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Degradation and Utilization of Cellulose and Straw by Three Different Anaerobic Fungi from the Ovine Rumen

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Three different ruminal fungi, a *Neocallimastix* sp. (strain LM-1), a *Piromonas* sp. (strain SM-1), and a *Sphaeromonas* sp. (strain NM-1), were grown anaerobically in liquid media which contained a suspension of either 1% (wt/vol) purified cellulose or finely milled wheat straw as the source of fermentable carbon. Fungal biomass was estimated by using cell wall chitin or cellular protein in cellulose cultures and chitin in straw cultures. Both strains LM-1 and SM-1 degraded cellulose with a concomitant increase in fungal biomass. Maximum growth of both fungi occurred after incubation for 4 days, and the final yield of protein was the same for both fungi. Cellulose degradation continued after growth ceased. Strain NM-1 failed to grow in the cellulose medium. All three anaerobic fungi grew in the straw-containing medium, and loss of dry weight from the cultures indicated degradation of straw to various degrees (LM-1 > SM-1 > NM-1). The total fiber component and the cellulose component of the straw were degraded in similar proportions, but the lignin component remained undegraded by any of the fungi. Maximum growth yield on straw occurred after 4 days for strain LM-1 and after 5 days for strains SM-1 and NM-1. The calculated yield of cellular protein for strain LM-1 was twice that of both strains SM-1 and NM-1. The cellular protein yield of strain SM-1 was the same in both cellulose and straw cultures. In contrast to cellulose, straw degradation ceased after the end of the growth phase. β-Glucosidase, carboxymethylcellulase (CM-cellulase), azure cellulase, β-xylosidase, and xylanase enzyme activities were detected in the supernatants from the medium containing straw after growth of the three fungi. The type of cellulase(s) produced by strain NM-1 probably will not degrade the form of purified cellulose used as the growth substrate in this study.

The degradation and fermentation of cellulosic fiber constitute a central process in the digestion of feed by ruminants. Bacteria, and perhaps ciliate protozoa, actively degrade fiber in the rumen (9, 13, 20). The close association of anaerobic fungi with fibrous plant particles in the rumen, together with the large numbers of these microorganisms which are found in ruminants that eat fibrous feeds, has suggested an important role for fungi as well as bacteria and protozoa in fiber degradation (6, 15, 31). Indeed, Akin and co-workers (3, 4) showed that the presence of anaerobic fungi in the rumen significantly contributed to voluntary intake and digestion of a fibrous feed by sheep.

Three major types of anaerobic fungi occur in the rumens of sheep and cattle, and they have been isolated in pure culture and have been described previously (6, 21, 23, 29, 30, 32, 39). The most attention has been paid to the genus *Neocallimastix*, and two species are recognized: *Neocallimastix frontalis* (17) and *Neocallimastix patriciarum* (37). *Sphaeromonas* and *Piromonas* are the other genera of anaerobic fungi which have been isolated from sheep rumen (30, 32, 39). Recently, several new types of anaerobic fungi have been isolated from the bovine rumen (2; M. W. Phillips, Abstr. Joint OECD—University of New England Seminar, Armidale, Australia, in press), but these organisms have not been extensively characterized.

The cellulolytic activity of *Neocallimastix* spp. was demonstrated when purified cellulose was degraded by pure cultures of *N. patriciarum* (originally called *N. frontalis* [36]), *N. frontalis* (7, 28, 38) and *Neocallimastix* sp. strain R1 (23). In all cases, the loss of cellulose from the culture medium was accompanied by growth of the fungi. This fungal growth was assessed either by the measurement of phospholipid accumulation in the culture (36) or by the measurement of the total amount of hydrogen evolved or formic acid produced by the culture as end products of carbohydrate fermentation (23, 28). Little is known about the ability of *Piromonas* and *Sphaeromonas* spp. to utilize purified cellulose for growth (39).

The effectiveness of anaerobic fungi in ruminal cellulolysis depends on their ability to degrade the complex polysaccharides which occur in plant cell walls. In addition to utilizing cellulose, *N. patriciarum* utilizes xylan and other grass hemicelluloses (36). Cultures of *N. patriciarum*, *Piromonas communis*, and *Sphaeromonas communis* (34), as well as several unnamed isolates of ruminal fungi which morphologically resemble *Neocallimastix* spp. or *S. communis* (15, 16), all degrade various polysaccharide components of wheat straw cell walls. About half of the total cell walls, including about half of the cellulose and hemicellulose components, are lost from 4- to 5-day-old cultures of *Neocallimastix* and *Piromonas* spp., whereas only smaller proportions of these cell wall components disappear from *Sphaeromonas* cultures grown for the same period of time (15, 34).

Because lipids from plant material in the growth media interfere with the measurement of lipid phosphorus in the fungal cells, this method cannot be used to estimate the growth of ruminal fungi on complex plant polysaccharides (36). Chitin is a component of the cell wall of anaerobic fungi (33), and as described in the accompanying article, we (40) have found that it is a convenient marker for estimation of growth of these fungi on cellobiose. We now report measurements of the growth of anaerobic fungi on both cellulose

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and straw, as well as a comparison of the degradation of both cellulose and some cell wall components of straw by three different fungi which belong to the genera *Neocallimastix*, *Piromonas*, and *Sphaeromonas*. Also, the activities of several enzymes involved in hydrolysis of cell wall polysaccharides were measured at intervals during growth of these three fungi.

MATERIALS AND METHODS

Organisms. Three strains of anaerobic chytridiomycete fungi were studied. The methods used to isolate them from the ovine rumen, as well as the maintenance of pure fungal cultures, have been described previously (39, 40). Strain LM-1 resembled *N. frontalis* (17, 29) and *N. patriciarum* (37), whereas strains SM-1 and NM-1, respectively, resembled *P. communis* (32) and *S. communis* (30).

Media and growth conditions. Anaerobic fungi were grown at 39°C in a semidefined ruminal-fluid-free medium originally developed for culturing ruminal bacteria (medium 10 [11]). A detailed description of the modifications to medium 10 and the method for preparation were reported earlier (39, 40). In the first experiment described, ruminal fluid (40% [vol/vol]) replaced the yeast extract, Trypticase, hemin, and volatile fatty acid mixture in medium 10. The ruminal fluid was prepared from digesta collected via a permanent ruminal fistula from an adult Merino wether sheep just before the daily ration (360 g of alfalfa hay and 240 g of oat grain; hammer milled and then pelleted) was given. Immediately after collection, the digesta was strained through nylon cloth (average pore size, 160 μm), and the fluid was centrifuged at 20,000 × *g* for 30 min at 4°C. This clarified fluid was stored at −20°C. After the fluid was thawed, the precipitate which had formed was removed by centrifugation at 1,000 × *g* for 5 min at room temperature, and the reclarified ruminal fluid was added to the medium prior to autoclaving. The initial pH values of all culture media were 6.3 to 6.4.

Wheat straw (senescent stems of *Triticum aestivum* L. with some leaves but free from grain and seed heads) was hammer milled to pass through a sieve with 1-mm pores. The straw or the cellulose substrate (fibrous powder, grade CF11; Whatman Chemical Separation Ltd., England) was present at 1% (wt/vol) in 25 ml of basal medium 10. Media were dispensed into 100-ml serum bottles fitted with butyl rubber septa and metal crimp seals (26) and then sterilized at 120°C for 15 min. When media containing glucose or cellobiose (both at 0.5% [wt/vol]) were required for enzyme studies, the sugars were separately sterilized in concentrated solutions as described above, but under a N_2 atmosphere, and they were aseptically added to basal medium 10 by syringe. Complete medium 10 contained glucose and cellobiose (each at 0.03% [wt/vol]) and xylan (oat spelt; Sigma Chemical Co., St. Louis, Mo.) and starch (each at 0.07% [wt/vol]) in basal medium 10 as described previously (3, 39).

Inocula for all growth, degradation, and enzyme studies were grown in complete medium 10 with the addition of 0.05% (wt/vol) agar to prevent ruminal fungi from adhering to the walls of the culture vessel. Cultures to be used as inocula were grown for either 4 days (*Neocallimastix* sp. strain LM-1) or 5 days (*Piromonas* sp. strain SM-1 and *Sphaeromonas* sp. strain NM-1). An inoculum of 4% (vol/vol) was always used. In order to assess the number of fungal CFU in an inoculum, 1-ml samples were serially diluted 10-fold in an anaerobic dilution solution (10) contained in Hungate-type anaerobic culture tubes fitted with butyl rubber septa (Bellco Glass, Inc., Vineland, N.J.). Small samples (0.2 ml) of 10^{-1} and 10^{-2} dilutions were separately inoculated into triplicate Hungate tubes containing 2.8 ml of complete medium 10 and 2% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) kept molten at 50°C. The tubes were rolled on ice immediately after inoculation and were incubated for 5 days before fungal colonies were counted through a dissecting microscope at a magnification of ×16. During growth and degradation experiments, six straw or three cellulose culture bottles were opened at each sampling time and the pH of the culture was measured immediately. The opened bottles were then stored at −20°C prior to further analysis.

Measurement of dry matter and plant fiber fractions in straw and cellulose cultures. The amount of dry matter remaining in triplicate straw or cellulose cultures was determined by washing the insoluble material three times with distilled water in tared glass centrifuge tubes (15 by 100 mm) and drying in an oven at 100°C. Washing was carried out at room temperature by centrifugation at 1,500 × *g* for 5 min. The dried material was retained for measurements of fungal growth.

The method of Goering and Van Soest (14) adapted to semimicro crucibles (10-ml capacity; Filtrex, Moorooka, Queensland, Australia) was used for analysis of plant fiber fractions in straw cultures. These crucibles were fitted with screw caps at each end, in addition to the sintered glass filter, so that the small sample (<0.25 g) could be extracted, filtered, and eventually ashed in the same crucible (27). The straw remaining in triplicate cultures was transferred to the crucibles, extracted with acid-detergent solution at 100°C, weighed, extracted with permanganate reagent, weighed again, ashed at 500°C, and weighed a third time. The straw used in this study was composed of 92.9% dry matter and, on a dry matter basis, 96.6% organic matter, 52.6% acid-detergent fiber (ADF), 43.5% cellulose, and 11.3% permanganate-oxidizable lignin.

Reducing sugars released into the medium in cellulose cultures were measured by the method of Nelson-Somogyi (5), with glucose as the standard.

Measurement of fungal growth on cellulose and on straw. Fungal growth in cellulose cultures were measured both by cellular protein and by cell wall chitin. Dried material from cellulose cultures was extracted with about 5 ml of 0.66 N NaOH at 39°C for 18 to 24 h. Cellulose was sedimented by centrifugation at 1,500 × *g* for 5 min at room temperature, and the pellet was washed twice with 0.66 N NaOH. All three supernatants were pooled, and the volume was adjusted to 25 ml with 0.66 N NaOH. The protein concentration in the alkali extracts was measured by a modified Lowry method with bovine plasma albumin as the standard, as previously described (22, 39, 40). After alkali extraction, the remaining cellulose and fungal mass was washed three times by centrifugation with distilled water, dried at 100°C, and retained for chitin estimation.

Fungal chitin was estimated as the total hexosamine content of straw and cellulose cultures after acid hydrolysis. Triplicate dried samples from either cellulose or straw cultures were hydrolyzed for 4 h at 100°C with 6 N HCl contained in tubes sealed with Teflon-lined screw caps. After the hydrolysates were dried in vacuo at 50 to 55°C, hexosamine was measured colorimetrically with Erhlich reagent by using D-glucosamine hydrochloride as the standard (12). The chitin content of the samples was calculated as the 1,4-anhydro-*N*-acetyl-2-deoxy-D-glucopyranose equivalent. The chitin contents of all straw and cellulose cultures were

corrected by subtracting the background chitin levels of straw (1.68 mg/g) and cellulose (0.34 mg/g), respectively.

Cellular protein could not be used as a measure of fungal growth in straw cultures because other substances extracted by the alkali interfered with the Lowry method. In these cultures the protein content was estimated from the chitin content. There is high positive correlation of cell protein to cell wall chitin derived for *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1, and *Sphaeromonas* sp. strain NM-1 grown on 0.5% cellobiose as described in the accompanying article (40). The cellular content of protein in the three fungi was estimated by using these correlations.

Assay of enzymes in culture supernatants. Fungal cultures were clarified by centrifugation at room temperature (1,500 × *g* for 10 min). The supernatants were dialyzed for 18 to 20 h at 4°C against 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH buffer (pH 6.0) containing 0.1 mM NaN_3. All enzyme assays were conducted at 39°C in MES buffer (pH 6.0).

Aryl-β-glucosidase and aryl-β-xylosidase assays were based on previously described methods (38, 44). *p*-Nitrophenyl derivatives of β-D-glucose or β-D-xylose (5 mM; Sigma) were incubated with 25 mM MES buffer (pH 6.0) and 20 to 200 μl of supernatant in a total volume of 2 ml for 30 min. The reactions were stopped by the addition of 2 ml of 0.2 M K_2CO_3, and the amount of *p*-nitrophenol produced was determined from a standard curve by measurement of A_{410}. Enzyme activity was expressed as nanomoles of product produced per minute.

CM-cellulase and xylanase assays were based on the method for CM-cellulase (25). CM-cellulose (sodium salt, low viscosity, C-8758) and xylan (from oat spelt, X-0376) were both purchased from Sigma and were added to 50 mM MES buffer (pH 6.0) at a concentration of 1% (wt/vol). Between 50 and 200 μl of supernatant (for CM-cellulase) or 2 to 50 μl of supernatant (for xylanase) was added to 1.5 ml of substrate solution in a final volume of 2.5 ml. The reactions were stopped by the addition of 2.5 ml 3,5-dinitrosalicylic acid reagent (24) after 30 min or 1 h of incubation for CM-cellulase or xylanase, respectively. The concentration of reducing sugars was measured with D-glucose or D-xylose as the appropriate standard. Enzyme activity was expressed as nanomoles of product produced per minute.

Azure cellulase was measured by using a modification of the method described by Coleman (13). Cellulose azure (C-8647; Sigma) was thoroughly washed in 50 mM MES buffer (pH 6.0) and suspended at a final concentration of 0.1% (wt/vol) in this buffer, which contained 0.1 mM NaN_3. Reaction mixtures containing 3.0 ml of azure cellulose suspension and either 0.3 or 0.5 ml of supernatant in a total volume of 3.5 ml were incubated for 7 h. The reaction was stopped by placing the tubes in ice. Control mixtures which lacked either the substrate or the culture supernatant were also incubated. The unreacted azure cellulose was pelleted by centrifugation (1,500 × *g* for 10 min), but it was necessary to pass the supernatants through glass fiber filters (grade GF/C; Whatman) to remove the last traces of the dyed cellulose. The amount of dye released from the substrate was determined by measuring A_{580}. Enzyme activity was expressed as the change in A_{580} per hour.

RESULTS

Influence of ruminal fluid medium and inoculum size on straw degradation by ruminal fungi. The anaerobic fungi *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1, and *Sphaeromonas* sp. strain NM-1 were grown in media containing straw, with or without added ruminal fluid. The quantities of two cell wall components of straw (ADF and cellulose) which were solubilized in the cultures are shown in Table 1. Strains LM-1 and SM-1 degraded similar amounts of either ADF or cellulose, whereas strain NM-1 degraded less plant fiber and cellulose. There was no change in the extent of breakdown of straw fiber by the three fungi when ruminal fluid was absent from the medium. All subsequent cultures used ruminal-fluid-free media.

TABLE 1. Breakdown of some components of wheat straw by ruminal fungi grown for 10 days in a semidefined medium or in a ruminal-fluid medium

Fungal strain	Ruminal fluid in medium	Final pH after growth[a]	% of component solubilized[b]	
			ADF	Cellulose
Neocallimastix sp. strain LM-1	No	5.8	47.0	54.1
	Yes	6.1	46.4	55.2
Piromonas sp. strain SM-1	No	6.0	45.6	53.8
	Yes	6.1	45.0	53.4
Sphaeromonas sp. strain NM-1	No	6.0	35.0	38.7
	Yes	6.2	32.8	38.4

[a] Initial pH values of medium 10 with straw and of ruminal-fluid-containing medium with straw were 6.4.

[b] Each culture (25 ml) initially contained 120 mg of ADF and 93 mg of cellulose. All values were corrected for washout from straw in uninoculated medium.

Growth and degradation of cellulose powder. Figure 1 shows the growth of strains LM-1 and SM-1 on cellulose powder, whereas strain NM-1 did not grow on this cellulose (data not shown). The growth of ruminal fungi in cellulose cultures was determined by measuring the amount of cellular protein and chitin produced in the cultures. Maximum growth occurred after 4 days for both strains LM-1 and SM-1, as determined by protein or chitin assays. Cultures of both fungi produced about the same maximal amount of protein, whereas the maximum chitin levels in strain LM-1

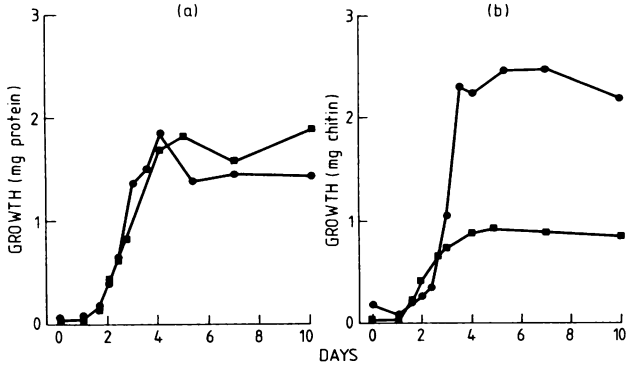

FIG. 1. Cellular protein (a) and cell wall chitin (b) produced by *Neocallimastix* sp. strain LM-1 (■) and *Piromonas* sp. strain SM-1 (●) growing on cellulose. Each value is the mean of three replicates.

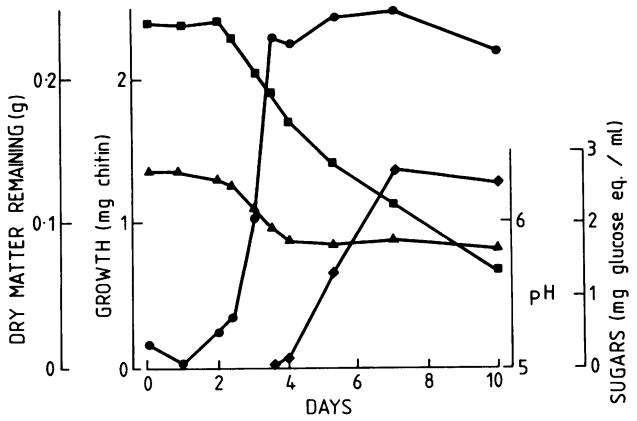

FIG. 2. Apparent loss of cellulose dry matter during growth of *Piromonas* sp. strain SM-1. Symbols: ■, dry matter; ●, fungal growth; ◆, reducing sugars; ▲, culture pH. Each value is the mean of three replicates.

cultures were about one-third of those produced by strain SM-1.

Growth of strain SM-1 on cellulose was accompanied by the loss of dry matter from the culture, and dry matter disappearance continued after growth had ceased (Fig. 2). During the period after growth had ceased, soluble sugars accumulated in the medium. The pH of the culture changed from 6.4 to 5.8 during growth. Similar data on the loss of cellulose dry matter, the decline in culture pH, and the accumulation of soluble sugars were obtained for strain LM-1 but are not shown.

Growth and degradation of straw. Chitin levels in cultures of *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1 and *Sphaeromonas* sp. strain NM-1 with straw as the only fermentable carbon source were determined (Fig. 3a). Strain NM-1 grew to the highest yield, whereas strains LM-1 and SM-1 had similar but lower yields of chitin. Maximum growth occurred after 3 days for strain LM-1 and 5 days for strains SM-1 and NM-1. When the chitin levels were converted to protein values, strain LM-1 had grown to the highest yield, followed by strains NM-1 and SM-1 (Fig. 3b).

All three fungi degraded the ADF and cellulose components of straw (Table 1). Detailed degradation curves for the

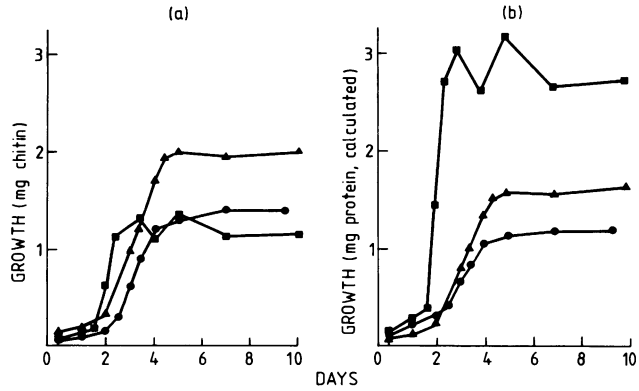

FIG. 3. Cell wall chitin (a) and calculated values for cellular protein (b) of *Neocallimastix* sp. strain LM-1 (■), *Piromonas* sp. strain SM-1 (●), and *Sphaeromonas* sp. strain NM-1 (▲) growing on milled wheat straw. Each value is the mean of three replicates.

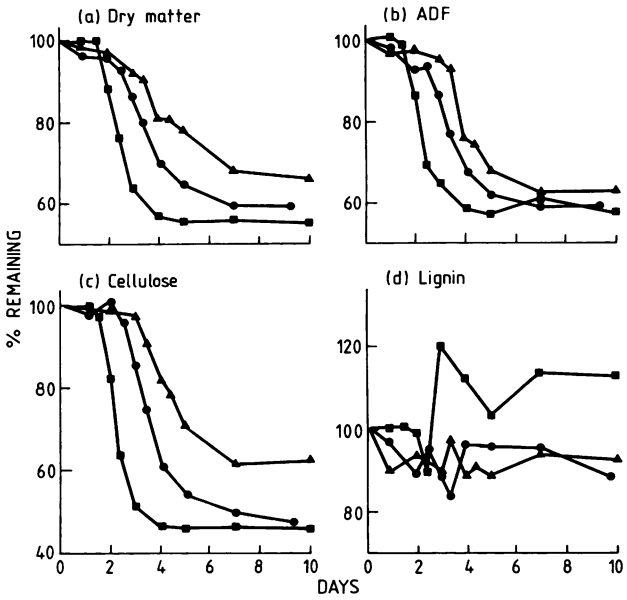

FIG. 4. Apparent degradation of straw dry matter and cell wall components by *Neocallimastix* sp. strain LM-1 (■), *Piromonas* sp. strain SM-1 (●), and *Sphaeromonas* sp. strain NM-1 (▲). Each value is the mean of three replicates. Each 25-ml culture contained 232 mg of dry matter, 122 mg of ADF, 101 mg of cellulose, and 26 mg of lignin.

dry matter, ADF, cellulose, and lignin components of straw are shown in Fig. 4a to d, respectively. Strain LM-1 degraded more dry matter, ADF, and cellulose than strain SM-1 did and strain NM-1 degraded these components the least. Similar results were observed for the rate of degradation of dry matter and cellulose (Fig. 4a and c). Because the datum points obtained for the disappearance of lignin during these experiments were scattered (Fig. 4d), comparison of lignin degradation between the three fungi was not possible.

The degradation of cellulose powder and that of straw dry matter have been compared for strains LM-1 and SM-1 (Fig. 5). Each fungus degraded cellulose powder and straw at the same approximate rate; it is only the extent of degradation after 10 days which differs, in part because of the continued hydrolysis of cellulose after fungal growth has ceased. However, a comparison of the growth of these two fungi on cellulose (Fig. 1b) and on straw (Fig. 3a) shows that strain LM-1 grew equally well on straw and on purified cellulose, whereas strain SM-1 grew better on cellulose than on straw.

Extracellular activities of some enzymes involved in degrading cellulose and xylan. The effects of several growth substrates on the activities of CM-cellulase, azure cellulase, xylanase, β-glucosidase, and β-xylosidase in culture supernatants of *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1, and *Sphaeromonas* sp. strain NM-1 are shown in Table 2. Straw-grown cultures of the three fungi contained high activities of all five of the enzymes assayed. More variable results were obtained with the other growth substrates. There was low enzyme activity in glucose and cellobiose cultures of strain LM-1, whereas SM-1 and NM-1 cultures on the same substrates generally possessed higher activities, which sometimes approached the enzyme activity found in straw-grown cultures (particularly strain SM-1).

The activities of β-glucosidase and CM-cellulase increased during the growth of strains LM-1 and SM-1 on straw or cellulose and the growth of strain NM-1 on straw (Fig. 6).

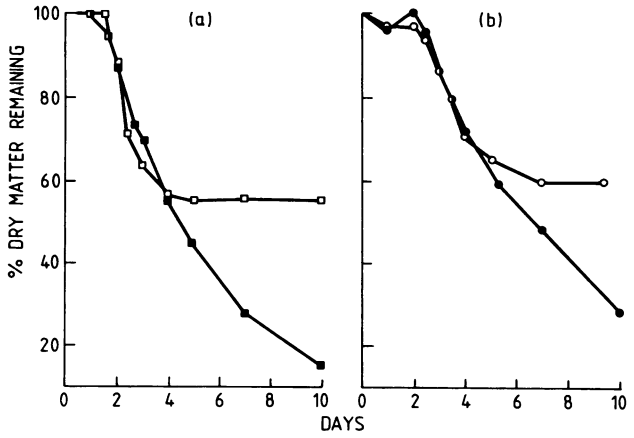

FIG. 5. Apparent dry matter loss in cultures of *Neocallimastix* sp. strain LM-1 (a) and *Piromonas* sp. strain SM-1 (b) growing either on cellulose (solid symbols) or on milled straw (open symbols). Each value is the mean of three replicates.

Xylanase and β-xylosidase activities also increased during the growth of all three fungi on straw. However these two enzymes increased in cellulose cultures of strain SM-1 but not in LM-1 cultures. Maximum extracellular activity for each enzyme coincided with maximum growth of all three fungi on straw and on cellulose (Fig. 1 and 3).

DISCUSSION

The rumen provides an environment rich in nutrients and cofactors, including amino acids, peptides, vitamins, and minerals, various combinations of which are required by microorganisms for fermentation and growth (20). Many ruminal bacteria require specific nutrients from ruminal fluid for digestion of cellulose, but they can be cultured in medium lacking ruminal fluid when it is amended with the appropriate growth factor(s) (9, 41). Members of the anaerobic fungus genus *Neocallimastix* are nutritionally nonfastidious, requiring only two vitamins in addition to sources of C, N, and S

TABLE 2. Extracellular enzyme activities in dialyzed culture fluids of three ruminal fungi grown for 7 days in media containing different substrates

Fungal strain	Growth substrate[a]	Enzyme activity (nmol/min per ml)[b]				
		β-Glucosidase	β-Xylosidase	CM-cellulase	Azure cellulase[c]	Xylanase
Neocallimastix sp. strain LM-1	Glucose	8	6	<10	6	97
	Cellobiose	7	3	<10	4	58
	Cellulose	26	5	180	36	170
	Straw	56	50	310	53	1,400
Piromonas sp. strain SM-1	Glucose	33	85	450	21	7,200
	Cellobiose	11	80	310	11	2,600
	Cellulose	14	41	380	19	2,500
	Straw	77	88	760	57	6,800
Sphaeromonas sp. strain NM-1	Glucose	10	26	180	63	410
	Cellobiose	19	33	400	99	750
	Straw	48	59	860	181	7,100

[a] Growth substrates were added to basal medium 10 at 0.5% (wt/vol) (glucose and cellobiose) or 1% (wt/vol) (cellulose and straw).
[b] Values are averages of duplicate assays.
[c] Azure cellulase activity is expressed as the change in A_{580} per hour per milliliter, 10^3.

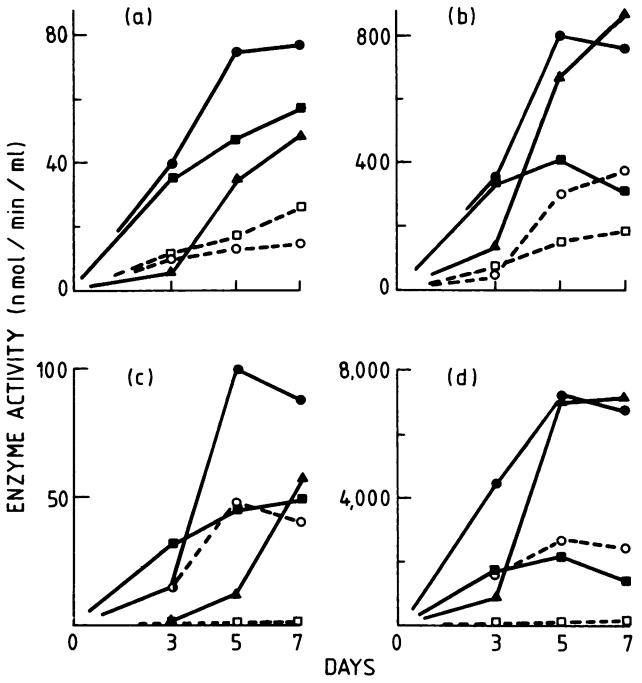

FIG. 6. Extracellular enzyme activities of β-glucosidase (a), CM-cellulase (b), β-xylosidase (c), and xylanase (d) in culture supernatants of *Neocallimastix* sp. strain LM-1 (■, □), *Piromonas* sp. strain SM-1 (●, ○), and *Sphaeromonas* sp. strain NM-1 (▲) growing on milled straw (solid symbols) or cellulose (open symbols).

(35), and they digest cellulose and plant cell walls when grown in media lacking ruminal fluid (23, 36). *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1, and *Sphaeromonas* sp. strain NM-1, the three anaerobic fungi used in this study, similarly did not require ruminal fluid for digestion of wheat straw cell walls and cellulose (in the cases of LM-1 and SM-1). Therefore it seems likely that *Piromonas* and *Sphaeromonas* strains are also nutritionally nonfastidious. This conclusion is also supported by data on the utilization of various plant polysaccharides by 17 strains of ruminal fungi in a semidefined culture medium (39). However, the precise minimal nutritional requirements of *Piromonas* and *Sphaeromonas* strains await discovery.

The degradation of cellulose by several *Neocallimastix* isolates has been reported (7, 23, 28, 36, 38), whereas representatives of the other two recognized genera of ruminal anaerobic fungi, *Piromonas* and *Sphaeromonas*, have received very little attention to date. *Neocallimastix* sp. strain LM-1 and *Piromonas* sp. strain SM-1 degraded purified cellulose and thus resembled the *Neocallimastix* spp. isolates studied previously. Extracellular β-glucosidase and CM-cellulase activities were maximal at the end of the growth cycle of both strains LM-1 and SM-1. Solubilization of cellulose continued after the cessation of growth, indicating that the cellulolytic enzymes produced by these two anaerobic fungi were capable of hydrolyzing the substrate to soluble products detectable as reducing sugars in the culture medium.

The failure of *Sphaeromonas* sp. strain NM-1 to degrade the purified cellulose used in our study was enigmatic, since this anaerobic fungus produced large amounts of β-glucosidase and CM-cellulase activities when growing on straw and solubilized about 40% of the cellulose component of straw

cell walls. It is possible that strain NM-1 produces an extracellular cellulase activity which is unable to degrade microcrystalline cellulose. However, *N. frontalis* is known to secrete into the culture medium a complex of cellulolytic enzymes which efficiently degrades highly ordered crystalline cellulose (45, 46). It is also possible that the rudimentary rhizoid produced by strain NM-1 (40) limited the ability of the organism to attach to cellulose particles prior to digestion by cellulase. Only further study of both the mechanism of attachment of *Sphaeromonas* spp. to cellulose and the extracellular cellulase(s) produced by *Sphaeromonas* spp. will provide an answer.

Unlike the degradation of cellulose by strains LM-1 and SM-1, which continued after growth had ceased, the degradation of wheat straw by these two anaerobic fungi, as well as by strain NM-1, did not continue after the end of the growth cycle. Similar results were obtained with *Neocallimastix* sp. strain R1 (23). *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1, and *Sphaeromonas* sp. strain NM-1 solubilized totals of 45, 40, and 30%, respectively, of straw dry matter. Therefore, degradation of straw by anaerobic fungi appears to require both the action of appropriate extracellular enzymes and active growth of the ruminal fungus. Physical disruption of plant particles by specialized structures of the fungal rhizoid, as observed by Ho et al. (19), would allow access of fibrolytic enzymes to susceptible plant cell walls located deep inside the particles.

Dry matter loss from straw was greatest for strain LM-1, which was followed by strain SM-1 and then strain NM-1, confirming preliminary data for these isolates (15, 16) and other related ruminal fungi (34). The same relative order applied to the rate and extent of degradation by these three fungi of the ADF and cellulose components of straw cell walls, suggesting that all of them are capable of contributing to fiber digestion in the rumen. However, the extent of this contribution is yet to be determined. Straw cell wall lignin was apparently not degraded by strain LM-1, SM-1, or NM-1. However, previous research showed that at least some anaerobic fungi solubilized small amounts of lignin (15, 34). More sensitive methods are required to further investigate the action of anaerobic fungi on the lignin component of plant cell walls. An appropriate method may be the solubilization of [^{14}C-lignin]lignocellulose, which has shown that partial degradation of lignin is caused by anaerobic fungi growing both as a mixed population selected in ruminal fluid by treatment with penicillin and streptomycin (1) and as pure cultures (G. L. R. Gordon, Abstr. 2nd Int. Symp. Nutr. Herbivores, 1987, p. 135–136).

Chitin is a structural component of the cell walls of anaerobic fungi (33, 40). This polysaccharide has now been assessed as an indicator of fungal biomass produced during growth in pure culture on an insoluble substrate, cellulose. Strains LM-1 and SM-1 grew at the expense of cellulose and reached maximum chitin levels after 4 days when the maximum amount of cellular protein was also produced in the cultures. The major difficulty with using chitin as a measure of fungal biomass in cultures is the variability in the proportion of chitin contained in the cell walls of different anaerobic fungi (33, 34, 40). Measuring the amount of cellular protein produced by the fungi allowed a more direct comparison of the growth of different fungi on cellulose, since the relationship between fungal dry mass and cellular protein was more constant for *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1, and *Sphaeromonas* sp. strain NM-1 than the relationship between fungal dry mass and the content of chitin in cell walls was (40).

Determination of fungal growth on a complex solid substrate such as straw must take into account the presence of substances in this material which interfere with the measurement of either phospholipid (36) or protein (this study). Chitin determinations were not similarly affected, so that the growth of strains LM-1, SM-1, and NM-1 in straw cultures has been successfully monitored by measuring this fungal polysaccharide. The results of the growth experiments with strain LM-1 are in general agreement with data previously obtained by measuring fermentation end products in cultures of several different *Neocallimastix* isolates (7, 23, 28, 38). Strain LM-1 produced the equivalent of about double the cellular protein produced by strains SM-1 and NM-1, which correlated with the relative extent of solubilization of straw cell wall components by the three anaerobic fungi after 10 days.

The extracellular activities of β-glucosidase, CM-cellulase, β-xylosidase, and xylanase reached maximum levels in 5- to 7-day-old cultures of *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1, and *Sphaeromonas* sp. strain NM-1, which is similar to the results obtained with *Neocallimastix* sp. strain R1 cellulase and xylanase (23) and *N. frontalis* cellulases (28, 38). Even though fine details of the enzyme assay methods may differ between laboratories, comparisons can be made between data obtained for several isolates of anaerobic fungi. CM-cellulase activities are similar for cellulose-grown *Neocallimastix* sp. strains R1 (23) and LM-1 (this study), whereas the activities are lower for *N. frontalis* PN2 (38) and 10-fold higher for *N. patriciarum* (42). Extracellular β-glucosidase activities are also similar for cellulose-grown strain LM-1 and other *Neocallimastix* isolates (18, 23, 38, 43). Cellulolytic enzyme activities are similar for the *Piromonas* isolates studied so far, *P. communis* (18, 42, 43) and strain SM-1 (this study), although the CM-cellulase activity was higher for *P. communis*. Recently, an independent study has shown that a *Sphaeromonas* sp. produced extracellular cellulase activity (18). Direct comparisons of xylanase activities produced by ruminal fungi as measured in other laboratories have not been made because of the various sources of supply, and hence composition (8), of the xylans used as enzyme substrates. However, active xylanases and β-xylosidases were produced by straw- and/or xylan-grown *Neocallimastix*, *Piromonas*, and *Sphaeromonas* spp. (18, 23, 42, 43).

Cellulolytic and xylanolytic enzymes like those from other *Neocallimastix* spp. (23, 43) and *P. communis* (42, 43) were inducible in strains LM-1 and NM-1. Xylanase from *N. patriciarum* appears to be constitutive (42), and our study has shown that all of the cellulolytic and xylanolytic enzymes were produced constitutively by strain SM-1. It is not known how this fungus may be affected by the constitutive production of fibrolytic enzymes, but is was unable to degrade more straw cell wall components in culture than strain LM-1 did.

Our study has elaborated on earlier work to show that *Neocallimastix* sp. strain LM-1, *Piromonas* sp. strain SM-1, and *Sphaeromonas* sp. strain NM-1 produce active fibrolytic enzymes and degrade cell wall components of straw in culture. However, it remains to be seen whether the fibrolytic enzymes produced by these anaerobic fungi are as active as the cellulase of *N. frontalis* RK21 (45, 46). Furthermore, the extent and significance of lignin degradation by these unusual microorganisms should be investigated further, as should methods for assessing the biomass of these fungi in the rumen.

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