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Exocellular β-mannanases from hemicellulolytic fungi

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Production of exocellular β-mannan- and xylan-degrading enzymes by eight wood rotting fungi was studied. Although all organisms excreted β-mannanase, endoxylanase and acetylxylan esterase, production of L-α-arabinosidase and 4-*O*-methylglucuronidase was variable. β-Mannanosidase was not detected in any culture filtrate. Highest β-mannanase and endoxylanase activities were observed in cultures of *Polyporus versicolor* and *Schizophyllum commune* grown in Avicel-supplemented media. While crude β-mannanases from *Linzites saepiria* and *S. commune* exhibited equivalent affinities for gluco- and galactomannan substrates, *P. versicolor* β-mannanase preferred a glucomannan substrate and did not use galactomannan from guar gum as a substrate.

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β-Mannanase [E.C. 3.2.1.78; mannan endo-1,4-β-D-mannanosidase, (1 → 4)-β-mannan mannanohydrolase] is the generic name given to enzyme activities that cleave randomly within the main chain of such homo- and hetero-β-D-mannans as mannan, galactomannan, glucomannan and galactoglucomannan. These enzymes are widely distributed in nature having been found in plants and animals (Dekker & Richards 1976; McCleary 1979; McCleary & Matheson 1983), as well as in a wide variety of bacteria and fungi (Civas *et al.* 1984; Takahashi *et al.* 1984; Rättö & Poutanen 1988). Interest in β-mannanase has arisen recently because of potential uses of enzyme treatments in the pulp and paper industry. Several studies of hemicellulase pre-treatment of pulps to facilitate lignin removal in subsequent chemical extractions or for modifications of pulp properties have been performed (Paice & Jurasek 1984; Mora *et al.* 1986; Noe *et al.* 1986; Viikari *et al.* 1987). It has been suggested that since softwoods contain 15 to 20% galactoglucomannan, β-mannanase activity, acting alone or in concert with other hemicellulases, might potentiate 'biobleaching' of pulps (Kantelinen *et al.* 1988) or have some utility in the complete hydrolysis of sulphite liquors to fermentable sugars (Rättö & Poutanen 1988). In the following study, a number of wood rotting fungi were screened in batch cultures for their capacity to produce β-mannan- and xylan-hydrolyzing enzymes. Growth conditions allowing high levels of enzyme activity in the best β-mannanase producers were determined, and preliminary characterization of the enzyme activities was undertaken.

Materials and Methods

Microorganisms and Growth Conditions

Coriolus versicolor NRCC 5906, *Haematosterium sanguinolentum* NRCC 5902, *Glionatix trabeum* NRCC 5907, *Lenzites saepiaria* NRCC 5910, *Polyporus versicolor* NRCC 5909, *Poria placenta* NRCC 5909, *Schizophyllum commune* NRCC 5911, and *Trichocladium candense* NRCC 5903 were maintained at 4°C on 1.5% (w/v) maltose agar (Difco) slants. Cultures for enzyme production studies were grown at 30°C for up to 10 days in the medium (1 l per 4 l baffled flask) described by Derochers *et al.* (1981) on a gyrorotary shaker operated at 150 rev/min. Polysaccharide supplements were added to growth media as detailed below. Inocula (5% v/v) were grown for 48 h at 30°C in 250 ml baffled flasks containing 1.5% (w/v) malt extract.

On a étudié la production de β-mannanases et de xylanases exo-cellulaires chez huit moisissures pourrissant le bois. Bien que tous les organismes excrètent la β-mannanase, l'endoxylanase et l'esterase de l'acétylxylane, la production de L-α-arabinosidase et de la 4-*O*-méthyl-glucuronisade a été variable. La β-mannanoxidase n'a été détectée dans aucun filtrat de culture. Les activités les plus élevées en β-mannanase et en endoxylanase ont été observées dans des cultures de *Polyporus versicolor* et de *Schizaphyllum commune*, développées en milieu supplémenté en Avicel. Alors que les β-mannanases brutes de *Linzites saepiria* et de *S. commune* ont montré des affinités équivalentes pour les substrats gluco- et galacto-mannanes, la β-mannanase de *P. versicolor* préfère un substrat gluco-mannane et n'a pas utilisé le galacto-mannane de la gomme guar comme substrat.

Polysaccharide Media Supplements

Oat-spelts xylan (Sigma), wheat bran prepared as previously described (Johnson *et al.* 1988a), Avicel PH 105, and acid-swollen Avicel prepared as previously described (Tansey 1971) were used at final concentrations 1% (w/v). Locust bean gum (Sigma), and konjacu glucomannan prepared by the method of Dea *et al.* (1977), were used at final concentrations of 0.1% (w/v).

Enzyme Assays

β-*Mannanase* was determined using konjacu root powder glucomannan. Locust bean gum, guar gum and yeast mannan (Sigma) were used in substrate specificity studies. Assay mixtures contained 0.2% (w/v) mannan substrate, 10 μmol potassium phosphate buffer pH 6.0, and appropriate enzyme dilutions in a total volume of 1.0 ml. After 15 min at 50°C, the amount of reducing sugars produced was measured. One unit of activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar (expressed as glucose) per minute at pH 6.0 and 50°C.

Endoxylanase was determined as previously described (MacKenzie *et al.* 1984) using larchwood xylan as substrate. Unit activity was defined as the amount of enzyme releasing 1 μmol reducing sugar (expressed as xylose) at pH 6.0 and 50°C.

Acetylxylan esterase was estimated as previously described (Johnson *et al.* 1988b) using acetylated larchwood xylan as substrate. One unit of activity was defined as the amount of enzyme releasing 1 μmol of acetic acid per minute at pH 6.0 and 50°C.

β-*Mannosidase and* L-α-*arabinofuranosidase* were estimated using *p*-nitrophenyl-β-mannopyranoside and *p*-nitrophenyl-L-arabinoside (Sigma) substrates respectively. Assay mixtures contained 1 μmol of the chromogenic substrate, 50 μmol potassium phosphate buffer, pH 6.0, and appropriate enzyme dilutions in a total volume of 1.0 ml. After designated periods of incubation at 50°C, reactions were terminated by addition of 1.0 ml of 1 M sodium carbonate, and the $A_{420\ nm}$ was determined. Unit activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute at pH 6.0 and 50°C.

4-O-*Methylglucuronidase* was measured as previously described (Johnson *et al.* 1988b) using larchwood xylan as substrate. One unit of activity was defined as the amount of enzyme releasing 1 μmol of 4-*O*-methylglucuronic acid at pH 6.0 and 50°C.

Protease was measured with solid Azocoll substrate (Calbiochem) as previously described (Johnson *et al.* 1986).

Enzyme Characterization

Following 18 h digestion at 50°C of 5 mg konjacu root powder glucomannan with 100 units of crude β-mannanase, reaction products were fractionated by high-performance liquid chromatography (HPLC) using three linked HPX-8H columns (Bio-Rad) operated at 45°C using 10 mM H_2SO_4 as eluant at a flow rate of 0.6 ml/min. Effluent components were detected with an SPG040 (Spectrophysics) differential refractometer. Dextran 150, maltotriose (Pharmacia), cellobiose (Sigma) and glucose were employed as molecular weight markers.

Optimal temperature was determined by measuring the activity in reaction mixtures incubated for 15 min at 22 to 75°C. Influence of H^+ concentration on enzyme activity was determined in 10 mM buffers of different pH values: pH 3.5 to 5.5 in sodium acetate, pH 6.0 to 8.0 in potassium phosphate, and pH 8.5 to 9.5 in Tris/HCl. K_m values were obtained from Lineweaver–Burk plots using substrate concentrations from 0.05 to 1.0% (w/v) of each indicated substrate.

Analytical Techniques

Reducing sugars were estimated by the Somogyi modification (1952) of the Nelson technique. Protein was measured by the method of Bradford (1976) using gamma globulin as standard.

Results

Enzyme Production

Activities of β-mannan- and xylan-hydrolyzing enzymes produced by fungal cultures grown for 7 days in 1% (w/v) wheat-bran-supplemented media appears in Table 1. While all cultures produced detectable activity of β-mannanase,

Table 1. Production of β-mannanase and xylan-hydrolysing enzymes by various fungi*.

Organism	Enzyme activity	U/ml	Specific activity†
	β-Mannanase	0.96	8.3
	Endoxylanase	4.02	34.6
P. versicolor	Acetylxylan esterase	2.12	18.4
	Arabinosidase	0.28	2.4
	4-*O*-methylglucuronidase	0.44	3.8
	β-Mannanase	0.82	2.4
	Endoxylanase	44.50	130.9
S. commune	Acetylxylan esterase	14.50	42.6
	Arabinosidase	0.04	0.1
	4-*O*-methylglucuronidase	0.25	0.7
	β-Mannanase	0.52	5.2
	Endoxylanase	4.86	48.6
L. saepiria	Acetylxylan esterase	2.69	26.9
	Arabinosidase	ND	—
	4-*O*-methylglucuronidase	0.04	0.4
	Endoxylanase	3.29	74.8
C. versicolor	Acetylxylan esterase	0.98	24.5
	Arabinosidase	0.16	3.7
	4-*O*-methylglucuronidase	ND	—
	β-Mannanase	0.39	10.9
	Endoxylanase	0.67	18.6
H. sanguinolentum	Acetylxylan esterase	0.11	3.0
	Arabinosidase	1.84	51.1
	4-*O*-methylglucuronidase	ND	—
	β-Mannanase	0.19	2.7
	Endoxylanase	0.22	3.2
G. trabeum	Acetylxylan esterase	0.06	0.9
	Arabinosidase	ND	—
	4-*O*-methylglucuronidase	0.042	0.6
	β-Mannanase	0.33	8.2
	Endoxylanase	0.57	14.2
P. placenta	Acetylxylan esterase	0.09	2.2
	Arabinosidase	ND	—
	4-*O*-methylglucuronidase	0.436	11.0
	β-Mannanase	0.29	3.5
	Endoxylanase	0.44	5.3
T. candense	Acetylxylan esterase	0.12	5.5
	Arabinosidase	ND	—
	4-*O*-methylglucuronidase	0.01	1.0

* All cultures were grown for 7 days at 30°C in media supplemented with 1.0% (w/v) wheat bran.

† Specific activity is expressed as U/mg protein.

ND, not detected.

endoxylanase and acetylxylan esterase, β-mannosidase was not detected in any filtrate at any time during 10 days of growth. Production of arabinosidase and 4-O-methylglucuronidase was variable, *P. versicolor* and *S. commune* being the only two organisms to produce the entire spectrum of enzyme activities studied. With all organisms, accumulation of cell mass attained or exceeded 5 mg/ml dry weight by 7 days.

Effect of Media Supplementation on Enzyme Production

Since *P. versicolor* and *S. commune* exhibited the highest absolute levels of β-mannanase and endoxylanase, they were selected for further experiments to study the effect of different supplements on enzyme production. As the results in Table 2 indicate, highest β-mannanase levels were observed when each organism was grown in Avicel-supplemented media. Although much higher endoxylanase levels were encountered in *S. commune*, overall absolute amounts and specific activities of β-mannanase were greater in *P. versicolor* under all tested cultural conditions.

Kinetics of Enzyme Production

Time-dependent evolution of β-mannanase and endoxylanase in cultures of *P. versicolor* (A) and *S. commune* (B) growth with 1% (w/v) Avicel appears in Figure 1. Under these conditions, cultures of both organisms exhibited sharp maximal production of both β-mannanase and endoxylanase at 8 days' growth. Following 8 days' cultivation, a marked drop in enzyme and exocellular protein content was observed, markedly so in cultures of *P. versicolor*. While some exocellular protease was detected in *P. versicolor* cultures between 2 and 5 days' growth, neither *P. versicolor* nor *S. commune* cultures excreted detectable protease from 6 to 10 days.

Substrate Specificity of β-Mannanases

Activity of 1% (w/v) Avicel-grown culture filtrates from *L. saepiria*, *P. versicolor* and *S. commune* was tested (Table 3) on β-glucomannan (from konjacu root powder) β-galactomannan (locust bean and guar gums) and α-mannan (yeast mannan). No α-mannanase activity was detectable in any culture filtrate. While crude enzymes from *L. saepiria* and *S. commune* degraded glucomannan and galactomannan with almost equal efficiency, and guar gum to a significantly lesser

Table 2. Effect of medium supplements on β-mannanase and endoxylanase activities in *Polyporus versicolor* and *Schizophyllum commune.**

Supplement	β-Mannanase		Endoxylanase	
	U/ml	Specific activity†	U/ml	Specific activity†
A. Polyporus versiocolor				
Avicel	1.92	3.99	2.29	8.37
Locust bean gum	1.68	3.42	1.07	2.18
Oat-spelts xylan	0.62	1.88	0.58	1.76
Acid-swollen Avicel	0.15	1.35	0.40	3.51
B. *Schizophyllum commune*				
Avicel	1.32	2.00	66.50	100.75
Locust bean gum	0.67	3.19	9.32	44.38
Oat-spelts xylan	0.19	2.31	24.54	79.92
Acid-swollen Avicel	0.59	1.90	26.85	86.60

* All cultures were grown for 8 days at 30°C in media containing 1% (w/v) of each supplement, except for locust bean gum which was used at a final concentration of 0.1% (w/v).
† Specific activity is expressed as U/mg protein.

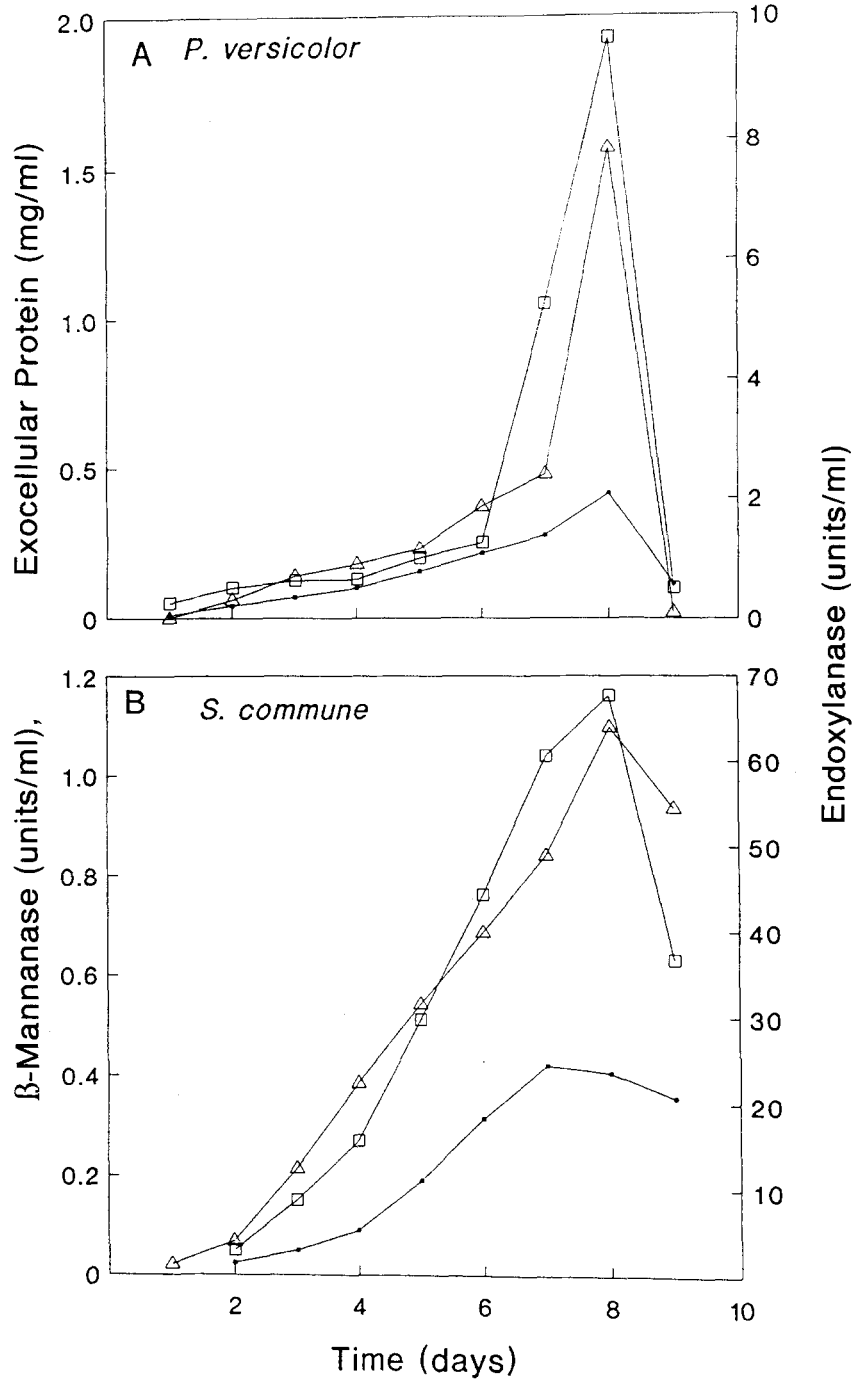

Figure 1. Time-course of β-mannanase and endoxylanase production by *P. versicolor* (A) and *S. commune* (B) grown in Avicel-supplemented media. ●— exocellular (protein; □— β-mannanase; △—endoxylanase.

extent, glucomannan was clearly the preferred substrate for crude β-mannanase from *P. versicolor* whose K_m for glucomannan was the lowest observed value. Guar gum did not serve as a substrate for crude *P. versicolor* β-mannanase.

Enzyme Characterization

The elution profiles of untreated konjacu root powder glucomannan and an 18 h *P. versicolor* digest of the same material fractionated by HPLC appears in Figure 2. While enzyme treatment resulted in significant depolymerization, no products occurring in the monosaccharide region of eluted materials were observed. Fractionation patterns were qualitatively the same for digests using crude enzyme from *L. saepiria* and *S. commune*.

Table 3. Activity of fungal β-mannanases on various mannans*.

Enzyme source	Substrate†	Activity‡	Relative % activity	K_m (%)
	Glucomannan	0.32	100	0.67
L. saepiria	Locust bean gum	0.34	106	0.72
	Guar	0.12	38	0.96
	Glucomannan	3.99	100	0.51
P. versicolor	Locust bean gum	3.42	86	3.42
	Guar	0	0	—
	Glucomannan	2.00	100	0.75
S. commune	Locust bean gum	2.12	106	0.79
	Guar	0.27	14	1.89

* Enzyme was from 8 day Avicel-supplemented culture filtrates.
† Glucomannan was prepared from konjacu root powder.
‡ Activity is expressed as μmol reducing power/min/mg/protein.

Crude β-mannanase activities from both *P. versicolor* and *S. commune* were active over a broad pH range from 4.0 to 9.0 with clear maxima at pH 5.5. Temperature optima were 65° and 55°C for *P. versicolor* and *S. commune* enzymes respectively.

Discussion

The need for multiple enzyme systems for the degradation of xylan wherein the backbone may be variously substituted with *O*-acetyl groups, arabinose, uronic acids and cinnamic-acid-based esters is now clearly recognized (Smith & Hartley 1983; Greve *et al.* 1984; van Soest *et al.* 1984; Biely 1985; Puls *et al.* 1987). Enzyme systems which remove such substituents are required to facilitate the complete action of endoxylanase. While the degradation of β-mannans appears less complex, hydrolysis is affected by the degree and pattern of main-chain α-D-galactosylation in galactomannan and galactoglucomannan, and by the pattern and distribution of main-chain D-glucosylation in glucomannan. In addition, presence of *O*-acetyl groups in glucomannan may adversely affect the action of β-mannanase (McCleary 1988). Although little information on the effect of β-mannan- and xylanase-degrading enzymes on wood pulps is available, preliminary studies infer the requirement for a multiplicity of activities to enhance lignin removal or to alter efficiently pulp properties (Viikari *et al.* 1987; Kantelinen *et al.* 1988). Industrial strategies utilizing such enzyme treatments would be, therefore, most economically vectored to the use of single microorganisms producing multiple enzyme systems.

Of several wood rotting fungi screened here, *P. versicolor* and *S. commune* were superior both in terms of the spectrum and levels of enzyme activities produced (Table 1). Although several of the other organisms produced enzymes having comparable or greater specific activity than those from *P. versicolor* and *S. commune*, this was a result of significantly lower levels of exocellular protein. Low activities of exocellular protein (less than 0.1 mg/ml) appeared to be a cultural characteristic in that all organisms produced copious cell mass when grown in wheat-bran-supplemented media. Altered nutritional conditions or use of other materials as inducers could possibly enhance enzyme production and/or levels of excreted protein in these organisms.

Nutritional supplements had a significant effect on the production of β-mannanase and endoxylanase by *P. versicolor* and *S. commune* (Table 2). Surprisingly, in both systems, Avicel was a more efficient inducer of β-mannanase and endoxylanase activities than either β-mannan from locust bean gum or xylan from oat spelts. β-Mannanase activities were 2- and 1.6-fold greater in Avicel-supplemented cultures of *P. versicolor* and *S. commune*, respectively, than in wheat-bran-

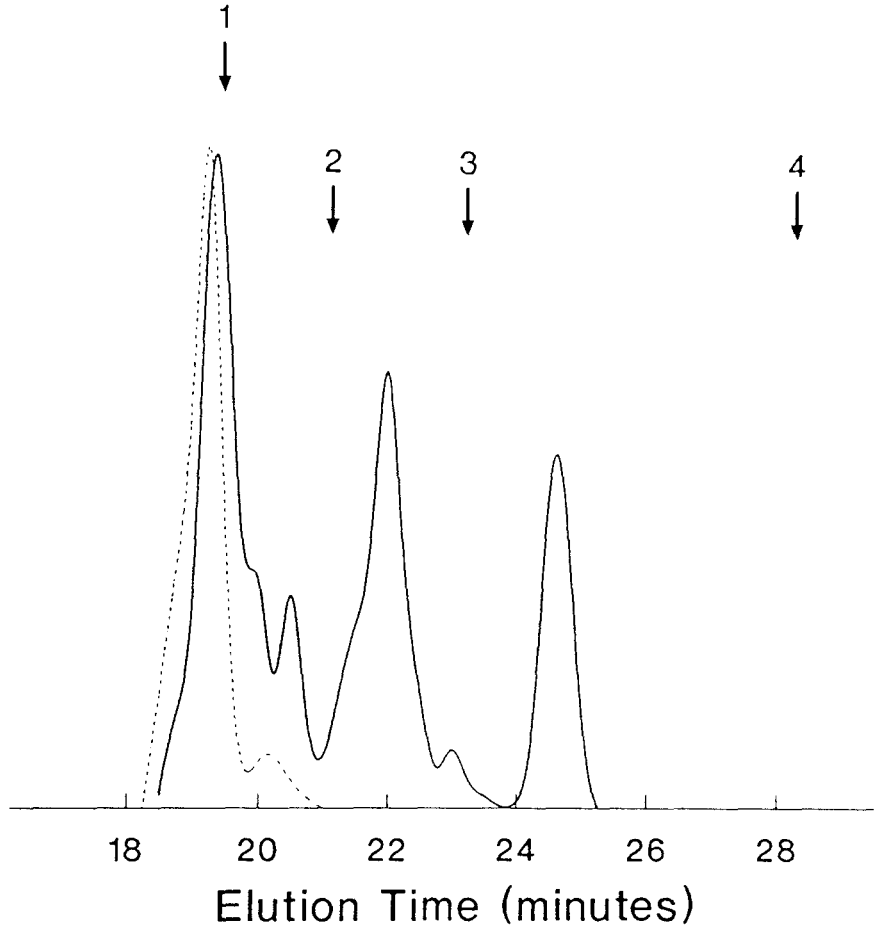

Figure 2. HPLC analysis of hydrolysis products of konjacu root powder glucomannan by crude *P. versicolor* β-mannanase. (----) untreated glucomannan; (—) enzyme digested glucomannan. Dextran 150 (1), maltotriose (2), cellobiose (3) and glucose (4) were used as standards in HPLC.

grown cultures. Nutritional studies with *Aspergillus awamori* and *Trichoderma reesei* indicated that supplementation of cultures with locust bean gum resulted in a 1.6- to 2.2-fold increases in β-mannanase levels, respectively (Rättö and Poutanen 1988), but the effect of Avicel was not determined. In any case, β-mannanase levels in Avicel-grown cultures of *P. versicolor* and *S. commune* were comparable to those observed in *A. awamori* and *T. reesei* (Rättö and Poutanen 1988). The precipitous decline in levels of measured culture parameters (Figure 1) after reaching their maxima is somewhat paradoxical in that sharp reductions in β-mannanase, endoxylanase and, to a lesser extent, exocellular protein occurred in the absence of detectable protease activity. Whether this drop resulted from an impairment of the excretion process, a switch-over to secondary metabolism or lack of reactivity of excreted proteases with the Azocoll substrate used to measure proteolytic activity is presently unclear.

Substrate specificity studies clearly differentiated the β-mannanase from *P. versicolor* from the activities produced by *S. commune* and *L. saepiria* (Table 3). On the basis of both activity and K_m, the order of substrate preference of *P. versicolor* β-mannanase was glucomannan > locust bean gum > guar. The K_m for galactomannan from locust bean gum was 6.7-fold greater than that for glucomannan. In contrast, the β-mannanases from *L. saepiria* and *S. commune* presented nearly equivalent relative activities and K_m values for glucomannan and locust bean galactomannan substrates. Overall, observed K_m values for galactomannan were 17- to 25-fold greater than those documented for *Aspergillus niger* β-mannanase acting on carob galactomannan but were comparable to Lucerne-seed β-D-mannanase acting on the same substrate (McCleary 1983). Predictably, the more-substituted galactomannan from guar gum was attacked to a lesser degree by

β-mannanases from *L. saepiria* and *S. commune*, and not at all by the activity from *P. versicolor*.

Fractionation of *P. versicolor* β-mannanase-digested glucomannan and similar digests with crude *L. saepiria* and *S. commune* disclosed considerable substrate depolymerization without concomitant monosaccharide formation, an observation consistent with the lack of detectable β-mannosidase activity. As such, culture filtrates from these organisms may be highly suitable for enhanced delignification or alteration of pulp properties where only limited hydrolysis of the main polymer chain is required. Purification and characterization of the β-mannanase activity from *P. versicolor* is in progress.

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