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Production of Hemicellulose- and Cellulose-Degrading Enzymes by Various Strains of *Sclerotium Rolfsii*

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ABSTRACT

A number of wild-type isolates of *Sclerotium rolfsii* were screened for their capacity to produce lignocellulolytic enzymes when grown on a cellulose-based medium. *S. rolfsii* proved to be an efficient producer of hemicellulolytic enzymes under the conditions selected for this screening, although there was a great variability in enzyme activities formed by the different isolates. In addition to xylanase and mannanase, which were produced in remarkably high levels, a number of accessory enzymes, which are important for the complete degradation of substituted hemicelluloses and include α-arabinosidase, acetyl esterase, and α-galactosidase, are formed by *S. rolfsii*. Efficient production of xylanase and mannanase was achieved when cellulose-based media were used for growth. Under these conditions, enhanced levels of endoglucanase were formed as well. Formation of xylanase and mannanase could be more specifically induced when using xylan or mannan as growth substrates, although the enzyme activities thus obtained were significantly lower compared to cultivations on cellulose as main inducing substrate.

Index Entries: *Sclerotium rolfsii*; xylanase; mannanase; cellulase; screening.

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INTRODUCTION

Plant cell walls are a major reservoir of fixed carbon in nature. In recent years there has been considerable interest in the utilization of plant material as a renewable source of fermentable sugars that could be subsequently converted into useful products such as liquid fuels, solvents, chemicals, food, or feed *(1)*. Such bioconversion processes are particularly attractive for the elimination of residues and wastes produced by agriculture and forestry. Hemicellulose is one of the three main polymeric constituents of lignocellulose and comprises several heterogeneous groups of polysaccharides that are combined to this group on essentially practical and historical reasons such as solubility in alkali and application of chemical extraction procedures.

Xylans are the most abundant noncellulosic polysaccharides in hardwood and annual plants, where they account for 15–30% of the total dry weight. In softwoods, they are found in lesser quantities, accounting for approx 7–10% of the tissue dry weight. The basic structure of xylans is a main chain of (1→4)-linked β-D-xylopyranosyl residues. Typically, the linear chains of the xylans carry short side chains to a varying extent. Hardwood xylans from dicotyledonous angiosperms grown in temperate zones are all remarkably alike, showing only little variation. Typical substituents for hardwood xylans are (1→2)-linked 4-*O*-methyl-α-D-glucuronic acid residues and acetyl groups, which are attached at the *O*-2 or *O*-3 sites. Softwood xylans typically carry 4-*O*-methyl-α-D-glucuronic acid and L-arabinofuranose side groups. These side groups are linked at *O*-2 and *O*-3 of the xylose units in the main chain. Uronic acid residues seem to be rather localized on contiguous xylose residues than randomly dispersed over the molecule *(2,3)*.

Mannans, or more appropriately galactoglucomannans, are the main hemicelluloses in softwoods, where their content varies between 15–20% of the total dry weight. In hardwood mannans are found in lesser quantities, constituting only up to 5%. The main chain of mannans is made up of β-(1→4)-linked D-mannopyranosyl and D-glucopyranosyl residues with widely varying proportions of these two types of sugar units. In softwood mannans α-D-galactosyl units are attached to the main chain units by (1→6)-bonds to a varying extent. The mannose and glucose units in the backbone are partially substituted at *O*-2 and *O*-3 by acetyl groups, whereas these side groups are not found in hardwood mannans *(2–4)*.

Because of this complex structure of hemicelluloses, several different enzymes are necessary for their complete enzymatic degradation. The two main glycanases hydrolyzing the hemicellulose main chains are endo-(1→4)-β-D-xylanase and endo-(1→4)-β-D-mannanase. Small oligosaccharides formed by the action of these glycanases are further cleaved by β-D-xylosidase, β-D-mannosidase, and β-D-glucosidase. The side group substituents are liberated by α-L-arabinosidase, α-D-glucuronidase, α-D-galactosidase, and various esterases including acetyl esterase *(5,6)*.

Sclerotium rolfsii (or *Athelia rolfsii*, which is used for the teleomorph) is an aggressive plant pathogen of many crop plants in the tropics and subtropics. The fungus colonizes organic matter in the soil from where it may parasitize certain plants. During its attack on plant material it forms large amounts of different enzymes that rapidly destroy host tissue and cell walls, thus enabling it to enter the host organism. *S. rolfsii* is known as an excellent producing strain of cellulolytic enzymes *(7,8)*. Furthermore, it forms high levels of a number of hemicellulolytic enzymes including xylanase and mannanase *(9)*.

It was the objective of our work to investigate the production of the spectrum of hemicellulose-degrading enzymes by different wild-type isolates of *S. rolfsii*. In addition, the effect of different carbon sources on the levels of xylanase and mannanase should be assessed. It was of special interest to investigate whether by selecting an appropriate inducing substrate high levels of hemicellulases with only low levels of concurrently produced cellulase could be attained, since hemicellulase preparations free of cellulase have gained significant interest during the last years because of their application in the pulp and paper industry *(10,11)*.

MATERIALS AND METHODS

Chemicals

α-Cellulose, *p*-nitrophenyl glycosides, α-naphthyl acetate, 2,6-dichlorophenol-indophenol, locust bean gum (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-xylose, and carboxymethylcellulose were from Fluka (Buchs, Switzerland). Konjac mannan, a glucomannan from *Amorphophallus konjac* with a mannose-to-glucose ratio of 1.8:1, was obtained from Arkopharma (Carros, France). Xylan from birchwood was from Roth (Karlsruhe, Germany) and peptone from meat was from Merck (Darmstadt, Germany). All other chemicals were analytical grade.

Organisms and Culture Conditions

Strains of *Sclerotium (Athelia) rolfsii* were obtained from Centraalbureau voor Schimmelcultures (CBS, Baarn, The Netherlands) or from the American Type Culture Collection (ATCC, Rockville, MD). 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.

All strains were 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) *(9)*. Unless otherwise indicated, 42.6 g/L α-cellulose were used as inducing substrate. All media were prepared with tap water. The pH-value was adjusted to 5.0 using phosphoric acid prior to sterilization. The trace element solution comprised (in g/L): $ZnSO_4 \cdot H_2O$, 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). Shake flasks were inoculated with a piece (1 cm^2) from an actively growing, 4–6-d-old colony of *S. rolfsii* on Sabouraud agar. The inoculated flasks were shaken continuously on an orbital shaker at 150 rpm (stroke 25 mm) and 30°C for 13 d. The culture was then centrifuged and the clear supernatant used for the estimation of enzyme activities.

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 disc 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. *Xylanase* (EC 3.2.1.8) activity was assayed using a 1% solution of xylan (4-*O*-methyl glucuronoxylan from birchwood; Roth Ltd., Karlsruhe, FRG) as a substrate *(12)*. The release of reducing sugars during 5 min at 50°C was measured as xylose equivalents using the dinitrosalicylic acid (DNS) method *(13)*. *Mannanase* (EC 3.2.1.78) and *endoglucanase* (carboxymethylcellulase, EC 3.2.1.4) activities were assayed similar to the determination of xylanase activity, using a 0.5% solution of locust bean gum galactomannan in 0.05*M* sodium citrate buffer, pH 4.0, or a 1% solution of carboxymethylcellulose (sodium salt, ultra low viscosity), respectively, as the substrates. Reducing sugars were assayed as mannose 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 liberating 1 μmol of xylose, mannose, or glucose equivalents per minute under the given conditions. 1 IU corresponds to 16.67 nkat.

α-*Arabinosidase* (α-L-arabinofuranosidase, EC 3.2.1.55), α-*galactosidase* (EC 3.2.1.22), β-*glucosidase* (EC 3.2.1.21), β-*mannosidase* (EC 3.2.1.25), and β-*xylosidase* (EC 3.2.1.37) were quantified in a similar manner, using the respective *p*-nitrophenyl-glycosides 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 (8 m*M*) at 50°C for 10 min. The reaction was stopped by adding 2.0 mL of 1*M* Na_2CO_3 and the absorbance 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-naphthylacetat as the substrate *(15)*. One unit of enzyme activity is

expressed as the amount of enzyme liberating 1μmol α-naphthol per minute.

The assay of *cellobiose dehydrogenase* (EC 1.1.99.18) activity was essentially as described by Sadana and Patil (*16*) using 1.8 m*M* cellobiose, 2.0 m*M* glucono-α-lactone, and 0.36 m*M* 2,6-dichlorophenol-indophenol in 100 m*M*-phosphate buffer, pH 6.3. Activities are expressed on the basis of the reduction of dichlorophenol-indophenol.

Protein Assays

Protein concentrations were determined according to the dye-binding method of Bradford (*17*) using bovine serum albumin (fraction V) as standard.

RESULTS

Screening of *S. rolfsii* Strains

Eight different strains of *S. rolfsii*, isolated worldwide at different geographic locations, were cultivated in shaken flasks using a cellulose-based medium. After 13 d of growth various hemicellulose- and cellulose-degrading enzyme activities produced by these different isolates were measured and compared. Results for xylanolytic, mannanolytic, and cellulolytic enzyme activities are shown in Figs. 1–3, respectively. The different strains produced all of the lignocellulose-degrading enzymes that were investigated in this study, albeit at greatly varying levels. Highest activities of xylanase were produced by *S. rolfsii* CBS 191.62 and *S. rolfsii* CBS 147.82. Furthermore, these two strains yielded the highest activities of β-xylosidase and acetyl esterase. On the other hand, xylanolytic enzyme activities formed by *S. rolfsii* CBS 149.82, which gave the lowest enzyme yields for most of the activities investigated, were only in the range of 15–25% of the maximal values produced by *S. rolfsii* CBS 191.62. Growth of *S. rolfsii* CBS 191.62 also resulted in the highest mannanase activity. Interestingly, the activity levels of the other mannan-degrading enzymes, i.e., β-mannosidase and α-galactosidase, produced by this strain were much lower in relation to the maximum values of 0.53 IU/mL for β-mannosidase formed by *S. rolfsii* CBS 151.31 and 26.4 IU/mL for α-galactosidase produced by *S. rolfsii* CBS 147.82. Highest activities of most cellulolytic enzymes were produced by *S. rolfsii* CBS 147.82. In addition to high activities of FP-cellulase (10.1 IU/mL) and endoglucanase (1800 IU/mL), a culture filtrate obtained after growth of this strain also contained high levels of β-glucosidase activity (21.2 IU/mL). Whereas the two former enzymes were produced in a similar range of activities (9.21 and 1640 IU/mL, respectively) by *S. rolfsii* CBS 191.62, β-glucosidase levels were considerably lower (2.8 IU/mL).

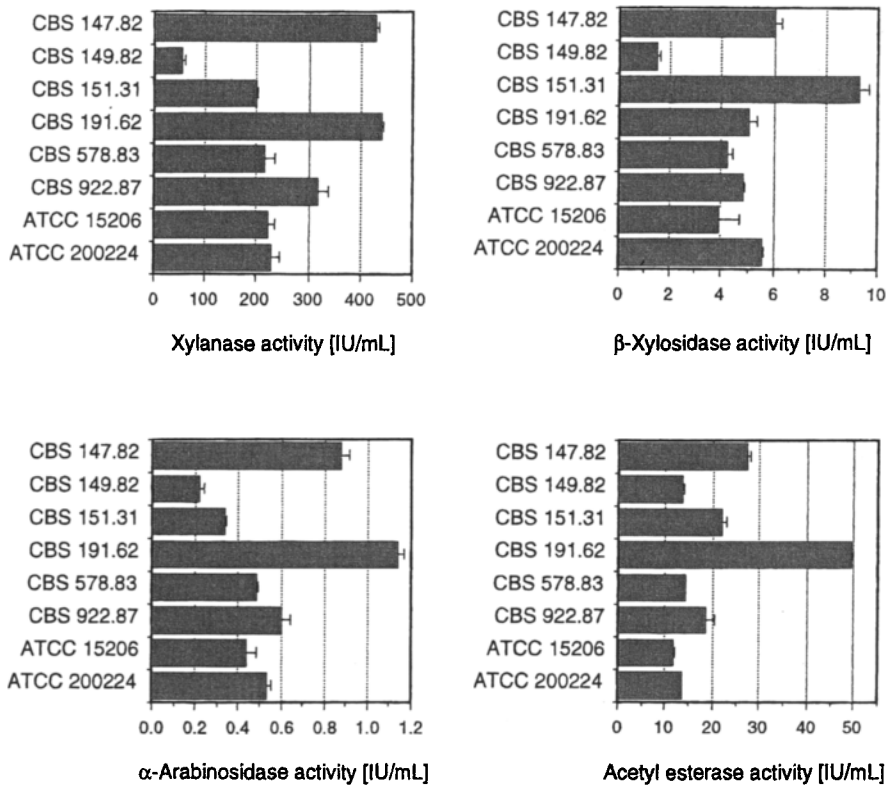

Fig. 1. Xylan-degrading enzyme activities produced by several strains of *Sclerotium rolfsii* when cultivated at 30°C on a cellulose-based medium for 13 d. Values are shown as the mean and standard deviation of two independent replicates.

Bioprocess Experiments

Strain *S. rolfsii* CBS 191.62 as the best producer of both xylanase and mannanase was selected for further studies. Production of xylanase, mannanase, and endoglucanase was followed in a 15-L laboratory fermentation using 42.6 g/L α-cellulose as the substrate. The inoculum was an 11-d-old shaken culture grown on the fermentation medium. The time course of this cultivation is shown in Fig. 4. Production of enzymes started after a lag of approx 30 h. This initial phase of growth was also accompanied by a rapid decrease in the pH from 4.8 to 3.3. Maximum xylanase and mannanase values of 192 and 568 IU/mL were reached after approx 165 h, corresponding to volumetric productivities of 1160 and 3440 IU/L · h, respectively. Thereafter, both enzyme activities remained constant for at least 3 d. Endoglucanase activity peaked after 213 h of cultivation reaching a value of 1290 IU/mL and then started to decrease slightly.

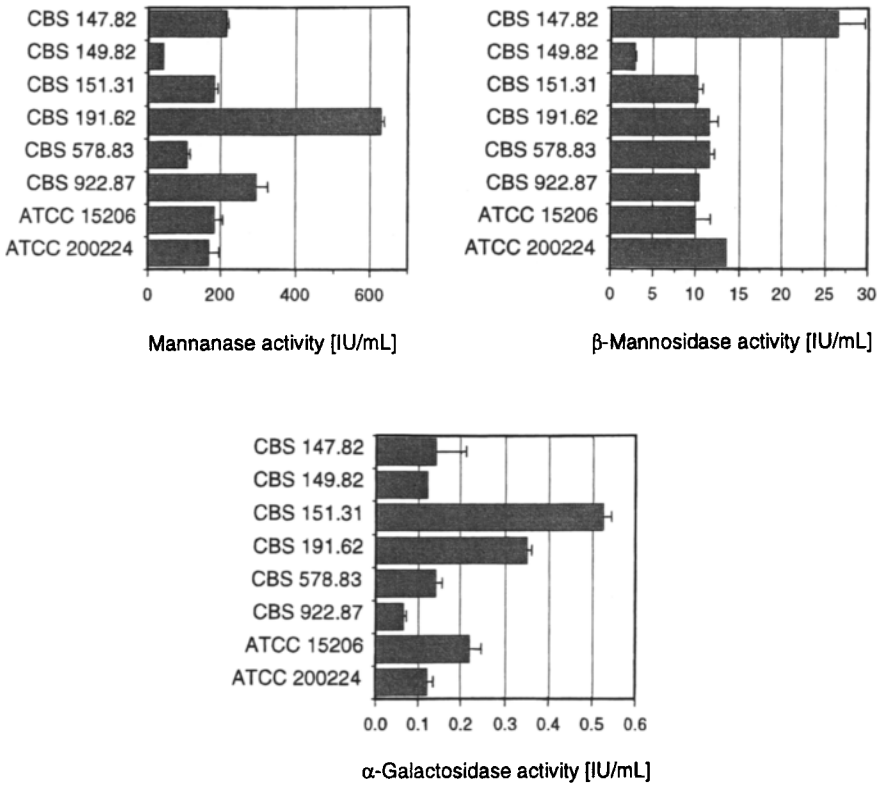

Fig. 2. Mannan-degrading enzyme activities produced by several strains of *Sclerotium rolfsii* when cultivated at 30°C on a cellulose-based medium for 13 d. Values are shown as the mean and standard deviation of two independent replicates.

Effect of Inducing Substrates

It was of special interest to investigate whether xylan or mannan when used as main carbon source could specifically provoke the synthesis of the enzyme activities necessary for the degradation of the respective polysaccharide by *S. rolfsii* CBS 191.62. Furthermore, small amounts of L-sorbose, D-xylose, or lactose, which were reported to enhance both xylanase and cellulase yields by *Trichoderma reesei (18,19)*, were added to the cellulose-based medium. The level of extracellular protein as well as xylanase, mannanase, and endoglucanase activities obtained after 13 d of growth on these different media are shown in Table 1. Highest levels of all three enzyme activities were obtained when α-cellulose was the main inducing substrate. Replacing a minute part of this C source by L-sorbose (1.5 g/L) resulted in a small, yet significant increase in xylanase activity. Formation of endoglucanase was slightly lower after growth on the medium containing L-sorbose, whereas mannanase activity was decreased by approx 20%. Whereas the supplementation of 4.3 g/L of lactose had no

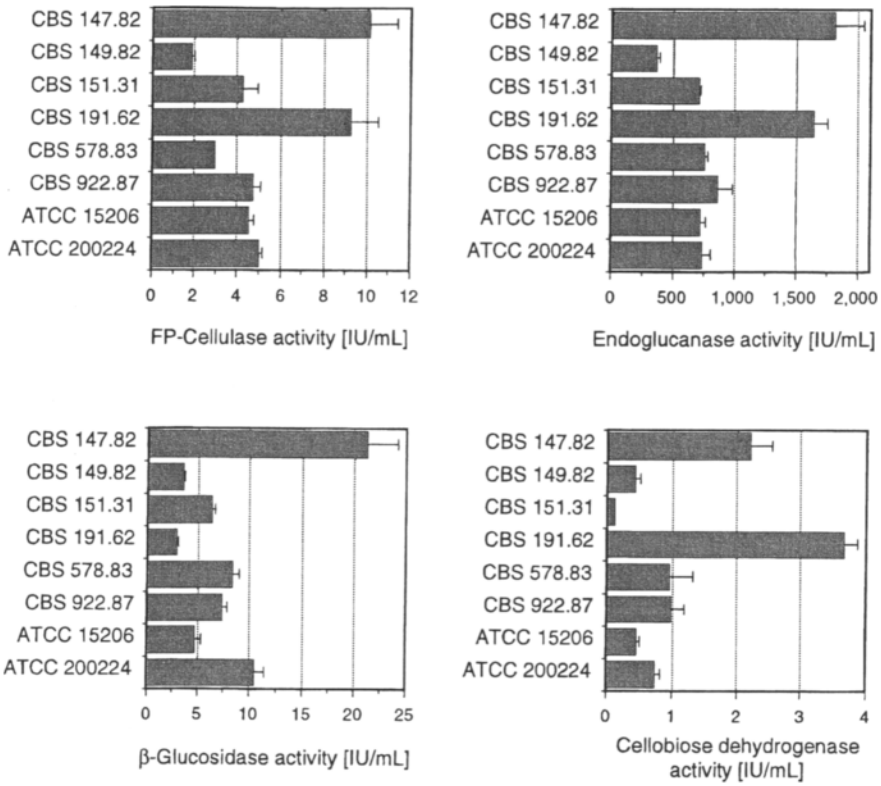

Fig. 3. Cellulose-degrading enzyme activities produced by several strains of *Sclerotium rolfsii* when cultivated at 30°C on a cellulose-based medium for 13 d. Values are shown as the mean and standard deviation of two independent replicates.

significant effect on the levels of enzymes formed by *S. rolfsii*, 4.3 g/L of D-xylose when added to the cellulose-based medium considerably reduced all three of the enzyme activities produced.

Production of mannanase could not be enhanced when several galacto- and glucomannans were used as sole inducing C sources or when these were added to cellulose. The mannans were each employed in a concentration of 21.3 g/L in these experiments. This resulted in a highly viscous culture medium that certainly caused severe mass transport limitations. However, this high viscosity was greatly reduced after only 2–3 d of cultivation because of the action of the endo-acting mannanases, which were excreted by the organism and cleaved the mannan main chains. In all cases, significantly higher mannanase activities were obtained when a mixture of mannan and α-cellulose (each at 21.3 g/L) was used as compared to a culture medium containing only mannan as main C source (Table 1). Similarly, the use of xylan did not yield higher levels of xylanase than a cultivation on an equal amount of α-cellulose.

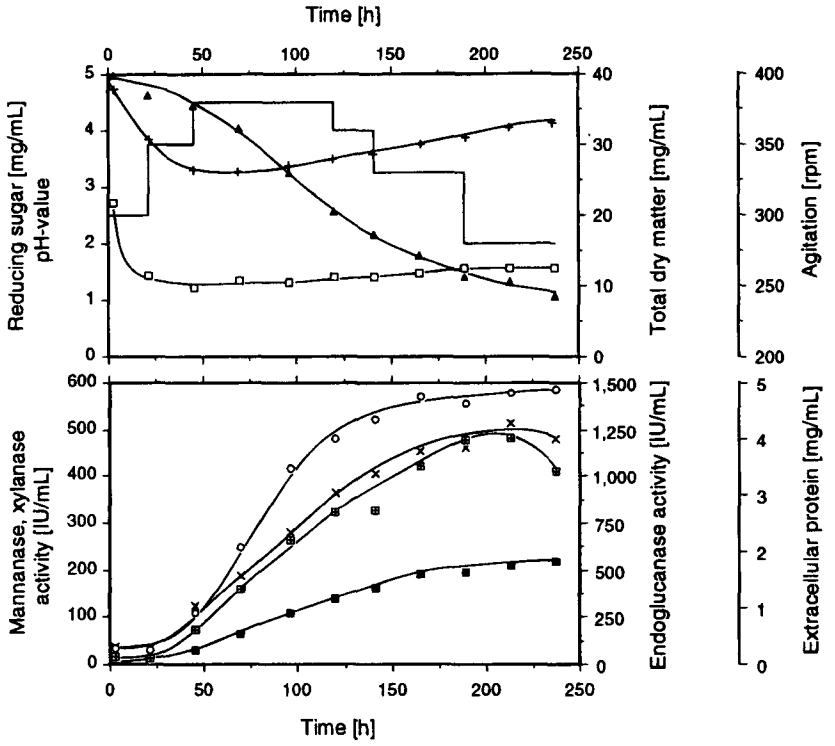

Fig. 4. Time course of xylanase, mannanase, and endoglucanase production by *Sclerotium rolfsii* CBS 191.62 in a 20-L stirred tank reactor (working volume 15 L). α-Cellulose (42.6 g/L) was used as the substrate (*9*). The temperature was controlled at 30°C and the pH, initially adjusted to 5.0, was allowed to float. Aeration was automatically varied from 0.1 to 1.0 vol of air per fluid vol per min to maintain a pO_2 of 40% of air saturation. Symbols : +, pH-value; ▲, total dry matter; □, reducing sugar; —, agitation; ⊞, extracellular protein; ○, mannanase; ■, xylanase; ×, endoglucanase.

Whereas xylan or mannan did not increase the production of xylanase or mannanase when employed as the main inducing substrates, these two polysaccharides could specifically induce the synthesis of the enzymes necessary for their hydrolysis. This is expressed by the ratios of xylanase, mannanase, and endoglucanase activities to each other that were calculated from the experimental data and are listed in Table 1. The ratio of xylanase to endoglucanase was fairly constant for the cellulose-based media (0.22–0.27). For the cultivation on xylan this value was significantly increased to 1.59, indicating that relatively more xylanase than endoglucanase is formed when *S. rolfsii* is grown on this substrate. In a similar manner, the ratio of mannanase to endoglucanase shows that mannans when employed as the only C source specifically provoke the synthesis of mannanase activity with only low levels of endoglucanase or xylanase formed. The ratio of mannanase to endoglucanase was increased to 4.06 and 5.49, respectively, when guar gum or locust bean gum galactomannan were

Table 1
Effect of Various Inducing Substrates used for Growth of *Sclerotium rolfsii* CBS 191.62 on the Formation of Extracellular Protein and Activities of Xylanase, Mannanase, and Endoglucanase[c]

Inducing substrate [g/L]	Extracellular protein [mg/mL]	Xylanase [IU/mL]	Mannanase [IU/mL]	Endo-glucanase [IU/mL]	Ratio xylanase to endoglucanase	Ratio mannanase to endoglucanase	Ratio mannanase to xylanase
Cellulose (42.6)	3.83 ± 0.11	257 ± 10	718 ± 9	1176 ± 59	0.22	0.61	2.79
Cellulose (38.3) + lactose (4.3)	4.04 ± 0.22	267 ± 7	724 ± 17	1165 ± 14	0.23	0.62	2.71
Cellulose (41.1) + sorbose (1.5)	3.83 ± 0.14	276 ± 18	580 ± 34	1104 ± 9	0.25	0.53	2.10
Cellulose (38.3) + xylose (4.3)	3.18 ± 0.10	181 ± 11	590 ± 11	765 ± 6	0.24	0.77	3.27
Cellulose (21.3) + guar gum (21.3)	3.24 ± 0.06	171 ± 6	528 ± 16	791 ± 38	0.22	0.67	3.09
α-Cellulose (21.3) + konjac GM[a] (21.3)	3.01 ± 0.30	176 ± 21	547 ± 28	645 ± 21	0.27	0.85	3.11
α-Cellulose (21.3) + LBG[b] (21.3)	2.98 ± 0.20	164 ± 18	535 ± 30	750 ± 124	0.22	0.71	3.26
Guar gum (21.3)	1.04 ± 0.07	12.2 ± 0.3	169 ± 17	41.6 ± 1.1	0.29	4.06	13.85
LBG[b] (21.3)	1.01 ± 0.06	7.8 ± 0.6	146 ± 6	26.6 ± 4.3	0.29	5.49	18.72
Xylan birchwood (42.6)	0.41 ± 0.04	4.6 ± 0.7	10.2 ± 2.5	2.9 ± 0.5	1.59	3.52	2.22

[a]Konjac glucomannan.

[b]Locust bean gum.

[c]Each value represents the mean ± standard deviation (n = 2). In addition, the ratio of these enzyme activities to each other are given.

used as the inducing substrates. This ratio was found to be in the range of 0.53–0.77 for the cellulose-based media, and even for a mixture of cellulose and mannan, both in equal concentrations, it was not significantly higher than this value.

DISCUSSION

Eight wild-type strains of *S. rolfsii*, which were screened for their capacity to produce various xylan-, mannan-, and cellulose-degrading enzymes, were all found to produce the enzyme activities investigated, although to a greatly varying extent. This variation, i.e., the ratio of the lowest to the highest values obtained, ranged from 4.3-fold for acetyl esterase to 38.5-fold for cellobiose dehydrogenase with the different strains studied.

Highest levels of cellulolytic enzyme activities were produced by *S. rolfsii* CBS 147.82. The ratio of β-glucosidase to FP-cellulase activity, which is important for efficient hydrolysis of cellulose, is significantly higher for an enzyme preparation obtained from this strain than that found for different *T. reesei* mutants *(20)*. It appears to be sufficient to hydrolyze cellulosic material at least at low substrate concentrations *(21)*, thus eliminating the need of supplementing additional β-glucosidase. The

β-glucosidase to FP-cellulase ratio varied considerably with the different fungal isolates considered in this study. The lowest value of 0.3 was found for strain CBS 191.62, whereas this ratio was calculated to be 2.8 for strain CBS 578.83.

Strain *S. rolfsii* CBS 191.62 was superior in terms of both xylanase and mannanase activities produced. Especially the maximum value of 600–700 IU/mL of mannanase activity is remarkable, since this value is significantly higher than mannanase activities produced by most other microorganisms and appears to be among the highest mannanase values ever reported *(22–25)*. In addition to glycanases, this strain also formed high levels of accessory enzyme activities that are needed for a complete degradation of substituted xylans or mannans. Interestingly, production of glycanases, which hydrolyze the polysaccharide main chains, and of auxiliary enzymes did not correlate with the different strains of *S. rolfsii* investigated in the screening. Production of relatively high activities of these glycanases does not necessarily have to be accompanied by relatively high levels of auxiliary enzymes. In the case that these accessory enzymes are of primary interest, certain strains of *S. rolfsii* could be better suited for the production of these enzymes.

The type of inducing substrate had an important effect on the production of xylanase, mannanase, and endoglucanase by *S. rolfsii* CBS 191.62. Surprisingly, highest levels of all three enzyme activities were produced when the organism was grown on a medium containing cellulose as the main substrate, whereas xylan from birchwood as well as several gluco- and galactomannans as C source yielded only low levels of xylanase or mannanase. Similar observations pertaining to mannanase production have been reported for *Polyporus versicolor, Schizophyllum commune,* and *Trichoderma reesei.* Culture filtrates from the organisms grown on cellulose showed a considerable increase in mannanase activity over those obtained after growth on various mannans *(23,26,27)*. Whereas cellulose seems to be necessary for obtaining high levels of xylanase or mannanase activity, a more specific induction of the synthesis of these two enzymes by *S. rolfsii* could be achieved when xylan or mannan were used as main C source. When employing galactomannans as growth substrates, formation of mannanase activity was reduced as compared to the values obtained after growth on cellulose, yet the ratios of mannanase to endoglucanase or xylanase were increased by a factor of approx 8, indicating that relatively more mannanase was produced. A similar effect was observed for the ratio of xylanase to endoglucanase when comparing the values obtained after growth on xylan or cellulose.

A possible explanation for this could be a complex regulatory control of the synthesis of the multiple mannanases in *S. rolfsii*, where some mannanases are specifically induced in the presence of mannans, whereas the synthesis of other isoforms could be under a common control together with cellulases and possible xylanases. An alternative expla-

nation could be the unspecificity of certain glycanases that not only show endoglucanase but mannanase and/or xylanase activity as well. This unspecificity of glycanases has been shown for certain enzymes from *Trichoderma harzianum* exhibiting mannanase and endoglucanase activities *(28)* as well as for *T. reesei* where unspecific glycanases hydrolyzed both xylan and cellulose *(29)*. However, at least one of the mannanases produced by *S. rolfsii* has recently been reported to be specific for various mannans *(30)*. The regulation of xylanase, mannanase, and endoglucanase synthesis in *S. rolfsii* is currently under investigation in the authors' laboratory.

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