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Production of Cellulolytic and Xylanolytic Enzymes During Growth of Anaerobic Fungi from Ruminant and Nonruminant Herbivores on Different Substrates

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ABSTRACT

Three anaerobic fungi, two *Neocallimastix* strains isolated from a ruminant (sheep) and one *Piromyces* strain isolated from a nonruminant (black rhinoceros), were tested for their ability to ferment a range of substrates. Bagasse, filter paper cellulose, fructose, and wheat straw were good inducers of celluloytic and xylanolytic enzymes. These enzymes were produced constitutively by all three strains, although enzyme activities were generally lower, expecially for both *Neocallimastix* strains, after growth on glucose and other soluble sugars. The isoenzyme patterns of extracellular enzyme preparations of *Neocallimastix* strains were influenced by the growth substrate.

Index Entries: Anaerobic fungi; *Neocallimastix; Piromyces;* cellulase; xylanase; activity staining.

INTRODUCTION

Anaerobic fungi inhabiting alimentary tracts of ruminant as well as nonruminant herbivorous mammals have an important role in the digestion of cellulose and xylan present in plant cell walls (*1,2*). These zoospore-forming fungi have been assigned to the genera: *Caecomyces, Neocallimastix,* and *Piromyces* (*2,3*), which have monocentric growth patterns, and *Anaeromyces* (*4*), *Orpinomyces* (*5*), and *Ruminomyces* (*6*), which

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have polycentric growth patterns. Anaerobic fungi have been shown to produce extracellular cellulolytic enzymes that degrade crystalline cellulose. Furthermore, comparative studies have shown that the extracellular cellulase of *Neocallimastix patriciarum* is more active against crystalline cellulose than the cellulase of the aerobic fungus *Trichoderma reesei* (*7*), which is considered as the cellulolytic fungus most suitable for possible industrial use in the conversion of cellulose to glucose.

From the point of view of economical cellulase and xylanase production, it is desirable to use inexpensive materials. Lignocellulosic biomasses, such as rice straw and bagasse, have been shown to be of value for production of cellulolytic and xylanolytic enzymes by aerobic fungi (*8*). Anaerobic rumen fungi are capable of fermenting a range of carbohydrates (*9,10*). The rumen fungi *Neocallimastix patriciarum, Piromyces communis,* and a *Piromyces* sp. produce a range of polysaccharide-degrading enzymes and glycoside hydrolase enzymes during growth on several carbohydrates (*11,12*). No information is given in literature with respect to these characteristics for anaerobic fungi isolated from nonruminants.

Neocallimastix frontalis strain N1, *Neocallimastix patriciarum* strain N2 isolated from a ruminant (sheep), and *Piromyces* strain R1, isolated from a black rhinoceros (a nonruminant) produce high amounts of cellulolytic enzymes when grown on filter paper cellulose (*13*). This article compares the ability of these anaerobic fungi to ferment a range of carbohydrates. The influence of different growth substrates on induction or repression of cellulolytic and xylanolytic enzyme synthesis was studied. The extracellular enzymes with β-glucosidase and endoglucanase activity were separated by SDS-PAGE and visualized by subsequent activity staining.

MATERIALS AND METHODS

Organisms and Growth Conditions

Neocallimastix patriciarum (N2) was obtained from the fungal collection of the Institute of Animal Physiology and Genetics Research of the Agriculture & Food Research Council (AFRC), Babraham, Cambridge, UK Isolation of *Neocallimastix* sp. (N1) and *Piromyces* strain R1 was described previously (*14*).

Anaerobic fungi were grown at 39°C in defined medium M2. Salt solutions A and B were used in medium preparation. Solution A contained ($g \cdot L^{-1}$) KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 3.0; NaCl, 6.0; $MgSO_4$, 0.6; and $CaCl_2$, 0.6; solution B contained K_2HPO_4, 3 $g \cdot L^{-1}$. The defined medium M2 contained: solution A, 150 mL; solution B, 150 mL; cellobiose, 4.275 g, $NaHCO_3$, 6 g; L-cysteine-HCl, 1 g; trace elements solution, 10 mL; hemin solution, 10 mL; resazurin solution (0.1%, w/v), 1 mL; and demineralized water to 950 mL. The medium was dispensed in 19.0-mL volumes in 50-mL serum bottles, and sealed with butyl rubber stoppers and aluminium crimp

caps (D. Prins B V., Schipluiden, the Netherlands). Where specified, the cellobiose was replaced by other carbohydrates (*see* Carbohydrate Utilization). The medium was then autoclaved for 20 min at 115°C. After cooling, 1.0% (v/v) vitamin solution was added. The trace element and hemin solutions were prepared as described by Lowe et al. (*15*). Vitamin solution contained ($mg \cdot L^{-1}$): thiamin-HCl, 5; riboflavin, 5; calcium D-pantothenate, 5; nicotinic acid, 5; folic acid, 2; cyanocobalamin, 1; biotin, 1; pyridoxin-HCl, 10; *p*-aminobenzoic acid, 5.

Stock cultures of anaerobic fungi were maintained on 0.1 g milled wheat straw in 19 mL medium M2. Cultures were inoculated with 1 mL of culture fluid (containing zoospores) and subcultured every 3 to 4 d (*13*).

Carbohydrate Utilization

Mono-, oligo-, and polysaccharides were tested as growth substrates in quadruplicate. Cellobiose, fructose, and oat spelt xylan were purchased from Sigma (St. Louis, MO); arabinose, galactose, D-glucose, inulin, lactose, mannose, raffinose, ribose, soluble starch, sucrose, and D-xylose from Merck (Merck, Darmstadt, FRG), and filter paper (no. 1) from Whatman (Whatman Limited, Maidstone, UK). Tests for carbohydrate utilization were conducted in medium M2 containing 150 m*M* fermentable carbon (30 m*M* C_5-sugar, 25 m*M* C_6-sugar, and so forth). Stock solutions of soluble carbohydrates (20 × concentrated) were sterilized separately in sealed bottles (butyl rubber septa, crimp seals; Rubber BV, Schipluiden, the Netherlands) under N_2:CO_2 (80:20, v/v) at 115°C for 20 min. Sterile carbohydrate solutions were added to the medium by syringe. Polysaccharides were included in the medium before sterilization, since no caramelization was observed. Bagasse, wheat bran, and wheat straw (4.6 $g \cdot L^{-1}$) were included in the medium before sterilization. Fermentable substrate was estimated after allowance for lignin and ash as determined by the proximate method of Goering and van Soest (*16*). The fermentable amounts of bagasse, wheat bran, and wheat straw were 3.9, 4.0, and 4.0 $g \cdot L^{-1}$, respectively. Inocula (5%, v/v) for experimental cultures were prepared by subculturing the fungus twice, at 3-d intervals, on the substrate to be tested.

Sample Collection and Treatment

Growth of the fungus was monitored by the production of hydrogen. After hydrogen production ceased, four parallel cultures were harvested by filtration over Whatman glass-fiber filters (grade C). Samples (1 mL) of the culture filtrate were stored at −20°C until used for analysis of soluble sugars and determination of enzyme activities. The filtrates of the parallel cultures were combined for SDS-PAGE with one aliquot (2 mL) being dialyzed against 25 m*M* MES buffer (pH 6.5, 24 h, 4°C) and another (5.5 mL) being concentrated by ammonium sulfate precipitation (85% saturation, 18 h, 4°C). The precipitated protein was collected by centrifugation

(20,000×*g*, 45 min, 4°C), dissolved in 0.5 mL 25 m*M* MES buffer (pH 6.5, 4°C), and dialyzed against the same buffer (18 h, 4°C).

Residual Sugar Analysis

Soluble (reducing) sugars were determined with dinitrosalicylic acid (DNS) reagent (*17*); the ratio of sample to DNS was 1:2, and after boiling for 15 min, the absorbance at 575 nm was measured against glucose, galactose, or xylose standards treated in the same way. Residual polysaccharides were hydrolyzed with 67% H_2SO_4 for 1 h at room temperature, and soluble sugars were determined after appropriate dilution.

Enzyme Assays

Enzyme assays with culture fluid were performed in duplicate at optimum pH and temperature. All enzyme reactions were linear over the period of the assays. Enzyme and substrate controls were included in all assays. Endoglucanase, β-glucosidase, and xylanase were assayed in 0.1*M* citrate-phosphate buffer (pH 6.0) at 50°C with carboxymethylcellulose (CM-cellulose sodium salt, low viscosity, Sigma C-8758), *p*-nitrophenyl-β-D-glucopyranoside (PNPG), and washed xylan (from oat spelts, Sigma X-0376), respectively, as substrate (*13*). Avicelase was assayed in the same buffer at 40°C with Avicel (microcrystalline cellulose; type PH105; Serva, Heidelberg, FRG) as substrate (*13*). Units of activity are defined as micromole of reducing sugar (glucose or xylose) released per minute (IU).

Electrophoresis

SDS-PAGE was performed in 10% polyacrylamide slab gels in the presence of SDS (0.1%, w/v) as described by Schwartz et al. (*18*). Enzyme samples were denatured in 5% (w/v) SDS by incubation for 5 min at 100°C (for gels stained for protein) or for 18 h at 20°C (for zymograms). Both the low and high denaturation temperature conditions resulted in a complete dissociation of proteins; the number of bands was the same, and apparent molecular masses of the bands were identical (*19*). The Low Molecular Weight Calibration Kit, High Molecular Weight SDS Kit (Pharmacia Fine Chemicals AB, Uppsala, Sweden), and the High Molecular Weight Standard Mixture SDS-6H (Sigma Chemical Company, St. Louis, MO) were used as molecular-size standards. Both high- and low-molecular-weight standards were applied to each gel. Electrophoresis was conducted at 15°C with a constant current of 40 mA until the tracking dye reached the bottom of the slab gels. Protein bands were detected by staining with Coomassie Brilliant blue G-250 (Serva, Heidelberg, FRG). The amount of protein applied to the gels varied from 5 μg (unconcentrated samples) to 50 μg (concentrated samples).

Enzyme activities in gels were detected by a modification (*19*) of the method described by Schwartz et al. (*18*). The substrate (0.2% w/v CMC) for endoglucanase was incorporated into the separation gel before addition of ammonium persulfate and polymerization. After electrophoresis, the gels were washed three times in 150 mL of 0.1*M* phosphate-citrate buffer (pH 6.0) for 30 min at 4°C, and were then submerged and incubated in the same buffer for 2.5 h at 40°C. Subsequently, gels were stained in 0.15% Congo Red (w/v, 20°C, 30 min) and destained overnight in 1*M* NaCl (4°C). To improve the resolution for photography, gels were submerged in 5% (v/v) acetic acid whereby the color changed from red to purple.

For activity staining of β-glucosidase activity, the gels were washed as described above. Subsequently, an overlay of Whatman no. 1 fliter paper saturated with 5 m*M* methylumbelliferyl-β-glucoside (MUG) in 0.1*M* phosphate-citrate buffer (pH 6.0) was put on the gels, and these were incubated for 30 min at 40°C. Positive bands were detected by fluorescence under UV illumination (long wavelength).

Protein Assay

The protein concentrations of culture filtrates were assayed with Bio-Rad protein reagent with bovine γ-globulin as a standard (Bio-Rad, Richmond, CA).

RESULTS

Growth of *Neocallimastix* Strain N1, *Neocallimastix* Strain N2, and *Piromyces* Strain R1 on Different Substrates

The anaerobic fungi tested were able to use a range of carbon sources in defined medium M2 (Table 1). All strains utilized cellobiose, fructose, glucose, lactose, bagasse, filter paper cellulose, soluble starch, wheat bran, wheat straw, and xylan, but not arabinose, galactose, mannose, and ribose. Only for *Neocallimastix strain* N2, no growth occurred on xylose. *Piromyces* strain R1 did not utilize raffinose, sucrose, and inulin. In contrast, both *Neocallimastix* strains utilized raffinose and sucrose, and *Neocallimastix* strain N2 utilized inulin as well. The period in which growth occurred (determined by H_2 production) varied from 120 to 164 h (results not shown). All mono- and disaccharides, except lactose, were used completely, as was determined by measurement of reducing sugars after growth. The reducing sugar remaining (approx 50% with galactose as standard) after growth of the fungi on lactose was probably galactose, since all fungi were unable to utilize this monosaccharide. Concerning the

Table 1
Enzyme Production by *Neocallimastix* Strains N1 and N2 and *Piromyces* Strain R1 during Growth on Different Substrates[a,b]

	Avicelase mIU·mL^{-1}			β-Glucosidase, mIU·mL^{-1}			CMCase, IU·mL^{-1}			Xylanase, IU·mL^{-1}			Protein, mg·mL^{-1}		
	N1	N2	R1	N1	N2	R1	N1	N2	R1	N1	N2	R1	N1	N2	R1
Soluble substrates															
Cellobiose	3	5	8	25	36	34	0.08	0.30	0.92	1.2	4.3	3.0	0.05	0.09	0.14
Fructose	7	5	11	49	44	22	0.36	0.25	0.92	3.9	3.4	2.6	0.12	0.12	0.09
Glucose	2	6	6	39	36	35	0.18	0.22	0.72	2.2	3.7	2.9	0.08	0.09	0.14
Lactose	1	2	1	30	24	45	0.08	0.15	0.99	1.9	3.4	3.0	0.04	0.02	0.05
Xylose	3	–	12	40	–	18	0.22	–	1.02	4.3	–	4.8	0.09	–	0.19
Raffinose	0	4	–	23	12	–	0.24	0.11	–	2.7	2.1	–	0.04	0.04	–
Sucrose	2	7	–	21	39	–	0.24	0.22	–	1.9	4.2	–	0.05	0.11	–
Inulin	–	6	–	–	28	–	–	0.21	–	–	3.2	–	–	0.10	–
Insoluble substrates															
Bagasse	4	5	2	62	44	18	0.50	0.42	0.57	6.3	6.8	4.8	0.12	0.12	0.14
Filter paper	15	24	19	65	81	47	1.12	1.12	1.16	4.6	7.3	5.5	0.10	0.12	0.18
Starch	4	8	8	76	62	30	0.54	0.42	0.80	4.0	6.1	4.8	0.06	0.05	0.11
Wheat bran	1	10	2	29	39	10	0.22	0.28	0.39	3.7	7.6	2.0	0.09	0.10	0.15
Wheat straw	3	9	3	69	52	23	0.34	0.54	0.41	7.2	9.0	5.3	0.08	0.11	0.12
Xylan	3	7	9	40	29	28	0.30	0.17	0.92	5.1	7.6	6.2	0.04	0.05	0.04

[a]Each value represents the mean of four replicate cultures.
[b]Indicates that no growth occurred.

growth on the complex substrates, microscopic examination revealed that filter paper cellulose was digested completely. Soluble starch and xylan were digested almost completely, since only small amounts of reducing sugars (< 2% of the substrate added) were found after acid hydrolysis of a complete culture. The complex substrates were digested to an extent of approx 60% by all three strains as was shown by dry weight measurements (results not shown).

Enzyme Production

The effect of growth substrate on cellulase and xylanase production by the three fungi was examined (Table 1). In general, the cellulolytic enzyme activities were highest for *Neocallimastix patriciarum* strain N2 cultures. Filter paper was a good inducer for both cellulolytic enzymes and xylanolytic enzymes. For both *Neocallimastix* species, the avicelase activities were highest after growth on filter paper cellulose, fructose, and starch. For *Piromyces* strain R1, highest avicelase activities were found in cultures grown on filter paper cellulose, fructose, and xylose. The growth substrate had a relatively small effect on β-glucosidase activity in contrast to the effects of endoglucanase activity. The latter varied from 0.08 to 1.16 $IU \cdot mL^{-1}$. Endoglucanase activity was for all strains high after growth on bagasse, filter paper cellulose, wheat straw, and xylan. For all three anaerobic fungi, high xylanase activity was found after growth on bagasse, filter paper cellulose, wheat straw, and xylan.

The amount of portein secreted is also given in Table 1. *Piromyces* strain R1 secreted, compared to both *Neocallimastix* strains, relatively high levels of protein; thus, specific enzyme activities were relatively low. The specific avicelase and endoglucanase activity of the three strains was highest after growth on filter paper cellulose. For β-glucosidase and xylanase, highest specific activities were found after growth on starch and xylan, respectively. The highest specific activity found for avicelase was 0.2 $IU \cdot mg^{-1}$ from filter-paper-grown *Piromyces* strain R1, for β-glucosidase, 1.3 $IU \cdot mg^{-1}$ from starch-grown *Neocallimastix* strain N1, for endoglucanase, 11.2 $IU \cdot mg^{-1}$ from filter-paper-grown *Neocallimastix* strain N1, and for xylanase, 152 $IU \cdot mg^{-1}$ from xylan-grown *Neocallimastix* strain N2.

Enzyme Activity Patterns Revealed by SDS-PAGE

Most microorganisms produce isoenzymes. Differences in enzyme activities obtained after growth on the different substrates could therefore be the result of variations in synthesis of isoenzymes. The influence of the growth substrate on the synthesis of enzymes with β-glucosidase or endoglucanase activity was examined by SDS-PAGE.

The endoglucanase activity patterns for samples obtained after growth of *Neocallimastix* strain N1, *Neocallimastix* strain N2, and *Piromyces* strain R1 on the different substrates are shown in Fig. 1. Endoglucanase activity resulted in clear hydrolysis zones against a dark background. Within some of the clearing zones (especially for the *Piromyces* strain), a dark band was formed during destaining probably because of the presence of an acid protein or the formation of an acid product by the enzymes. For *Neocallimastix* strain N1, isoenzymes having molecular masses of 110, 55, and 42 kDa were detected in most enzyme preparations. In enzyme preparations of wheat straw-, wheat bran-, and xylan-grown cultures, activity bands were relatively faint, although the endoglucanase activity applied was higher compared to the enzyme preparation from cellobiose-grown cultures in which bands were clearly visible. For *Neocallimastix* strain N2, the most intense endoglucanase bands had molecular masses of 120, 42, and 40 kDa. More than 30 endoglucanase bands were found for the extracellular enzyme preparation of *Piromyces* strain R1. The most intense stained endoglucanase bands ranged in molecular mass from 300 to 65 kDa. For both *Neocallimastix* strains, the endoglucanase pattern was influenced by the growth substrate, whereas for *Piromyces* no significant differences were found.

The β-glucosidase activity patterns for samples obtained after growth of the fungi on the different substrates are shown in Fig. 2. β-Glucosidase banding patterns were identical for both *Neocallimastix* strains. Therefore, only the banding pattern of *Neocallimastix* strain N2 is shown. The most distinct β-glucosidase activity bands had a molecular mass of 110, 74, 55, 50, and 42 kDa. Two fainter β-glucosidase bands having molecular masses of 38 and 32 kDa were also detected. In the samples obtained from the cellulose-grown cultures, the three β-glucosidase bands with the highest molecular masses were relatively more intense. In the bagasse- and wheat-straw-grown cultures, the low-molecular-mass β-glucosidases were relatively more intensely stained. The β-glucosidase banding patterns for the *Piromyces* strain R1 showed an intense band with a molecular mass of 110 kDa, and fainter bands with a molecular mass of 75, 60, 54, 59, and 45 kDa. The difference of the 42-kDa band of the *Neocallimastix* strains and the 45-kDa band of the *Piromyces* strains was significant, since their R_f values were different when they were applied in one separation gel (results not shown). The 38- and 32-kDa β-glucosidases were absent in the enzyme preparation of *Piromyces* strain R1. The culture substrate had no significant effect on β-glucosidase banding pattern of *Piromyces* strain R1.

DISCUSSION

Anaerobic fungi, isolated from ruminants, produce high levels of cellulolytic and xylanolytic enzymes when grown on a range of substrates

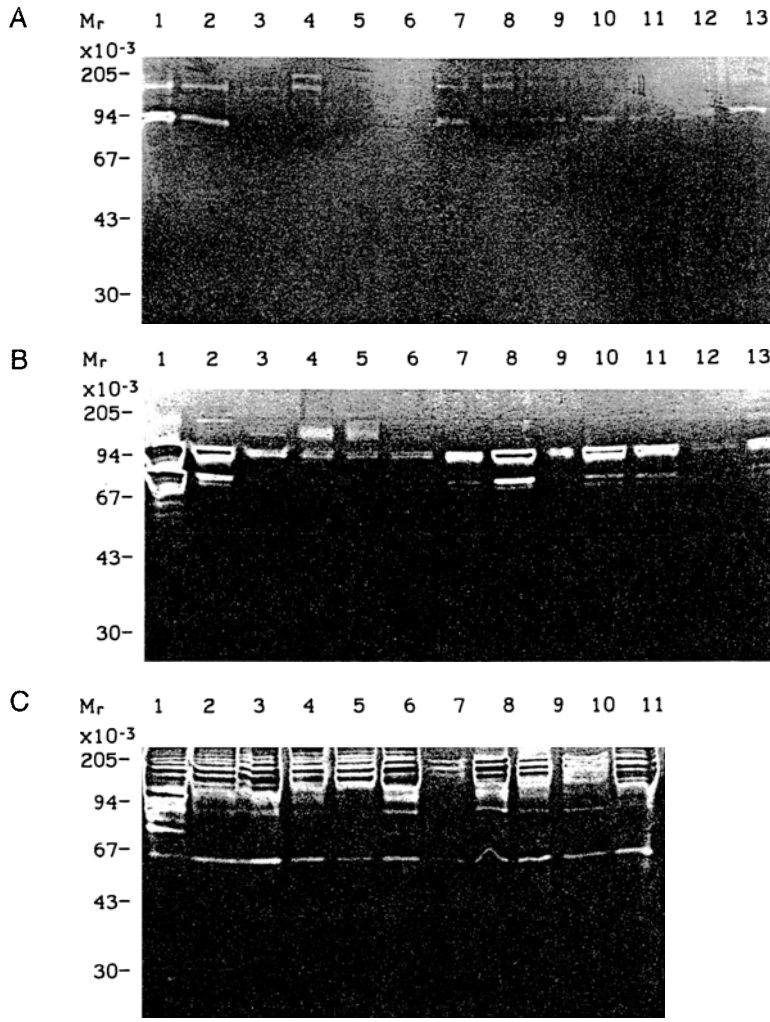

Fig. 1. Endoglucanase activity banding patterns of extracellular enzymes from *Neocallimastix* strain N1 (A), *Neocallimastix* strain N2 (B), and *Piromyces* strain R1 (C) grown on several substrates. The amount of protein applied to lanes 1-3 and 7-12 was 5 μg. To lanes 4, 5, and 6, 10 μg of protein were applied. Migration was from the top to the bottom. At the left, position of molecular mass markers is indicated. The samples of lanes 1-10 were obtained from cultures grown on the following substrates: 1, filter paper; 2, starch; 3, xylan; 4, bagasse; 5, wheat straw; 6, wheat bran; 7, fructose; 8, cellobiose; 9, lactose; 10, glucose. For *Neocallimastix* strain N1, samples applied to lanes 11-13 were obtained from cultures grown on xylose, sucrose, and raffinose, and for *Neocallimastix* strain N2, for cultures grown on sucrose, raffinose, and inulin. For *Piromyces* strain R1, the sample applied to lane 11 was obtained from a xylose-grown culture.

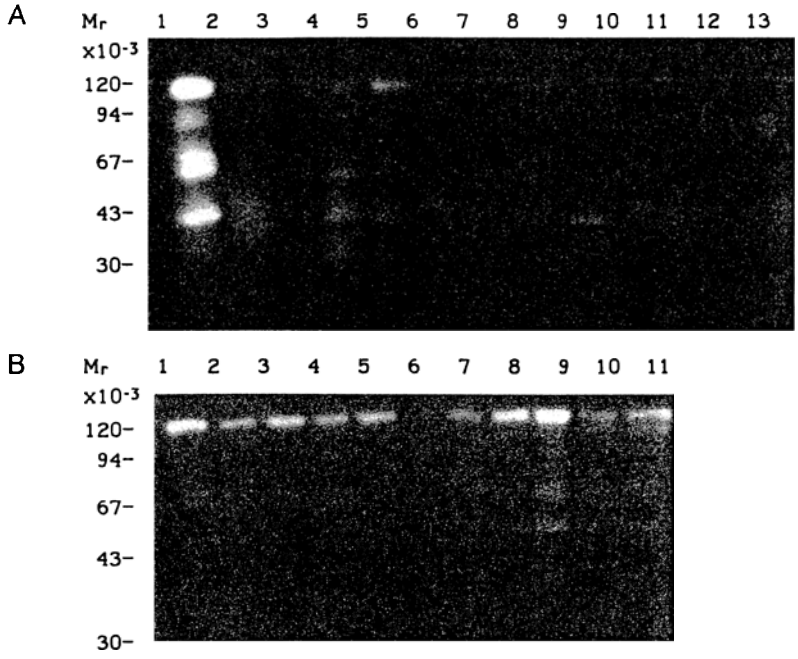

Fig. 2. β-Glucosidase activity banding patterns of extracellular proteins from *Neocallimastix* strain N2 (A) and *Piromyces*strain R1 (B) grown on several substrates. The amount of protein applied to lanes 1–3 and 7–12 was 25 μg. To lanes 4, 5, and 6, 50 μg of protein were applied. Migration was from the top to the bottom. At the left, position of molecular mass markers is indicated. The samples of lanes 1–10 were obtained from cultures grown on the following substrates: 1, filter paper; 2, starch; 3, xylan; 4, bagasse; 5, wheat straw; 6, wheat bran; 7, fructose; 8, cellobiose; 9, lactose; 10, glucose. For *Neocallimastix* strain N1, samples applied to lanes 11–13 were obtained from cultures grown on xylose, sucrose, and raffinose, and for *Neocallimastix* strain N2, from cultures grown on sucrose, raffinose, and inulin. For *Piromyces* strain R1, the sample applied to lane 11 was obtained from a xylose-grown culture.

(*1,11,12,20*). However, the ability of anaerobic fungi from nonruminants to utilize different carbohydrate substrates and the effects of different substrates on enzyme production are not well documented. Furthermore, the effect of the substrate on β-glucosidase and endoglucanase banding patterns, indicating isoenzymes, has not been described for anaerobic fungi.

Piromyces strain R1 did not grow on mannose, galactose, inulin, raffinose, and sucrose. The eight ruminal *Piromyces* species tested by Phillips and Gordon (*10*) likewise did not grow on inulin, but the ability to ferment raffinose and sucrose was shown to be strain dependent. Phillips and Gordon (*10*) did not test these *Piromyces* species for their ability to utilize galactose and mannose. *Neocallimastix* strain N1 did not grow on galactose, mannose, inulin, and raffinose, whereas *Neocallimastix* (*patriciarum*)

strain N2 did not grow on galactose, mannose, and xylose. In contrast, the *Neocallimastix patriciarum* strain used by Orpin and Bountiff (*20*) used galactose. Two other ruminal *Neocallimastix* spp. (*9.21*) did not grow on this substrate. The ability of *Neocallimastix* strains to utilize inulin, raffinose, and xylose is strain dependent. The inability of *Neocallimastix* strain N2 to grow on xylose seems to be in contrast with its ability to grow on xylan.

Cellulolytic and xylanolytic enzymes were produced by the three anaerobic fungal strains during growth on monosaccharide, disaccharide, polysaccharide, or complex carbon sources. Enzyme production was substrate dependent, as was found for ruminal *Neocallimastix* and *Piromyces* species (*11,12*). Studies with *Neocallimastix frontalis* (*20*) and *Neocallimastix patriciarum* (*11,12*) indicated that enzyme production was substrate dependent and that soluble sugars were less effective inducers of cellulase than cellulose. Furthermore, Mountfort and Asher (*21*) demonstrated that the production of endoglucanase by *Neocallimastix frontalis* was repressed by glucose and other soluble sugars. Enzyme production was high for fructose-, starch-, wheat straw-, and xylan-grown cultures. In this study, cellobiose was shown to be not so effective as inducer of cellulases as reported for *Neocallimastix frontalis* (*21*). Neither fructose nor starch has been shown before to be an inducer of cellulolytic enzymes. Enzyme production during growth on these substrates may be high because of the absence of repression. The complex substrates were relatively good inducers of cellulolytic enzymes, which is promising for commercial production of these enzymes.

Xylanase production was not repressed by xylose. Both repression (*22*) and induction (*23*) of xylanase synthesis by xylose for, an aerobic fungus and an actinomycete, respectively, have been described. During growth on complex substrates and filter paper cellulose, production of xylanolytic enzymes was high. For the aerobic fungus *Trichoderma koningii*, crystalline cellulose induced highly active xylanases in addition to all components of the cellulase system (*24*). It was apparent that xylanolytic enzyme production was constitutive, but enzyme activities were lower after growth on glucose and other soluble sugars. Similar results were obtained for ruminal *Neocallimastix* (*1,11,12*) and *Piromyces* strains (*11,12*).

Little is known of the molecular masses of cellulolytic enzymes of anaerobic fungi. PAGE and activity staining of extracellular enzymes of *Neocallimastix frontalis* gave multiple bands for endoglucanase activity, but molecular details could not be obtained by this technique (*25*). Two β-glucosidases from the rumen fungus *Neocallimastix frontalis* have been purified, and differed in mole w and p*I* being 120 kDa and 3.85 (*26*) and 125.5 kDa and 7.10 (*27*). These molecular masses coincide with one of the distinct activity bands found for the *Neocallimastix* strains and the *Piromyces* strain R1 used in this study. However, the resolution of the SDS-PAGE technique is not high enough to differentiate between these β-glucosidases.

Samples from *Piromyces* strain R1 cultured on various substrates gave almost identical β-glucosidase and endoglucanase activity patterns after SDS-PAGE. This indicates for this fungus that the differences in enzyme production on different substrates were not caused by secretion of different mixtures of isoenzymes. However, for both *Neocallimastix* strains, the banding patterns were influenced, and therefore, the differences in enzyme activity could also be caused by the secretion of different mixtures of isoenzymes. PAGE separation of the extracellular endoglucanases of *Neocallimastix frontalis* confirmed that several activity bands present in cellulose-grown cultures were not present or were less active in cellobiose- and glucose-grown cultures (*25*).

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REFERENCES

1. Lowe, S. E., Theodorou, M. K., and Trinci, A. P. J. (1987), *Appl. Environ. Microbiol.* **53,** 1216–1223.
2. Orpin, C. G. (1988), *BioSystems* **21,** 365–370.
3. Gold, J. J., Heath, I. B., and Bauchop, T. (1988), *BioSystems* **21,** 403–415.
4. Breton, A., Bernalier, A., Dusser, M., Fonty, G., Gaillard-Martini, B., and Guillot, J. (1990), *FEMS Microbiol. Lett.* **70,** 177–182.
5. Barr, D. J. S., Kudo, H., Jakober, K., and Cheng, K.-J. (1989), *Can. J. Bot.* **67,** 2815–2824.
6. Ho, Y. W., Abdullah, N., and Jalaludin, S. (1990), *Mycotaxon* **38,** 397–405.
7. Wood, T. M., Wilson, C. A., McCrae, S. I., and Joblin, K. N. (1986), *FEMS Microbiol. Lett.* **34,** 37–40.
8. Kawamori, M., Morikawa, Y., Ado, Y., and Takasawa, S. (1986), *Appl. Microbiol. Biotechnol.* **24,** 454–458.
9. Lowe, S. E., Theodorou, M. K., and Trinci, A. P. J. (1987), *Appl. Environ. Microbiol.* **53,** 1210–1215.
10. Phillips, M. W. and Gordon, G. L. R. (1988), *BioSystems* **21,** 377–383.
11. Williams, A. G. and Orpin, C. G. (1987), *Can. J. Microbiol.* **33,** 418–426.
12. Williams, A. G. and Orpin, C. G. (1987), *Can. J. Microbiol.* **33,** 427–434.
13. Teunissen, M. J., Smits, A. A. M., Op den Camp, H. J. M., Huis in 't Veld, J. H. J., and Vogels, G. D. (1991), *Arch. Microbiol.* **156,** 290–296.
14. Teunissen, M. J., Op den Camp, H. J. M., Orpin, C. G., Huis in 't Veld, J. H. J., and Vogels, G. D. (1991), *J. Gen. Microbiol.* **137,** 1401–1408.
15. Lowe, S. E., Theodorou, M. K., Trinci, A. P. J., and Hespell, R. B. (1985), *J. Gen. Microbiol.* **131,** 2225–2229.
16. Goering, H. K. and van Soest, P. J. (1970), Agricultural Research Service Agricultural Handbook no. 379. US Department of Agriculture, Washington, D.C.

17. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
18. Schwartz, W. H., Bronnenmeier, K., Gräbnitz, F., and Staudenbauer, W. L. (1987), *Anal. Biochem.* **164,** 72–77.
19. Teunissen, M. J., Huis in 't Veld, J. H. J., and Vogels, G. D. (1993), *Arch. Microbiol.* submitted for publication.
20. Orpin, C. G. and Bountiff, L. (1978), *J. Gen. Microbiol.* **104,** 113–122.
21. Mountfort, D. O. and Asher, R. A. (1985), *Appl. Environ. Microbiol.* **49,** 1314–1322.
22. Richards, G. N. and Shambe, T. (1976), *Carbohydr. Res.* **49,** 371–391.
23. Kluepfel, D., Vats-Mehta, S., Aumont, F. Sharbeck, F., and Morosoli, R. (1990), *Biochem. J.* **267,** 45–50.
24. Huang. L., Hseu, T.-H., and Wey T.-T. (1991), *Biochem. J.* **278,** 329–333.
25. Barichiviech, E. M. and Calza, R. E. (1990), *Appl. Environ. Microbiol.* **56,** 43–48.
26. Hebraud, M. and Fevre, M. (1990), *Appl. Environ. Microbiol.* **56,** 3164–3169.
27. Li, X. and Calza, R. E. (1991), *Enzyme Microb. Technol.* **13,** 622–628.