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# Xylanase Production by *Aspergillus tamarii*

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## ABSTRACT

*Aspergillus tamarii* has been found to grow well and to produce high cellulase-free xylanase activity when growing on corn cob powder as the principal substrate. Maximum xylanase production (285–350 U/mL) was obtained when the strain was grown in media supplemented with high corn cob concentration (5–8%, w/v) for 5 d. The presence of constitutive levels of xylanase was detected in cultures with glucose as the carbon source. Zymogram analysis for detection of xylanase activity after electrophoresis in polyacrylamide gels has shown that *A. tamarii* produces at least two xylanases under the conditions utilized. The hydrolysis patterns of xylan demonstrated that the xylanases were endoenzymes, yielding mainly xylobiose, xylotriose, and higher xylooligosaccharides with traces of xylose.

**Index Entries:** Xylanolytic fungi; cellulase-free xylanase; *Aspergillus tamarii*; β-xylosidase; zymogram analysis.

## INTRODUCTION

β-1,4 xylanases (1,4 β-D-xylan-xylanohydrolase, EC 3.2.1.8) catalyze the hydrolysis of xylan, the major component of hemicellulose of plant cell walls to xylo-oligosaccharides and xylose. In combination with other polysaccharides, especially cellulases, xylanases appear to open new possibilities for the food industry and for bioconversion of agricultural wastes into easy fermentable products (*1,2*). In addition, cellulase-free xylanases can be utilized for the selective hydrolysis of the hemicellulose components in paper and pulp (*3,4*). The use of purified xylan as an inducer increases the

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cost of enzyme production. For this reason, different lignocellulosic residues have been utilized as growth substrate in cultures to produce xylanolytic enzymes (*5–9*). A great number of different *Aspergillus* species have been reported to produce xylanases: *A. niger* (*10*), *A. ochraceus* (*6*), *A. oryzae* (*11*), and *A. awamori* (*12*). Recently, a strain of *A. tamarii* able to produce high cellulase-free xylanase when growing on corn cob powder as the major substrate was isolated from soil. The purpose of this study was to investigate some of the cultural variables that influence the production of xylanase by this *A. tamarii* strain.

## MATERIALS AND METHODS

### Microorganism

*Aspergillus tamarii* was isolated from soil during a screening program for xylanase-producing microorganisms in this laboratory. The organism was maintained on potato dextrose agar slants (*13*) at 40°C and subcultured in intervals from 10 to 30. The cultures were incubated for spore production on potato dextrose agar slants at 30°C for 5 d. The spore suspensions were prepared by adding 10 mL of sterilized water to slant cultures and the surface was gently rubbed with a sterilized wire loop.

### Media and Cultivation Conditions

Enzyme production was studied in 250-mL Erlenmeyer flasks containing 50 mL of Vogel salts (*14*) and the appropriate carbon source. One milliliter of spore suspension ($10^4$ spores) was used as the inoculum. The cultures were incubated at 30°C on a rotary shaker at 120 rpm for 5 d.

### Enzyme Assays

The mycelium was removed from the medium by filtration. Xylanase activity was assayed using birchwood xylan (Sigma, St. Louis, MO) as substrate. A 1.0-mL reaction mixture contained 0.5 mL of appropriately diluted enzyme solution and 0.5 mL of a 1% suspension of xylan in citrate-HCl buffer, 0.05*M*, pH 5.4. The mixture was incubated at 50°C for 10 min and the reducing sugars were assayed by the dinitrosalicylic acid method (*15*), using xylose as standard. A similar method was utilized to assay carboxymethylcellulolytic activity (CMCase), utilizing 1% (w/v) carboxymethylcellulose as substrate and glucose as the standard. In both cases, an enzymatic unit was defined as the amount that produced 1 μmol of reducing sugars per minute. β-xylosidase activity was assayed by incubating 1 mL of 0.1% (w/v) p-nitrophenyl-β-D-xylopyranoside (PNPX) with 0.1 m*M* of suitable diluted enzyme in citrate-HCl buffer (0.05*M*, pH 5.4) at 50°C for 30 min. The reaction was stopped by

addition of 2 mL of 1*M* $Na_2CO_3$. The liberated p-nitrophenol was measured at 410 nm.

### Determination of Protein

Mycelial pads were ground in a porcelain mortar with twice their weight of glass beads (0.1-mm diameter) at 0°C with cold 0.05*M* citrate-HCl buffer, pH 5.4 (15 mL/g mycelium). The slurry was then centrifuged at 9000*g* for 30 min. The supernatant fraction was utilized for mycelial protein determination (*16*). Extracellular protein was determined from TCA precipitates (*17*) of culture filtrates utilizing the same method above (16). Bovine serum albumin was used as the standard.

### Detection of Xylanase Activity in Polyacrylamide Gels

Electrophoresis under nondenaturing conditions was carried out by the method of Davis (*18*). The culture filtrates were concentrated by acetone precipitation. The xylanase zymogram technique utilized was a modification of the method of Biely et al. (*19*), using remazol brilliant blue (RBB) dyed xylan (Sigma) as the substrate. To prepare substrate gel, 150 mg of xylan was dissolved in 10 mL of deionized water at 50°C and mixed with 20 mL of melted 2.5% (w/v) agar in 0.05*M* citrate-HCl buffer, pH 5.4. The mixture was cooled to approx 55°C and poured on a glass. After hardening, the substrate gel was carefully overlaid with a protein gel and incubated at 50°C for 30 min. The gels were finally separated and the protein gel was fixed and stained with Coomassie Blue. The substrate gel was immersed in ethanol-citrate-HCl buffer (2:1) for 48 h to enhance zones of clearing against a blue background. The RBB-dyed gel was photographed on a light table.

### Chromatography of Xylan Hydrolysis Products

Xylanase I or II eluted from polyacrylamide gel was incubated with 1.0 mL of 1% birchwood xylan in 0.05*M* citrate buffer pH 5.4. The aliquots at different time intervals were analyzed for the sugar products formed by descendent paper chromatography in a solvent system containing (1:5:3:3) benzene:n-butanol:piridine:water (*20*). The chromatograms were sprayed as described by Trevelyn et al. (*21*).

### Other Methods

Residual xylose and glucose in the media were determined by the orcinol method (*22*) and by the peroxidase-glucose oxidase method (*23*), respectively.

## RESULTS

*A. tamarii* was grown in 250-mL Erlenmeyer flasks containing 50 mL Vogel salts and different carbon sources (1%, w/v) for 5 d. Xylanase,

Table 1
Effect of Carbon Source on the Production of Xylanase, β-Xylosidase, and CMCase by *Aspergillus tamarii*

| carbon source | Enzymatic activity (U/mL) | | |
|---|---|---|---|
| | xylanase | β-xylosidase | CMCase |
| glucose | 1.76±0.9 | 0.08±0.03 | nd |
| xylose | 8.21±3.0 | 0.16±0.05 | nd |
| lactose | 6.81±1.9 | 0.10±0.03 | nd |
| cellobiose | 5.23±1.8 | 0.09±0.04 | nd |
| birchwood xylan | 43.42±8.3 | 0.29±0.10 | 0.10±0.05 |
| oat spelt xylan | 94.63±13.8 | 0.42±0.18 | 0.14±0.06 |
| corn cob | 76.61±10.8 | 0.24±0.10 | 0.10±0.03 |
| wheat bran | 54.32±7.8 | 0.51±0.19 | 0.12±0.05 |
| sugar cane bagasse | 16.68±4.2 | 0.28±0.10 | 0.07±0.02 |
| CMcellulose | 3.48±1.2 | 0.08±0.02 | 0.06±0.02 |

Values are given as means ± S.D. of three replicated cultures; nd, not detectable.

β-xylosidase, and CMCase activities were determined in the early stationary growth phase. In the case of soluble substrates, the enzymatic activities were determined on dialyzed culture samples. As shown in Table 1, low xylanase and β-xylosidase and no measurable amounts of CMCase were observed after growth on glucose, xylose, lactose, cellobiose, and CMCellulose. High xylanase activities were observed when the organism was grown on commercial xylan (from birchwood or from oat spelt) or on lignocellulosic residues (corn cob, wheat bran, and sugar cane bagasse). Extracellular β-xylosidase activities were obtained in these cultures, especially with wheat bran as growth substrate. However, the CMCase activities were very low.

The microorganism was grown on 1% corn cob as the carbon source and the production of xylanase was determined as a function of time. As shown in Fig. 1, xylanase activity reached a value of 6 U/mL after 48 h of incubation; at this stage xylose or glucose (0.5 or 1.0%, w/v) were added. The addition of these sugars decreased xylanase production, and after the

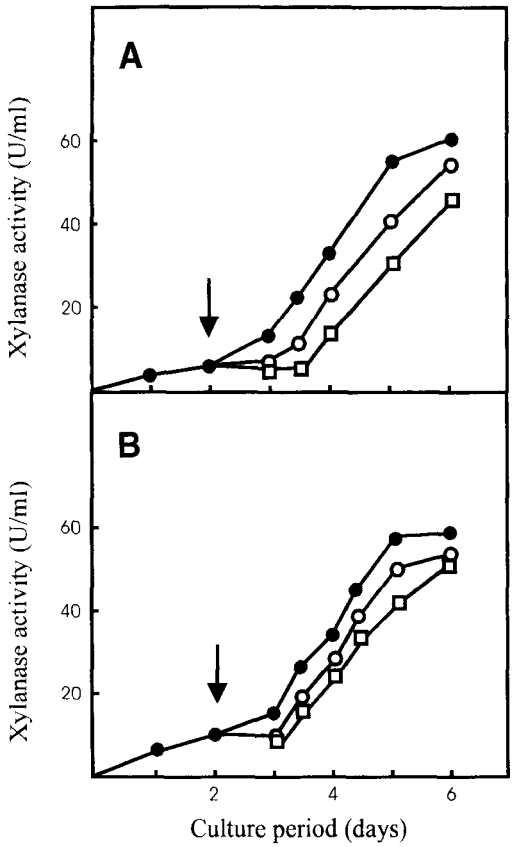


Fig. 1. Inhibition of xylanase production by glucose and xylose. *A. tamarii* was grow in 1% corn cob medium. At the time indicated by the arrows, soluble sugars were added. (△) control; **(A)** (○) 0.5% glucose; (□) 1.0% glucose; **(B)** (○) 0.5% xylose; (□) 1.0% xylose.

soluble sugars had been consumed, enzyme synthesis was resumed. After 24 h these cultures presented less than 5% of initial concentration of sugars added (data not shown).

The effect of varying substrate concentration (corn cob or oat spelt xylan) on the production of xylanase, β-xylosidase, and CMCase was investigated (Fig. 2). For lower substrate concentrations (until 2%, w/v), the cultures utilizing xylan as substrate produced more xylanase than those grown on corn cob (Fig. 2A). However, for an initial substrate concentration greater than 2%, higher xylanase activities were obtained with corn cob cultures. When xylan was used as substrate, high levels of reducing sugars were obtained in the culture filtrates, but not in the corn cob culture filtrates (Fig. 2A). An analysis of xylan filtrates by paper chromatography showed the presence of glucose, xylose and different xylo-oligosaccharides (data not shown). For all substrate concentrations

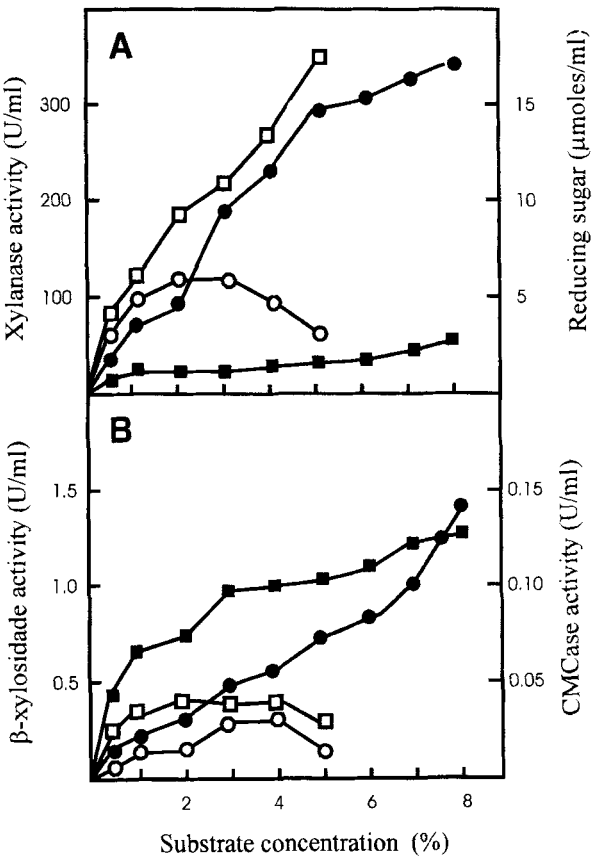


Fig. 2. Effect of substrate concentration in the production of enzymes by *A. tamarii*. open symbols, oat spelts xylan; close symbols, corn cob. **(A)** (○, ●) xylanase; (□, ■) reducing sugars; **(B)** (○, ●) β-xylosidase; (□, ■) CMCase.

utilized, corn cob cultures produced more β-xylosidase and CMCase activities than the xylan cultures (Fig. 2B).

Two major phases of xylanase production were observed during growth of *A. tamarii* on 5% corn cob (Fig. 3). The first phase occurred during the first 84 h of incubation and it was characterized by a great increase in the production of enzyme and an increase in pH values from 5.6 to 6.9. This phase included an initial 24 h lag period followed by the exponential growth phase. The second phase was characterized by a maintenance of xylanase levels, a drop in pH values from 6.9 to 6.1, and an increase in protein secretion.

Zymogram analysis of cell-free cultures obtained with different carbon source (oat spelt xylan, corn cob, wheat bran, and sugar cane bagasse), showed that *A. tamarii* had produced at least two xylanase activities

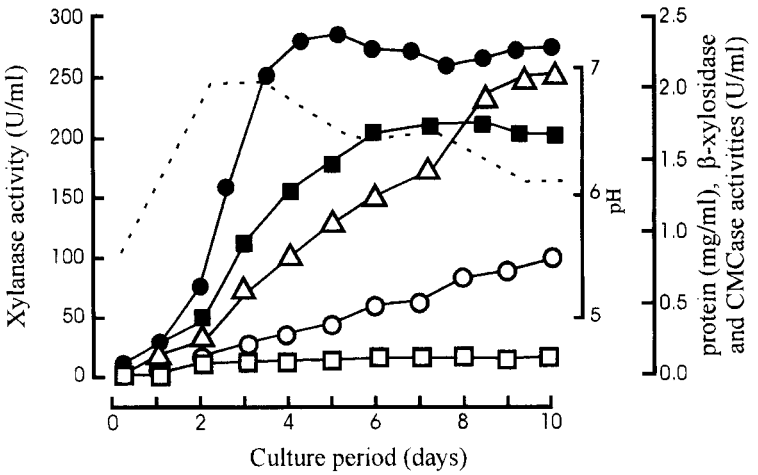


Fig. 3. Time course for the formation of extracellular enzymes in relation to growth of *A. tamarii* on corn cob. (●) xylanase; (○) β-xylosidase; (□) CMCase; (△) extracellular protein; (■) mycelial protein; broken line pH.

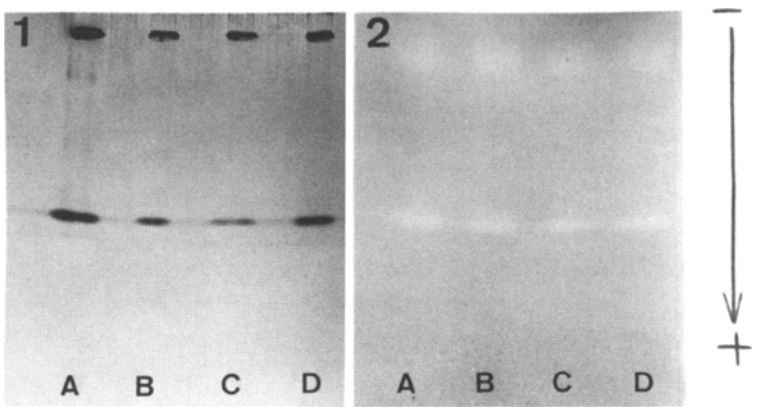


Fig. 4. Comparison of xylanase production by different inducers. Cell-free samples obtained with different inducers were submitted to electrophoresis under nondenaturing conditions and the gel was used to generate a zymogram using the technique RBB-xylan. 1: Proteins staining with Coomassie brillant blue; 2: Xylanase zymogram with RBB-xylan. The enzymes were obtained in cultures with oat spelt xylan **(A)**, corn cob **(B)**, wheat bran **(C)**, and sugar cane bagasse **(D)** as inducer.

(Fig. 4). The xylanase of lower electrophoretic mobility was designed I and the other xylanase was designed II.

The hydrolysis products of xylan were analyzed by paper chromatography after treatment with *A. tamarii* xylanase I and II eluted from the polyacrylamide gel (Fig. 5). The products obtained were xylobiose, higher xylooligosaccharides, and low amounts of xylose.

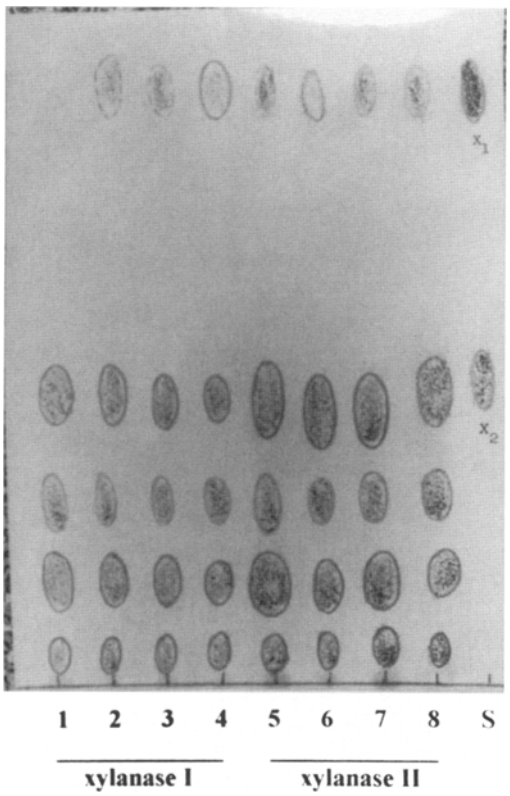


Fig. 5. Paper chromatography of hydrolysis products of xylan by xylanases I and II. Xylanases I and II were eluted from polyacrylamide gel. 1: oat spelt xylan; 2, 3 and 4: xylan plus xylanase I after 10, 30, and 60 min, respectively; 5, 6, and 7: xylan plus xylanase II after 10, 30, and 60 min, respectively; Xylose ($X_1$), and Xylobiose ($X_2$) are standards.

## DISCUSSION

The inductive nature of production of xylanase and β-xylosidase by *A. tamarii* was suggested by the high levels of these enzymes in cultures with xylan or lignocellulosic residues as carbon sources, whereas they were low on glucose-, xylose-, cellobiose-, lactose-, and CMCellulose-containing cultures (Table 1). However, the fact that xylanase could be formed in the absence of xylan seems to indicate that a fraction of the enzyme was formed constitutively. The results show that xylanase biosynthesis probably decreased when glucose or xylose were added during growth on corn cob and resumed after depletion of these sugars (Fig. 1). This inhibition of xylanase synthesis can be interpreted in terms of catabolite repression likewise described for other xylanolytic microorganisms *(24,25)*. The objective of this work was to significantly increase the xylanase production without

increasing the CMCase production. Thus, the effect of substrate concentration on xylanase production was studied. When the corn cob concentration was increased from 1 to 8%, the xylanase activity increased from 76 to 330 U/mL, whereas the CMCase production remained low (0.12 U/mL). However, the xylanase production was characterized by a steady decline at higher xylan concentrations (Fig. 2A). This decline can be caused by the presence of accumulated hydrolysis products resulting in catabolite repression of enzyme production at higher substrate concentration (Fig. 2A). Similar results were obtained in *Trichoderma harzianum* cultures *(25)*. The maximum production of xylanase (285–350 U/mL) was obtained when *A. tamarii* was grown on media containing 5–8% (w/v) corn cob after 5 d of incubation. The maintenance of xylanase levels after 10 d incubation suggests high stability of the enzyme and low production of proteolytic enzymes in these cultures (Fig. 3). Two xylanases characterized as endoenzymes were detected by electrophoresis in polyacrylamide gels and the zymogram technique (Fig. 4 and 5). Multiple xylanases have been reported in numerous micro-organisms *(1)*. More detailed studies are required before the basis of xylanases multiplicity in *A. tamarii* can be properly assessed.

In the last few years, the bulk of the research in the production of xylanases has been stimulated by their utility in a variety of biotechnological applications *(26)*. The xylanase activity reported in this paper (350 U/mL) is higher than those reported by other researchers using cheaper raw materials such as sugar cane bagasse, wheat straw, or canola meal *(27–29)*. The data presented in this paper indicate that corn cob is a convenient substrate for the production of cellulase-free xylanase activity by *A. tamarii*. The characteristics obtained in the cultures of *A. tamarii* such as the ease of cultivation and the high production of cellulase-free xylanases in cultures utilizing a cheap growth substrate make this soil isolate a potential source of xylanases for the selective hydrolysis of xylan residues in lignocellulosic biomass.

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## REFERENCES

1. Wong, K. K. Y., Tan, L. U. L., and Saddler, J. N. (1988), *Microbiol. Rev.* **52,** 305–317.
2. Biely, P. (1985), *Trends Biotechnol.* **3,** 286–290.
3. Jurasek, L. and Paice, M. (1986), *Chem. Tech. (Heidelberg)* **16,** 360–365.
4. Wong, K. K. Y. and Saddler, J. N. (1992), *Crit. Rev. Biotechnol.* **12,** 413–435.

5. Blanco, A. and Pastor, F. I. J. (1993), *Can. J. Microbiol.* **39,** 1162–1165.
6. Biswas, S. R., Mishra, A. K., and Nanda, G. (1988), *Folia Microbiol.* **33,** 355–359.
7. Bailey, M. J. and Poutanen, K. (1989), *Appl. Microbiol.* **30,** 5–10.
8. Chaudri, S., Thakur, I. S., Reeta, G., and Johri, B. N. (1988), *Biochem. Internat.* **17,** 563–575.
9. Warzywoda, W., Ferre, V., and Pourquie, J. (1983), *Biotechnol. Bioeng.* **25,** 3005–3011.
10. Costa-Ferreira, M., Dias, A., Maximo, C., Morgado, M. J., Sena-Martins, G., and Cardoso Duarte, J. (1994), *Appl. Biochem. Biotechnol.* **44,** 231–242.
11. Bailey, M. J., Puls, J., and Poutanen, K. (1991), *Biotechnol. Appl. Biochem.* **13,** 380–389.
12. Smith, D. C. and Wood, T. M. (1991), *Biotechnol. Bioeng.* **38,** 883–890.
13. Smith, D. and Onions, A. H. S. (1983), *The preservation and maintenance of living fungi.* Commonwealth Mycological Institute, Kew, Surrey, UK.
14. Montenecourt, B. S. and Eveileigh, D. E. (1977), *Appl. Environ. Microbiol.* **34,** 777–784.
15. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* **13,** 265–275.
17. Tan, L. U. L., Chan, M. K.-H., and Saddler, J. N. (1984), *Biotechnol. Lett.* **6,** 199–204.
18. Davis, B. J. (1964), *Ann. N Acad. Sci.* **121,** 404–427.
19. Biely, P., Markovic, O., and Mislovicova, C. (1985), *Anal. Biochem.* **144,** 147–151.
20. Gaillard, B. D. E. (1953), *Nature* **171,** 1160.
21. Trevelyn, W., Procter, P. D., and Harrison, J. S. (1950), *Nature* **166,** 444, 445.
22. Herbert, D., Phipps, P. J., and Strange, R. E. (1971), in *Methods in Microbiology,* vol. 5b, pp. 289–292.
23. Bergmeyer, H. U. and Bernt, E. (1974), in *Methods of enzimatic analysis.* vol. 3, Bergmeyer, H. U., ed., Academic Press, London and New York, pp. 1205–1212.
24. Bérenger, J.-F., Frixon, C., Bigliardi, J., and Creuzet, N. (1985), *Can. J. Microbiol.* **31,** 635–643.
25. Senior, D. J., Mayers, P. R., and Saddler, J. N. (1989), *Appl. Microbiol. Biotechnol.* **32,** 137–142.
26. Coughlan, M. P. and Hazlewood, G. P. (1993), *Biotechnol. Appl. Biochem.* **17,** 259–289.
27. Biely, P., Mckenzie, C. R., and Schneider. H (1988), *Can. J. Microbiol.* **34,** 767–772.
28. Szczodrak, J. (1988), *Acta Biotechnol.* **8,** 509–515.
29. Gattinger, L. D., Duvnjak, Z., and Khan, A. W. (1990), *Appl. Microbiol. Biotecnol.* **33,** 21–25.