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pH Control of the production of recombinant glucose oxidase in *Aspergillus nidulans*

R. Luque[1,2], M. Orejas[3], N.I. Perotti[1,2], D. Ramón[3,4] and M.E. Lucca[1,5]

[1]Planta Piloto de Procesos Industriales Microbiológicos PROIMI, Consejo Nacional de Investigaciones Científicas y Técnicas, Tucumán, [2]Cátedra de Microbiología General e Industrial, Facultad de Ciencias Exactas y Tecnología, Universidad Nacional de Tucumán, Tucumán, Argentina, [3]Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas, Valencia, [4]Departamento de Medicina Preventiva y Salud Pública, Bromatología, Toxicología y Medicina Legal, Facultad de Farmacia, Universitàt de Valencia, Valencia, Spain, and [5]Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán, Argentina

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ABSTRACT

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Aims: Recombinant *Aspergillus nidulans* sVAL040, capable of synthesizing and secreting glucose oxidase derived from *Aspergillus niger* was used to study the influence of pH and carbon source on enzyme production.

Methods and Results: Glucose oxidase gene (*goxC*) was expressed under transcriptional regulation by using the promoter of *A. nidulans xlnB* gene (encoding an acidic xylanase). A maximum specific glucose oxidase activity of approx. 10 U mg^{-1} protein and a maximum volumetric productivity of 29·9 U l^{-1} h^{-1} were obtained at pH 5·5, after 80 h of growth by using xylose as inducer. Enzyme volumetric productivity increased when xylans were used instead of xylose; however, specific glucose oxidase activity did not differ significantly.

Conclusions: Specific GOX activity obtained at pH 5·5 are two to three times more than those previously described for *goxC* multicopy transformants of *A. nidulans*. Xylans were a more powerful inducer than xylose although fungal growth was lower when the polymers were used.

Significance and Impact of the Study: The obtained results by using *xlnB* promoter in *A. nidulans* could be useful in improving heterologous enzyme production by using genetic- and process-engineering strategies.

Keywords: *Aspergillus nidulans*, glucose oxidase, pH regulation.

INTRODUCTION

Filamentous fungi are of considerable biotechnological importance as producers of a broad range of metabolites and enzymes. Many species of these micro-organisms can produce and secrete large quantities of proteins, which has resulted in an increasing interest in studying and using filamentous fungi in industrial processes. In general, many enzymatic activities of industrial interest are obtained from fungal cultures as components in mixtures containing other nonrequired activities. In order to improve the production levels of specific extracellular enzymatic activities a number of molecular-biological strategies have been developed. The understanding of the molecular basis of gene expression in filamentous fungi is helpful to design and optimize these strategies. Fungal species that present known regulatory mechanisms of gene expression were chosen as appropriate host expression systems to produce high levels of a target protein with high specific activity.

Aspergillus nidulans is a useful model organism in metabolic and developmental regulation studies, although not employed in industry. This fungus is able to use a wide variety of nitrogen and carbon sources and grow over a wide range of pH values. Therefore, syntheses of a number of permeases, secreted enzymes and exported metabolites are

Correspondence to: María Ester Lucca, Av. Belgrano y Pje Caseros, Tucumán, 4000, Argentina (e-mail: proimiservicios@arnet.com.ar).

appropriately regulated by ambient conditions. Most native proteins secreted by *A. nidulans* are catabolic enzymes, essential for growth on complex substrates. Among these, the xylanolytic complex is responsible for the degradation of arabinoxylan (Fernández-Espinar *et al.* 1992). *A. nidulans* produces three xylanases when grown on D-xylose as the sole carbon source: one minor xylanase (X_{24}) encoded by *xlnB* gene, and two major xylanases (X_{22} and X_{34}) encoded by the genes *xlnA* and *xlnC*, respectively. The transcription of *xlnB* gene of *A. nidulans* that encodes an acidic endo-1,4-β-xylanase (X_{24}) is regulated by at least three mechanisms: specific induction by xylan or xylose, carbon catabolite repression and regulation by ambient pH (MacCabe *et al.* 1998; Orejas *et al.* 1999, 2001). It appears to be attractive to exploit these regulatory mechanisms to produce heterologous enzymes in specific conditions that improve yields, purity and stability.

Glucose oxidase (GOX, β-D-glucose:oxygen, 1-oxidoreductase, EC 1.1.3.4) catalyses the oxidation of β-D glucose to D-glucono-δ lactone and H_2O_2 using molecular oxygen as electron acceptor. GOX is used in the food industry for removal of residual glucose and as an antioxidant in the production of beer and soft drinks. The enzyme forms the basis of methods for the detection and quantitation of glucose in industrial solutions and body fluids. GOX is a glycosylated, dimeric flavoprotein with a molecular mass of 150 000 Da (Pazur *et al.* 1984). The enzyme was first isolated from *A. niger* and, although it is present in a number of *Aspergillus* and *Penicillium* species, the highest levels are observed in strains of this species (Fiedurek *et al.* 1986). Relatively little is known about the regulation of GOX synthesis and secretion. For industrial purposes it is treated as an intracellular enzyme requiring disruption of the mycelium as a first step in its preparation, increasing significantly purification costs.

This paper deal with the study of the influence of pH and carbon source on extracellular GOX production in a recombinant *A. nidulans* strain, capable of synthesizing and secreting GOX derived from *A. niger* under *xlnB* gene promoter control.

MATERIALS AND METHODS

Fungal strains and media

Aspergillus nidulans sVAL040 (*xlnBp::goxC*, *argB2/argB*$^+$, *metG1*, *biA1*) was employed as the model system in this study. The sVAL040 strain was previously obtained by transformation of *A. nidulans argB*$^-$ (*argB2*, *metG1*, *biA1)* with plasmid pVAL040 (Orejas *et al.* 2001) and selection for arginine prototrophy. Single-copy integration at the *argB* site located at chromosome III was established using Southern blotting.

The media used for fungal growth, based on *Aspergillus* minimal medium (Pontecorvo *et al.* 1953), contains (per litre): 10 g fructose, 1·5 g casaminoacids, 0·52 g KCl, 0·52 g $MgSO_4.7H_2O$, 1·52 g KH_2PO_4, 0·04 mg $Na_2B_4O_7$, 0·4 mg $CuSO_4$, 0·8 mg $FeSO_4.7H_2O$, 0·8 mg $MnSO_4$, 0·8 mg Na_2MoO_4 and 0·008 mg $ZnSO_4$. Xylose, birch wood xylan and oat spelt xylan used as inducers were from Sigma (St Louis, MO, USA).

Culture conditions

Studies of growth and enzyme production kinetics were carried out in a 2-l stirred-tank bioreactor (LH S 210, LH Fermentation Inc., Emeryville, CA, USA) with 1 l of working volume at 37°C and 350 rev min^{-1}. Dissolved oxygen was maintained at 20% saturation by supplying air automatically via a proportional, integrative and derivative (PID) controller. pH was adjusted with 0·2 mol l^{-1} NaOH and 0·2 mol l^{-1} HCl by using a PID controller.

Bioreactor cultures were inoculated with a 10-day-old spore suspension to give a final concentration of 10^6 spores ml^{-1}. *A. nidulans* sVAL040 was grown initially at pH 5·5 with fructose as carbon source during 15 h. Then, ambient pH was changed to different values and at 20 h of growth 10 g l^{-1} of the corresponding carbon source pulse was added to induce the expression of GOX.

Analytical determinations

Samples (5 ml) were periodically withdrawn and filtered to analyse GOX activity and total protein concentration. GOX activity was determined as in Bergmeyer (1974).The enzyme reaction was carried out in a final volume of 750 μl containing sodium phosphate buffer pH 6·0 20 mmol l^{-1}, *o*-dianisidine 0·7 mmol l^{-1} 1, D-glucose 100 mmol l^{-1}, horseradish peroxidase 20 μg ml^{-1} and 100 μl of the conveniently diluted sample. Reactions were performed for 10 min at 25°C and stopped with an equal volume of 4 mol l^{-1} HCl solution. Absorbance at 540 nm was measured. The value was converted to GOX activity (U ml^{-1}) by comparison with a standard curve of 0–0·05 (U ml^{-1}) prepared with commercially available GOX (Sigma). One unit of GOX activity was defined as the amount of enzyme required to oxidize 1 μmol of glucose per minute at 25°C and pH 6·0. Protein concentration was measured with the Coomassie dye-binding assay (Bradford 1976) using bovine serum albumin as a standard. For biomass determination, 10 ml of the culture broth was filtered, washed twice with distilled water and dried at 105°C until constant weight was achieved.

All determinations were made in triplicate and results reported are the average of two independent experiments.

RESULTS

Effect of ambient pH on extracellular production of GOX

The effect of ambient pH was studied in batch cultures using different pH values in stirred-tank bioreactors. In order to obtain fungal biomass, *A. nidulans* sVAL040 was pregrown at pH 5·5 using D-fructose 10 g l^{-1} as the sole carbon source. After 15 h of growth, the culture pH was changed to different pH values (3·5, 4·5, 5·5 and 6·5). At 20 h of cultivation D-fructose was depleted (data not shown) and a pulse of D-xylose 10 g l^{-1} was added to induce the expression of GOX. The influence of pH on growth kinetics is presented in Fig. 1a. Optimal cell growth after pulse addition was achieved at pH 5·5 (X_{max}, maximum dry cell weight of 8·7 g l^{-1} and μ, maximum specific growth rate of 0·08 h^{-1}). As it can be seen, X_{max} and μ were virtually unaffected at pH 6·5 (8·2 g l^{-1}, 0·08 h^{-1}) and 4·5 (7·3 g l^{-1}, 0·07 h^{-1}). How-

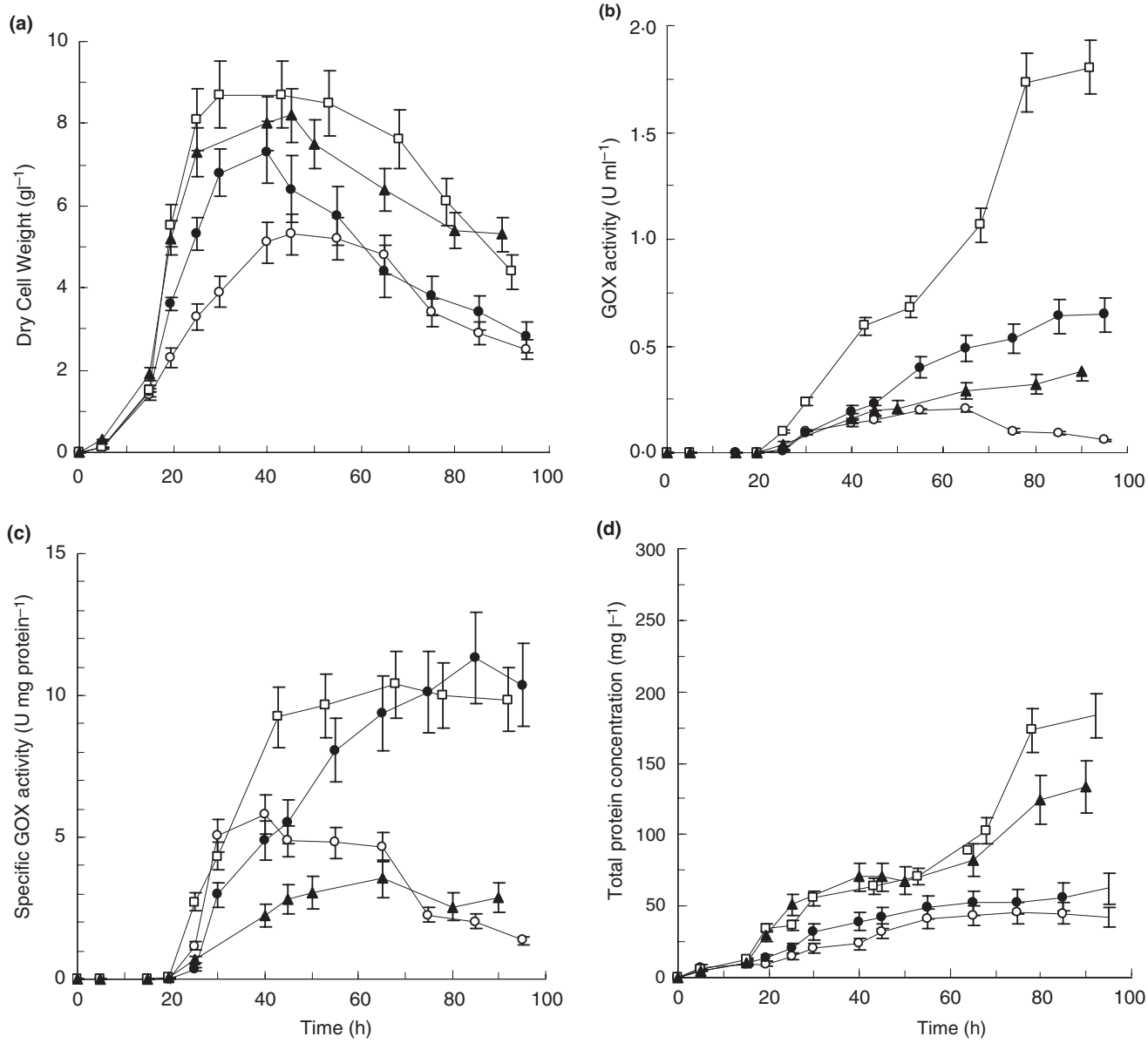

Fig. 1 Effect of ambient pH on GOX production. *A. nidulans* sVAL040 was first grown at pH 5.5 in a medium containing D-fructose as sole carbon source. After 15 h of growth ambient pH was changed, and at 20 h D-xylose (10 g l^{-1}) was added to induce GOX production. pH values tested: (○) 3.5, (●) 4.5, (□) 5.5 and (▲) 6.5. (a) Cell growth, (b) extracellular GOX activity, (c) specific GOX activity and (d) total protein concentration. Bar markers represent S.D. from mean values of three independent experiments

ever, at pH 3·5 X_{max} and μ decreased significantly (5·3 g l^{-1}, 0·04 h^{-1}) compared with those obtained at pH 5·5.

As expected, no extracellular GOX production was detected before D-xylose addition (Fig. 1b). Maximum extracellular GOX activity was obtained at pH 5·5 (1·8 U ml^{-1}, 80 h of growth). Markedly, lower GOX activities were observed at pH 3·5, 4·5 and 6·5. As shown in Fig. 1c, maximum specific activity was obtained at pH 4·5 (11·3 U mg^{-1} protein, 85 h of growth). At pH 5·5 similar values of specific GOX activity were reached before (9·3 U mg^{-1} protein, 45 h of growth). In addition, the highest volumetric productivity was obtained for the culture performed at pH 5·5 (29·9 U l^{-1} h^{-1}, 80 h of growth). Total amount of protein in culture medium is presented in Fig. 1d. Comparison of maximum specific GOX activities for the cultures revealed a greater amount of extracellular protein secreted at higher pH. It is interesting to note that the values of specific GOX activity obtained at pH 5·5 are

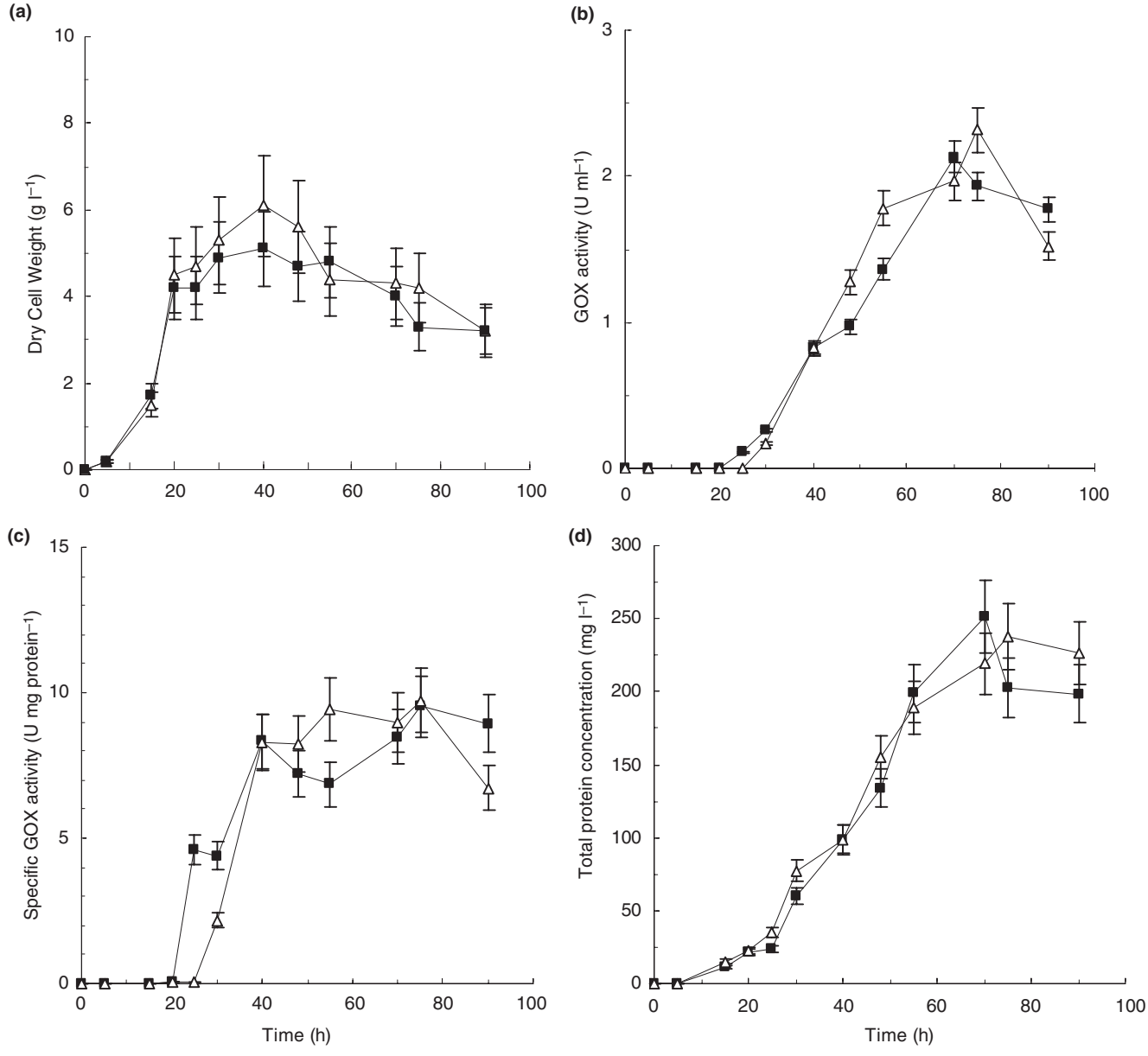

Fig. 2 Effect of carbon source on GOX production. Batch cultures were carried out at pH 5.5. *A. nidulans* sVAL040 was first grown in a medium containing D-fructose as sole carbon source. After 20 h of growth, xylans (10 g l^{-1}) were added to induce GOX production: (■) xylan from birch wood and (△) xylan from oat spelt. (a) Cell growth, (b) extracellular GOX activity, (c) specific GOX activity and (d) total protein concentration. Bar markers represent S.D. from mean values of three independent experiments

two to three times more than those previously described for *goxC* multicopy transformants of *A. nidulans* and similar to those described for multicopy transformants of *A. niger* (Whittington *et al.* 1990). Taking into account all these results, a pH value of 5·5 was chosen for the next set of experiments.

Effect of carbon source on extracellular production of glucose oxidase

Batch cultivations were carried out in stirred-tank bioreactor as described previously. Strain sVAL040 was pregrown with D-fructose 10 g l^{-1}. After 20 h of growth a pulse of the corresponding carbon source (oat spelt xylan and birch wood xylan) at 10 g l^{-1} concentration was added to induce the expression of recombinant GOX.

Figure 2(a) shows that cell mass diminished by 30% in cultures with xylan as carbon source. Induction with the polymers increased volumetric extracellular GOX production (Fig. 2b). Maximum GOX activity was obtained in the presence of oat spelt xylan as carbon source (2·3 U ml^{-1}, 80 h of growth). Similar results were obtained with birch wood xylan. Volumetric enzyme productivity increased when xylans were used instead of xylose, mainly at early stages of culture. After 55 h of growth GOX productivities obtained were 50·9, 39·0 and 20·7 U l^{-1} h^{-1} for cultures in the presence of oat spelt xylan, birch wood xylan and xylose, respectively (Fig. 2c). However, specific GOX activity did not differ significantly. As expected, total extracellular protein concentration increased in cultures containing xylan as inducer (Fig. 2d).

DISCUSSION

Aspergillus nidulans produces three xylanases when grown on D-xylose as the sole carbon source: one minor xylanase (X_{24}) encoded by *xlnB* gene, and two major xylanases (X_{22} and X_{34}) encoded by *xlnA* and *xlnC*, respectively. It has been demonstrated by MacCabe *et al.* (1998) that the expression of *xlnA* and *xlnB* are regulated by ambient pH via the wide-domain zinc finger transcription factor PacC. Specific transcription of *xlnA* and *xlnB* genes requires both the presence of an inducer and alkaline and acidic ambient pH, respectively. It is interesting to note that *A. nidulans* appears to lack detectable acid protease activity (van Kuyk *et al.* 2000) suggesting that the expression of heterologous protein at acidic pH might increase yields in enzyme production by preventing proteolysis. The results obtained in this study shows that fungal growth was markedly diminished at extremely acidic ambient pH and, therefore, enzyme production was lower in these conditions. However, it is possible to use *xlnB* promoter to produce heterologous proteins of fungal origin under pH conditions that may be favourable for their stability.

Although the production of xylanases in *A. nidulans* is induced by xylose, the latter is also a repressing carbon source at high concentrations and induction of extracellular xylanase activity by xylan is considerably stronger (Piñaga *et al.* 1994). However, results obtained with the strain sVAL040 indicated that growth with xylose was higher than those with xylans. In addition, higher amounts of extracellular protein were secreted by using polymers as carbon sources.

In conclusion, observed culture responses to induction and step changes in pH could be extremely useful in improving heterologous enzyme production by using genetic- and process-engineering strategies.

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