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Glycosidases of the Rumen Anaerobic Fungus *Neocallimastix frontalis* Grown on Cellulosic Substrates

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The rumen anaerobic fungus *Neocallimastix frontalis* was grown on cellulosic substrates, and the cellular distribution and types of glycosidases produced by the organism were studied. Fungal cultures were fractionated into extracellular, insoluble (membrane), and intracellular fractions and assayed for glycosidase activity by using Avicel, carboxymethylcellulose, xylan, starch, polygalacturonic acid, and the *p*-nitrophenyl derivatives of galactose, glucose, and xylose as substrates. Enzymic activity was highest in the extracellular fraction; however, the membrane fraction also displayed appreciable activity. The intracellular fraction was inactive towards all substrates. Polygalacturonic acid was the only substrate not hydrolyzed by the active fractions, indicating that pectinase was absent. The results show that *N. frontalis*, a common rumen anaerobic fungus, produces enzymes for degrading cellulose and hemicellulose, key components of plant fiber.

Microbial digestion of cellulose and other plant fiber components is central to the utilization of natural diets by the ruminant animal. Until recently, the dense complex microbial population of the rumen was believed to comprise only bacteria and protozoa. More recently, large populations of fungi which possess the exceptional property of being obligate anaerobes have been shown to form part of this microbiota (2–5, 16, 17). The fungi specifically colonize fibrous plant fragments in the rumen, and the apparent magnitude of their populations suggests that they may have a role in fiber digestion as initial colonizers in lignocellulose breakdown (2, 5, 6). Additional data concerning these colonization sites have recently been reported which support this concept (1). Confirmation of cellulose digestion was obtained with pure cultures of fungi (3, 18), and later these cultures were shown to carry out an active fermentation of cellulose (7).

Anaerobic fungi have been cultured on cellulose, starch, xylan, and other hemicelluloses (3, 6, 18), but the glycosidases produced by these unusual fungi have not been investigated. Information on the nature and range of the fungal enzymic hydrolytic activities is required to assess the importance of anaerobic fungi relative to other microbes in the rumen microbiota. We studied here the types and quantities of glycosidase produced by *Neocallimastix frontalis*, a common rumen anaerobic fungus, grown on cellulosic substrates. The results show that *N. frontalis* produces the range of enzymes required for the degradation of cellulose and hemicellulose, major components of plant fiber and ruminant diets.

MATERIALS AND METHODS

Isolation procedures. *N. frontalis* PN2 was isolated by serial dilution of strained bovine rumen contents in anaerobic medium containing 15% (vol/vol) clarified rumen fluid in roll tubes. The medium was as previously described (2) with the addition of 1.5% (wt/vol) agar (Davis). Tubes of molten agar were maintained at 46°C in a waterbath, and an antibiotic mixture (2) was added immediately before inoculation. Rolled tubes were incubated at 39°C, and by 48 h a number of large fungal colonies had developed (13). Colonies were picked and transferred to standard broth medium (2) containing antibiotics. Reinoculation into antibiotic-free roll tubes and reisolation into standard broth medium produced a pure culture which was designated PN2. The fungus was transferred to cellulose medium (50 mg/7 ml), where strips of Whatman no. 1 filter paper replaced glucose as the substrate. The organism was maintained by transfer every 4 to 6 days.

PN2 possessed polyflagellate zoospores, and on the basis of gross morphology, it was identified as *N. frontalis*, an organism isolated from sheep and recently assigned (10) to the family *Neocallimasticaceae* of the *Spizellomycetales* (*Chytridiomycetes*).

Culture conditions. The anaerobic techniques described by Hungate (12) were used for the maintenance and subculturing of the fungus cultures. Cultures were grown anaerobically at 39°C in tubes containing 7.0 ml of medium (2) with substitution of cellulose for glucose. The cellulose substrates used were filter paper (as described above) or Avicel PH-102 (0.3%, wt/vol) (FMC Corp., Philadelphia, Pa.). Experimental media were inoculated by transferring 1/20 of the volume of a 6-day-old culture. In a typical experiment, 20 inoculated and 10 uninoculated (control) tubes of media were incubated. Each day one control tube and two inoculated tubes were removed for analysis of the headspace gases and measurement of the enzymic activities. Experiments with paper as the substrate were performed three times and twice with Avicel as substrate. All incubations were at 39°C with the tubes maintained in a vertical position without shaking.

Gas analysis. The production of gases during growth was monitored by using a 10-ml glass syringe with the barrel lubricated with water. Gas volumes were measured at 21°C. The headspace gases were analyzed on an Aerograph 660 gas chromatograph fitted with a Porapak QS80/100 column. Nitrogen was the carrier gas.

Fractionation of growth cultures. The contents of the two inoculated tubes were pooled and fractionated as follows. The fungi and undigested cellulose were centrifuged for 5 min at 1,800 × g_{max} in a bench centrifuge. The supernatant was removed with a Pasteur pipette and filtered through a Whatman GF/C glass-fiber filter to produce the extracellular fraction. The pellet was washed by resuspension in 10 ml of cold 50 mM sodium acetate buffer (pH 5.1) and recentrifuged. This procedure was repeated twice, and the resultant

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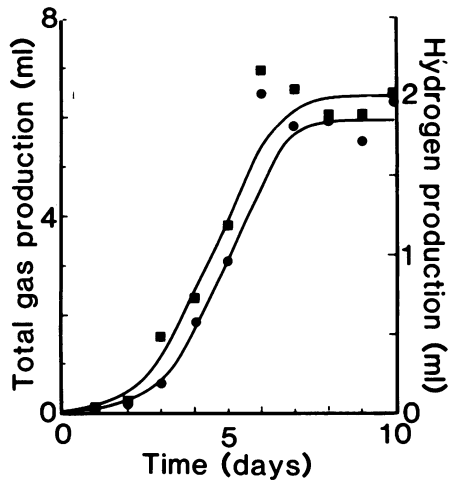

FIG. 1. Hydrogen and total gas production by *N. frontalis* grown on paper. Symbols: ■, Hydrogen production; ●, total gas production.

pellet was resuspended in 14.6 ml of cold buffer and homogenized in a Potter-Elvejheim homogenizer. The pellet wash fluids were discarded unassayed. The homogenate was centrifuged at 200,000 × g_{max} for 30 min at 4°C, the supernatant was decanted (intracellular fraction), and the pellet was resuspended in 14.6 ml of the acetate buffer (insoluble fraction).

Enzyme assays. Enzyme assays were performed in duplicate at 37°C in 50 mM sodium acetate buffer (pH 5.1). The variation between duplicate assays was less than 5%. Preliminary experiments showed that the optimum pH and temperature for enzymic activity towards Avicel and carboxymethylcellulose (CMC) were 5.1 and 37°C, respectively. A shaking water bath was used for assays involving insoluble substrates. Reducing sugars were measured by using an arsenomolybdate reagent (15) with appropriate monosaccharides as standards. Glucose was measured by using glucose oxidase-peroxidase with 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) as a chromogen (8). One unit of enzyme is defined as the quantity required to produce 1 μmol of product per min.

Cellulase activity was measured by using Avicel PH-101 and CMC as substrates. With Avicel as the substrate, sample (250 to 500 μl) was incubated with 100 mg of Avicel in a total volume of 2.5 ml for 2 h. The undigested cellulose was pelleted by centrifugation, and a sample of the supernatant (250 to 500 μl) was assayed for glucose and reducing sugars. With CMC as the substrate, a sample (50 to 250 μl) was incubated with 10 mg of low-viscosity sodium CMC (Sigma Chemical Co., St. Louis, Mo.) in a total volume of 1.0 ml for 2 h, and the reducing sugars were measured.

Xylanase activity was measured by incubating 100 to 500 μl of the sample with 20 mg of larch wood xylan (Sigma) in a total volume of 2.0 ml for 2 h. Undigested xylan was pelleted by centrifugation, and samples (250 to 500 μl) of the supernatant were assayed for reducing sugars.

Starch degrading activity was measured by incubating 50 to 250 μl of sample with 2.5 mg of soluble starch (BDH, Poole, England) in a total volume of 1.0 ml for 2 h, and the reducing sugars were measured.

Pectinase activity was measured by incubating 250 μl of sample with 2 mg of polygalacturonic acid (Sigma) in a total volume of 1.0 ml for 2 h, and the reducing sugars were measured.

β-Glucosidase, β-galactosidase, and β-xylosidase were measured by incubating 50 to 250 μl of sample with 5 mg of the *p*-nitrophenyl derivatives of β-D-glucose, β-D-galactose, and β-D-xylose, respectively, in a total volume of 2.0 ml for 30 min. The reactions were stopped by the addition of 3.0 ml of 0.1 M NaOH, and the amount of *p*-nitrophenol (pNP) liberated was determined by measuring A_{405} (9).

Partial digestion products from starch and xylan. Partial digestions of starch and xylan were performed to determine which enzymes were responsible for hydrolysis. Samples (3 ml) of the extracellular fraction were incubated at 37°C with 37.5 mg of soluble starch or 120 mg of xylan in 15 ml of 50 mM acetate buffer (pH 5.1). Portions (1.5 ml) were removed at 0, 0.5, 2, 5, 9, and 20 h, boiled for 5 min, and stored at −20°C before analysis by gel filtration.

Gel filtration. A column (1.6 by 70 cm) packed with Bio-Gel P2 (Bio-Rad Laboratories, Richmond, Calif.) was used for analysis of the partial digestion products (14). A 1-ml sample was applied to the column, which was eluted with 130 ml of distilled water at a flow rate of 20 ml h^{-1}. The column was maintained at 37°C with a water jacket. Fractions (1.0 ml) were collected, and on completion of the elution, 5% (wt/vol) aqueous phenol (1 ml) and concentrated sulfuric acid (5.0 ml) were added to each tube, and after cooling, the A_{490} was measured (11). The column was calibrated by using xylose, glucose, maltose, and raffinose as standards.

Protein assays. The modified Lowry procedure developed by Petersen (19) was used to measure protein concentrations.

RESULTS

Enzyme production by *N. frontalis* grown on filter paper. Hydrogen production was used to monitor growth together with total gas production (Fig. 1). After a lag phase, hydrogen and total gas production levels were parallel to each other, reaching maxima at 6 days. As it was not possible to separate fungal tissue from undigested filter paper, only the

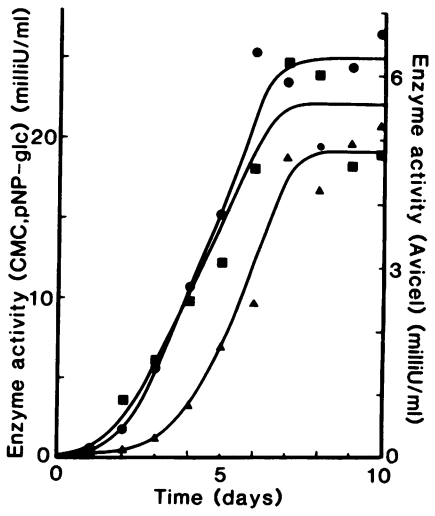

FIG. 2. Production of soluble glycosidases by *N. frontalis* PN2 grown on paper. Enzymic activity towards: ■, Avicel; ●, CMC; and ▲, *p*-nitrophenyl-β-D-glucoside.

TABLE 1. Maximum activities and specific activities of culture supernatant obtained from *N. frontalis* grown on filter paper

Substrate	Maximum activity (U/ml)	Maximum sp act (U/mg)
Avicel	0.006	0.076
CMC	0.025	0.33
pNP-glucose	0.021	0.24

culture supernatants were assayed for enzymic activity towards Avicel, CMC, and pNP-glucose. The glycosidase activity curves were parallel to the growth curves, reaching maxima after 6 days of growth (Fig. 2). The maximum activities and specific activities towards the three substrates tested are shown in Table 1.

After growth of the fungus on paper for 6 days, the concentration of reducing sugar in the culture supernatant increased rapidly (Fig. 3.) This reducing sugar was shown to be glucose by using glucose oxidase-peroxidase. After 10 days the glucose concentration reached 2.25 mg/ml.

Enzyme production by *N. frontalis* grown on Avicel. Growth was again monitored by measuring hydrogen production (Fig. 4). By 6 days, 2.4 ml of hydrogen had been produced, but thereafter the volume of hydrogen in the headspace gas apparently declined. The volume of total gas evolved reached a plateau at 5.7 ml after day 6 (Fig. 4). Glucose and other reducing sugars did not accumulate when Avicel was the substrate.

Avicel-grown fungus was fractionated into extracellular, intracellular, and insoluble fractions, which were assayed for enzymic activity. The activities of the extracellular and insoluble fractions with the cellulosic substrates Avicel and CMC are shown in Fig. 5, and the activities towards starch and xylan are shown in Fig. 6. The activities of the fractions towards disaccharides were measured by using pNP derivatives (Fig. 7). The intracellular fraction lacked activity with all of the substrates tested.

Enzymic activities in the extracellular fraction generally reached plateau levels after 6 or 7 days, and the maximum activities and specific activities towards the different substrates are shown in Table 2. In the insoluble fraction, enzymic activity reached a maximum level after 4 days and then declined to a plateau level. The activities and specific

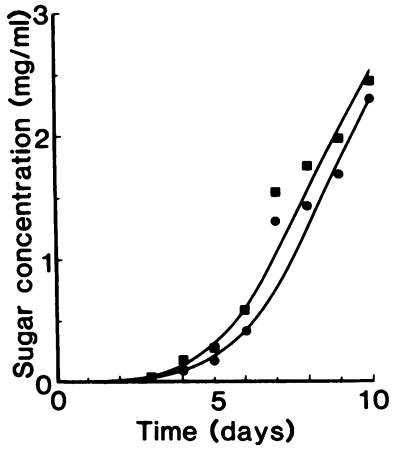

FIG. 3. Concentration of glucose and reducing sugars in culture supernatant obtained from *N. frontalis* PN2 grown on paper. Symbols: ■, Reducing sugars; ●, glucose.

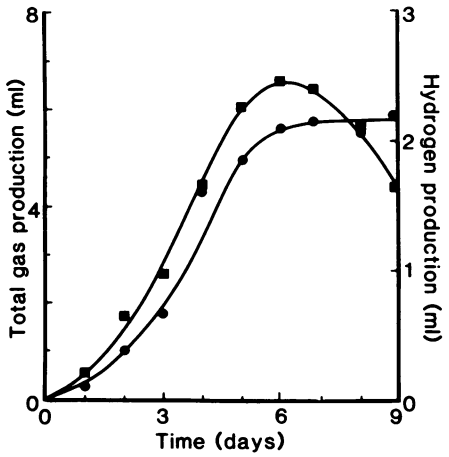

FIG. 4. Hydrogen and total gas production by *N. frontalis* grown on Avicel. Symbols: ■, Hydrogen production; ●, total gas production.

activities of the insoluble fractions after 7 days of growth are shown in Table 2.

Products of enzymic digestion of starch and xylan. To determine the nature of the enzymes involved in xylan and starch digestion, the soluble fraction of *N. frontalis* grown on Avicel was used to carry out partial digestion of these polysaccharides. With xylan as the substrate, oligomers with chain lengths of 3 to 10 were produced before xylose could be detected, a result consistent with the hydrolysis proceeding via xylanase and β-D-xylosidase. With starch as the substrate, oligomers and maltose were not detected and glucose was the only product, indicating that hydrolysis was catalyzed by an amyloglucosidase.

DISCUSSION

The main interest for rumen microbiologists with respect to the gut anaerobic fungi has been the extent of their role in ruminant digestion. The nature and range of the fungal enzymic activities as well as the quantity of fungal tissue

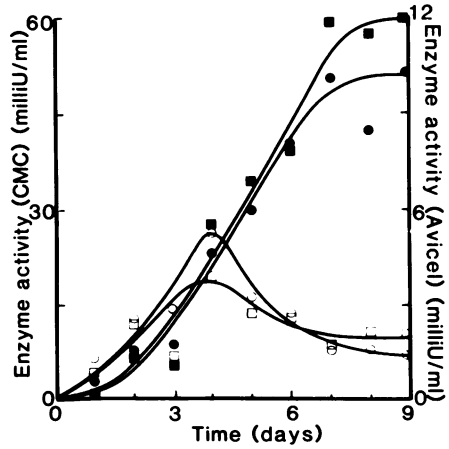

FIG. 5. Enzymic activities towards Avicel and CMC of the extracellular and insoluble (membrane) fractions obtained from *N. frontalis* grown on Avicel. Symbols: ■, Extracellular fraction Avicel activity; □, insoluble fraction Avicel activity; ●, extracellular fraction CMC activity; ○, insoluble fraction CMC activity.

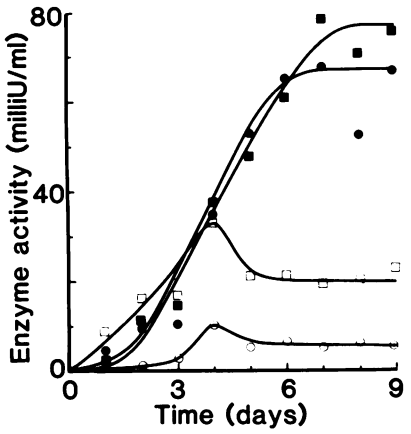

FIG. 6. Enzymic activities towards starch and xylan of the extracellular and insoluble (membrane) fractions obtained from *N. frontalis* grown on Avicel. Symbols: ●, Extracellular fraction starch activity; ○, insoluble fraction starch activity; ■, extracellular fraction xylan activity; □, insoluble fraction xylan activity.

present in the rumen are key factors to be examined. In this work, we examined the nature of the fungal enzymic activities and characterized the production of hydrolytic enzymes in a common rumen fungus, *N. frontalis*, when grown on cellulosic substrates.

When cultured on Avicel, *N. frontalis* produced enzymes capable of hydrolyzing cellulose, xylan, and starch to their component monosaccharides. The fungal enzymes were not able to digest polygalacturonic acid, however, showing that *N. frontalis* does not secrete pectinase when grown on cellulose. This is in agreement with the finding of Orpin and Letcher (18). Results of fermentation studies with several as yet unclassified anaerobic fungi have shown consistently that growth is not obtained with pectin or polygalacturonic acid (T. Bauchop, unpublished data). Of the major plant fiber components consumed in forage, pectin is the most rapidly fermented by the rumen microbiota. The lack of pectinase in anaerobic fungi may relate to the relatively long time scale (ca. 24 h) required for the fungal life cycle in the rumen (3, 4). Preferential colonization and utilization of the more refractory plant components permits the increased rumen retention required for the survival of the fungi in the rumen. In sheep receiving diets relatively low in fiber, fungi are absent from the rumen (3, 6).

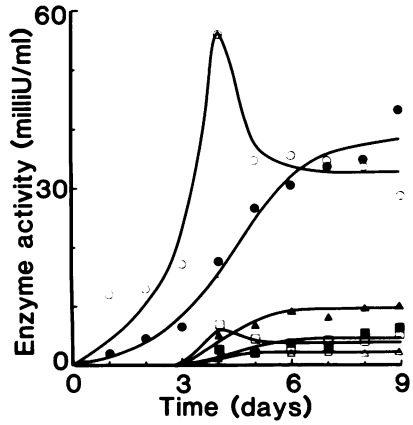

FIG. 7. Enzymic activities towards *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-galactoside, and *p*-nitrophenyl-β-D-xyloside of the extracellular and insoluble (membrane) fractions obtained from *N. frontalis* grown on Avicel. Symbols: ●, Extracellular fraction pNP-glucoside; ○, insoluble fraction pNP-glucoside; ■, extracellular fraction pNP-galactoside; □, insoluble fraction pNP-galactoside; ▲, extracellular fraction pNP-xyloside; △, insoluble fraction pNP-xyloside.

With Avicel as the substrate, the major digestion product obtained from cellulose was glucose and not cellobiose, suggesting that excess β-glucosidase was produced relative to cellulase. This contrasts with the situation found in *Trichoderma* viride which secrete small quantities of β-glucosidase (21). The secretion of xylanase and β-xylosidase during growth on cellulose is similar to the situation found in several aerobic fungi which likewise produce these enzymes when grown on cellulose.

The finding that glucose was the only product from the partial digestion of starch appears unusual. The absence of maltose or limit dextrins suggests that an amyloglucosidase is responsible for starch hydrolysis. The secretion of an amyloglucosidase by fungi grown on cellulose has not been reported previously.

Enzymic activity towards pNP-β-D-galactoside suggests that a β-galactosidase is produced by *N. frontalis*. However, some β-glucosidases are relatively nonspecific and display activity towards β-galactosides (23). Further studies are thus required to confirm the identity of the β-galactosidase.

The enzymic activities of the extracellular fractions generally reached plateau levels after 6 or 7 days of growth, whereas the activities associated with the insoluble fraction peaked at 4 days and then declined to plateau levels (Fig. 2, 5, 6, and 7). The plateau levels in the insoluble fraction were attributed to membrane-bound enzymes. The early peaks of activity for the insoluble fractions were attributed to extracellular enzymes adsorbed to the undigested cellulose present. By 6 or 7 days of growth, when most of the cellulose had been digested as indicated by microscopic examination, the activities of the insoluble fractions had declined. Enzymic activities in the extracellular fractions were 1.3 times (β-glucosidase) to 13 times higher (amyloglucosidase) than those found in the insoluble (membrane) fractions (Table 2).

The inactivity of the intracellular fraction towards any of the substrates tested was in agreement with the findings that such hydrolytic activities are either extracellular or membrane bound (20).

The results of Bauchop and Mountfort (7) demonstrated that in *N. frontalis* PN1, an ovine anaerobic fungus, hydro-

TABLE 2. Maximum activities and specific activities of the extracellular fraction and activities and specific activities of the insoluble (membrane) fraction from *N. frontalis* after 7 days of growth on Avicel

Substrate	Extracellular fraction		Insoluble fraction	
	Maximum activity (U/ml)	Maximum Sp act (U/mg)	Activity (U/ml)	Sp act (U/mg)
Avicel	0.012	0.12	0.002	0.016
CMC	0.051	0.51	0.007	0.06
Starch	0.066	0.58	0.005	0.04
Xylan	0.075	0.75	0.019	0.15
Polygalacturonic acid	0	0	0	0
pNP-galactose	0.010	0.1	0.004	0.032
pNP-glucose	0.041	0.41	0.031	0.25
pNP-xylose	0.006	0.06	0.002	0.016

gen production was closely parallel to cellulose utilization and growth. In the present study similar effects were observed. With Avicel as the substrate, the maximum amount of hydrogen was produced at 6 or 7 days, and the amount of hydrogen then apparently declined. After 6 days, 5.6 ml of gas was produced containing 2.45 ml of hydrogen. The decrease in the hydrogen content of the headspace gas after 6 days may be explained by a hydrogenase reaction (22).

Cellulose utilization was not measured directly, but microscopic examination of the cultures after 6 days of growth on Avicel showed that most of the cellulose had been digested. With paper as the substrate, cellulose fiber was still obvious after 10 days. By assuming complete digestion of the Avicel (95% dry matter), the ratio of moles of hydrogen produced per 100 mol of hexose fermented was calculated to be 83. This ratio is higher than the 35.3 ratio reported for *N. frontalis* PN1 (7) grown on filter paper. In the present work we have not attempted to calculate the ratio for *N. frontalis* grown on filter paper because of the difficulty in separating fungal tissue from undigested filter paper.

The maximum activities of the extracellular fraction enzymes produced by the fungus grown on Avicel were nearly double the activities of the corresponding fraction when paper was the substrate (Tables 1 and 2). Similarly, the maximum specific activities of the Avicel-derived enzymes were 1.6 to 1.7 times greater than those obtained from the paper-grown organisms. The reduced amounts of enzymes produced with paper as the substrate may be caused by repression of enzyme synthesis as a result of the increased amount of glucose formed in the cultures after 6 days.

Large populations of anaerobic fungi have been shown to be present in the rumen of cattle and sheep on fibrous diets (1, 2, 5), and these fungi specifically colonize and grow on the fibrous plant fragments present. A role as initial colonizers in lignocellulose digestion has been suggested (2), and the ability of fungi to penetrate deeply into tissues inaccessible to other rumen microbes may be significant in this regard (5, 6).

In the present work we have shown that a common rumen fungus, *N. frontalis*, produces the range of enzymes necessary to degrade the important plant structural polysaccharides, cellulose and hemicellulose, to simple sugars. The highest enzymic activities were in the extracellular fractions, although the membrane fractions also possessed appreciable activities. The nature of these glycosidases is consistent with a fungal role in fiber digestion in the rumen.

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