protein. Sequence alignment between the deduced amino acid sequence, and xylanase proteins from different filamentous fungi showed high identity among them (Fig. 2a). The phylogenetic tree showed that ApXLN is close to xylanases B from other filamentous fungi (Fig. 2b).

Expression of the *Apxln* gene

The xylanase gene expression in *A. phoenicis* mycelium was analysed in cultures submitted to experimental conditions known to promote the induction or repression of xylanase activity. Total RNA extracted from mycelium submitted to the different culture conditions was analysed by Northern blot using the 300 bp fragment as probe. RNA analysis showed that the gene was expressed as a full-length RNA transcript of approximately 1.2 kb, which corresponded to the transcript size of xylanase genes from different fungi.

Induction of transcription was observed when cells were exposed to the medium containing xylan or xylose as unique carbon sources (Fig. 3, lanes 2 and 8). There was no gene transcription in the absence of the inducers (Fig. 3, lane 1). Glucose strongly represses the induction by xylan or xylose (Fig. 3, lanes 3 and 9). However, the glucose repression was completely abolished by the addition of

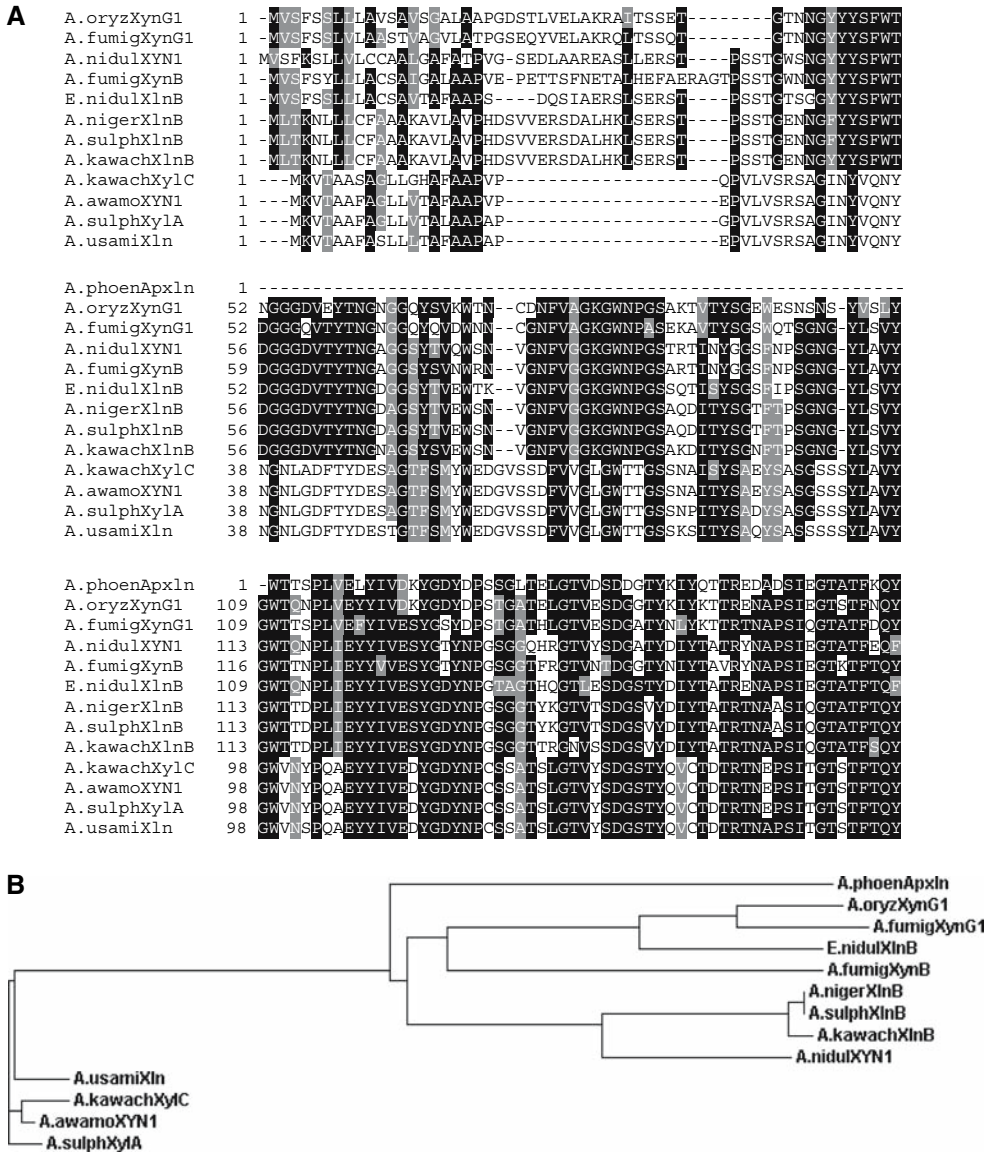

Fig. 2 **a** Amino acid sequences alignment of xylanases from different filamentous fungi. Identical amino acids are represented in *black* and conservative changes in *gray background*. Sequences were aligned using the ClustalW 1.8 program at the BCM Search Launcher and similarities were determined by using the BoxShade 3.21 program (http://www.ch.embnet.org/software/BOX_form.html). The accession numbers at the NCBI website are: *A. oryzae* XynG1 (BAA19744), *A. phoenicis* (DQ306885), *A. fumigatus* XynG1 (EAL89062), *A. niger* XlnB (AAS46914), *A. sulphureus* XlnB (AAZ95432), *A. kawachii* XlnB (AAS46914), and *E. nidulans* XlnB (CAA90074). **b** Phylogenetic analysis based on polypeptide sequences of xylanase proteins from filamentous fungi (http://www.ebi.ac.uk/clustalw)

cAMP (Fig. 3, lanes 4 and 10) or its analogue dibutyryl-cAMP (Fig. 3, lanes 6 and 13). Addition of these cyclic nucleotides does not interfere in the gene transcription induced by xylan or xylose (Fig. 3, compare lanes 5 and 7 to lane 3 for induction by xylan, and lanes 11 and 12 to 8 for induction by xylose).

Discussion

The xylanase produced by *A. phoenicis* was induced by xylan, xylose and *β*-methylxyloside. Xylanolytic enzymes induced by xylan or xylose have been reported in other microorganisms such as *Aspergillus sydowii* [6], *A. terreus* [8] and *A. tubingensis* [7]. In *A. nidulans* xylose does not induce xylanolytic activity [22], suggesting the existence of different mechanisms of enzymatic induction in the *Aspergillus* genus.

The regulation studies carried out with *Aspergillus phoenicis* showed reduction in the enzymatic levels when glucose was added to xylan or xylose, which was reverted by addition of cAMP or dibutyryl-cAMP. Similar results were described by Ghosh and Nanda [6] for *A. sydowii*, that is, glucose inhibited xylanase production, and the inhibition

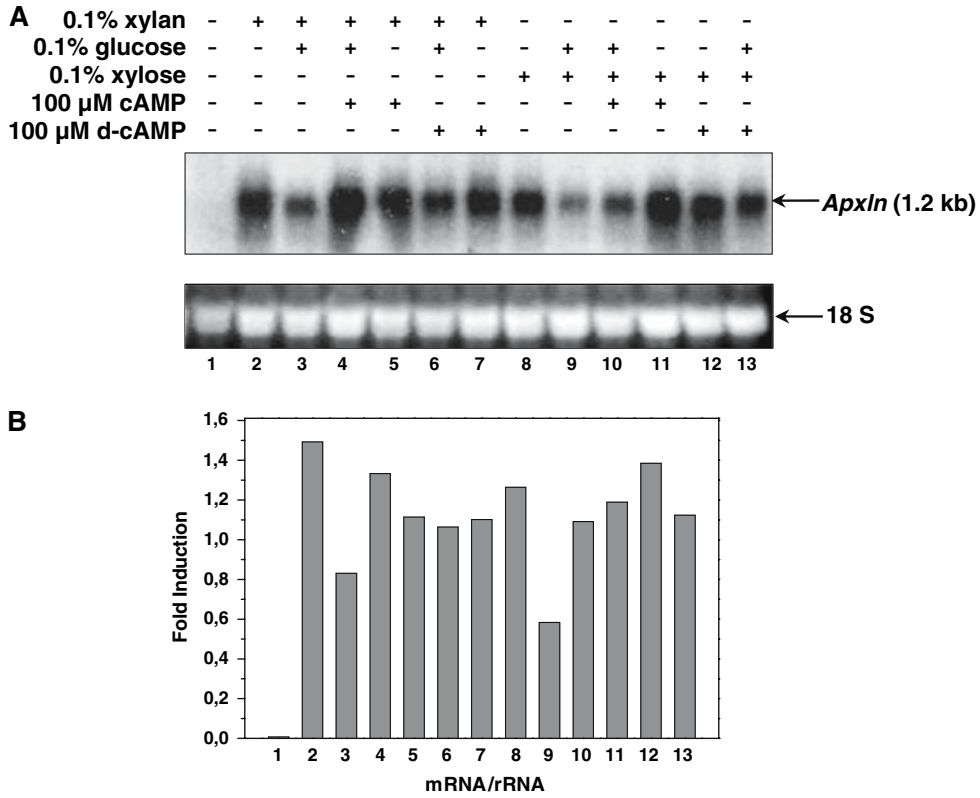

Fig. 3 Northern blot hybridization of the *A. phoenicis* gene *Apxln*. Analysis was performed by using 10 µg of total RNA extracted from cells exposed to different growth conditions. **a** *Upper panel* shows the autoradiography using the genomic DNA fragment of 300 bp as probe. *Lower panel* shows the 18S rRNA used as quantitative loading control. **b** Densitometry analysis of the relative induction. *Lane 1*, total RNA extracted before treatment. *Lanes 2* to *13*, total RNA extracted after growth in different culture conditions; *lane 2*, 0.1% xylan; *lane 3*, 0.1% xylan + 0.1% glucose; *lane 4*, 0.1% xylan + 0.1% glucose + 100 µM cAMP; *lane 5*, 0.1% xylan + 100 µM cAMP; *lane 6*, 0.1% xylan + 0,1% glucose + 100 µM dibutyryl-cAMP; *lane 7*, 0.1% xylan + 100 µM dibutyryl-cAMP; *lane 8*, 0.1% xylose; *lane 9*, 0.1% xylose + 0.1% glucose; *lane 10*, 0.1% xylose + 0.1% glucose + 100 µM cAMP; *lane 11*, 0.1% xylose + 100 µM cAMP; *lane 12*, 0.1% xylose + 100 µM dibutyryl-cAMP; *lane 13*, 0.1% xylose + 0.1% glucose + 100 µM dibutyryl-cAMP

was reverted by addition of exogenous cAMP. In *Streptomyces* sp. cAMP causes a repression in xylanase synthesis [18]. Repression by glucose seems to be a common phenomenon observed on xylanases biosynthesis [16], but a few studies reported the influence of cAMP in this process [10].

As cited by Orejas et al. [20] and Ruijter and Visser [26] in *A. nidulans* and *A. niger*, in the presence of glucose, the CREA protein inhibits gene transcription probably due its joining to the promoter region from xylanolytic genes. The CREA protein from *A. nidulans* presents specific regions (as zinc fingers) that probably allow its binding to gene promoter sequences inhibiting this gene expression. Probably, when glucose is present occurs an increasing of CREA affinity by DNA and when the glucose levels decrease, also decreases the CREA affinity for its binding sites and the carbon repression reverts [26].

Not only CREA protein participates in the carbon repression mechanisms, but also cyclic AMP (cAMP) seems to be involved. In *S. cerevisiae* the addition of glucose to cells resulted in a transient level of cAMP and, this transient increase seems to be a signal of the presence of easily fermentable carbon sources in order to change the metabolism accordingly [32]. In *A. nidulans* the level of cAMP increases when glucose is present and decreases after glucose depletion [40]. The addition of cAMP also resulted in a two-fold decreased of glucoamylase mRNA level produced by *A. awamori* [1].

The physiological results observed in this work for *A. phoenicis* were confirmed at molecular level by analysing the presence of the xylanase gene transcript under conditions known to induce or repress xylanase activity. It was observed that glucose decreased the mRNA levels, and that the effect was abolished in the presence of cyclic nucleotides. This is an important result, since few studies have carefully shown the transcription regulation of xylanase, and indicates that, in *A. phoenicis*, cyclic nucleotide regulates xylanase gene expression at transcription level.

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