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Production of Extracellular Xylanases by *Penicillium janthinellum*

Effect of Selected Growth Conditions

EMILIA CUROTTO,*[,1] MARISOL CONCHA,[1] VICTORIANO CAMPOS,[2] ADRIANE M. F. MILAGRES,[3,4] AND NELSON DURAN[4]

[1]*Instituto de Quimica and* [2]*Instituto de Biologia, Universidad Católica de Valparaiso, Casilla 4059, Valparaiso, Chile;* [3]*Biotecnology and Chemistry Center, FAENQUIL, Lorena, S.P., Brazil; and* [4]*Instituto de Quimica, Biological Chemistry Laboratory, Universidades Estadual de Campinas, Campinas CEP, SP Brazil*

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ABSTRACT

Xylanase production by *Penicillium janthinellum* using 10–100 m*M* of 2,2-dimethylsuccinate (DMS) buffer, in a range of pH 4.5–6.0 was studied. The enzyme activity was enhanced using oat xylan as the carbon source. Under these conditions a culture produced 1.14 μmol/min (11.4 U/mL or 84.4 U/mg) of β-xylanase after 5 d of growth in a 10-m*M* buffer solution at pH 4.5. Protease was absent in the DMS buffer except when 100 m*M* phosphate buffer at pH 6.0 was used (4 U/mL). β-Xylosidase was only found at a pH of 4.5 in all the buffer concentrations. At a 50 m*M* DMS buffer concentration at pH 4.5 β-xylanases were induced by both oat and birch xylans, having a greater effect with oat spelt xylans.

Electrophoretic analyses showed that the birchwood xylan induction exhibited different proteins profiles. No β-xylosidase or β-glucosidase was induced until d 5. The β-xylanases were rapidly inactivated at 50°C, however, birch xylanase appeared to be more stable than oat xylanase.

*Author to whom all correspondence and reprint requests should be addressed.

Using oat xylan as an inductor, the β-xylosidase and β-glucosidase were 85 and 91 U/L, respectively, on d 7. The xylanase produced by induction from sugar cane bagasse hydrolyzate was used for pulp biobleaching. A 20% decrease on the Kappa value in Kraft pulp using the culture extract was obtained. These selective growth conditions led us to modulate the xylanase production for pulp delignification.

Index Entries: *Penicillium janthinellum;* xylanases; xylosidase; protease; biobleaching.

INTRODUCTION

The sugar composition and the structure of hemicelluloses and their content in wood vary from species to species, however, xylans, mannans, and galactans are the major components. Using alkaline pulping conditions, xylan, arabinose, and other branching substituents are released from wood and are dissolved into the cooking liquor. Some of this dissolved xylan has been shown to precipitate back onto the surface of the cellulose fibrils. This is an indication that endoxylanase and probably endomannase are the only enzymes necessary for biobleaching (*1–4*).

Recently, xylanases have been used by several groups for the biobleaching of different kind of pulps (*1,2,5–8*). Eriksson (*1*) described a microorganism, *Aureobasidium pullulans,* that produced essentially a cellulase-free xylanase solution when grown on xylan containing substrate. *Aureobasidium pullulans,* grown on oat spelt xylan as the sole carbon source, produced a high amount of endoxylanase, but low levels of endoglucanase and mannase activity. The enzymatic treatment, largely on the action of endoxylanases, removes some of the precipitated xylan, increasing the permeability of the fibers thus facilitating the extraction of lignin (*2*). Glucomannan, which is present in large amounts in softwood pulps, has not been shown to precipitate onto fibril surfaces in the same way as xylan. In addition, mannanase enzymes have not exhibited the same effect on the bleachability of the pulp (*9*).

Based on this information, it is of paramount importance to select a fungus that has a high endoxylanase activity but a low endocellulase production for pulp biobleaching.

We have been studying the production of xylanase by *Penicillium janthinellum* for several years with the aim of developing a technology for the production of enzymes using indigenous microbial strains (*10*). This particular fungus produces a high xylanase enzyme activity in culture broth, but a low endocellulase activity was found under all conditions tested (*11*).

The aim of this work was to study the xylanase production induced by oat spelt, birchwood xylans, and hydrolyzate from sugar cane bagasse in order to select conditions for maximal endoxylanase production and minimal cellulases activities.

MATERIALS AND METHODS

Chemicals

Birchwood 4-*O*-methyl-β-D-glucoroxylan (90% xylose) and oat spelt arabinoxylan (10% arabinose, 15% glucose) were obtained from Sigma (St. Louis, MO). All the other chemicals were of analytical grade. Hydrolyzate from sugar cane bagasse was obtained as previously described (*8*).

Fungus

P. janthinellum (Strain 87-115) was obtained from the Biotechnology and Chemical Center Collection at Lorena (Sao Paulo, Brazil) (*8,10*).

Growth Conditions

The basic medium for growth and induction contained Difco (E. Molesley, Surrey, UK) yeast extract (2.5 g/L), a mineral solution based on Vogel's medium (20 mL/L) (*13*), phosphate buffer (100 m*M*, pH 6.0), or 2,2-dimethyl succinate buffer (DMS 10-100 m*M*, pH 4.5–6.0), and different xylans as the carbohydrate source (0.8%). Induction experiments involved two growth phases: Transfer the inoculum to a 125-mL flask containing 24 mL of inducing medium; and incubate the flasks at 28°C with subsequent agitation on a rotatory shaker at 150 rpm. Enzymatic activities were measured after 24 h during 7 d unless otherwise specified.

Previous published methods on xylanases induction by sugar cane bagasse hydrolyzate was used (*8*). The utilization of this substrate is justified because of economical aspects.

Xylanase was produced by *P. janthinellum* using a Microferm (4 L) containing 2.8 L of sugar can bagasse hydrolyzate, supplemented with 0.1% yeast extract and a 2.0% salt solution based on Vogel's medium. The bioreactor was inoculated with 5×10^5 spores/mL. The culture was incubated at 30°C at pH 6.0 with an agitation of 300 rpm and with an aeration of 0.2 vvm. After 96 h of culturing, it was filtered using a millipore membrane (45 μm). The characteristics of this extract was the following: 16 U/mL xylanase (assay with oat spelt xylan, soluble part) and 0.08 U/mL Carboximetil cellulase (CMCase), Avicelase and FPase activities were absent.

Enzymatic Activities and Protein Analyses

The β-xylanase activity was determined by measuring the liberated reducing sugar birch or oat spelt xylan (soluble and insoluble part) at 50°C with 0.1*M* phosphate buffer (pH 6.0). Only soluble hydrolyzate from sugar cane bagasse was used for the xylan assay. Reducing sugars were determined by a 3,5-dinitrosalicilic acid method (DNS) with xylose as the standard (*14*).

The β-xylosidase activity was determined by measuring the *p*-nitrophenol released by the enzyme from *p*-nitrophenyl-β-xylopiranoside at 40°C with 50 m*M* acetate buffer pH 5.0 (*15*). One unit of β-xylanase or β-xylosidase was defined as one μmol of xylose or *p*-nitrophenolate released per minute, respectively. β-Glucosidase activity was measured by the method of *p*-nitrophenyl-β-D-glucopiranoside (*15*).

Protease activity was measured with azocollageno as the substrate. One unit of protease was defined as U′ = 3.5 × Absorbance (*16*).

Protein content was measured by the method of Bradford (*17*).

The electrophoretic analyses were carried out by the method previously published (*18*).

Temperature Stability

The remaining β-xylanase activity was determined at various time intervals after preincubation of the enzyme at 30–55°C using a 100-m*M* phosphate buffer pH 6.0. The enzyme inactivation kinetic constant (k_i) was determined by applying a first order reaction equation.

Pulp Treatment

The substrate for pulp treatment was of a 3% consistency (Kraft pulp from *Eucalytus grandis*) for 8 h at 30°C at pH 5.0. Following enzyme treatment (50 U/mL of xylanase from hydrolyzate), a concentration of 1*M* NaOH on pulp extraction was used at 2.0% consistency for 60 min at 65°C. The Kappa value of Kraft pulp was measured by a standard method (*19*).

RESULTS AND DISCUSSION

Several cultures of *P. janthinellum* were carried out using oat spelt and birch xylan as the carbon sources in 2.2-dimethyl succinate buffer (DMS 10–100 m*M*), at pH range 4.5–6.0. Table 1 shows these results and the comparison with those carried out in 100 m*M* phosphate buffer at pH 6.0. This data shows that higher β-xylanase activity was found with DMS buffer concentrations between 10 m*M* (1.14 μmol/min) and 50 m*M* (1.12 μmol/min) at pH 4.5. At higher DMS concentrations, although the activities were lower, the culture pH maintained was constant (Fig. 1). It seems that the most important condition in xylanase production is the initial pH and not the pH during growth.

Under our experimental conditions the presence of protease was only observed with 100 m*M* phosphate buffer at pH 6.0 (Fig. 2). β-Xylanase disappearance correlates with the protease activity during growth. Protease activity was triggered in the presence of phosphate buffer, which affected xylanolitic enzymes. This seems to be the major impediment to the continuous production of xylanases. The use of an appropriate buffer should eliminate this problem. Similar effects in *Phanerochaete chrysosporium* ligninolytic enzymes was also observed (*20*).

Table 1
Xylanase Activity at Different pH and Buffer Concentrations

DMS buffer (m*M*)	pH	Activity at d 5, μmol/min	Maximum activity,[a] μmol/min
10.0	4.5	1.14	1.14 (5)
	5.0	0.44	0.48 (7)
	6.0	0.68	0.68 (5)
50.0	4.5	1.08	1.08 (5)
	5.0	0.15	0.68 (9)
	6.0	0.56	0.56 (5)
100.0	4.5	1.00	1.00 (5)
	5.0	0.54	0.91 (9)
	6.0	0.56	0.75 (7)
100.0 Phosphate buffer	6.0	0.58	0.80 (7)

[a]In parenthesis day of maximal activity.

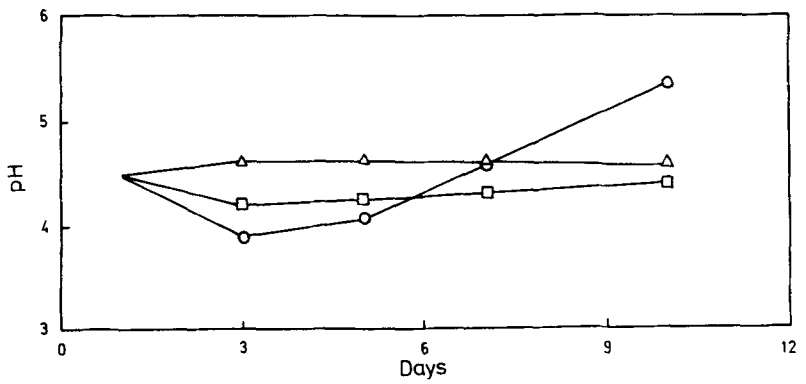

Fig. 1. pH Changes during growth in different DMS buffer concentrations. (—○—) 10 m*M*; (—□—) 50 m*M*; and (—△—) 100 m*M*.

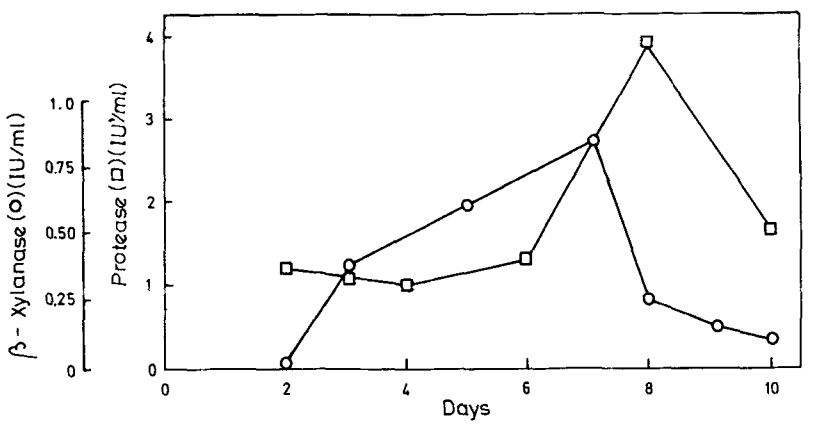

Fig. 2. Production of protease (—□—) and β-xylanase (—○—) in 100 m*M* phosphate buffer at pH 6.0.

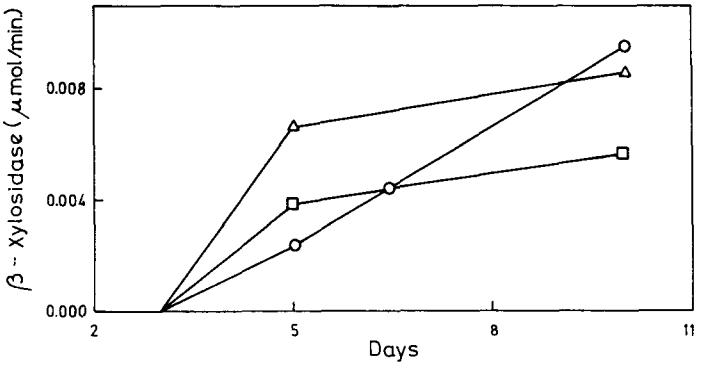

Fig. 3. Production of β-xylosidase at different DMS buffer concentration at pH 4.5. (—○—) 10 m*M*; (—□—) 50 m*M*; and (—△—) 100 m*M*.

β-Xylosidase activity was found in the culture at all the DMS concentrations at pH 4.5 (Fig. 3). No xylosidase activity was seen in assays done with any pH other than 4.5.

Induction of β-xylanase with 50 m*M* DMS at pH 4.5 was carried out using two kinds of substrates. Oat spelt xylan and birchwood xylan induced different kinds of enzymes with distinct specific activities. Figure 4A–C shows the production kinetics of these experiments. In culture of *P. janthinellum* β-xylanase activity was lower on birchwood xylan than in oat spelt xylan. These results are probably owing to the different inductor structures of these substrates, which are able to produce different types of β-xylanases. The bases for these differences were evaluated through the inactivation constants and their half-life at the assayed temperature. The inactivation constants of β-xylanase induced by oat and birch xylans were different (Table 2), with those coming from birch being more stable.

When birchwood xylan was used as an inductor, only β-glucosidase (Fig. 5B) and β-xylosidase appeared at d 6 (Fig. 5A), but at very low levels. Relative higher activities in the presence of oal spelt xylan than in birchwood xylan induction were found on d 7. β-Xylosidase and β-glucosidase induced by oat spelt xylan were 85 U/L and 91 U/L, respectively.

The electrophoretic results on polyacrylamide and sodium dodecylsulfate (SDS) show a profile of the different proteins for each culture (Fig. 6). This figure shows that both types of carbon source induced various types of proteins and, probably, different xylanases, from the values of stability constants. Since both birchwood xylan and the hydrolyzate from sugar cane bagasse (*10*) induced high xylanase activities, we decided to run a preliminary experiment with the later on pulp biobleaching.

A hemicellulolytic fraction from sugar cane bagasse was shown to be a good inductor of xylanase and the electrophoretic profile was similar with those obtained with birchwood. An experiment in pulp biobleaching using xylanase induction by hydrolyzate from sugar cane bagasse was carried out. Mainly this filtrate exhibited a xylanase activity of 16 U/mL.

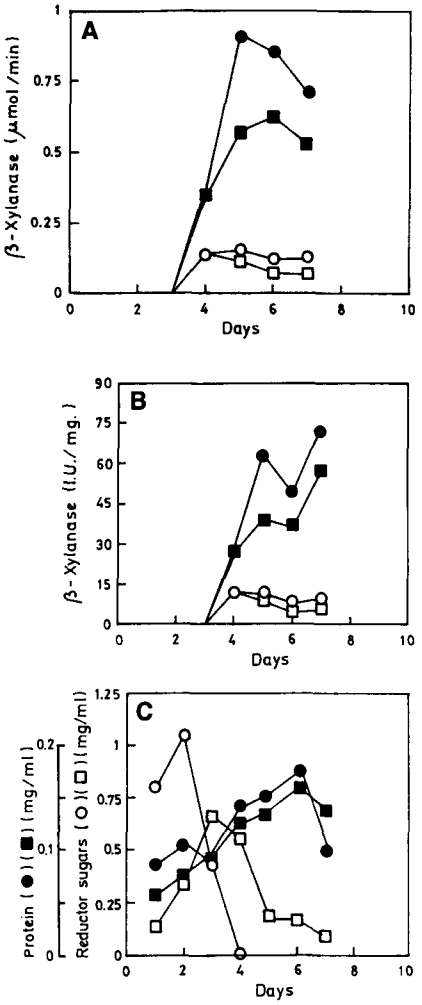

Fig. 4. Production of β-xylanase induced by birchwood xylan and oat spelt xylan in 50 m*M* DMS buffer at pH 4.5. (**A**) In birchwood xylan source using birchwood xylan assay (—○—) and oat spelt xylan (—□—). In oat spelt xylan source using birchwood xylan assay (—●—) and oat spelt xylan (—■—) in μmol/min. (**B**) The same as A but in IU/mg of protein. (**C**) Reducing sugar disappearance and protein production using birchwood xylan and oat spelt xylan as inductors. Reducing sugar: (—○—) induced by birchwood and (—□—) oat spelt xylan. Protein: (—■—) induced by birchwood and (— —) induced by oat spelt xylan.

Table 2
Thermal Stability of β-Xylanase Induced by Birchwood or Oat Spelt Xylan

Temperature, °C	$k_1 \cdot 10^2$, [a] min^{-1}	$t_{½}$, [a] min	$k_1 \cdot 10^2$, [b] min^{-1}	$t_{½}$, [b] min
45	$1.25 \pm 8.9 \cdot 10^{-4}$	55.3	$3.2 \pm 1.8 \cdot 10^{-2}$	21.0
50	$5.12 \pm 1.3 \cdot 10^{-3}$	13.5	$11.0 \pm 1.1 \cdot 10^{-2}$	6.3
55	$13.40 \pm 2.2 \cdot 10^{-2}$	5.0	$42.8 \pm 4.6 \cdot 10^{-2}$	1.6
60	$37.80 \pm 2.3 \cdot 10^{-2}$	1.8	$86.5 \pm 5.0 \cdot 10^{-2}$	0.8

[a] Birchwood xylan as inductor.
[b] Oat spelt xylan as inductor, the assays are in triplicate and from two different cultures.

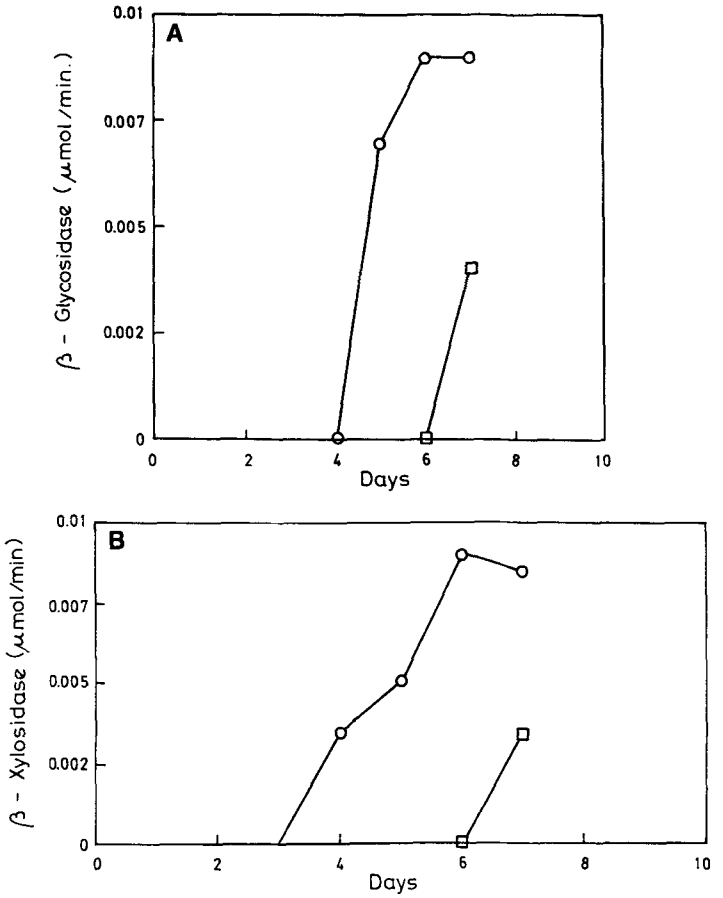

Fig. 5. Production of β-xylosidase and β-glucosidase in 50 m*M* DMS buffer at pH 4.5: (**A**) β-xylosidase induced by oat spelt (— —) and by birchwood xylan (—□—). (**B**) β-glucosidase induced by oat spelt (—○—) and by birchwood xylan (—■—).

Treatment of unbleached Kraft pulp (Kappa value of 14.1) with 50 U/mL of xylanase filtrate gave a Kappa value of 11.3 ± 1.2 (6% decrease) and after basic treatment a Kappa value of 10.0 ± 0.1 (29% decrease) was obtained. An optimization study showed that at 25 U/mL of xylanase for 24 h there was a decrease of Kappa value from 16.0 to 12.8 (20% decrease with a 98.7% pulp yield). Using xylanase alone and after alkaline extraction the Kappa value decreased from 12.0 to 10 (17% decrease with 94.4% pulp yield). This process exhibited a total efficiency of 37% of Kappa value decrease.

Since high specific activities of endoxylanase were obtained in the xylanase induction by hydrolyzate from sugar cane bagasse, this carbon source appears to be a potential inductor for industrial xylanases. Experiments with pulp biobleaching using xylanases induced by oat spelt and birchwood xylans are in progress.

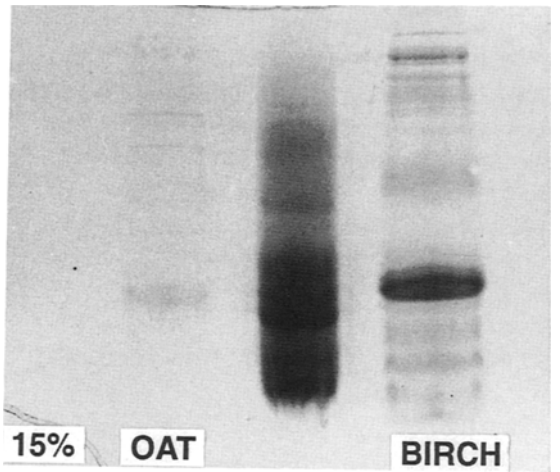

Fig. 6. SDS-polyacrylamide gel electrophoretic profile of the production of β-xylanase induced by oat spelt xylan (lane 1), standard molecular weight (lane 2) and birchwood xylan (lane 3).

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