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NOTE

Glucose-induced production of a *Penicillium purpurogenum* xylanase by *Aspergillus nidulans*

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Abstract The heterologous secretion of xylanase B from *Penicillium purpurogenum* using glucose as inducer was performed in *Aspergillus nidulans*. For this purpose, plasmid pEVXB, containing the xylanase B cDNA (including its own signal peptide) under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter, was constructed and used to transform *A. nidulans*. Analysis of transformed clones showed that several of them secreted extracellular xylanase activity when grown in a medium containing glucose. The clone showing the highest xylanase activity was chosen for further work. When this clone was grown on glucose, xylanase activity (0.72 U/ml), was detected in the culture supernatant. This was confirmed by a zymogram analysis and by the amplification of *xynB* cDNA from this clone. To our knowledge, this is the first example of the production of a xylanase from *Penicillium* in heterologous fungal hosts using glucose as inducer.

Keywords Glucose induction · Heterologous expression · *Penicillium purpurogenum* · Xylanase

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Xylan, the main polysaccharide from hemicelluloses, is degraded by an array of enzymes called xylanases. Among them are endoxylanases, which hydrolyze the main chain of xylan to oligosaccharides, which in turn are degraded to xylose by β-xylosidase (Chávez et al. 2006 and references therein). Xylanases have great biotechnological potential in the development of environment-friendly technologies in the paper and pulp industry, and for the generation of liquid fuels and chemicals from lignocellulose, but these potential biotechnological applications require cellulase-free xylanases (Kurzatkowski et al. 1996; Ahmed et al. 2009). However, most xylanolytic organisms, including filamentous fungi, simultaneously produce xylanolytic and cellulolytic enzymes (Stricker et al. 2008; Noguchi et al. 2009).

We are currently studying the xylanolytic enzyme system from *Penicillium purpurogenum*. This fungus co-produces several endoxylanases (Belancic et al. 1995) and cellulases (Steiner et al. 1994) when the fungus is grown on media containing inducers such as xylan. Thus, to selectively produce *P. purpurogenum* cellulase-free endoxylanases, recombinant DNA technology can be utilized. Since the production of xylanases and cellulases is repressed by glucose, a suitable strategy could be the use of a glucose-induced promoter that ensures the production of a specific endoxylanase with the simultaneous repression of cellulases and other xylanases (Kurzatkowski et al. 1996). However, there is no a homologous experimental system available in *P. purpurogenum* to produce recombinant proteins in the conditions described. Thus, to overcome this difficulty, a heterologous approach can be used. The availability of the glucose-induced glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter from *Aspergillus nidulans* (Punt et al. 1987) would make it possible to produce in this heterologous host a specific xylanase from *P. purpurogenum* using glucose. In the genus *Penicillium*,

Table 1 Xylanase activity of culture supernatants of *Aspergillus nidulans* transformants and *Penicillium purpurogenum*

Organism	Carbon source	Enzymatic activity (U/ml)
A. nidulans pEVXB clone 1	Glucose	0.72 ± 0.06
A. nidulans pScaI (control)	Glucose	Not detected
P. purpurogenum	Oat spelts xylan	24.0 ± 2.0

Xylanase activity of *P. purpurogenum* was induced after 6 days of growth in liquid cultures according to Belancic et al. (1995). *A. nidulans* transformants were grown for 4 days on modified liquid Czapeck medium (1% glucose, 0.2% $NaNO_3$, 0.05% K_2HPO_4, 0.05% $MgSO_4 \times 7H_2O$, 0.001% $FeSO_4 \times 7H_2O$, and 0.0002% *p*-amino benzoic acid). The remaining glucose was eliminated from culture supernatants by Amicon ultrafiltration (3 kDa cutoff membrane) against sodium citrate buffer 50 mM pH 5.3 before the xylanase and zymogram assays. The final volume of concentration was 5 mL. Xylanase activity was assayed using the dinitrosalicylate method (Bailey et al. 1992). Enzymatic activity is the average of three independent experiments with the respective standard error

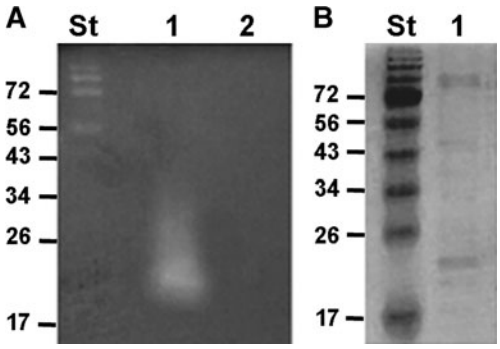

Fig. 1 **a** Zymogram showing xylanase B (XynB) activity secreted by *Aspergillus nidulans* clone 1 grown in a medium containing glucose. The zymogram was carried out using culture supernatants of *A. nidulans* clone 1 (*lane 1*); a band of the expected size for xylanase B (around 23 kDa) was obtained based on the pre-stained standards used in the gel. No activity was detected using the supernatants from the *A. nidulans* control transformed with empty pScaI plasmid (*lane 2*). The zymogram was carried out according to Chávez et al. (2002). **b** SDS-PAGE in the absence of xylan showing the protein pattern of clone 1 (*lane 1*). *St* pre-stained molecular weight standards (Fermentas). Sizes are indicated in kDa

examples of production of active recombinant xylanases are scarce and there are no examples of production of xylanases from *Penicillium* origin in heterologous fungal hosts. In this report, we describe the heterologous production of xylanase B from *P. purpurogenum* in *A. nidulans* using glucose as inducer.

Strains used in this work were *P. purpurogenum* ATCC MYA-38 and *A. nidulans* A722, a *pyrG*-deficient strain (Fungal Genetics Stock Center, Kansas, USA). *P. purpurogenum* was grown on xylan, its total RNA was extracted, and cDNA was synthesized as previously described (Díaz et al. 1997). The *xynB* cDNA amplification, including its own signal peptide coding sequence, was carried out using

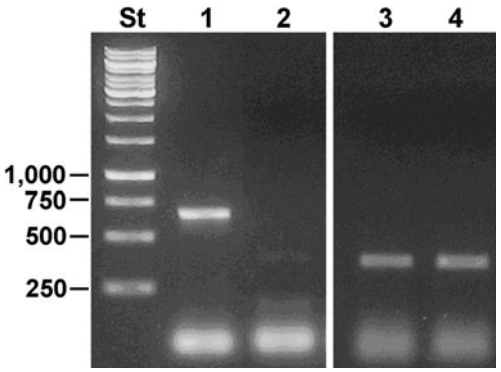

Fig. 2 Expression of *xynB* cDNA induced by glucose in *Aspergillus nidulans* clone 1. Clone 1 and the control (transformant without insert) were grown as described in Table 1. Mycelia were washed, ground in liquid nitrogen, and total RNA was extracted using the "RNeasy Plant" kit (Qiagen) according to the manufacturer's instruction. cDNA was synthesized using the "SuperScript RT" kit (Invitrogen) and *xynB* cDNA was amplified by PCR using primers XYNBFW and XYNBRV (see text for sequence and PCR conditions). PCR products were subjected to electrophoresis in agarose gels. The *xynB* cDNA amplified from clone 1 was observed at the expected size (626 bp; *lane 1*), but was absent in the *A. nidulans* control transformed with empty pScaI plasmid (*lane 2*). A segment of the constitutive GAPDH gene (around 350 bp) was amplified from clone 1 (*lane 3*) and from the *A. nidulans* control transformant (*lane 4*), confirming the specificity of *xynB* induction. GAPDH was amplified in the same experiment using primers GPDFW and GPDRV described by Espinosa et al. (2011). *St* GeneRuler 1 kb DNA ladder standard (Fermentas). Sizes are indicated (bp)

the protocol and PCR program previously described (Díaz et al. 1997). Primers XYNBFW (5′AGACTTAGTACTATGAAGGTCACTGC3′; *Sca*I restriction site underlined) and XYNBRV (5′AGACTTGGATCCCTAAGCTGAGACCGTGA3′; *Bam*HI restriction site underlined) were used.

For the expression of *P. purpurogenum* xylanase B in *A. nidulans*, plasmid pEVXB was constructed from the previously described plasmid pAN7-1 (Punt et al. 1987). pAN7-1 contains the hygromycin resistance gene (*hph*) under the control of the *gpdA* promoter from *A. nidulans*, and the *trpC* terminator. The *hph* gene is flanked by *Sca*I and *Bam*HI sites, and can be removed using these enzymes; however, another *Sca*I site is present in the ampicillin resistance gene (Ap^r) present in the plasmid, so this last site must be previously eliminated before removing the *hph* gene. Thus, pEVXB was constructed as follows: (1) the *Sca*I site located in the Ap^r gene in plasmid pAN7-1 was eliminated by site-directed mutagenesis using the QuickChange Site-directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions; (2) The resulting plasmid (named pScaI) was digested with *Sca*I and *Bam*HI, and thus the *hph* gene was eliminated. In its place, the *xynB* cDNA previously amplified (see above) digested with the same restriction enzymes, was cloned. As result, pEVXB contains the *xynB* cDNA located under the

control of the *gpdA* promoter from *A. nidulans*, followed by the *trpC* terminator.

A. nidulans A722 was co-transformed with pEVXB and ppyrG (containing the selection marker *pyrG*, Fungal Genetics Stock Center, Kansas, USA) according to Navarrete et al. (2009), and transformants were selected in a suitable medium lacking uridine. Several of these transformants were grown on glucose as the only carbon source and analyzed. One of them, named clone 1, showed the highest relative activity and was selected for further characterization. Table 1 shows xylanase activity production by *A. nidulans* clone 1 when grown on glucose. Since xylanase production in *A. nidulans* (and fungi in general) is repressed by glucose, this activity must correspond to xylanase B, under the control of the glycolytic *gpdA* promoter. This was confirmed by the absence of xylanasic activity in the control transformed with empty pScaI plasmid (Table 1). The xylanase activity of clone 1 in culture supernatants is 0.72 U/ml, about 33 times lower than the total xylanase activity detected in *P. purpurogenum* culture supernatants when grown in the presence of a suitable inducer (Table 1). Several facts may explain this difference in activities. In contrast to *A. nidulans* clone 1, whose activity corresponds to the production of the single secreted xylanase B, the xylanasic activity measured in *P. purpurogenum* includes several xylanase isoforms (Chávez et al. 2002). On the other hand, inefficient secretion of proteins induced by glucose has been previously observed in *Aspergillus* (Punt et al. 1991), and although the reason for this is unknown, is has been postulated that the so-called "constitutive secretory pathway" has a limited capability for the secretion of proteins induced by glucose (Kurzatkowski et al. 1996). In addition, the use of the own signal peptide of *P. purpurogenum* xylanase B may contribute to the low level of secretion. To obtain the suitable secretion of recombinant proteins in *Aspergillus*, other authors have used optimized signal peptides (Cardoza et al. 2003). Finally, extracellular heterologous proteins are readily degraded by *Aspergillus* supernatants by a combination of high medium acidification and the production of potent extracellular proteases (de Vries et al. 2004).

The zymogram of *A. nidulans* clone 1 supernatant confirmed the presence of an active xylanase with a size similar to that of xylanase B (23 kDa; Belancic et al. 1995). This band is absent in the control transformed with empty pScaI plasmid (Fig. 1). On the other hand, a RT-PCR experiment was carried out using RNA from the same clone. As expected, cDNA from *xynB* was amplified only in clone 1, but was absent in the control transformed with empty pScaI plasmid (Fig. 2). These results confirm that *xynB* is expressed in clone 1 when grown on glucose.

In summary, by means of recombinant DNA technologies, it is feasible to produce a single *P. purpurogenum* xylanase in fungi using glucose as inducer. With the aim of obtaining enough recombinant protein for its purification, currently we are trying to improve our yields by the use of more suitable hosts (including the own *P. purpurogenum*) and other promoters and signal peptides.

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