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pH regulation of enzyme production in *Aspergillus nidulans* growing in aerobic batch fermenter

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Abstract

The physiological response to ambient pH on the regulation of the production of the xylanolytic enzyme complex was investigated using two modified strains of *Aspergillus nidulans.* Transcriptional gene fusions were constructed between the promoters *xlnA* and *xlnB* (alkaline and acid expressed, respectively) and the *Aspergillus niger goxC* (encoding glucose oxidase), and *A. nidulans* transformants possessing single integrations at the *argB* locus were selected. Changing the pH from 5.5 to 4.5 or 7 after induction resulted in differential expression of the homologous and heterologous proteins. Thus, versatile industrial strains capable of differentially producing mixtures of extracellular enzymes in response to ambient pH can be produced.

Introduction

Enzymes from the filamentous fungi, *Aspergillus* and *Trichoderma*, are widely used in the agro-food industry. For production purposes, genetic engineering techniques have been applied to construct new fungal strains producing increased amounts of the secreted enzyme (Teuber 1993). Nevertheless, secretion of heterologous proteins in fungi is much less efficient than that of homologous proteins (Calmels *et al.* 1991, Jeenes *et al.* 1991, Archer *et al.* 1994).

The ascomycete *Aspergillus nidulans* is a model organism for the study of gene regulation in filamentous fungi and is related to the industrial fungus *Aspergillus niger* (van Brunt, 1986).When *A. nidulans* is grown on xylan as sole carbon source it produces a xylanolytic complex composed of at least three different endo-β-(1,4)-xylanases (X_{22}, X_{24} and X_{34}, the subscript numbers refer to the molecular masses in kDa) and one β-xylosidase (Fernández-Espinar *et al.* 1992, Piñaga *et al.* 1994, Kumar & Ramón 1996). Two of these enzymes (X_{22} and X_{24}) are of interest in wine and bread production, respectively (Monfort *et al.* 1996, Ganga *et al.* 1999, 2001). The four enzymes have been purified to homogeneity and the corresponding genes have been cloned and sequenced (Fernández-Espinar *et al.* 1993, 1994, 1996, Kumar & Ramón 1996, Pérez-González *et al.* 1996, 1998, MacCabe *et al.* 1996).

Regulatory studies have demonstrated that the synthesis of the xylanolytic complex is controlled at the level of transcription by at least three different mechanisms: (i) specific induction by xylan or xylose, (ii) carbon catabolite repression mediated by the transcriptional factor CreA, and (iii) ambient pH regulation controlled by the PacC transcriptional factor (Piñaga

et al. 1994, MacCabe *et al.* 1996, 1998, Pérez-González *et al.* 1998, MacCabe & Ramón 2001). With regard to CreA control, the promoters of the *xlnA* and *xlnB* genes (coding for X_{22} and X_{24}, respectively) have been molecularly dissected and the *in vivo* CreA binding sites have been defined (Orejas *et al.* 1999, 2001). The genes have opposite patterns of expression with respect to ambient pH (MacCabe *et al.* 1998). The principal goal of the present study was to use the ambient pH response properties of the *xlnA* and *xlnB* promoters as regulatory signals in pilot plant fermentations. To this end, we constructed gene fusions of both promoters with the *Aspergillus niger goxC* gene which encodes glucose oxidase, an enzyme of relevance to the food industry. This article reports analysis of fermentations conducted by *A. nidulans* transformants containing the corresponding gene fusion as a single integration event at the *argB* gene *locus.*

Materials and methods

Strains and culture conditions

The *A. nidulans* (*argB2*, *metG1*, *biA1*) strain was used as the recipient for transformations. Different arginine prototrophic transformants of *A. nidulans* carrying the *goxC* gene under the control of the *xlnA* ($xlnA_p$) or the *xlnB* ($xlnB_p$) gene promoters were selected. Strains sVAL039 (*argB2/argB*$^+$, *metG1, biA1,* $xlnA_p$*::goxC*) and sVAL040 (*argB2/argB*$^+$, *metG1, biA1,* $xlnB_p$*::goxC*) were constructed during previous work (Orejas *et al.* 1999, 2001). Fermentation growth medium was minimal selective medium (MSA) (Cove 1966), supplemented with 6.8 g KH_2PO_4 l^{-1}, 5 g NH_4NO_3 l^{-1}, 1.5 g Casamino acids l^{-1}, 100 mg methionine l^{-1}, 100 μg biotin l^{-1} and 10 g fructose l^{-1} as sole carbon source. In each case, the reactor was inoculated with a 6 day-old spore suspension of the original strain (in sterile distilled water with 0.01% (w/v) Tween 80) to give 10^6 conidiospores ml^{-1}. After growth of mycelial biomass for 18 h, xylose was added at 1% (w/v) from a 20% (w/v) sterile stock solution. Samples were taken periodically after addition of the carbon source until the xylose was almost completely consumed (approximately 50 h). Growth experiments were conducted with 5 l reactors (Biostat B, Germany) with 2 l of medium. The pH, temperature and dissolved O_2 controllers were set at 5.5, 37 °C and 40%, respectively. Prior to addition of inducer the pH was shifted to either 4.5 $\pm$ 0.1 or 7.0 $\pm$ 0.1 by automatic addition of 1.5 NH_4OH or 0.1 M H_3PO_4. The fermenters were stirred at 250 rpm during the overnight (18 h) pre-growth with fructose (1% w/v) and shifted to 550 rpm during the induction with xylose (1% w/v). Aeration (40% saturation) was controlled by the airflow through the medium.

Enzyme determinations

Glucose oxidase C (β-D-glucose-1-oxidereductase EC 1.1.3.4) (GOX), α-L-arabinofuranosidase, endo-β-(1-4)-endoglucanase, β-xylosidase and endo-β-(1-4)-xylanases activities were determined in culture supernatants. All determinations were done in triplicate. Endo-β-(1-4)-xylanase activity was assayed using carboxymethyl-cellulose (CMC) and birchwood xylan as substrates. CMCase and xylanase activities were determined by incubating a 500 μl reaction mixture containing 50 μl appropriately diluted enzyme and 100 μl 2.8% (w/v) CMC or 3% (w/v) xylan suspension in 50 mM sodium acetate buffer (pH 4.5) for 25 min at 50 °C. The reaction was stopped with 750 μl 1% (w/v) 3,5 dinitrosalicylic acid and the reaction mixture was boiled for 10 min and subsequently cooled in ice. Absorbance of the supernatant was measured at 590 nm. One unit of CMCase or xylanase activity was defined as the amount of enzyme that released 1 μmol glucose or xylose equivalents min^{-1} under the established conditions. The hydrolytic enzyme activities β-xylosidase and α-L-arabinofuranosidase were determined by measuring the release of *p*-nitrophenol (pNP) at 405 nm during incubation in 50 mM citric acid or Na_2HPO_4 buffer solution (pH 4 or pH 5, respectively) at 30 °C for 15 min in the presence of the substrates pNP-Xylose (0.04 w/v) or pNP-Arabinose (0.04 w/v), respectively. Reactions were stopped with 250 μl of 0.25 M Na_2CO_3. All reactions were carried out in 500 μl final volume and contained 50 μl enzyme. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol pNP min^{-1} under the established conditions. GOX activity was measured as described by Orejas *et al.* (1999). One unit of GOX was defined as the amount of enzyme that released 1 μmol glucose min^{-1} under the established conditions.

Protein and biomass determination

Protein concentrations were measured by the Bradford method using lysozyme as standard (Bradford 1976). Biomass dry weight was determined by filtering 10 ml

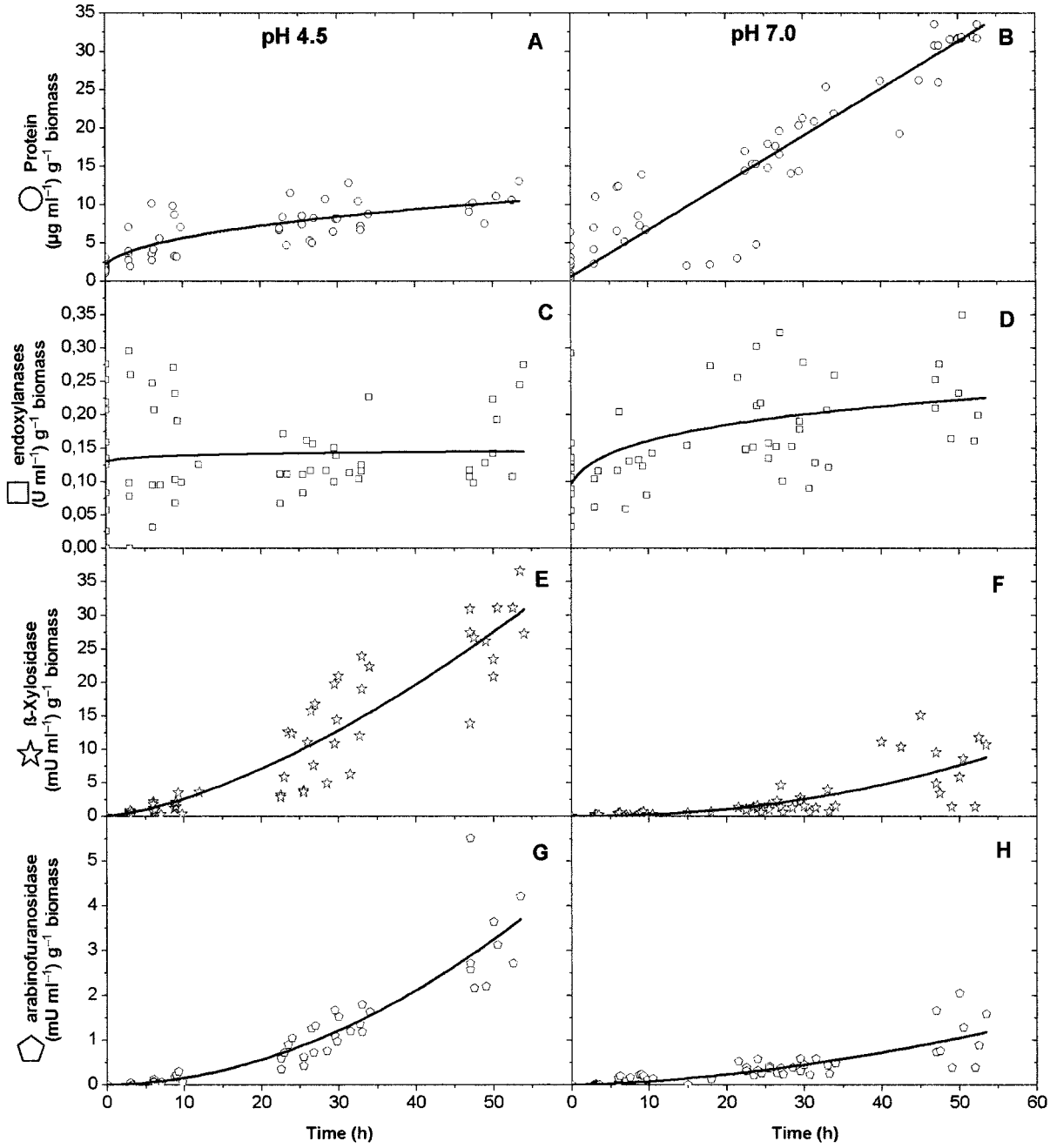

Fig. 1. Fermentation profile experiments for *A. nidulans* OTCase (*argB2*, *metG1*, *biA1*) in a 5-l bioreactor at acid (4.5) and alkaline (7) controlled pH. Mycelial biomass was obtained by growth on fructose (1% w/v) for 18 h. At zero time xylose was added to a final concentration in the medium of 1% (w/v). (A) and (B) Protein concentration of culture filtrates; (C) and (D) endo-β-(1-4)-xylanases activity; (E) and (F) β-xylosidase activity; (G) and (H) α-L-arabinofuranosidase activity.

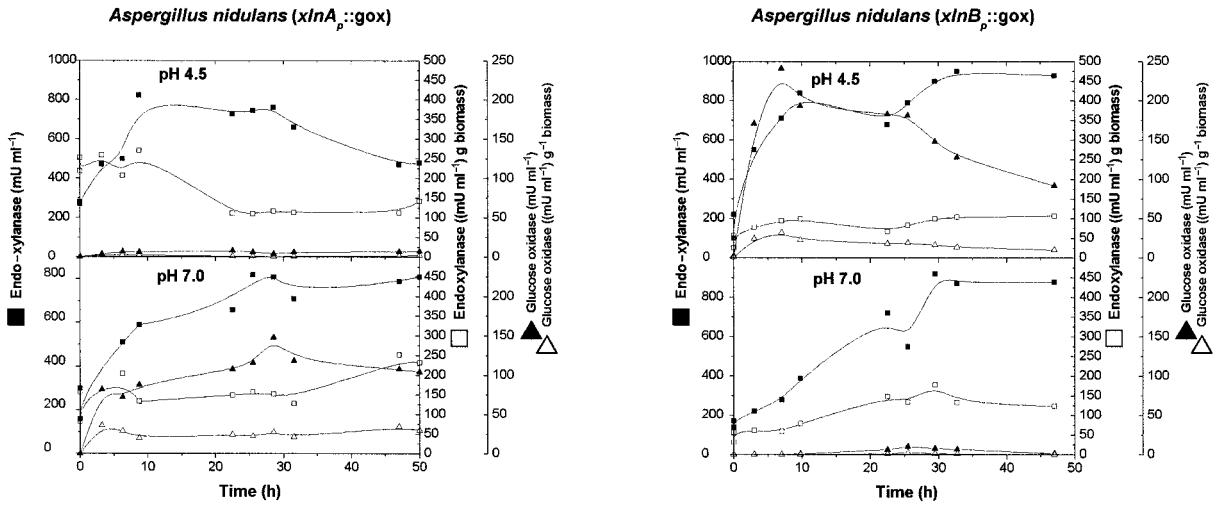

Fig. 2. Fermentation profile experiments for *A. nidulans* sVAL039 and sVAL040 strains in a 5-l bioreactor at acid (4.5) and alkaline (7) controlled pH. Mycelial biomass was obtained by growth on fructose (1% w/v) for 18 h. At zero time xylose was added to a final concentration in the medium of 1% (w/v). Secretion of homologous endo-β-(1-4)-xylanase activity and heterologous GOX activity was determined.

of culture sample through a Whatman No. 3 filter followed by washing with distilled water and then drying to constant weight at 80 °C. The filtrates collected were stored at −20 °C until further analysis.

Results and discussion

The xylanolytic complex of *A. nidulans* has been extensively characterized at the molecular level (MacCabe *et al.* 2001). To characterize the physiological response of *A. nidulans* in pilot plant fermentation using xylose as inducer, this fungus was cultivated under the inducing conditions described in Materials and methods. Figure 1 shows the results of six independent assays for two pH conditions studied (acid or alkaline). It is important to note that these data may be influenced by the form of growth of the microorganism. Since the filamentous growth resulted in the tendency of organism to accumulate on the walls and sensors of the culture vessel thus acting as a filter, allowing medium to flow through, while mycelium partially remained. Nevertheless, it is clear that after induction by xylose at different pH values, the rate of protein secretion in *A. nidulans* is higher when the fungus is grown under alkaline inducing conditions (Figures 1A and 1B). This is a different situation to that described for other aspergilli in which acidic pH found to be the optimal condition for protein secretion (Jeenes *et al.* 1991). The maximum rate of protein secretion under our conditions was 30 μg protein g^{-1} mycelia·ml at alkaline pH. In comparison with other filamentous fungi such as *A. niger* or *Trichoderma reesei*, this is a very low rate reinforcing the idea that *A. nidulans* is not an optimal producer for industrial purposes.

Transcription of the *xlnA* and *xlnB* genes is enhanced by alkaline and acidic pH, respectively (MacCabe *et al.* 1998). However, the total amount of endo-xylanase activity in culture supernatants was not influenced by the pH of the media (Figures 1C and 1D). As xylanases X_{22} and X_{24} are major and minor components, respectively, of the *A. nidulans* xylanolytic complex, this result could indicate a major role for the third component, the X_{34} xylanase, the synthesis of which is not apparently under pH control (MacCabe & Ramón 2001).

The amounts of α-L-arabinofuranosidase and β-xylosidase, being the other enzymatic components of the xylanolytic complex, were favored by growth at acidic pH (Figure 1E–H). This was particularly important in the case of β-xylosidase because the obtained results defined adequate conditions for the production of this enzyme.

In order to assay the relative extracellular production of different components of the *A. nidulans* xylanolytic complex, transformant strains containing single copies of either the *xlnA$_p$::goxC* or *xlnB$_p$::goxC* gene fusions integrated at the *argB2 locus* were studied. These transformant strains defined conditions of expression by induction with xylose and acidic pH (*xlnB$_p$::goxC*) or xylose and alkaline pH (*xlnA$_p$::goxC*), respectively. As can be seen in Fig-

ure 2, depending on the *xln* promoter used, GOX was detected under acidic or alkaline conditions following kinetics similar to those of the appearance of the endoxylanases. In the case of the $xlnA_p$*::goxC* construction, GOX was detected as soon as xylose was added to the alkaline medium and remained stable in the culture supernatant until the end of the fermentation. In contrast, in the strain containing the $xlnB_p$*::goxC* construction, GOX was detected during the first 10 h of induction at acidic pH and markedly decreased after 50 h of batch culture.

The amount of GOX secreted relative to biomass was nearly the same in both types of transformants when grown at the corresponding optimal pH values. This is a surprising result because previous data indicate that *xlnB* transcript accumulation is less than that of the *xlnA* gene (MacCabe *et al.* 1998).

Using the results presented in this work it is possible to define pH batch conditions in which genes under the control of *A. nidulans xln* gene promoters are differentially expressed during fermentation. In this context, our results demonstrate the possibility of generating versatile industrial strains which can produce complex extracellular enzyme mixtures by ambient pH regulation. In addition, the recent cloning of the gene coding for the *A. nidulans* regulator of the synthesis of the xylanolytic complex (Tamayo & Orejas, personal communication) will permit the construction of strains in which the transcription of the genes under its control will be increased.

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