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Efficient Production of Mannan-Degrading Enzymes by the Basidiomycete *Sclerotium rolfsii*

ALOIS SACHSLEHNER,[1] DIETMAR HALTRICH,*,[1] GEORG GÜBITZ,[2] BERND NIDETZKY[1] AND KLAUS D. KULBE[1]

[1] *Abteilung Biochemische Technologie, Institut für Lebensmitteltechnologie, Universität für Bodenkultur BOKU (University of Agricultural Sciences Vienna), Muthgasse 18, A-1190 Wien, Austria;* [2] *Institut für Mikrobiologie, Technische Universität Graz, Petersgasse 12, A-8010 Graz, Austria*

ABSTRACT

Sclerotium rolfsii CBS 191.62 was cultivated on a number of carbon (C) sources, including mono- and disaccharides, as well as on polysaccharides, to study the formation of different mannan-degrading enzyme activities. Highest levels of mannanase activity were obtained when α-cellulose-based media were used for growth, but formation of mannanase could not be enhanced by employing galactomannan as the only carbon source. Although both xylanase and cellulase formation was almost completely repressed when *S. rolfsii* was grown on more readily metabolizable carbohydrates, including glucose or mannose, considerable amounts of mannanase activity were secreted under these growth conditions. Enhanced mannanase production only commenced when glucose was depleted in the medium. The maximal mannanase activity of 240 IU/mL obtained in a laboratory fermentation is remarkable. Mannanase activity formed under these derepressed conditions could be mainly attributed to one major, acidic mannanase isoenzyme with a pI value of 2.75.

Index Entries: *Sclerotium rolfsii;* mannanase; xylanase; endoglucanase; regulation.

INTRODUCTION

Hemicellulose forms, together with cellulose and lignin, the main polymeric constituents of lignocellulose, which is a major reservoir of fixed carbon (C) in nature. Hemicellulose comprises several heterogeneous groups

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

of polysaccharides, which are combined in this group on essentially practical and historical reasons, such as solubility in alkali and application of chemical extraction procedures. These are usually named according to the main sugar residues in the backbone chain of the polymer, e.g., xylans, mannans, and galactans. Depending on the source from which they have been obtained, and the physicochemical extraction procedure, hemicelluloses vary significantly in their structures and mol wt *(1)*.

The major hemicelluloses in softwoods are galactoglucomannans; their content varies between 15–20% of the total dry wt. In hardwood, they are found in quantities up to 5%. Glucomannans, and especially galactomannans, also occur in annual plants, mainly in seeds and tubers, in which they serve as storage carbohydrates. The mannose and glucose units in the backbone of softwood mannans are partially substituted at the *O*-2 and *O*-3 position by acetyl groups. Additionally, D-galactosyl units are attached to the main chain by α-(1,6)-bonds to a varying extent, but these side groups are not found in hardwood mannans *(1–3)*.

Owing to the complex structure of mannans, several different enzymes are necessary for their complete enzymatic degradation. The backbone is hydrolyzed by the action of endo-(1,4)-β-D-mannanase, yielding mannobiose and mannotriose, and various mixed oligosaccharides, which are further cleaved by β-D-mannosidase and β-D-glucosidase. The side group substituents are removed by α-D-galactosidase and various esterases, including acetylesterase *(4)*.

β-Mannanases have been reported to be isolated from a wide spectrum of organisms, including bacteria, fungi, germinating seeds of terrestrial plants, marine algae, and animals *(5)*, as well as yeasts and yeast-like microorganisms *(6)*. Similarly, the occurrence of β-mannosidase, α-galactosidase, and various esterases has been described for a large range of plant and animal tissues, as well as for many microorganisms *(7)*.

Mannan-degrading enzymes can find numerous applications in the food, feed, and pulp and paper industries. Their employment is probably most useful when the selective removal of mannans is required. Limited endohydrolysis or removal of side groups may be applied for the modification of mannans used as thickeners in the food industry. Mannanases can also be used in the processing of instant coffee, in which they reduce the viscosity of the coffee extracts by hydrolyzing galactomannans, which decreases the costs for subsequent evaporation and drying *(8)*. In the past few years, the employment of mannan-degrading enzymes in the pulp and paper industry gained much interest. In combination with xylanases, mannanases are used to partially hydrolyze mannan and xylan in kraft pulps. This leads to an increase in brightness and to a significant decrease in the amount of chemicals required for bleaching *(9)*.

Sclerotium rolfsii (or *Athelia rolfsii*, which is used for the teleomorph) is an aggressive plant pathogen of many crops in the tropics and subtropics. The fungus colonizes organic matter in the soil, from where it may

parasitize certain plants. During its attack to plant material, it forms several different enzymes that rapidly destroy cell walls, thus enabling it to enter the host. *S. rolfsii* is known as a good producer of cellulolytic and hemicellulolytic enzymes, including mannanases *(10,11)*. It was the aim of our work to investigate the formation of mannan-degrading enzymes in more detail.

MATERIALS AND METHODS

Chemicals

α-Cellulose, *p*-nitrophenyl glycosides, α-naphthyl acetate, locust bean gum (LBG; a galactomannan from *Ceratonia siliqua*, with a mannose-to-galactose ratio of 4:1), and guar gum (a galactomannan from *Cyamopsis tetragonobola*, with a mannose-to-galactose ratio of 2:1) were from Sigma (St. Louis, MO); lactose, L-sorbose, D-mannose, D-melibiose, D-raffinose, and carboxymethylcellulose were from Fluka (Buchs, Switzerland). Xylo-oligosaccharides containing more than 95% xylobiose (xylooligo-95) were a kind gift from Suntory (Tokyo, Japan). The hydrolysate of LBG was prepared as follows. Fifty g of LBG were dissolved in 1 L 0.05 *M* sodium citrate buffer, pH 4.0, and incubated with a crude culture filtrate of *S. rolfsii* (20 U mannanase activity/g LBG) for 24 h on an orbital shaker (170 rpm, 50°C). The hydrolysate, consisting mostly of mannobiose and mannotriose, was then lyophilized. Azo-carob galactomannan (covalently dyed with Remazol brilliant blue) was purchased from Megazyme (Sydney, Australia). Xylan from birchwood was from Roth (Karlsruhe, Germany), and peptone from meat was from Merck (Darmstadt, Germany). All other chemicals were analytical grade.

Organism and Culture Conditions

Sclerotium (Athelia) rolfsii CBS 191.62 (Centraalbureau voor Schimmelcultures, Baarn, The Netherlands) was used throughout this study. Stock cultures were maintained on glucose-maltose Sabouraud agar, and routinely subcultured every 4 wk. Inoculated plates were incubated at 30°C for 4–6 d, and then stored at 4°C.

The strain was cultivated in unbaffled 300-mL Erlenmeyer flasks at 30°C for 13 d on a medium containing (in g/L) peptone from meat, 80; NH_4NO_3, 2.5; $MgSO_4 \cdot 7H_2O$, 1.5; KH_2PO_4, 1.2; KCl, 0.6, and trace element solution, 0.3 mL/L *(12)*. The trace element solution is comprised of (in g/L): $ZnSO_4 \cdot 7H_2O$, 1.0; $MnCl_2 \cdot 4H_2O$, 0.3; H_3BO_3, 3.0; $CoCl_2 \cdot 6H_2O$, 2.0; $CuSO_4 \cdot 5H_2O$, 0.1; $NiCl_2 \cdot 6H_2O$, 0.2, and H_2SO_4 conc., 4.0 mL/L. Carbon sources were added as indicated at a concentration of 42.6 g/L, unless otherwise stated. All media were prepared with tap water. The pH was adjusted to 5.0 using phosphoric acid prior to sterilization. Flasks were inoculated with a 1-cm^2 piece from an actively growing, 4–6-d-old culture of *S. rolfsii* on Sabouraud agar. All media not containing insoluble compo-

nents were homogenized with a laboratory homogenizer (Polytron, Kinematica, Kriens, Switzerland) at 9,500 rpm for 15 s after inoculation. This was necessary to break up the piece of agar with the mycelium to obtain homogeneous growth. The inoculated flasks were incubated at 30°C, with continuous shaking at 150 rpm (stroke 25 mm), for 13 d. Each culture was then centrifuged, and the clear supernatant was used for the estimation of enzyme activities. Results given are the mean of at least duplicate experiments.

Fermentation studies were carried out in a 20-L laboratory fermenter (MBR Bio Reactor, Wetzikon, Switzerland) with a working volume of 15 L, and equipped with four disk turbine impellers, each with six flat blades.

Enzyme Activity Assays

All activity assays were carried out in 0.05 *M* sodium citrate buffer, pH 4.5, unless otherwise stated. Mannanase (EC 3.2.1.78) activity was assayed using a 0.5% solution of LBG galactomannan in 0.05 *M* sodium citrate buffer, pH 4.0, as a substrate. The release of reducing sugars in 5 min at 50°C was measured as mannose equivalents, using the dinitrosalicylic acid (DNS) method *(13)*. Xylanase (EC 3.2.1.8) and endoglucanase (carboxymethylcellulase, EC 3.2.1.4) activities were assayed similar to mannanase activity, using a 1% solution of xylan (4-*O*-methyl glucuronoxylan from birchwood) or of carboxymethylcellulose (sodium salt, ultra-low viscosity) respectively, as the substrates. Reducing sugars were assayed as xylose or glucose, using the DNS method. Filter paper cellulase activity was measured according to IUPAC recommendations, employing filter paper (Whatman No. 1, Maidstone, UK) as a substrate *(14)*. One unit (IU) of enzyme activity is defined as the amount of enzyme releasing 1 μmol of xylose, mannose, or glucose equivalents per min under the given conditions. One IU corresponds to 16.67 nkat.

α-Galactosidase (EC 3.2.1.22), β-glucosidase (EC 3.2.1.21), and β-mannosidase (EC 3.2.1.25) were quantified in a similar manner, using the respective *p*-nitrophenyl-glycosides (8 m*M* final concentration) as substrates. Buffer (0.5 mL) was incubated with 0.25 mL of the appropriately diluted enzyme solution and 0.25 mL of substrate solution at 50°C for 10 min. The reaction was stopped by adding 2.0 mL of 1 *M* Na_2CO_3, and the absorbance was measured at 405 nm. Activities are expressed on the basis of the liberation of *p*-nitrophenol.

Acetyl esterase (EC 3.1.1.6) activity was determined using 1 m*M* α-naphthylacetate as the substrate *(15)*. One unit of enzyme activity is expressed as the amount of enzyme liberating 1 μmol α-naphthol per min.

Protein Assays

Protein concentrations were determined according to the dye-binding method of Bradford *(16)*, using bovine serum albumin (fraction V, United States Biochemical Corp., Cleveland, OH) as standard.

Analytical Isoelectrical Focusing (IEF) and Activity Stains

Isoelectric focusing was carried out on the Pharmacia Phast System using precast, dry gels (PhastGel dry IEF, Pharmacia, Uppsala, Sweden), rehydrated with carrier ampholytes (7.5 parts Pharmalyte, pH 2.5–5, and 2.5 parts Ampholine, pH 3.5–5.0; Pharmacia), as described by the manufacturer. Mannanase activity in IEF gels was detected by active staining (zymogram technique), using covalently dyed mannan overlays, as described by Biely *(17)*. The pI values of the mannanase isoenzymes were determined by comparison with marker proteins (Pharmacia, low pI kit, pH range 2.8–6.5), which were run simultaneously, and were visualized by silver staining, as recommended by the manufacturer.

RESULTS

Growth Experiments

Sclerotium rolfsii CBS 191.62, which was identified as an outstanding producing strain of mannanase activity when grown on cellulose-based media *(11)*, was cultivated on a number of different substrates, to investigate, in detail, the formation of mannan-degrading enzymes. These substrates included various polysaccharides, which structurally resemble the main carbohydrate constituents of lignocellulose, and disaccharides, which are liberated from these polysaccharides by the action of the respective endoglycanases. Furthermore, several well-known inducers of cellulolytic or hemicellulolytic enzymes, including lactose and sorbose *(18,19)*, as well as a number of more easily metabolized sugars were employed. A blank containing no carbohydrate supplemented to the medium was used. After 13 d of growth, the mycelia were separated by centrifugation, and several mannan-degrading enzymes, as well as endoxylanase and endoglucanase, were assayed in the culture supernatants (Tables 1 and 2). Growth of *S. rolfsii* on α-cellulose resulted in the highest activities of all three endoglycanases investigated. This stimulating effect of α-cellulose is especially pronounced for both xylanase and endoglucanase, since activities resulting from growth on the other substrates used in this experiment were lower by at least one order of magnitude. Titers of mannanase could not be enhanced when α-cellulose was substituted by several mannans as the inducing substrate. However, these latter C sources were employed in lower concentrations, because they drastically increased the viscosity of the culture medium. The superiority of cellulose as inducer of mannanase is also reflected when comparing results obtained for cellobiose and the manno-oligosaccharides (LBG hydrolysate). The former substrate clearly stimulated the formation of higher mannanase activities. Although growth of the fungus on more readily metabolized carbohydrates, e.g., glucose or mannose, resulted in only low, presumably constitutive levels of both

Table 1
Formation of Extracellular Protein and Endoglycanase Activities by *S. rolfsii* CBS 191.62 when Grown in Shaken Flasks on Different Substrates (Conditions: 30°C, 150 rpm, 13 d)

Inducing substrate[a] (g/L)	Extracellular protein (mg/mL)	Activities (IU/mL)			Ratios		
		Mannanase	Xylanase	Endoglucanase	Mannanase to endoglucanase	Mannanase to xylanase	Xylanase to endoglucanase
Polysaccharides							
Cellulose	3.83	675	228	1090	0.62	2.96	0.21
Guar gum (21.3)	1.04	169	12.2	41.6	4.06	13.9	0.29
LBG[b] (21.3)	1.01	146	7.8	26.6	5.49	18.7	0.29
Xylan birchwood	0.41	10.2	4.6	2.9	3.52	2.22	1.59
Oligosaccharides							
Cellobiose	1.12	286	11.0	127	2.25	26	0.09
Lactose	0.90	135	1.4	10.6	12.7	96.4	0.13
Xylooligo-95	0.52	98.2	4.2	8.0	12.3	23.4	0.53
Hydrolysate of LBG[b]	0.72	167	1.6	22.1	7.56	104	0.07
Melibiose	0.64	94.4	1.4	8.4	11.2	67.4	0.17
Raffinose	0.76	122	1.0	10.9	11.2	122	0.09
Monosaccharides							
D-Glucose	0.62	81.5	0.59	6.8	12.0	138	0.09
D-Mannose	0.60	113	0.71	6.4	17.7	159	0.11
L-Sorbose	0.30	98.8	7.1	15.8	6.25	13.9	0.45
D-Galactose	0.25	31.9	0.94	3.5	9.11	33.9	0.27
D-Fructose	0.70	25.1	0.24	2.0	12.6	104	0.12
Blank	0.26	6.3	0.16	0.33	19.1	39.4	0.48

[a] Concentration of substrates was 42.6 g/L, unless otherwise indicated.
[b] Locust bean gum.

Table 2
Effect of Different Substrates on Production of Auxiliary Mannan-Degrading Enzymes by *S. rolfsii* when Grown in Shaken Flasks

Inducing substrate[a] (g/L)	Activities (IU/mL)			
	β-Mannosidase	β-Glucosidase	α-Galactosidase	Acetylesterase
Polysaccharides				
Cellulose	0.50	1.31	7.44	35.8
Guar gum (21.3)	0.61	1.17	5.63	16.5
LBG[b] (21.3)	0.57	1.41	5.44	15.9
Xylan birchwood	0.40	1.57	2.52	5.40
Oligosaccharides				
Cellobiose	0.49	0.72	3.94	13.7
Lactose	0.48	0.57	2.65	7.60
Xylooligo-95	0.43	0.70	2.76	6.10
Hydrolysate of LBG[b]	0.57	0.93	3.99	13.1
Melibiose	0.47	0.70	2.81	9.24
Raffinose	0.56	0.57	3.48	11.7
Monosaccharides				
D-Glucose	0.28	0.57	2.36	8.60
D-Mannose	0.22	0.45	2.40	8.20
L-Sorbose	0.25	1.16	1.66	5.40
D-Galactose	0.20	0.60	1.42	2.80
Blank	0.20	0.63	1.71	1.90

[a] Concentration of inducing substrate was 42.6 g/L, unless otherwise indicated.
[b] Locust bean gum.

xylanase and endoglucanase, considerable activities of mannanase were formed in the cultivations on these monosaccharides.

Even though *S. rolfsii* did not produce increased levels of mannanase when cultured on mannan-based media, the galactomannans specifically provoked the synthesis of mannanase activity, with relatively lower concomitant formation of endoglucanase and xylanase, when compared to cultures using α-cellulose or cellobiose. This is obvious from the ratios of mannanase to endoglucanase or xylanase, which were calculated from the experimental data, and are given in Table 1. The value for the mannanase-to-endoglucanase ratio of 0.62, which was relatively constant for cellulose-based cultures *(11)*, increased significantly for the cultivations on the galactomannans. An even further increase of this ratio, indicating that relatively more mannanase than endoglucanase activity is secreted by the fungus, was observed for several oligosaccharides, including manno-oligosaccharides, as well as for most of the more easily metabolizable monosaccharides. The highest value of this ratio, 17.7, was obtained for the mannose-based medium. A similar conclusion can be drawn when considering the mannanase-to-xylanase ratio. Cultivations on various mannans and manno-oligosaccharides, as well as on a number of monosaccharides, resulted in a relatively increased formation of mannanase, compared to the simultaneously produced xylanase activity (Table 1).

As is evident from Table 2, the effect of the inducing substrate employed in the cultivations is less pronounced with respect to the formation of the auxiliary mannan-degrading enzymes, which are necessary for the complete hydrolysis of substituted mannans, than for the endoglycanases. However, the experimental data suggest that the synthesis of these hydrolases is inducible as well. Highest activities of β-mannosidase were obtained when the fungus was grown on mannan or on the manno-oligosaccharides; the formation of elevated activities of β-glucosidase was provoked by growth on cellulose, cellobiose, or sorbose. The latter is a known inducer of cellulases in fungi *(18,20)*. Similarly, the oligosaccharides melibiose and raffinose, which contain an α-1,6-linked galactosyl moiety, as well as galactomannan or the LBG hydrolysate, when employed as substrates, stimulated the synthesis of α-galactosidase. Interestingly, α-cellulose was clearly the best inducer for the formation of this enzyme which is part of the mannan-degrading enzyme system of the fungus.

Fermentation Studies

To study the effect of the more readily metabolized sugars on the formation of the mannan-degrading enzyme system by *S. rolfsii*, and to evaluate the use of these sugars as cheap substrates for the production of mannanases, *S. rolfsii* was cultivated in a 20-L laboratory fermenter, using 42.6 g/L glucose as the main C and energy source. The inoculum was an

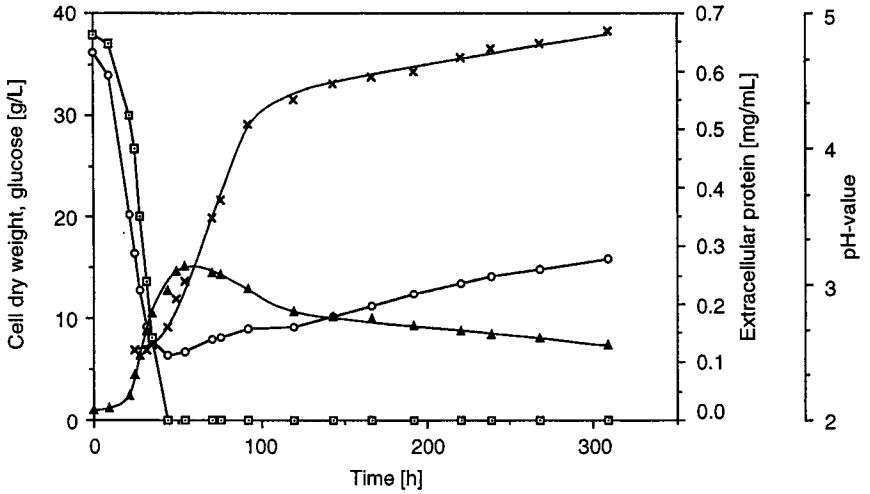

Fig. 1. Time-course of a laboratory cultivation of *S. rolfsii* CBS 191.62 in a 20-L stirred-tank reactor (working volume 15 L) using a medium based on glucose (42.6 g/L). The temperature was controlled at 30°C, and the pH, initially adjusted to 5.0, was allowed to float. Aeration was automatically varied from 0.1 to 1.0 vol of air/fluid vol/min to maintain a pO_2 of 30% of air saturation. Symbols: (□), glucose; (○), culture pH; (×), extracellular protein; (▲), cell dry wt.

11-d-old shaken culture grown on the glucose-based fermentation medium. The time-course of this cultivation is shown in Fig. 1. Glucose was rapidly consumed during the initial phase of the cultivation, and was depleted after 44 h. This initial phase of glucose consumption was accompanied by a characteristic decrease in the culture pH from 4.7 to 2.5. The maximum amount of biomass formed also coincides with the depletion of glucose. Production of extracellular enzymes, however, was not significant in this first phase of the cultivation (Figs. 2 and 3). It only started after glucose was spent in the medium. The maximum value of mannanase activity of 240 IU/mL was reached after approx 10 d, and remained constant thereafter (Fig. 2). This corresponds to a volumetric productivity of 1,000 IU/L/h. Simultaneously, very low activities of endoglucanase and xylanase were secreted by the organism. The maximum activities of 5.6 and 3.0 IU/mL, respectively, were obtained after approx 140 h, and again remained constant for at least 170 h. Furthermore, filter paper cellulase activity could not be detected in appreciable amounts during the course of this fermentation. Compared to the shaken-flask cultivations, production of acetylesterase and several glycosidases was significantly increased in the fermentation process (Fig. 3). The maximum activities of β-mannosidase (0.65 IU/mL), β-glucosidase (1.95 IU/mL), and α-galactosidase (8.30 IU/mL) produced in this experiment were even higher than the values obtained by growth on galactomannan or α-cellulose in shaken flasks.

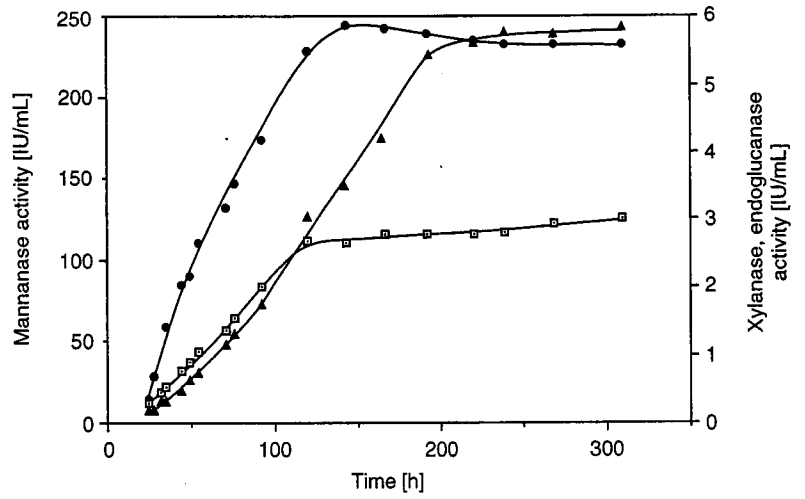

Fig. 2. Production of mannanase, xylanase, and endoglucanase by *S. rolfsii* in a laboratory fermenter. Symbols: (▲), mannanase; (□), xylanase; (●), endoglucanase.

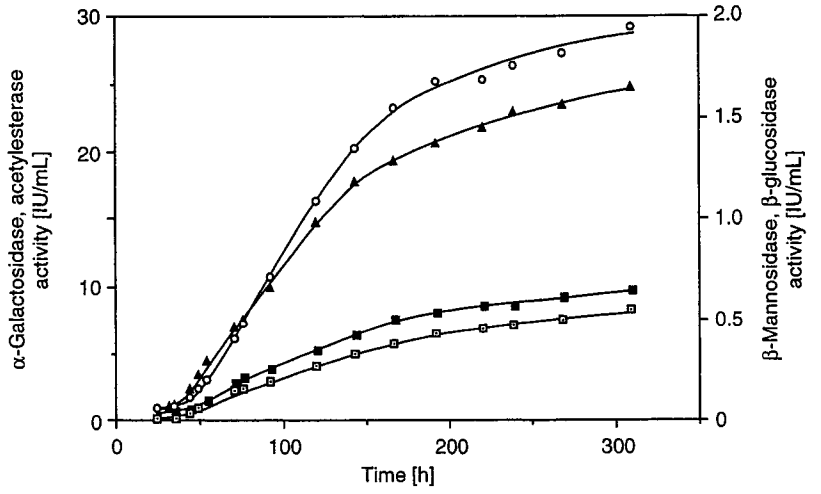

Fig. 3. Formation of β-mannosidase, β-glucosidase, α-galactosidase, and acetylesterase by *S. rolfsii*. Symbols: (▲), β-mannosidase; (○), β-glucosidase; (□), α-galactosidase; (▲), acetylesterase.

Analysis of Multiple Mannanases

S. rolfsii has recently been reported to form at least five multiple isoforms of mannanase when grown on a cellulose-based medium supplemented with konjac glucomannan *(21,22)*. To investigate the mannanase isoenzymes secreted by the organism when cultivated on glucose, the culture filtrates obtained at various cultivation times were separated by ana-

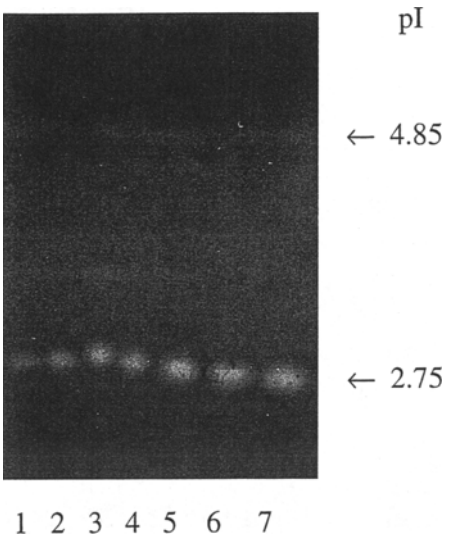

Fig. 4. Activity staining of mannanase in culture filtrates obtained at different times during a laboratory cultivation using glucose as the substrates. Lanes 1–7, samples taken at 28, 49, 71, 92, 143, 221, and 309 h, respectively. Equal activities of 0.05 IU mannanase were applied for each of the samples to the separation gel.

lytical IEF and subsequently monitored by active staining with Remazol brilliant blue (RBB) mannan overlays (Fig. 4). Mannanase activity could be attributed mostly to one protein showing a pI value of 2.75. In addition, a second minor mannanase of pI 4.85 could be detected by the zymogram analysis. Judging from the activity staining, in which equal mannanase activity in each sample was applied to the separation gel, the relative amount of these two isoformic mannanases remained relatively constant for the entire cultivation period.

DISCUSSION

In several recent studies *(11,12,23)* the basidiomycete *S. rolfsii* has been identified as an outstanding producer of mannan-degrading enzymes. Especially, the levels of endomannanase activity secreted by this plant pathogenic fungus are exceptional. In this investigation, the production of mannan-hydrolyzing enzymes was further studied. *S. rolfsii* was cultivated on a number of different substrates, including mono-, oligo-, and polysaccharides, and the resulting mannanolytic enzyme activities, together with endoglucanase and xylanase, were determined. Pure cellulose clearly is the best substrate for the production of mannanase, as well as for the other two endoglycanases by *S. rolfsii.* Levels of mannanase activities could not be enhanced when replacing cellulose by various mannans as the inducing substrate. A similar observation has been made for

several other fungi and bacteria *(10,23–26)*; *Trichoderma harzianum* produces almost the same mannanase activities during cultivation on crystalline cellulose or LBG *(27)*. When several of the low-mol-wt compounds that are described as inducers of cellulase or xylanase activity in different fungi, e.g., cellobiose or sorbose *(18,20,28)*, were used as growth substrates, elevated endoglycanase levels were obtained as well. These were, however, significantly lower than those resulting from growth on cellulose. Glucose, an easily metabolizable sugar, was used as a control in these growth experiments, and resulted in only very low activities of xylanase and endoglucanase formed, but levels of mannanase activity were surprisingly high. A similar effect was found when several other easily metabolizable, typically repressive carbohydrates, including fructose or mannose, were used as growth substrates.

From these results, it is evident that the synthesis of mannanase, xylanase, and endoglucanase is inducible in *S. rolfsii*. This is in agreement with reports on several other fungi that have been closely investigated in this respect *(18,19,28,29)*. Contrary to the reports on most other fungi, however, this induction is closely related in *S. rolfsii*, and seems to depend on the presence of cellulose. This assumption is further confirmed when comparing the enzyme activities resulting from growth on cellobiose, xylobiose (xylooligo-95), and manno-oligosaccharides (LBG hydrolysate). Highest titers of all three endoglycanase activities were unequivocally obtained in the cellobiose-based medium. Cellobiose, which is one of the end products of the enzymatic degradation of cellulose, could be a common in vivo inducer of these three endoglycanases. In accordance with the generally accepted model on induction of polysaccharide-degrading enzymes *(18,20,28)*, cellobiose is released from the substrate cellulose by the action of constitutive amounts of cellulases secreted by the organism, is taken up by the fungal cell, and finally triggers the elevated synthesis of the three enzyme activities.

Although the induction is closely related, there seems to be no common regulatory mechanism for mannanase, xylanase, and endoglucanase activity in *S. rolfsii*, since certain significant differences pertaining to the regulation of these three enzyme activities exist. The most obvious one certainly concerns the formation of mannanase, which is synthesized by the organism, even when easily metabolizable sugars are used as substrates. As was shown in a laboratory fermentation using glucose as the substrate, elevated levels of mannanase were formed under derepressed conditions, i.e., when glucose was depleted in the medium; simultaneously, only low, presumably constitutive levels of endoglucanase and xylanase were secreted by the organism. For the enhanced formation of these two latter endoglycanases, the presence of an appropriate inducer seems to be a prerequisite, indicating that the synthesis of both endoglucanase and xylanase is more tightly controlled in *S. rolfsii* than that of mannanase.

This apparent difference in the regulation, i.e., the increased formation of mannanase under derepressed, noninducing conditions, also explains

the increase in the ratios of mannanase to endoglucanase or xylanase (Table 1), which indicate that relatively higher activities of mannanase are secreted in the presence of certain carbohydrates as growth substrates. Cellulose is relatively resistant to enzymatic degradation, and therefore cellobiose will only be slowly released, which will ensure the prolonged availability of the inducer, and presumably does not lead to repressive conditions. On the other hand, enzymatic hydrolysis of the disaccharides, or even of the soluble polysaccharides mannan or xylan, will be considerably faster, which results in a more rapid degradation of the low-mol-wt inducers caused by the action of glycosidases. Under these conditions, mannanase formation will be favored.

In contrast to this, the xylanase-to-endoglucanase ratio was found to be relatively constant when different carbohydrates were used as growth substrates for *S. rolfsii* (Table 1). This indicates that the regulation of their synthesis is more closely linked. However, even here certain differences exist. The values of this ratio, calculated for the enzyme activities secreted in response to xylan or xylobiose, are significantly higher, suggesting that xylanase is preferentially formed in their presence.

When *S. rolfsii* was cultivated on glucose, only two isoforms of mannanase could be detected by the activity staining. The major mannanase produced under these growth conditions was found to have a pI value of 2.75. This acidic pI is notable, since, for most of the fungal mannanases characterized to date, this value has been reported to be in the range of 3.2–5.8 *(7)*. In addition, formation of only two isoformic mannanases under derepressed conditions is in contrast to results obtained for a wild-type isolate of *S. rolfsii*, when grown on a cellulose-based medium supplemented with konjac mannan. Under these culture conditions, five multiple mannanase isoforms were formed by this isolate *(21,22)*. Furthermore, it was found that resting mycelia of *S. rolfsii* CBS 191.62 secreted at least seven mannanase isoforms in response to bacterial cellulose, which is formed by the bacterium *Acetobacter xylinum*, and contains no traces of hemicellulose (unpublished results). This indicates that the regulation of the synthesis of mannanases in *S. rolfsii* is even more complicated. Whereas some of the multiple mannanases are formed under derepressed conditions, certain isoforms are apparently separately regulated and only efficiently secreted under inducing conditions. This complex regulation seems to correspond to the different inducibility of endoglucanases, as well as the two major xylanases of *Trichoderma reesei* QM9414 *(30,31)*.

The findings reported in this study are of technological significance. By selecting appropriate culture conditions, mannanase preparations practically free of cellulase activity can be easily produced. In this respect, the maximum mannanase activity of 240 IU/mL, obtained in a laboratory cultivation of *S. rolfsii* employing a glucose-based medium, is remarkable. This value is significantly higher when compared to data reported in the literature on the production of mannanases by other microorganisms,

using cellulose or mannan as inducing substrates *(23,25,32)*. Such enzyme preparations have gained increased interest because of new areas of application within the pulp and paper industry. This absence of cellulase activity, which would negatively affect pulp properties, is essential for these applications *(7,33)*. On the other hand, enzyme preparations containing appreciable amounts of cellulase and xylanase can be obtained when cellulose or suitable lignocellulosic material is used as the substrate *(10,11)*. Since *S. rolfsii* also forms considerable amounts of auxiliary hemicellulolytic enzymes, such enzyme preparations can be used when the complete hydrolysis of lignocellulose is the aim.

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